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Humoral and Cellular Immune Responses to Pre-Erythrocytic Malaria Vaccination and Impact of Epidemiological Factors

Robert Andrew Mitchell

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Humoral and Cellular Immune Responses to Pre-Erythrocytic Malaria Vaccination and Impact of Epidemiological Factors

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List of abbreviations

ACTs:	Artemisinin-based combination therapy
ADCC:	Antibody-dependent cellular cytotoxicity
ADCI:	Antibody-dependent cellular inhibition
aMBC:	Atypical memory B cells
AMA1:	Apical membrane antigen 1
APCs:	Antigen presenting cells
BCG:	Bacillus-Calmette Guérin
BCR:	B cell receptor
BREGS:	Regulatory B cells
CD:	Cluster of Differentiation
CHMI:	Controlled human malaria infection
ChAd63:	Chimpanzee adenovirus
COVID-19:	Coronavirus disease 2019
CSP:	Circumsporozoite protein
CTLA4:	Cytotoxic T-lympocyte assoiated protein 4
CyRPA:	Cysteine-rich protective antigen
DBL:	Duffy binding-like
DC:	Dendritic cell
DDT:	Dichlorodiphenyltrichloroethane
EBA:	Erythrocyte binding antigens
ELISA:	Enzyme-linked immunosorbent assay
Fc:	Fragment crystallizable
FOXP3:	Forkhead box P3 protein
GC:	Germinal center
GPI:	Glycophosphatidylinositol
γδ T cells:	Gamma delta T cells
HBsAg:	Hepatitis B surface antigen
HSPG:	Heparan sulfate proteoglycans

ICOS:	Inducible T-cell COStimulator
IFA:	Immunofluorescence assay
IFN:	Interferon
IgA:	Immunoglobulin A
IgD:	Immunoglobulin D
IgE:	Immunoglobulin E
IgG:	Immunoglobulin G
IgM:	Immunoglobulin M
IL:	Interleukin
IP-10:	Inducible protein 10
IPTp:	Intermittent preventive therapy in pregnancy
KC:	Kupffer cell
LAG3:	Lymphocyte-activation gene-3
LSA-1:	Liver stage antigen-1
M:	Month
MHC:	Major histocompatibility complex
MPL:	Monophosphoryl lipid
mAbs:	Monoclonal Antibodies
ME-TRAP:	Multiple-epitope thrombospondin-related adhesion protein
MHC-I:	Major histocompatibility complex class I
MHC-II:	Major histocompatibility complex class II
MSP:	Merozoite surface protein
MTI:	Malaria Transmission Intensity
NAI:	Naturally acquired immunity
NK:	Natural killer
PAMPs:	Pathogen-associated molecular patterns
PBMC:	Peripheral blood mononuclear cell
PCD:	Passive case detection
PCR:	Polymerase chain reaction
PD1:	Programmed cell death-1
PfEMP1:	Plasmodium falciparum erythrocyte membrane protein 1

PMC:	Perennial malaria chemoprophylaxis
PNG:	Papua New Guinea
PS:	Phosphatidylserine
RAS:	Radiation-attenuated sporozoites
RDTs:	Rapid diagnostic tests
RBC:	Red blood cells
Rhs:	Reticulocyte-binding-like homologs
RON:	Rhoptry neck protein
SHM:	Somatic hypermutation
SMC:	Seasonal malaria chemoprophylaxis
TGF-β:	Transforming growth factor-β
TFH:	T follicular helper
TH:	T helper
TIM3:	T-cell immunoglobulin and mucin domain-3
TLRs:	Toll-like receptors
TMV:	Tobacco mosaic virus
TNF:	Tumor necrosis factor
TR1:	Type 1 regulatory T cells
TR1:	T regulatory type 1
TRAP:	Thrombospondin-related adhesive protein
TREG:	Regulatory T cells
VE:	Vaccine efficacy
VLP:	Virus-like particles
WHO:	World Health Organization

List of publications

This thesis is in the format of a compendium of articles. The thesis consists of three objectives and three articles:

Article 1

This article addresses the first objective to assess the fine specificity and sporozoite neutralizing capacity of polyclonal and monoclonal antibodies targeting circumsporozoite epitopes. The results of this research have been published in the following article:

J. Mauricio Calvo-Calle*, **Robert Mitchell***, Rita Altszuler, Caroline Othoro, and Elizabeth Nardin. ***Identification of a neutralizing epitope within minor repeat region of Plasmodium falciparum CS protein***. npj Vaccines. 2021 Jan 18;6(1):10. *co-first authors.

Impact factor (2021): 9.2 Quartile: 1. Category: Immunology

Article 2

This article addresses the second objective to characterize the cellular responses elicited by the RTS,S/AS01$_E$ vaccine booster dose at the peak response and 11 months later and identify immune correlates of malaria protection or risk. The sub-objectives to evaluate the effect of epidemiological factors on immunogenicity and protection, and to assess associations between cytokine responses and antibodies were also addressed. The results of this research have been accepted in the following article:

Robert A. Mitchell, Dídac Macià*, Chenjerai Jairoce*, Maxmillian Mpina, Akshayata Naidu, Ana Chopo-Pizarro, Miquel Vázquez-Santiago, Joseph J. Campo, Pedro Aide, Ruth Aguilar, Claudia Daubenberger, Carlota Dobaño, Gemma Moncunill. ***Effect of RTS,S/AS01$_E$ vaccine booster dose on cellular immune responses in African infants and children.*** *Accepted by npj Vaccines.*

Impact factor (2023): 6.9 Quartile: 1. Category: Immunology

Article 3

This article addresses the third objective to characterize the immunophenotypic changes induced by chronic malaria exposure associated with T-cell exhaustion and its association with B cell subsets previously found to be altered by malaria exposure. The results of this research have been published in the following article:

Robert A. Mitchell, Itziar Ubillos, Pilar Requena**,** Joseph J. Campo, Maria Ome-Kaius, Sarah Hanieh, Alexandra Umbers, Paula Samol, Diana Barrios, Alfons Jiménez, Azucena Bardají, Ivo Mueller, Clara Menéndez, Stephen Rogerson, Carlota Dobaño, and Gemma Moncunill. ***Chronic malaria exposure is associated with inhibitory markers on T cells that correlate with atypical memory and marginal zone-like B cells.*** Clinical and Experimental Immunology. 2024 Apr 23;216(2):172-191.

Impact factor (2023): 3.4 Quartile: 2. Category: Immunology

Resum de la Tesi

Títol: Respostes Immunitàries Humorals i Cel·lulars a la Vacunació enfront la fase Pre-eritrocítica de la Malària i l'Impacte dels Factors Epidemiològics

Introducció

La immunitat adquirida naturalment enfront la malària es desenvolupa després de diverses exposicions al paràsit, depenent de la intensitat de la transmissió i l'edat. Les exposicions repetides i cròniques a la malària, però poden conduir a fenotips cel·lulars immunitaris alterats que poden afectar la immunitat. Les primeres vacunes aprovades contra la malària tenen la proteïna del circumsporozoït (CSP) que envolta l'esporozoït de *Plasmodium falciparum* com a diana. La vacuna RTS,S/AS01E (RTS,S), composta pels epítops repetits majors basats en la seqüència d'aminoàcids NANP i el fragment C-terminal de la CSP, està recomanada per a nens Africans. En l'assaig clínic de fase 3, les respostes d'anticossos induïdes per RTS,S i les respostes de limfòcits T auxiliars de tipus 1 després de la immunització primària s'associaven amb la protecció. Tanmateix, amb i sense una dosi de reforç de la vacuna, les respostes d'anticossos i l'eficàcia de la vacuna van ser modestes i de curta durada, plantejant preguntes sobre la inclusió de diferents epítops a la vacuna, la immunitat cel·lular post-reforç, i els factors que poden afectar les respostes vacunals, informació que podria ajudar a millorar el disseny de la propera generació de vacunes.

Hipòtesi

1. Els anticossos contra la CSP de *P. falciparum* estan associats amb la protecció enfront la malària, però la especificitat fina als diferents epítops, inclosos o no a la vacuna RTS,S, està associada amb diferents activitats funcionals de neutralització d'esporozoïts.
2. La dosi de reforç de la vacuna RTS,S/AS01$_E$ indueix respostes immunitàries cel·lulars que sostenen les respostes protectores d'anticossos. Els factors epidemiològics afecten la immunogenicitat cel·lular de la dosi de reforç i la seva associació amb la protecció.

3. L’exposició crònica a la malària provoca l’"esgotament" dels limfòcits T, resultant en una expressió augmentada de marcadors inhibidors i de senescència, que correlaciona amb alteracions en els limfòcits B i pot afectar la resposta immune a infeccions naturals i a la vacunació.

Objectius

Identificar les respostes immunitàries a CSP de *P. falciparum* induïdes per l'exposició natural a la malària o per la vacunació, incloent els factors que poden afectar aquestes respostes.

Específicament:

1. Avaluar l’especificitat fina i la capacitat de neutralització d’esporozoïts d’anticossos policlonals i monoclonals que reconeixen epítops de la CSP.
2. Caracteritzar la resposta cel·lular induïda per la dosi de reforç de la vacuna RTS,S/AS01$_E$ i identificar correlats immunitaris de protecció o risc de malària.

    2.1. Avaluar l'efecte dels factors epidemiològics sobre la immunogenicitat i la protecció.

    2.2. Avaluar l’associació entre la resposta de citocines i els anticossos.

3. Caracteritzar els canvis immunofenotípics induïts per l'exposició crònica a la malària relacionats amb l’esgotament limfòcits T i la seva associació amb subpoblacions de limfòcits B que estan alterats per l'exposició a la malària.

Mètodes i Resultats

Els resultats es presenten en el format de tres articles. **L’article 1** avalua l’especificitat i la capacitat de neutralització d’esporozoïts dels anticossos policlonals i monoclonals dirigits als epítops de la CSP. Es van utilitzar assaigs immunològics i de neutralització in vitro i infeccions de malària en ratolins per mostrar que els anticossos monoclonals específics per als epítops repetitius menors de la CSP tenien activitat de neutralització,

mentre que els anticossos contra els epítops repetitius majors tenien una activitat de neutralització menor o nul·la. **L'article 2** aborda les respostes immunitàries cel·lulars induïdes per la dosi de reforç de RTS,S, avaluant la immunogenicitat de la vacuna, examinant la secreció de citocines per part de cèl·lules mononuclears de sang perifèrica (PBMC) estimulades per l'antigen de la vacuna amb la plataforma Luminex. Els resultats van mostrar que IL-2, IFN-γ, IL-17, IL-5 i IL-13 estaven associats amb la vacunació de reforç i IL-2 es estava elevada fins a un any després. No es va trobar cap factor epidemiològic que tingués un efecte sobre les respostes de la vacuna. IL-2 estava associada amb un risc reduït de malària en nens de Mahniça (Moçambic) però no de Bagamoyo (Tanzània), indicant que la relació de la resposta a la vacuna i la protecció pot ser diferent segons l'àrea. A més, IL-2 correlacionava positivament amb els anticossos IgG anti-CSP. **En l'article 3** es va analitzar els marcadors inhibidors en supoblacions de limfòcits T mitjançant citometria de flux en PBMC d'individus afectats per l'exposició crònica a la malària. Aquests individus tenien freqüències elevades de limfòcits T que expressaven marcadors inhibidors, i que correlacionaven amb anticossos contra antígens de la malària, limfòcits B de memòria atípics i anèmia.

Conclusions

1. La regió dels epítops repetitius menors de CSP de *P. falciparum*, composta per tres còpies de repeticions de tetràmers alternants NANP i NVDP, conté un epítop reconegut per anticossos neutralitzadors d'esporozoïts i, en canvi, els anticossos monoclonals específics d'epítops repetitius majors (NANP)n van demostrar una activitat neutralitzadora reduïda o nul·la. Aquestes troballes indiquen la importància d'avaluar l'especificitat fina i les funcions dels anticossos induïts per vacunes basades en CSP i suggereixen que la inclusió d'epítops repetitius menors a la vacuna podria millorar l'eficàcia d'aquesta.

2. La dosi de reforç de la vacuna RTS,S/AS01$_E$ va induir IL-2 en concentracions relativament baixes però encara detectables 12 mesos després. La IL-2 va correlacionar positivament amb la IgG anti-CSP i inversament amb les IgG2 i IgG4 no protectores, i es va associar amb una reducció del risc de malària en nens de

Manhiça. Modificar el disseny de la vacuna per induir respostes de limfòcits T de memòria més altes que puguin sostenir els limfòcits B podria augmentar l'eficàcia i la durabilitat de la vacuna, mentre que les diferències entre àrees haurien de ser estudiades i considerades per a l'estratègia de vacunació.

3. Un augment del risc de malària es va associar amb la IL-10 en nadons independentment de la vacunació amb RTS,S/AS01$_E$, indicant que les respostes immunoreguladores específiques d'edat poden afectar la susceptibilitat a la malària.

4. L'exposició crònica a la malària es va associar a freqüències més elevades de subpoblacions de limfòcits T amb marcadors inhibidors associats amb l'esgotament immunitari i correlacionaven amb limfòcits B de memòria atípics i anèmia. Aquests fenotips alterats podrien afectar les respostes a les vacunes.

INTRODUCTION

1. Malaria: biology and the global disease burden

Malaria is a significant mosquito-borne disease caused by a protozoan parasite from the genus *Plasmodium*. While many *Plasmodium* species can infect birds, reptiles, and mammals, five species are known to infect humans including *P. falciparum, P. vivax*, *P. malariae*, *P. ovale* (two subspecies *P. ovale curtisi* and *P. ovale wallikeri*), and *P. knowlesi* (1). *P. falciparum* is considered to be most deadly and causes the most severe form of malaria, and predominantly occurs in sub-Saharan Africa (1).

1.1 *Plasmodium falciparum* life cycle

The *P. falciparum* life cycle is complex, involving a female *Anopheles* mosquito vector and having both extracellular and intracellular zoite forms, which are required to traverse and invade various cell types at different stages (2) (**Figure 1**).

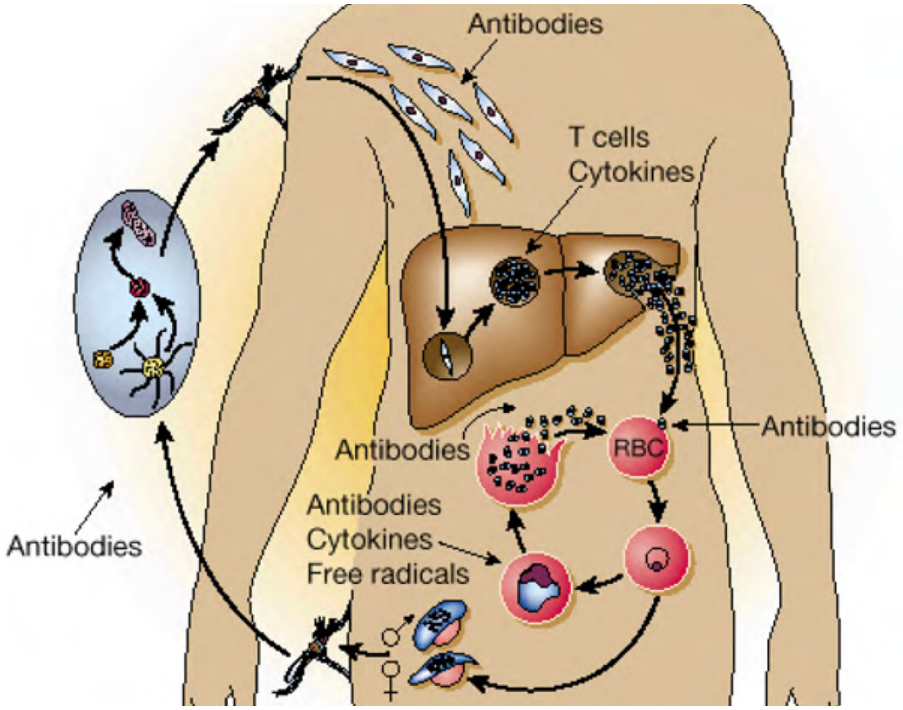

Figure 1. *P. falciparum* life cycle. Arrows indicating types of immune responses known to target parasites at different stages. Adapted from (3).

Pre-erythrocytic stage. The asymptomatic pre-erythrocytic stage of infection begins with the inoculation of motile sporozoites into the dermis of the host when an infected *Anopheles* mosquito probes for a blood meal (4–6). Estimates of the number of

sporozoites deposited into the skin of mice using the murine parasite *P. berghei* (7,8) or *P. falciparum* (9) varies widely from a dozen up to hundreds of sporozoites, which may stay at the inoculation site or within the dermis for minutes up to several hours before migrating (10,11). Sporozoites traverse through the skin tissue until they reach a capillary and enter into the bloodstream, which carries them rapidly to the liver (12–14).

Circumsporozoite protein (CSP), the major surface protein covering the sporozoite, is critical for sporozoite migration and invasion (15). Sporozoites rely upon CSP and the sulfation level of heparan sulfate proteoglycans (HSPG) on host cells to help navigate through different tissue types (16). Thrombospondin-related adhesive protein (TRAP) expressed on the sporozoite surface, is also required for motility and invasion (17).

Once a sporozoites reaches the liver, its surface CSP engages HSPG of hepatic stellate cells, then it glides along the sinusoidal endothelial barrier until it reaches a Kupffer cell (KC) which it exploits to cross the endothelium and invade a hepatocyte (18). The sporozoite traverses through several hepatocytes, which may be a necessary activation step (19), until it enters a final hepatocyte by establishing a parasitophorous vacuole (12). After settling into the vacuole, the sporozoite transforms into trophozoites, an exo-erythrocytic schizogony development (20). Within ten days of development, infected hepatocytes release tens of thousands of exo-erythrocytic merozoites encased in host-membrane derived vesicles called merosomes into the bloodstream that consequently infect red blood cells (RBC) (2).

Blood-stage. Merozoites released from the liver into the bloodstream rapidly enter a multi-step process including a pre-invasion phase involving parasite actomyosin driven deformation of the host RBC upon adhesion, preparation for active invasion, followed by echinocytosis (21). Merozoite surface protein 1 (MSP1), a major glycophosphatidylinositol (GPI)-anchored antigen, permits interactions between the merozoite and the RBC membrane (22). Deformation of the erythrocyte occurs following contact, then the merozoite positions its apical end towards the RBD membrane enabled by erythrocyte binding antigens (EBA), as well as reticulocyte-binding-like homologs

(Rhs) (2). Rh5 interacting with Ripr (Rh5-interacting protein) and CyRPA (cysteine-rich protective antigen) form a tightly bound complex that interacts with the host receptor basigin, which is required for erythrocyte invasion (23,24).

Apical membrane antigen (AMA1) produced by micronemes (secretory organelles) is translocated to the parasite surface after invasion has been initiated, while rhoptry neck protein (RON) is secreted by the rhoptry organelles (25). A tight moving junction formed between AMA1 and the RON2 complex, which is deposited on the RBC membrane, is required for invasion (25). A parasitophorous vacuole is formed around the merozoite as it enters the erythrocyte, (26). Following *P. falciparum* invasion of erythrocytes, during the next 48 hours, merozoites develop inside the vacuole, undergoing schizogony through several stages by first entering the ring form, then trophozoite, which becomes a mature blood schizont, and after several rounds of divisions yields 16 to 36 merozoites before rupturing the RBC, releasing the new merozoites into the bloodstream to repeat the asexual cycle (2).

During the asexual erythrocytic cycle, a subpopulation of parasites commits towards developing into male and female sexual gametocyte forms, and for *P. falciparum* gametocytes it takes up to 11 days to mature for transmission to mosquitoes (2). To avoid splenic clearance, gametocytes have been found to sequester in the bone marrow until they release back into circulation to be taken up by mosquitoes during a blood meal (27).

Mosquito stage. While taking a blood meal the mosquito ingests gametocytes, which then turn into either one female macrogamete or up to eight male microgametes (28). The male gametocyte goes through an exflagellation process becoming a motile male microgamete which must locate and fertilize the female macrogamete. Fusion of the female and male gametes produces a zygote which develops into an ookinete, a motile form that enters the mosquito midgut wall to form oocysts (28). The oocysts further develop, enlarge, and erupt into sporozoites that migrate to the mosquito salivary glands, closing the cycle with an infectious mosquito capable of transmitting parasites to the vertebrate host during its next blood meal (29).

1.2 Epidemiology

Malaria endemic regions are localized to tropical and subtropical zones **(Figure 2)**, however this has not always been its geographic distribution, as it had once reached more temperate regions (1). A number of factors determine the distribution of *Plasmodium spp.* including environment, climate, host, and mosquito vector.

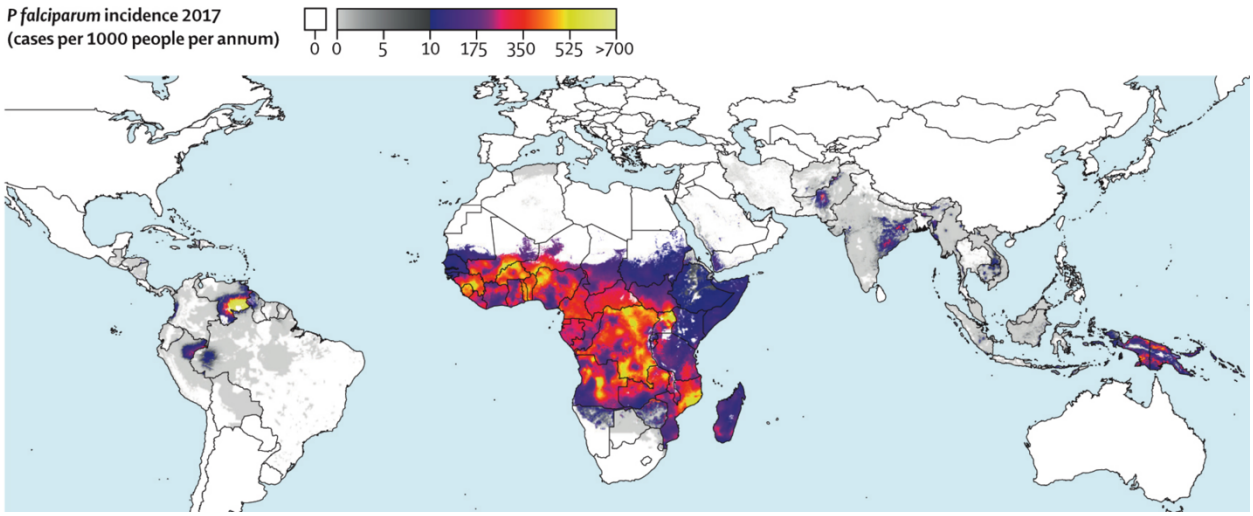

Figure 2. Global distribution of *P. falciparum*. Map showing incidence in all age groups during 2017. Adapted from (30).

From 2000 until 2015, there was a steady decline in global cases of malaria from an estimated 245 million to 230 million within the 108 countries found to be malaria endemic in 2000 (31). *P. falciparum* has had the greatest impact of cases and mortality in sub-Saharan Africa (32). The World Health Organization (WHO) created the Global Technical Strategy for Malaria 2016-2030 in 2015 to help guide countries towards malaria elimination with the goal of reducing malaria by at least 90% globally by 2030 (33). Since 2016 however, malaria cases have risen with the greatest annual increase of 13 million cases occurring between 2019-2020 during the initial year of the COVID-19 pandemic (31).

Malaria deaths remain above pre-pandemic levels with an estimated 610,000 deaths worldwide in 2021 and 608,000 deaths in 2022 (34). Cases continued to rise from 244

million cases estimated during 2021 to 245 million in 2022 (34). The WHO African Region has the highest rate *of P. falciparum* malaria globally with 95% of cases and 96% of all deaths, of which 80% of deaths attributed to children under 5 years of age (34).

Clinical pathology and diagnosis

The pre-erythrocytic stages of *Plasmodium* infection are considered to be “clinically silent” while the malaria-associated disease pathology occurs during blood-stage infection (35). The characteristic symptoms of malaria onset are cyclical fever and elevated levels of toxins and immune responses by cell mediators that coincide with schizont rupture from infected RBC and release of merozoites into circulation (36). Symptoms of uncomplicated malaria include fever, chills, body-aches, cough, and diarrhea (1). Clinical indicators for severe malaria include anemia, cerebral malaria, respiratory distress, kidney injury, and coma (1).

Malaria surveillance efforts in endemic areas can involve passive case detection when patients presenting with symptoms such as fever go to the clinic or through active case detection where community health workers make home visits in high risk populations and screen for fevers followed by parasitological examination (36). Conventional diagnostic methods used to detect malaria parasites are by Giemsa-stained blood smears and examination by light microscopy (37). An alternative to microscopy, instrument-free rapid diagnostic tests (RDTs) can be used at point of care to detect blood-stage parasite antigens such as histidine-rich protein II for *P. falciparum* (1). Advantages of RTDs are that they provide quick results that do not require complex equipment or extensive training to use and interpret (38). Submicroscopic or low-level parasitemia, however, can be common in adult populations and more advanced molecular techniques such as polymerase chain reaction (PCR) is required to confirm positive cases where microscopy or RDTs are less sensitive (39,40). PCR-based screening is limited by the higher cost of equipment, such as thermocyclers, and trained personnel (41).

Treatment and prevention tools

Efforts to prevent malaria have been addressed by several key strategies such as vector control, bite prevention, chemoprevention, vaccine development and monoclonal antibodies (vaccines and monoclonal antibodies will be covered in section 3).

The Malaria Eradication Programme in 1955 used dichlorodiphenyltrichloroethane (DDT) for insecticide spraying (42), but its use decreased due to cost and environmental concerns. More recent methods include bed nets treated with insecticide and indoor residual spraying (1), but mosquito resistance to insecticides is increasing (43). Advances in CRISPR-Cas9 gene editing allow for population suppression and modification of mosquitoes to make them sterile or resistant to *Plasmodium* infection (38). Studies are also being conducted to use endectocides like ivermectin to reduce mosquito lifespan after feeding (44). Additionally, there are early-stage efforts to engineer a bacterium, *P. agglomerans*, to produce anti-malarial proteins in the mosquito midgut (45).

With the spread of *P. falciparum* drug resistance to chloroquine and antifolate compounds, the current option is artemisinin-based combination therapy (ACTs) recommended for the treatment of *P. falciparum* malaria except during the first trimester of pregnancy (1). ACTs consist of an artemisinin derivative that quickly reduces parasitemia and an additional drug that eliminates residual parasites over an extended period (1). The current leading ACTs are artemether–lumefantrine, artesunate–amodiaquine, dihydroartemisinin–piperaquine, artesunate–mefloquine, and artesunate plus sulfadoxine–pyrimethamine (1).

The groups recommended for temporary chemoprophylaxis are pregnant women, young children, and travelers. Intermittent preventive therapy in pregnancy (IPTp) and for infants has been slow to initiate in many areas, in addition to the concern of sulfadoxine-pyrimethamine resistance (1). Early detection of malaria in pregnancy is crucial for preventing potential complications. A seven-day course of quinine and clindamycin is the recommended treatment for malaria during the first trimester and after 12 weeks of gestation, the treatment protocol is the same as for non-pregnant patients (1). Children in

Africa can also benefit of targeted drug-based preventive interventions with the application of seasonal malaria chemoprophylaxis (SMC) (46,47) or perennial malaria chemoprophylaxis (PMC) (48), based in the earlier concept of IPT in infants (IPTi), depending on the local transmission patterns. In endemic populations it may be necessary to monitor the impact of such interventions on acquisition of malaria immunity. To prevent malaria in travelers, Atovaquone-proguanil, doxycycline, and primaquine are prescribed as prophylaxis frequently, and mefloquine is an option, but unpopular due to neurotoxicity concerns (1).

2. Immunity to Malaria

Following years of repeated exposure to *Plasmodium* parasites, individuals living in malaria endemic regions develop a level of naturally acquired immunity (NAI) that is typically capable of preventing clinical disease, but sterile immunity to infection is rarely developed (49,50). A variety of factors impact NAI including repeated exposures, and age as was evidenced by early sero-epidemiology studies (49,51). Naturally acquired *P. falciparum* sporozoite-specific antibodies were first detected in children and adults from a hyperendemic region of The Gambia, which demonstrated age-dependent responses of >90% of adult serum samples giving positive CSP precipitation or immunofluorescence assay (IFA) reactions, and either low or negative reactions from children (52). Similarly, another study reported age-dependent findings in Thailand, with a higher percentage of adults exhibiting sporozoite-specific IFA reactivity than the younger cohort (53). A study of individuals living in low and high malaria transmission intensity (MTI) settings further demonstrated the levels of *P. falciparum* sporozoite-specific antibody responses differed by age and MTI, with no detectable antibodies in the younger cohort compared to >90% positivity rate among adults aged 50-80 years, while in the high MTI site, all individuals aged 2-80 years had positive IFA reactions (54).

Acquired immunity to *P. falciparum* malaria develops in stages depending on exposure and age, with the initial phase being protection from severe disease, which can develop after one or two infections; the second stage is immunity to clinical symptoms, and the third, partial protection against parasites, both developing during childhood (55). NAI

appears to depend on constant transmission, and when transmission levels decrease, immunity can wane (56). A lack of continuous exposure has been demonstrated to result in loss of immunity to malaria (57), mostly to the parasite but not as much to severe disease.

Mechanisms of NAI involve proinflammatory responses often generated by innate immune effectors that respond quickly to infection to control parasitemia and other regulatory effectors that are required to protect the host from excessive proinflammatory cytokine responses that have a role in anti-disease (49). Antibodies have a central role in NAI, particularly during the blood stage (49), and T cell-mediated responses are needed to support B cells and help effector cells.

2.1 Innate Immunity

Innate immune cells hold a critical position as the first responders during the early stages of infection, helping to control parasite replication in the liver and reducing the blood-stage infection by clearing parasites, in addition to working in synergy to induce, support and regulate adaptive immunity (58) **(Figure 3)**. Myeloid cells such as dendritic cells (DCs), monocytes, macrophages, and neutrophils are key members of innate immune populations that detect pathogens through pattern recognition receptors such as Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) (59,60). Lymphocytes such as natural killer (NK) cells and semi-innate γδ T cells are also involved in the innate response to malaria. Severe malaria has been attributed to exacervated innate immune response and associated inflammatory mediators and therefore considered in part to be an immune-mediated disease. Accordingly, anti-disease immunity includes tolerogenic mechanisms to regulate and protect the host (61–63).

2.1.1 Innate cell types and cytokines

Granulocytes, also known as polymorphonuclear cells, are the most abundant type of leukocyte and consist of several populations, including neutrophils, eosinophils, and mast cells. They are critical players in innate immunity, responding rapidly and being one of the

first cell types to defend against invading pathogens. **Neutrophils**, the most dominant granulocyte in circulation, play a crucial role during malaria infection and operate by phagocytizing pathogens, degranulation, and creating neutrophil extracellular traps (64). **Eosinophils** produce granule proteins with antimicrobial properties and have been shown to kill *P. falciparum* parasites inside erythrocytes in vitro (65). Eosinophils can also promote the degranulation of mast cells and have been shown to modulate T-cell activity (66) and support plasma B cell survival in the bone marrow (67). The role of eosinophils in human malaria has yet to be extensively studied. A small human cohort study in Ghana showed significant increases in eosinophilia with *P. falciparum* infection and that eosinophil granule proteins were detected at higher concentrations in cases of cerebral malaria, indicating a potential pathogenic association (68). **Mast cells**, located in the mucosal, epithelial, and vascularized connective tissues, can encounter antigens or invading pathogens and quickly release potent mediators, including vasodilators, histamine, proteases, cytokines, chemokines, and growth factors that can modify the innate and adaptive immune responses (69). Few studies have been conducted on human mast cell response to malaria. A study from Thailand studied the skin of *P. falciparum*-infected patients and found the number of mast cells and degree of degranulation correlated with parasitemia and disease severity (70).

Monocytes are involved throughout different stages of the parasite cycle and play a key role in innate and adaptive immunity. Monocytes can detect pathogens through TLRs which recognize conserved PAMPs, control parasite burden through phagocytosis and can modulate innate and adaptive immunity through cytokine production and antigen presentation (71). Monocytes are classified into three subsets depending on their level of CD14 and CD16 expression, associated function, and behavioral differences (72) Classical monocytes ($CD14^{++}CD16^{-}$), the most abundant subset, are recruited to inflamed sites where they may differentiate into macrophages or DCs. Non-classical monocytes ($CD14dimCD16^{++}$) are known for patrolling and resolving inflammation in damaged tissues (71). Intermediate monocytes ($CD14^{++}CD16^{+}$) are more efficient than the other subsets at phagocytosing antibody or complement opsonized *P. falciparum* (73) Circulating monocytes clear merozoites and infected RBC either by antibody or

complement-mediated opsonic phagocytosis via Fcγ and CR1 receptors, respectively, or by non-opsonic phagocytosis mediated through CD36 (71).

Secretion by monocytes of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1β, IL-6, interferon-gamma inducible protein 10 (IP-10), and interferon (IFN)-γ can help control parasitemia; however, excessive production can also lead to pathogenesis. Monocytes along with γδ T cells have been identified as dominant cellular sources of cytokines and chemokines associated with severe malaria (74). In particular, monocyte production of IP-10 correlated with intravascular inflammation in the brain and identified as a biomarker for cerebral malaria (75). A year-long longitudinal study of (PBMCs) from Malian children starting at baseline (before malaria season) and found an altered response to *P. falciparum* re-exposure with downregulation of proinflammatory cytokines from monocytes/macrophages and upregulation of $CD4^+$ T cell regulatory and proinflammatory responses and mechanisms to control parasitemia (76). This indicates how children undergoing repeated infections may appear to stay afebrile while also controlling parasite replication (76). Interestingly a recent study found baseline blood transcriptome modules related to monocytes that have been associated with higher risk of malaria infection in children who were enrolled in a malaria vaccine study, suggesting that antigen recall responses at baseline could impact the response to malaria vaccination (77).

Macrophages are antigen presenting cells (APCs) that are major effectors of the innate immune system. They can be found in all tissues, exhibiting diverse roles, including homeostasis, repair, phagocytosis, cytokine and chemokine production, inflammation, and antigen presentation (78). Tissue macrophages such as KC in the liver allow sporozoite traversal of the liver sinusoidal barrier but have also been shown in rats during *P. berghei* infection to produce inflammatory cytokines to control merozoite egress from hepatocytes (79). Mouse models have demonstrated that macrophages are critical for the clearance of blood-stage parasitemia in the spleen (80) and to control infection by phagocytosing infected RBC (81,82). Direct binding of the CD36 receptor to infected RBC

allows non-opsonic phagocytosis (82,83). Opsonic phagocytosis of infected RBC has been demonstrated to be directed through Fcγ receptors (73,84).

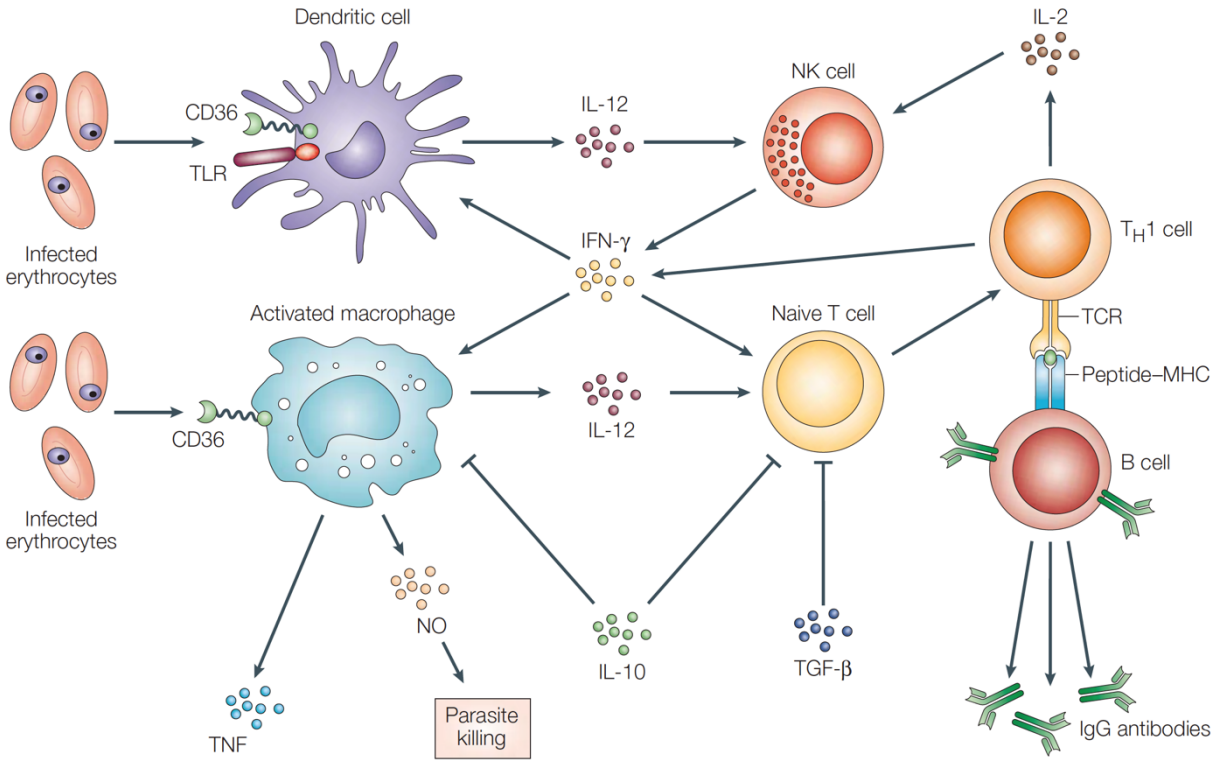

Figure 3. Innate immunity linking with adaptive immunity during blood-stage malaria. Upon recognition of parasite ligands by pattern-recognition receptors such as TLRs, DCs mature and migrate to the draining lymph nodes or spleen. Maturation involves the upregulation of various molecules and the production of proinflammatory cytokines. The production of IFN-γ by NK cells results in DC maturation, activation of macrophages, and enhances the antigen-specific maturation and expansion of naive $CD4^+$ T cells. Cytokines such as IL-10 and TGF-β negatively regulate both innate and adaptive responses. Figure from (61).

DCs are professional APCs critical for regulating the innate immune response and initiating and modulating adaptive immune responses (85). Immature DCs phagocytize antigens or pathogens and are exposed to PAMPs that are detected by pattern recognition receptors (PRR) such as TLRs, which induces developmental maturation and functional changes, and redistribution of major histocompatibility complex (MHC) molecules from the endocytic compartment to the cell membrane (85,86). Mature DCs upregulate CD40, CD80, and CD86 co-stimulation receptors and migrate to draining lymph nodes to present processed peptides on MHC molecules to naïve T cells to initiate

the adaptive immune response (85). DCs are distributed throughout the body, and interactions between *Plasmodium* and DCs occur during the pre-erythrocytic and blood-stage infection. Murine studies have demonstrated that during pre-erythrocytic infection, sporozoites that do not exit the skin or that enter the lymphatics and are directed to draining lymph nodes can encounter DCs, which phagocytose and process sporozoite antigens to present to $CD8^+$ T cells (87). Within the liver, a murine study found that irradiated sporozoites recruit DCs to the liver, where they phagocytose infected apoptotic hepatocytes that provide parasite antigens to present to T cells (88). Studies of naturally exposed individuals have suggested that DC function may be reduced in high-transmission settings, causing persistent infections (85), through decreased antigen uptake and increased apoptosis in adults during blood-stage malaria (89).

NK cells are considered innate effector cells and interact with other immune cell types, such as macrophages, DCs, and T cells during infections (90). A predominant mechanism NK cells use for controlling pathogens is the production of IFN-γ, which then activates macrophages to clear pathogens (91). NK cells have demonstrated adaptive, "memory-like" characteristics, such as increased cytokine production or enhanced cytotoxic responses upon repeated antigenic stimulations (92). A controlled human malaria infection (CHMI) study in humans found memory-like responses of elevated IFN-γ production by NK cells over 4 months after infection that was dependent on T cells producing IL-2 (93). During *P. falciparum* malaria, NK cells have exhibited direct parasite recognition (94) and antibody-dependent cellular cytotoxicity (ADCC) (95). A study of anti-merozoite antibodies showed that antibody-dependent NK cell activity increased *P. falciparum* RBC invasion inhibition, and was associated with protection after CHMI in African adults, and increased with age following natural infections in children (96).

Gamma delta (γδ) T cells are an unconventional population of T cells that do not require antigen processing and MHC presentation. γδ T cells are infrequently found in secondary lymphoid organs, but reside in abundance in many peripheral tissues and, with their innate-like and adaptive-like features, can provide early responses to infection by producing high levels of cytokines, direct killing of infected cells, recruiting neutrophils,

and activating phagocytes (97). γδ T cells have been found to produce higher levels of IFN-γ to *P. falciparum* malaria than NK cells and αβ-T cells (98). Mouse studies of malaria have demonstrated that γδ T cells support pre-erythrocytic immune responses during liver-stage infection (99). Early studies of circulating γδ T cells found an expansion of this population during acute *P. falciparum* malaria (100,101). Recently, a study of adult patients with natural *P. falciparum* blood-stage infection showed in vitro that γδ T cells directly bound and lysed infected RBC by delivering granzymes and were additionally able to phagocytose and degrade opsonized infected RBC (102). A subset of γδ T cells (Vδ1effector γδ T cell) has also been shown to clonally expand and exhibit adaptive-like features following repeat *P. falciparum* infection in vivo (103).

Cytokine responses. A tight balance between proinflammatory responses initiated to control infection and regulatory responses is necessary to protect the host from excessive inflammatory damage (104). Monocytes and macrophages in the spleen are major producers of proinflammatory cytokines such as IL-1β and TNF during acute blood-stage malaria (60). A study of plasma cytokine levels in *P. falciparum* malaria patients showed that while TNF was associated with severe malaria compared to uncomplicated malaria, it correlated with rapid parasite clearance (105). A study of severe malaria in Mozambican children found an imbalance between proinflammatory and anti-inflammatory cytokines, with significantly higher IL-6, detectable levels of IL-1β, and lower transforming growth factor-β (TGF-β) and IL-12 plasma concentrations in children with severe malaria compared to those with uncomplicated malaria (106). Natural and experimentally-induced *P. falciparum* infections showed increased serum levels of pro-inflammatory cytokines, including IFN-γ, IL-12p40, and IL-8, following merozoite release from the liver and early onset of blood-stage infection (107). IFN-γ is a key mediator of proinflammatory immune responses induced predominantly by IL-12 during *Plasmodium* infection and can be produced by various innate and adaptive immune cell subsets during different stages of infection (108). Pro-inflammatory IL-18 has demonstrated a protective role in host defense and, along with IL-12, can stimulate IFN-γ production (109,110). Immunoregulatory cytokines such as IL-10 and TGF-β can be produced by innate cells

such as monocytes/macrophages, as well as adaptive immune cells, and may counter excessive proinflammatory responses that can lead to severe malaria (61).

Our group investigated age and exposure-dependent cytokine responses to a first infection of *P. falciparum* in naïve adults (European travelers) and naïve Mozambican children, and found that compared to malaria-experienced adults, children and travelers had higher concentrations of IFN-γ, IL-2, and IL-8. Additionally, children had elevated IL-12, IL-4, and IL-1β (111). A balanced proinflammatory and anti-inflammatory cytokine signature most likely by innate cells around two years of age was associated with reduced risk of clinical malaria during childhood (112). Another study by our group assessed the outcome of interrupted malaria exposure in malaria-experienced immigrants and found that loss of exposure was accompanied by a reduction in proinflammatory responses and tolerance to *P. falciparum* (113).

2.1.2 Trained Immunity

Although immunological memory has been a hallmark of the adaptive immune system, recently, it has been hypothesized that the innate immune system may exhibit memory-like responses called trained Immunity (114). The proposed view is that extended functional conditioning of innate cells, such as monocytes, macrophages, and NK cells, upon first exposure to a stimulus, results in a modified response, faster and stronger, during the secondary challenge, following a rest period between exposures (115). While the adaptive immune response relies upon gene recombination, innate immune cells undergo epigenetic reprogramming during trained immunity. A study using *P. falciparum*-infected RBCs or malaria crystal hemozoin to prime human adherent PBMCs induced elevated proinflammatory and decreased anti-inflammatory cytokine production upon secondary TLR ligand stimulation (116). The hyper-responsiveness correlated with epigenetic remodeling with increased levels of H3K4me3 at promoters associated with inflammation found in Kenyan children during acute malaria infection (116). Continuing from this previous work, the same group conducted a recent study using in vitro cultures and trans-well experiments testing infected RBC or hemozoin-stimulated PBMC cultures depleted of T cells or purified monocytes alone, and neither produced hyperproduction of IL-6 and TNF proinflammatory cytokines under the training conditions (117). Their

findings indicate that the trained immune response to *P. falciparum* is not inherent to the monocytes but requires soluble lymphocyte signaling (117).

Trained immunity has implications for vaccinology, as was found during a controlled human malaria infection study after the anti-tuberculosis Bacillus-Calmette Guérin (BCG) vaccination (118). The study found that BCG vaccination alters the clinical and immunological response to *P. falciparum* challenge with volunteers developing earlier and stronger symptoms, as well as memory-like immune responses of NK cells and monocytes correlating with reduced parasitemia (118). The same group then investigated whether *P. falciparum* infection can induce innate immune memory by measuring monocyte-derived cytokine responses during CHMI and found that during acute infection, monocytes produced lower levels of inflammatory cytokines upon secondary stimulation; however, after 36 days post malaria infection, production of IL-6 and TNF was elevated following stimulation with non-malaria stimuli (119). The study further showed that during trained immunity generated in vivo by human malaria, epigenetic reprogramming of monocytes occurs with changes in H3K4me3 at promoter regions of inflammatory genes that remain long-term following parasite clearance (119).

Recently, trained immunity and tolerogenic responses have been described in innate cell populations during malaria, where following one or more infections, immunoregulatory pathways are activated including epigenetic and transcriptional changes resulting in decreased proinflammatory responses (120). A study in Malian adults and children found an epigenetic reprogramming of monocytes/macrophages driven towards a regulatory phenotype that mitigated inflammatory responses during re-exposure to *P. falciparum* (121).

2.2 Adaptive Immunity

Adaptive immune responses are mediated by lymphocytes such as B and T cells, which are responsible for humoral and cell-mediated immunity, respectively. These lymphocytes are defined by exhibiting variable cell-surface receptors for antigens and the ability to generate antigen-specific responses and immunological memory.

Humoral immune responses are induced in the follicles of secondary lymphoid organs such as lymph nodes and spleen, where naive B cells are activated after engaging antigen with the B cell receptor (BCR) resulting in a cascade of signaling and internalization, processing, and presentation of the bound antigen on MHC class II molecules (122). Activated naive B cells migrate to the T cell zone where they interact by binding antigen-MHC class II complex and CD40 on B cells with TCR and CD40L ligand on antigen-specific T follicular helper (T_{FH}) cells that were previously primed by APCs (122). Multipotent naïve B cells are able to differentiate into short-lived plasma cells that rapidly produce antigen-specific antibodies that are primarily unswitched IgM, but also class switched IgG are produced (122). Naïve B cells can differentiate into germinal center (GC) B cells that will further become memory B cells (MBC) and long-lived plasma cells (122) when supported by T_{FH} cells.

B cells can produce five antibody isotypes that differ in their constant region and effector function, and these include IgM, IgD, IgA, IgE, and IgG, which is the most abundant in human serum (123). IgG antibody has four subclasses (IgG1, IgG2, IgG3, and IgG4) with each functioning differently to bind antigen, form immune complexes, activate complement, and activate effector cells (123). Antibodies bridge adaptive immunity with effector mechanism of the innate immune system and these mechanisms vary between IgG subclasses. In general, IgG1 and IgG3 are more potent activators of effector mechanisms, while IgG2 and IgG4 tend to be subtler (123). IgG (and IgM) can activate complement through binding and activation of C1q with the strongest activation found for IgG3 then IgG1 and less for IgG2 with little interaction for monovalent IgG4 (123). FcγRs on APCs such as macrophages and DCs facilitate uptake and presentation of immune complexed antigens which can activate APC maturation, attack pathogens, and regulate T cells (124). ADCC utilized by cytotoxic effector cells, such as NK cells, targets and kills infected cells coated with antibodies by interacting with the IgG Fc-receptor expressed on the effector cell and initiating signaling and activation (124,125). Antibody-dependent cellular inhibition (ADCI) is a mechanism where antibody bids to target cells and monocytes are engaged through Fc-receptors and release soluble factors that inhibit

parasite function (126). IgG1 and IgG3 are the most effective at opsonization and bind all FcγRs, while IgG2 and IgG4 are more limited (123).

Cell-mediated immunity to malaria has been defined by the presence and function of the two main classes of T cells, $CD4^+$ and $CD8^+$ T cells (127). T cells become activated following a primary signal where their T cell receptor (TCR) engages with antigen-specific peptides presented on MHC molecules by APC. $CD8^+$ T cells recognize peptides bound to MHC class I molecules, which are expressed on the majority of cells and can present antigen derived from proteins in the cytosol typically if a cell is infected with a pathogen. $CD4^+$ T cells recognize peptides bound MHC class II molecules, which are expressed by APCs such as DCs, macrophages, and B cells (128). A second signal is required for naïve T cell activation which involves binding of costimulatory molecules such as CD28 on T cells with CD80/CD86 on APCs and a third signal consisting of cytokines which can direct and amplify T cell differentiation and expansion (129). The plasticity of primed $CD4^+$ T cells during clonal expansion permits differentiation into several effector T cell subsets including T helper (T_H)1, T_H2, T_H17, T_{FH}, regulatory T cells (T_{REG}) and Type 1 regulatory T cells (TR1) cells, with each subset able to sense different cytokines, program different transcription factors, produce specific cytokines/chemokines and express receptors to control infections and prevent pathology (130) For example, $CD4^+$ T cell production of cytokines including IFN-γ, IL-4, IL-5, and IL-10 which support B cell development and class-switching (131).

Both humoral and cell-mediated adaptive immune responses are required during pre-erythrocytic and blood-stage infection to control parasite invasion, replication, and disease severity (127) **(Figure 4).**

2.2.1 Humoral immunity to pre-erythrocytic infection

Sporozoites can linger in the skin for several hours (10) and can enter the lymphatic system and skin-draining nodes (6), where they can be intercepted by DCs that prime the adaptive immune response. Whether sporozoite-specific B cells are also activated and undergo differentiation in the draining lymph node remains to be determined (132).

Similarly, while there have been studies defining the role of antibodies against liver-stage antigens, the development of liver-stage malaria-specific B cells have not been extensively investigated (132).

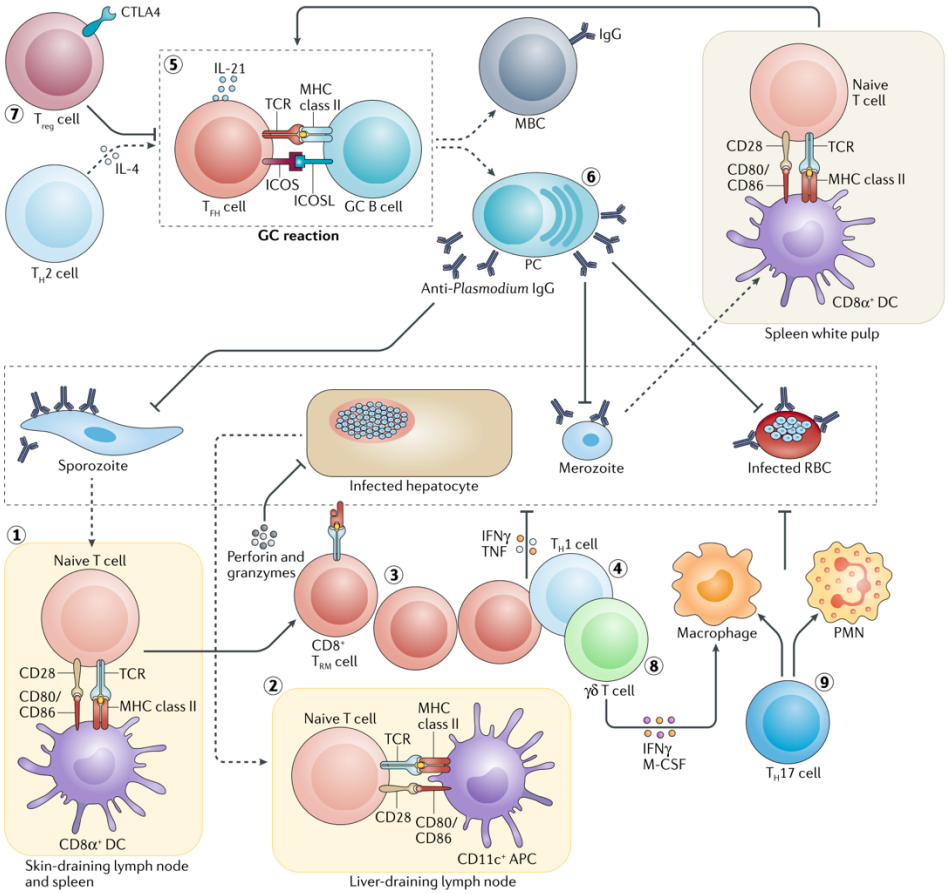

Figure 4. Humoral and cell-mediated responses during *Plasmodium* infection. Immune response to *Plasmodium* infection involves a complex network of immune cell interactions. Dendritic cells have a central role in activating T cells by presenting *Plasmodium* antigens in the skin- and liver-draining lymph nodes and the spleen. DCs producing IL-6 and IL-12 can skew naive T cells to differentiate into T_H1 or T_{FH} subsets, producing cytokines that activate other innate immune cells or engage B cells. B cells produce antibodies that target the parasite, while $CD8^+$ T cells can kill infected cells. T_{REGS} can help control the immune response, and other T cell subsets such as T_H2 and T_H17 have less defined roles; they may be involved in B cell support in the germinal center and recruiting and activating phagocytes. Figure from (127).

Early in vitro studies demonstrated that antibodies targeting CSP blocked invasion (133). Studies in mice have demonstrated that antibodies exhibit their protective effect in the dermis after inoculation from infected mosquitoes during feeding, consequently reducing parasite mobility and entry into blood vessels (5,134). Studies using attenuated sporozoite inoculation have established that antibodies are the predominant effectors targeting the sporozoite stage and there is limited evidence demonstrating the same level of protection is obtained by NAI (135). A study in Kenyan children found that antibodies to multiple antigens expressed on sporozoites, specifically CSP, liver stage antigen (LSA-1), and TRAP, correlated with reduced risk of developing clinical malaria which may be mediated by prevention of high parasitemia (136). While NAI directed towards pre-erythrocytic stage antigens such as CSP has been detected (57,137), antibody levels are low and not very immunogenic. The majority of antibody-mediated response is against blood-stage infection (49).

2.2.2. Cell-mediated immunity to pre-erythrocytic infection

As mentioned, animal models have helped define the diversity of immune cells that constitute the skin environment, including neutrophils, monocytes, DCs, and mast cells that infiltrate the infection site and can induce T and B cell recruitment to draining lymph nodes (138,139), which is central to DC priming of T cells to induce adaptive immunity against pre-erythrocytic malaria (87,140,141). Early murine studies using $CD8^+$ T cell depletion and adoptive transfer of a CSP-specific CD8+ T cell clone demonstrated that $CD8^+$ T cells can protect mice from sporozoite challenge (142) independently from B cells or $CD4^+$ T cells (143). $CD4^+$ T cells could also protect against pre-erythrocytic mouse malaria infection without $CD8^+$ T cells, which established protective immunity can be mediated by class II-restricted immune effector mechanisms (144). When $CD4^+$ and $CD8^+$ T were depleted in another murine study, a significant reversal of immunity was found upon sporozoite challenge (145). Since IgG antibodies and memory B-cells have been shown to target pre-erythrocytic stage antigens, this would require the induction and support of $CD4^+$ T cells (146).

Studies of NAI in humans demonstrated $CD4^+$ T cells specific for pre-erythrocytic stage antigens correlating with protection (147). Early field studies using tetramers (R32tet32) or full-length CSP synthetic peptides aided in the identification of $CD4^+$ T cell responses in field studies and helped define the immunodominant T cell epitopes of CSP (51). Human volunteers exposed to sporozoites under chloroquine cover developed antibodies and T cell responses against sporozoites and blood-stage antigens and protection against a challenge that was associated with effector memory T cells producing IFN-γ, TNF, and IL-2 (148). Another study of humans immunized by bites from *P. falciparum*-infected mosquitoes during chloroquine prophylaxis found evidence of cytotoxic markers associated with protection, specifically $CD4^+$ T cells producing the degranulation marker CD107a and $CD8^+$ cells producing granzyme B (149). A longitudinal study by the same group studying immunized volunteers under chloroquine cover assessed the activation and differentiation kinetics and found increases in $CD4^+$ and $CD8^+$ T cells, γδT cells, and NK cell proliferation and activation, as well as increases in regulatory T cell proliferation (150).

$CD8^+$ T cells rapidly expanded following sporozoite immunization (151–153) and were the primary effector cells found against pre-erythrocytic stages during murine, non-human primate, and human studies (154–157). Naïve CD8+ T cells require priming by APCs before differentiating and being capable of exerting antiparasitic activity (158). Priming leads naïve CD8+ T cells to differentiate into short-lived effector or precursor memory cells depending on cytokine signaling (152). The short-lived effector CD8+ T cells migrate to the liver to exert their effector functions (152). Activated CD8+ T cells can undergo clonal expansion with the help of IL-2 and IL-4 produced by CD4+ T cells (159). While the pre-erythrocytic stage is relatively short, which limits the newly primed CD8+ T to contribute to parasite clearance, memory CD8+ T cells generated by previous exposures or vaccinations can provide rapid and more effective protection against subsequent liver-stage infections (160).

2.2.3 Humoral immunity to blood-stage infection

A seminal study by Cohen et al. established that antibodies are central to blood-stage malaria immunity when purified IgG from malaria immune adults were transferred to children with acute malaria, resulting in significant reductions in parasitemia and symptoms (161). Other passive transfer studies involving IgG extracted from African adult immune sera to *P. falciparum*-infected non-immune Thai patients, demonstrated a clearance or reduction in parasitemia with antibody cross-reactivity to genetically distinct parasites (162,163).

Parasite genetic diversity is thought to play a role, in part, for the slow acquisition of protective antibodies to malaria (164) and the ability of *P. falciparum* to clonally vary *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) proteins expressed on infected erythrocytes, requires years of exposure to build a sufficient antibody repertoire covering the diversity of expressed parasite antigens (165). Studies in malaria endemic areas found PfEMP1 to be a target of humoral immunity (166). Antibodies to blood-stage antigens, such as AMA1, MSP, EBA-175, and the VAR2CSA Duffy binding-like (DBL) domains associated with placental malaria, have been used as markers of malaria exposure (167–170). Studies on antibody responses against blood-stage antigens can have controversial results since antibodies are good markers of exposure, and therefore are associate with risk rather than protection (168). Therefore, immunoepidemiologic studies investigating immune mechanisms of protection need to integrate prior exposure and evaluate multiple immune responses within the analysis (168).

Efforts to identify a conserved blood-stage protein identified PfRH5, which is required for merozoite invasion of erythrocytes (171,172). RH5-specific IgG was able to inhibit parasite growth in vitro and was associated with protection from malaria (173). A study evaluating antibody responses in Kenyan children to several *P. falciparum* merozoite antigens found AMA-1 and MSP antigens (MSP-1 block 2, MSP-2, and MSP-3) to be associated with protection, while MSP-1_{19} and EBA-175 were not (174). Additionally, IgG subclasses need to be considered as well, since they have different functions, contrasting

associations with protection or risk, and half-lives (175,176). High levels of AMA-1-specific IgG3 and $MSP1_{19}$-specific IgG1 were predictive of reduced risk of symptomatic *P. falciparum* malaria in children from Papua New Guinea (PNG) (177). Naturally acquired IgG antibodies against crude asexual *P. falciparum* lysates found associations of IgG1 with protection and IgG2 with disease (178). A sero-epidemiological study in Tanzania exploring factors that might influence IgG subclass switching during anti-malaria immune acquisition found that IgG1 and IgG3 class switching was independent from antigen, exposure and age of the individual (179).

Several mechanisms of antibody-mediated protection against blood-stage infection have been identified including blocking RBC invasion by antibodies targeting merozoites (180), through opsonic phagocytosis of infected RBC (181–183), and antibody fixation of complement on merozoites to inhibit erythrocyte invasion and lyse merozoites (184–186). A recent study leveraging CHMI to study mechanism of acquired immunity found that IgG Fc-mediated effector functions targeting merozoites and ring stages is a strong predictor of protection and can serve as a correlate of clinical immunity by discriminating between clinically immune and non-immune individuals (183).

2.2.4 Cell-mediated immunity to blood-stage infection

T_H1 cells specific for merozoite antigens have been detected in both humans and mouse models (187). Murine studies indicate that T cells besides NK cells, and antibodies are required for the control and clearance of blood-stage parasites, and presumably, IFN-γ produced by NK cells and T cells could enhance phagocytosis of infected RBC by macrophages (61). Murine studies demonstrated that IFN-γ is critical for activating macrophages (188), as well as inducing class-switched recombination of *Plasmodium*-specific B cells (189). $CD4^+$ T cells producing IL-2 can activate NK cells and directly lyse *Plasmodium*-infected erythrocytes (190).

A role for T_H1 effector cells in asexual blood-stage immunity was demonstrated by inoculating a small group of adults with low doses of unattenuated infected RBCs followed by drug treatment which later resulted in cell-mediated protection against CHMI (191).

Experimental human immunization studies using repeated infected mosquito bites under chloroquine cover yielded protection of participants against homologous challenge and identified IFN-γ-, IL-2-, and TNF-producing effector memory cells following in vitro stimulation with sporozoites or infected RBCs (148). The same cohort was followed with a long-term study of protection and found that four out of 6 individuals who were re-challenged 28 months later were protected and again detected IFN-γ and IL-2-producing effector memory T cells following stimulation with both sporozoites and infected RBCs (93). In field studies, a balance between T_H1- and T_H2-type cellular cytokine responses was observed during acute uncomplicated malaria in hyperendemic Gabonese individuals of different age groups (192) and during uncomplicated malaria in African immigrants (193). Studies have predominantly focused on the T_H1 response.

T_H2 cells also play a role in malaria immune responses with several studies highlighting IL-4 secreting T cells. Murine malaria models have demonstrated that protection from malaria is dependent on both T_H1 and T_H 2 responses, where T_H1 IFN-γ producing CD4+ T cells have been shown to control the acute phase of parasitemia while the induction of T_H2 cells with B cells facilitate parasite clearance (194). Human studies have shown antigen-specific $IL4^+$ T cells were associated with increased serum levels of malaria-specific IgG (195).

T_H17 cells have not been extensively examined for their role in malaria but have been gaining some attention due to their role in chronic inflammation, autoimmune diseases, and responses to extracellular pathogens (146). T_H17 cells produce IL-17A (IL-17), IL-17F, IL-21, and IL-22 (196). Since T_H17 cells have been shown to secrete IL-21, which is essential for GC reactions, it may indicate a potential association of support for B cells and antibody responses (197). Several murine malaria models have shown that T_H17 are activated during infection, but their function and role have yet to be defined (146).

T_{FH} cells have a critical role in adaptive immunity in generating immunological memory responses by providing support to B cells, class-switching, somatic hypermutation (SHM) in germinal centers (GCs), and development of memory and long-lived antibody

producing plasma cells (198). Some markers defining T_{FH} cells include IL-21, chemokine receptor CXCR5, programmed cell death protein 1 (PD1), Inducible T-cell COStimulator (ICOS), and Bcl-6 (199). T_{FH} cells expand during blood-stage infections and are critical for supporting protective antibody responses (200). Similar to $CD4^+$ T cell subsets, T_{FH} cells can also have characteristics and functions similar to T_H1, T_H2, T_H17, and T_{REG} type cells (127). Circulating T_H1-like T_{FH} cells expressing $CXCR3^+$ in Malian children with acute malaria exhibited impaired help to B cells (201). $FoxP3^+$ T follicular regulatory (T_{FR}) cells localized to the GC have been found to suppress humoral responses through expression of PD1 and cytotoxic T-lymphocyte-associated antigen-4 (CTLA4) (202–204).

2.4 Immune Regulation and Dysfunction

2.4.1 Immune Regulation

Disease pathology is often a consequence of excessive or uncontrolled proinflammatory immune responses; however, unbalanced immune regulation can also allow pathogen persistence (205). In humans and animal models there is some indication that malaria parasites can subvert host responses by stimulating regulatory T cells, which down-regulate protective immune responses and enable parasite persistence (206).

Innate cells including DCs, NK cells, γδ T cells and macrophages are capable of producing regulatory cytokines that can modulate adaptive immunity (61). A recent study used single cell transcriptomics to obtain an overview of malaria-induced immunoregulatory responses across multiple cell subsets and found altered immune cell profiles including immunosuppressive monocytes and DCs, and activation/regulation of NK and γδT cells (62). Studies of repeat malaria exposure suggest immunosuppressive changes and potential epigenetic modifications occur within innate populations such as monocytes, Vδ2+ γδ T cells, and $CD56^-$ NK cells, resulting in increased expression of inhibitory receptors and reduced production of inflammatory cytokines (120) (**Figure 5**)

$CD4^+FOXP3^+$ regulatory T (T_{REGS}) cells play a prominent role in maintaining tissue homeostasis, controlling innate and adaptive immune responses, and restraining bystander cell responses (207). In a CHMI study increased $CD14^+$ monocyte-derived

TGF-β production and the presence of $CD4^+CD25^+FOXP3^+$ T_{REGS} were associated with increased rates of *P. falciparum* parasites growth in vivo (208). Expansion of $CTLA4^+$ T_{REGS} were found during blood-stage infection, impeding both acute and long-term immunity by interfering with T helper responses and T_{FH} cell interactions with B cells in germinal centers (203). A study of prenatal exposure to *P. falciparum* blood-stage antigens reported induction of T_{REG} cells that suppress recall responses of T_H1-type $CD4^+$ T cells to blood-stage antigen stimulation, thus increasing a child's risk of infection and disease progression (209).

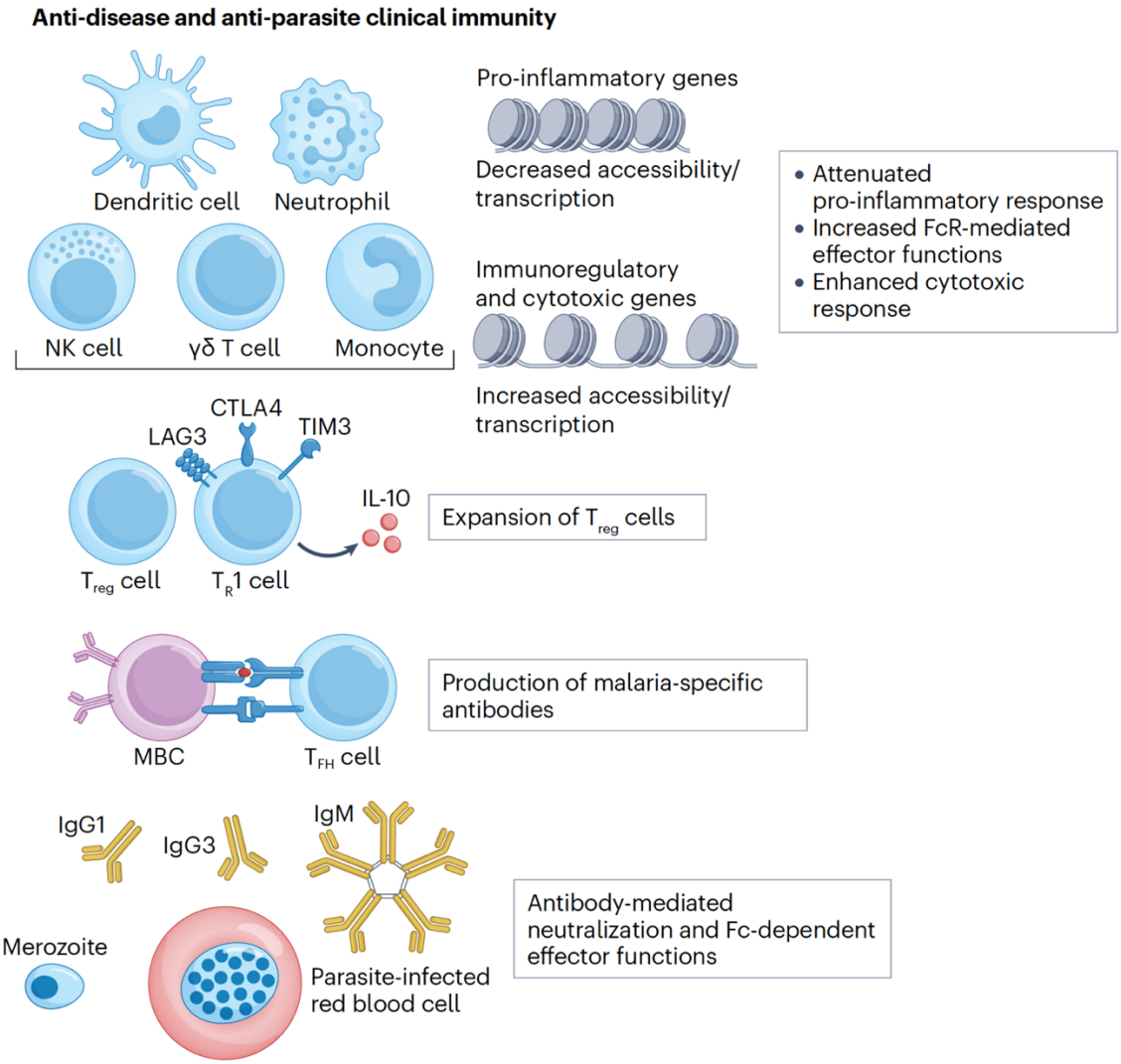

Figure 5. Anti-disease and anti-parasite clinal immunity to malaria. Following repeated *Plasmodium* exposures, various pathways in the immune system are activated, leading to changes in innate and adaptive immune cells, including decreased expression of

pro-inflammatory genes and increased expression of immunoregulatory and cytotoxic genes in innate immune cells. Additionally, NK cells and γδ T cells exhibit enhanced antibody Fc receptor-mediated functions and cytotoxic responses to combat parasites. TREG populations expand, producing anti-inflammatory cytokines to regulate immune responses upon re-exposure to malaria parasites. The adaptive immune response is mediated by MBC and T_{FH} cells, producing malaria-specific antibodies, which play a significant role in maintaining anti-parasite immunity. Functional antibodies, including specific subclasses like IgG1, IgG3, and IgM, are critical for effectively killing parasites through neutralization and Fc-dependent effector functions. Figure adapted from (120).

Initially, IL-10 was thought to be produced predominately by T_H2 and FoxP3$^+$ T_{REGS} CD4$^+$ T cells; however, it has been shown that effector memory T cell subsets expressing T-bet co-produce IFN-γ /IL-10 during continuous antigen exposure (210). **T regulatory type 1 (TR1)** cells are a population of FoxP3$^-$ T cells induced in the periphery following antigen stimulation and are associated with tolerogenic APCs and co-express IFN-γ and immunoregulatory IL-10 (211,212). TR1 cells have been shown to limit parasite control in mice (212) and constrain protective immunity by suppressing humoral responses in experimental mouse studies (200). IL-10 can suppress innate cell populations such as DCs and macrophages and limit T cell helper and effector responses (212). Early murine studies showed that IL-10 downregulates inflammatory responses, resulting in a lower risk of experimental cerebral malaria (213). In several studies involving African children, an insufficient IL-10 response to high TNF concentrations has been reported to be associated with severe malarial anemia (68,214). However, increased *P. falciparum*-specific IL-10 levels have also been connected to less effective clearance of parasitemia, consequently perpetuating infection (215). IFN-γ/IL-10 co-producing CD4$^+$ T cells were associated with increased malaria risk in Ugandan children (216); although, during another study, the same group also found an association with a lower risk of severe malaria (217). Coproduction of IL-10 and IFN-g by T_H1 cells may permit simultaneous pathogen clearance and suppression of pathology (212). Increased frequencies of TR1 and T_{REG}-IFN-γ$^+$ producing proinflammatory responses and IL-10 during active *P. falciparum* infections in PNG pregnant women correlated with protection and better delivery outcomes (218). A study of study Gabonese neonates born to mothers with

placental *P. falciparum* infection at delivery found increased frequencies of IL-10 producing *P. falciparum* antigen-specific $CD4^+$ T regulatory cells that suppressed T_H1 and CD8+ T cell IFN-γ responses and MHC class I and II expression on monocytes in the cord blood (219).

Regulatory and tolerogenic immune responses to pre-erythrocytic stages have been studied, although to a lesser extent than the role of T_{REG} cells during blood-stage infection. *Plasmodium*-infected mosquito bites have been shown to induce the mobilization of DCs and T_{REGS} cells in the skin of mice followed by a reduction in the influx of DCs to skin-draining lymph and reduced homeostatic proliferation of $CD4^+$ T cells (220). *Plasmodium* sporozoites have been shown to induce regulatory macrophages that produce IL-10 and increased tolerogenic surface expression of PD-L1, and IFN-γ production by antigen specific $CD8^+$ T cells (221). *Plasmodium* is able to exploit the host liver environment that is typically under a state of perpetual tolerance due to constant exposure of trace microbial products via systemic circulation which engage TLRs and modulate the innate immunity (222). Additionally, the hepatic environment is immunosuppressive with several liver cell populations including liver sinusoidal endothelial cells that express inhibitory ligand PD-L1 and secrete IL-10 upon TLR ligation, resulting in a state of T cell tolerance (222).

Malaria-specific IL-10 producing **regulatory B cells (B_{REGS})** have been reported in murine studies and have associated with protection from cerebral malaria (223,224). Human B_{REGS} were recently described in a study using single cell transcriptomics showing that *P. falciparum* malaria produces immunoregulatory and suppressive immune cell types (62). IL-10^+ B_{REGS} induced by malaria were found to not express significant levels of IL-6 or TNF, which had been reported in a previous study that found B_{REGS} co-expressing IL-10, IL-6 and TNF (62,225). The proportion of IL-10^+ B_{REGS} found were comparable to $CD3^+$ cells indicating that B_{REGS} may have a significant role in immune regulation and tolerance during human malaria (62).

2.4.2 Immune Dysfunction and Exhaustion

Persistent immune activation caused by chronic infections, cancer, and autoimmune disorders can lead to immune dysfunction, which can be classified into several different states that share some overlapping characteristics including anergy, tolerance, exhaustion, and senescence (226–228). The various states of dysfunction are defined by an increased expression of inhibitory molecules, the loss or alteration of immune cell function, dampened production of proinflammatory cytokines, diminished cell proliferation, production of autoantibodies and reduction in neutralizing antibodies (226,228,229). In malaria, during both acute and chronic exposures, a dysregulated immune response has been implicated in the hampering of NAI and contributing to parasite persistence (135).

Innate immune suppression has been suggested in a study where PfEMP-1 expressed on the surfaces of infected red blood cells can bind to CD36 receptor on DCs, inhibiting their maturation and resulting in immune dysregulation and reduced ability to stimulate T cells (230). A study of *P. falciparum* and *vivax* malaria in Papuan adults reported a significant reduction in circulating DCs due to apoptosis that was associated with plasma IL-10 levels (89). *Plasmodium*-infected blood-stage interactions with DC have been found to affect maturation and the ability to initiate adaptive responses, and suppress protective $CD8^+$ T cell responses against the liver stage in mice (231). In addition to myeloid DCs, this immunosuppressive effect by adhesion of infected erythrocytes has also been demonstrated in monocytes and macrophages (232). An in vitro study using human primary DCs and *P. falciparum* iRBC found atypical patterns of activation on DCs including increase maturation markers and chemokine production with almost complete lack of inflammatory cytokine secretion (233).

Anergic T cells are hypo-responsive, do not proliferate or produce IL-2 following ligation of TCR (228). A study of newborns exposed to malaria blood-stage antigens in utero exhibited a more tolerogenic state that persisted into childhood, characterized by

exhibiting anergic T cells, increased production of IL-10 and reduced production of IL-2 and IFN-γ by cord blood mononuclear cells upon stimulation with blood-stage antigens (234).

T cell exhaustion is characterized by the expression of inhibitory markers including PD1, lymphocyte-activation gene-3 (LAG3), T-cell immunoglobulin and mucin domain-3 (TIM3), CTLA4, and 2B4 and a decrease in production of effector cytokines such as IL-2, IFN-γ, and TNF (226,228). Chronic *P. falciparum* exposure of Kenyan children has been associated with expression of PD1 and LAG3 on $CD4^+$ T cells (235). Co-expression of PD1 and CTLA4 on $CD4^+$ T cells has been reported in children in Ghana during malaria infection (236). Additionally, immune senescence resulting in the loss of costimulatory molecule CD28 expression on T cells, has been reported along with co-expression of PD1 and CTLA4 on $CD4^+$ and $CD8^+$ T cells during acute symptomatic *P. falciparum* infection (237). Nonetheless, not all studies are reporting an exhausted phenotype, when inhibitory receptors are expressed as some recent murine studies have demonstrated that T-cells expressing inhibitory markers still maintained a functional capacity (238,239).

Atypical memory B cells (aMBC), a distinct MBC subset lacking the CD27 memory marker, have been reported to increase in frequency during chronic malaria exposure (240,241) along with the upregulation of inhibitory receptors FcRL3, FcRL4, and FcRL5 and have been associated with immune exhaustion (242,243). Kenyan children experiencing chronic *P. falciparum* exposure had increased frequencies of aMBC and an expansion of PD1-expressing and PD1/LAG3-co-expressing $CD4^+$ T cells (235). Nevertheless, aMBC have not always been linked to immune exhaustion and a study of exposed and partially immune Gabonese adults reported that aMBCs had the capacity to produce protective neutralizing antibodies against *P. falciparum* blood-stage parasites (244). More recently, a study of chronic malaria exposure in Malian donors reported that aMBC are not hyporesponsive to malaria antigens and maintain the capacity of BCR signaling, antigen internalization, and plasma cell differentiation (245).

3. Vaccines and Monoclonal Antibodies (mAbs)

Vaccines have proven to be invaluable public health tools for preventing a number of infectious diseases. Malaria vaccine efforts have been ongoing for decades, with many candidates in the pipeline at different stages of development. Recently, two malaria vaccines achieved WHO approval. Research interests have also increased in mAbs targeting the malaria parasite to help define antigenic targets and use them as malaria prophylactic tools. mAbs are being developed for all stages of the parasite cycle, however, only mAbs targeting pre-erythrocytic CSP are discussed below.

3.1 Malaria Vaccines

The complexity of the *Plasmodium* parasite life cycle, the high number of antigens and their variability expressed during different stages, and parasite immune evasion mechanisms have made malaria vaccine development difficult (246). Each stage of the *Plasmodium* life cycle provides unique targets for malaria vaccines including **pre-erythrocytic stage vaccines**, asexual **blood-stage vaccines**, and sexual stage within the mosquito in **transmission-blocking vaccines** (247)**.** An advantage of pre-erythrocytic stage and transmission-blocking vaccines is that these target the parasite at bottlenecks in the life cycle, as opposed to the high-density parasite burden occurring in the host during the blood-stage (248) **(Figure 6)**.

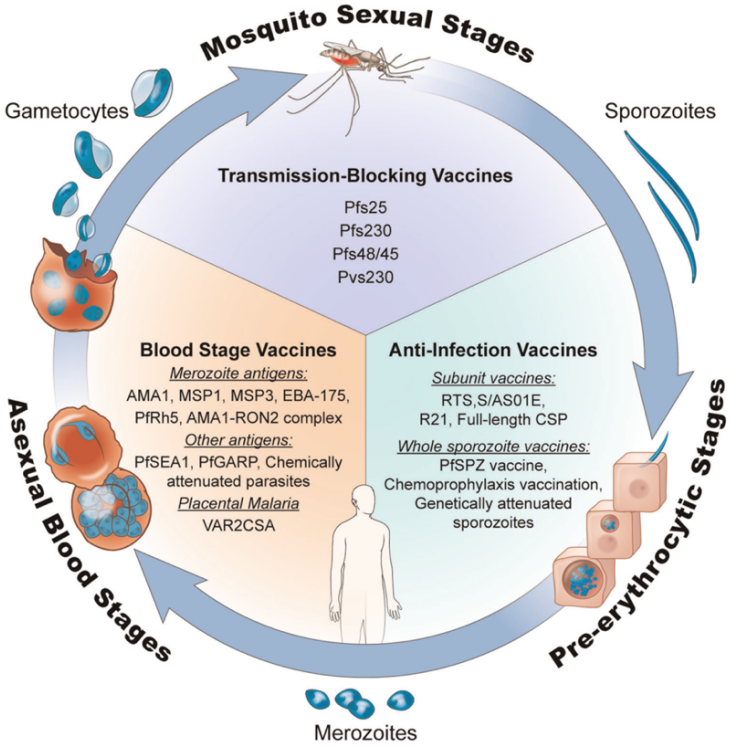

Figure 6. Malaria vaccines according to parasite stage. Vaccine candidates targeting different stages of the *Plasmodium* life cycle. RTS,S/AS01$_E$ and R21 vaccines have been approved for use by the WHO. Figure from (247).

3.1.1 Pre-erythrocytic Stage Vaccines

Pre-erythrocytic stage vaccines target the sporozoite and liver parasite stages with the aim of inhibiting hepatocyte invasion and blocking further development of blood-stage parasites and clinical malaria. Early pivotal studies in mice and human volunteers using radiation-attenuated sporozoites (RAS) demonstrated protective immunity and established that a malaria vaccine could be attainable (249–251). While attenuated sporozoite immunization demonstrated a high level of sterile protection, the approach was considered to be impractical for a wide-spread vaccination campaign due to several hurdles including delivery by infected mosquito bite and that sporozoites could only be reared within the mosquito (3). Researchers then turned focus towards developing subunit vaccine approaches based on CSP, which had been identified as a key target of protective immune responses generated by sporozoite immunization (252,253).

CSP-based subunit vaccines

Several advantages of the subunit vaccine approach include safety, scalability, and cost-effectiveness. However, purified synthetic peptide or recombinant proteins lack the PAMPs required to stimulate the innate immune response needed to initiate antigen-specific adaptive immunity. Potent adjuvants are often needed to overcome this deficit when designing formulations for subunit vaccine approaches (254).

The RTS,S/AS01$_E$ vaccine (RTS,S), the first malaria vaccine to pass a phase 3 trial, was approved by the WHO in 2021 for use in children living in moderate-to-high malaria-endemic regions in sub-Saharan Africa (255). RTS,S comprises a portion of the central repeating amino acid sequence (NANP) and the C-terminal region of *P. falciparum* CSP, fused to and co-expressed with unfused hepatitis B surface antigen (HBsAg) in yeast forming virus-like particles (VLP) **(Figure 7)** and formulated with AS01$_E$ liposomal-based adjuvant containing Monophosphoryl lipid (MPL) A and QS-21 (256).

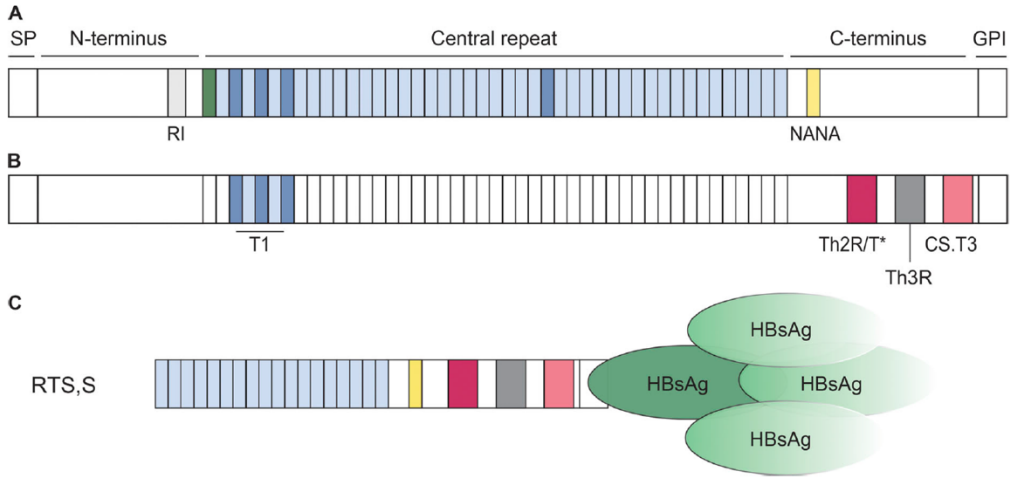

Figure 7. Schematic representation of *P. falciparum* CSP and RTS,S immunogen. (A) *P. falciparum* CSP showing N-terminus region linked to the central repeat region containing NANP major repeats (light blue) with NVDP minor repeats (dark blue), by a junctional region containing NPDP (green). These amino acid motifs have been recognized by protective antibodies, as well as a single NANA motif (yellow) in the C-terminus. **(B)** T cell epitopes identified in the junctional region (T1) and the C-terminus. **(C)** RTS,S immunogen showing NANP repeats and completed *P. falciparum* CSP 3D7 C-terminal fused to HBsAg and unfused HBsAg. Figure from (257).

RTS,S, was designed to elicit robust humoral and cell-mediated immune responses (258,259) and thus far, anti-CSP antibodies appear to be one of the main correlates of protection. Efforts continue to define RTS,S' immunogenicity and correlates of protection, to improve upon the vaccine efficacy (VE) which was found to be moderate during the phase 3 trial (260). The recent completion of a phase 3 trial in Burkina Faso and Mali utilizing seasonal RTS,S vaccination with or without seasonal malaria chemoprevention found significant protection maintained over five years of follow-up (261). RTS,S is covered in more detail below in section 4.

R21/Matrix-M vaccine (R21) became the second malaria vaccine to be recommended by the WHO in October, 2023. R21 is a VLP, similar in design to RTS,S, consisting of a portion of the central repeats and C-terminus regions of *P. falciparum* CSP, fused to HBsAg. R21 differs from RTS,S by forming CSP-HBsAg fusion proteins without the excess of unfused HBsAg, resulting in a higher density of CSP, formulated with a saponin

Matrix-M adjuvant (262). Preclinical murine challenge studies demonstrated that R21 produced neutralizing antibodies that could inhibit infection of transgenic *P. berghei* sporozoites that express both *P. berghei* and *P. falciparum* CSP (262). A phase 3 trial in children aged 5-36 months living in either seasonal or perennial malaria endemic regions in Africa, found that R21 had a VE of 75% and 68%, respectively, for time to first clinical malaria episode (263).

Whole sporozoite vaccines

Studies in mice immunizing with RAS demonstrated the induction of protective antibodies and $CD8^+$ T cells (155,264). Considered the "gold standard" for sterilizing immunity, pursuit of the RAS vaccine approach was revisited in the early 2000s with studies showing high efficacy when irradiated *P. falciparum* sporozoites were delivered by bites of 1000s of infected mosquitoes (265). Efforts to mass-produce aseptically purified attenuated and cryopreserved *P. falciparum* were led by Sanaria (266) which has recently demonstrated complete in vitro production of the *P. falciparum* parasite cycle (267). This was a major hurdle for the whole sporozoite approach which had depended upon sporozoite production in the mosquito and require harvesting sporozoites under sterile conditions. Intravenous delivery of attenuated sporozoites "PfSPZ vaccine" to malaria naïve individuals followed by CHMI challenge demonstrated high efficacy (268,269). However, more recent field studies have not shown the same high level of efficacy. A phase 1 trial evaluating PfSPZ administered intravenously, demonstrated 48% protective efficacy by time to first positive blood smear and 29% efficacy by proportion of participants in malaria-exposed adults living in Mali (270). A phase 2 trial assessing PfSPZ administered intravenously to children aged 5-12 months living in high transmission western Kenya, found dose dependent antibody responses that correlated with efficacy three months after vaccination, but did not detect T cells responses and no significant protection was demonstrated at six months following immunization (271).

CVac immunization has advantages in that it allows for the parasite to progress through all liver developmental stages while under chemoprophylaxis, thus exposing the immune system to a broader range of antigens, whereas RAS arrest at the onset of liver stage

infection (272). CVac has demonstrated sterile protection against human malaria infection in several studies (273,274). Increased protection was shown to be associated with antibody responses and expansion of Vδ2 γδ T cells, which may induce protective $CD8^+$ T cells that target intrahepatocytic parasites (274).

Besides RAS and CVac immunization, other whole sporozoite vaccine approaches are being investigated and at various stages of development and clinical trials including the delivery of genetically-attenuated (275,276) or chemically-attenuated sporozoites (277), and transgenic rodent *Plasmodium*-based immunization platform (*Pb*Vac) expressing *P. falciparum* CSP (278).

Viral vector vaccines

The viral vector vaccine approach efficiently delivers genetic material that can encode antigens into host cells to produce antigenic proteins (279). Viral vectors have several advantages including the ability to elicit strong humoral and cellular responses without requiring additional adjuvants, by signaling through toll-like receptors, and have the capacity to deliver multiple genes, allowing a broader immune response (280). Vectors from nonhuman primates have been used to overcome the drawback of preexisting immunity that humans have to many common viruses (279). Viral vectors such as chimpanzee adenovirus (ChAd63) and modified vaccinia Ankara (MVA) encoding TRAP have demonstrated the ability to generate increased frequencies of $CD8^+$ T cells (which no subunit vaccine has induced) targeting the pre-erythrocytic parasite liver stage antigen ME-TRAP (Multiple-epitope thrombospondin-related adhesion protein) (281). A heterologous prime-boost immunization with CHAd63 and MVA vectored vaccines has shown partial protection from *P. falciparum* infection in Kenyan adults within a two-month follow-up (282). However, a recent field efficacy trial of a heterologous prime boost immunization with CHAd63 and MVA vectored vaccines in infants and children living in high malaria-endemic Burkina Faso, did not show protective efficacy (283).

3.1.2 Blood-stage, Transmission-blocking, and Multi-stage vaccines

As previously mentioned, passive antibody transfer studies (161) and NAI following repeated blood-stage infection provided the motivation to develop **blood-stage vaccines** (161,163). A major obstacle for blood-stage vaccines is high level of antigenic polymorphism of the parasite molecules on the parasitized red blood cell (284). RH5.1/Matrix-M, a leading blood-stage vaccine candidate has completed a phase 1b trial in Tanzanian adults and children reporting acceptable safety and reactogenicity profiles and all children reached growth inhibition activity levels that had previously been associated with protection against *P. falciparum* blood-stage in non-human primates (285).

The **transmission-blocking vaccine** strategy targets parasite sexual stages aiming at interrupting transmission from hosts to mosquitoes, thus shielding the larger community by herd immunity and not by directly protecting the host from infection or clinical symptoms (286). A potential logistical challenge to transmission-blocking vaccines is that a high level of coverage is needed to achieve herd immunity, including all population age groups (287). Two leading transmission-blocking vaccines candidates Pfs25 and Pfs230 have been tested in various adjuvant formulations and constructs that are undergoing phase 1 clinical trials (288).

Multi-stage vaccines have several potential advantages over a single-stage vaccine in that it may provide broader immune coverage and reduced opportunity for parasite escape. If a pre-erythrocytic stage vaccine allows a single sporozoite to invade a hepatocyte, it is enough to proliferate and continue with blood-stage infection. A viral-vectored vaccine AdHu5-Pfs25-PfCSP, which combines pre-erythrocytic stage *P. falciparum* CSP antigen with transmission blocking Pfs25 has been studied in mice (289). Recently, the mRNA-based vaccine approach, with its advantages including faster development, flexibility in antigen design, and scalability, has been applied to malaria vaccine efforts (290,291). Several mRNA vaccine candidates are in development that target different stages of the parasite life cycle including pre-erythrocytic and sexual stages. A multi-stage mRNA-LNP vaccine candidate expressing *P. falciparum* CSP and

Pfs25 has been evaluated for immunogenicity and protection against challenge in mice (292). Multi-stage vaccine studies having the objective of delivering multiple vaccines targeting different parasite stages are currently underway in a phase 1 clinical trial investigating the co-delivery of R21 vaccine and RH5.2-VLP (ClinicalTrials.gov ID: NCT05357560).

3.2 Monoclonal antibodies

Early studies in mice developed the mAb Pb44, specific for the surface antigen of rodent parasite *P. berghei* (293) and established that passive transfer of Pb44 could protect against sporozoite challenge (294). Soon after, mAbs specific for *P. falciparum* CSP were generated (253) and used to characterize the immunodominant region of CSP (295). Research interests have gained traction in the application of mAbs to better define protective antigenic epitopes and their potential use as malaria prophylactics.

Human mAbs specific for NANP repeats, generated from volunteers immunized with RTS,S were found to protect mice against challenge with transgenic *P. berghei* sporozoites that express *P. falciparum* CSP (296,297). Studies demonstrated human mAbs isolated from sporozoite-immunized Tanzanian (298) and malaria-naïve (299) adults were dual-specific and bound a unique junctional epitope within the sequence KQPADGNPDPNANP between the N-terminus and central repeat region of *P. falciparum* CSP. Passive transfer of dual specific mAbs reactive for the junctional epitope protected humanized mice from challenge with *P. falciparum* sporozoites (298) and from challenge against transgenic *P. berghei* sporozoites expressing *P. falciparum* CSP in mice (299). Another study demonstrated mAb L9, isolated from an individual immunized with radiation attenuated PfSPZ, to preferentially bind NVDP minor repeats with high affinity and cross-reacted with NANP major repeats; moreover, they showed sporozoite neutralizing activity in the livers of mice (300). Further studies of these mAbs preferentially binding the junctional tetrapeptide NPDP, CIS43 (299), NVDP minor repeats, L9 (300), and NANP central repeat binding mAb 317 (297) were assessed for protection in mice against in vivo challenge with *P. berghei* transgenic parasites expressing *P. falciparum* CSP with either the junctional and minor repeats knocked in or knocked out and it was demonstrated that

these human mAbs prevent malaria by targeting three different protective epitopes in the CSP repeat region (301).

Aiming to characterize different mAbs for future potential vaccine and immuno-prophylactic development, Livingstone et al. compared in vitro and in vivo activities of six CSP-specific mAbs derived from humans either immunized with RTS,S (mAbs 317 and 311); an irradiated whole sporozoite vaccine PfSPZ (mAbs CIS43 and MGG4); or individuals exposed to malaria (mAbs 580 and 663) (302). RTS,S mAb 317, which specifically binds $(NPNA)_n$, exhibited the highest affinity and sterile protection in mice, while sporozoite immunized mAb CIS43, which has dual-specific binding at the junctional epitope and $(NPNA)_n$ was best at inhibiting sporozoites in vitro (302). Another study investigating the potential of combining repeat-specific mAbs (CIS43, L9, and 317) that target the three tetrapeptides (NPDP/NVDP/NANP) respectively, with a mAb specific for the C-terminal region of *P. falciparum* CSP, did not provide increased protection in mouse in vivo experiments (303). However, repeat mAbs (but not C-terminal) administered to mice immunized with R21 vaccine enhanced protection against sporozoite challenge as compared to R21 or mAbs alone (303). Recently, a study of mAbs derived from RTS,S immunized volunteers specific to the C-terminal domain of CSP, identified an antibody targeting a previously unknown conserved epitope that demonstrated inhibition against malaria infection in a mouse malaria model (304).

Efforts to assess mAb have now entered clinical trials. CIS343LS (“LS” for leucine and serine point mutations in the Fc domain to increase antibody half-life (305), entered phase 1 clinical trial of human malaria mAb in malaria-naïve adults and demonstrated sterile protection against CHMI infection (306). L9LS specific for the minor repeats entered a Phase 1/2a clinical trial in US adults and demonstrated 88% protection following CHMI (307). Recently, a study characterized a large panel of mAbs from RTS,S vaccinees and using binding assays and mouse malaria models, they identified sequences in *P. falciparum* CSP that suggest RTS,S may induce immunodominant antibody response that limit more protective subdominant responses (308). From the panel of mAbs, AB-000224

(MAM01) was selected and has advanced into a phase 1 trial currently underway (ClinicalTrials.gov Identifier: NCT05891236) (308).

4. RTS,S Malaria Vaccine

Following the initial proof of concept in the 1970's that RAS could confer sterile protection, a collaboration between GlaxoSmithKline (GSK) and the Walther Reed Army Institute of Research (WRAIR) that began in the 1980's led to the development of the RTS,S malaria vaccine (309). The RTS,S program was based on earlier vaccine constructs that were designed to elicit antibodies against the repeat epitopes of CSP and demonstrated that humans could be protected by a subunit vaccine (310). RTS,S differed from previous CSP subunit vaccine candidates since it comprised both the CSP repeat region, as well as, portions of the C-terminus region containing T cells epitopes found to target cell-mediated immunity (CMI) (311).

A number of CHMI trials were performed in malaria-naïve adult volunteers assessing safety and efficacy of various RTS,S-adjuvant formulations, dosing and immunization strategies such as heterologous prime-boost (3). RTS,S formulated in AS02, a squalene-in-water emulsion containing MPL and QS21(saponin), demonstrated the highest level of protection with six out of seven volunteers (85.7%) protected (312). The positive results and safety profile determined by the earlier studies using CHMI led the way to RTS,S field trials in malaria-endemic countries. However, evaluating VE in the partially protective RTS,S vaccine in field trials was more difficult than CHMI studies in malaria naïve volunteers, since variables such as MTI, exposure, age, NAI, and other factors introduce greater complexity (3). Field studies in adults and children using RTS,S/AS02 and RTS,S/AS01 formulations showed good safety profiles and partial protection (3,313,314). RTS,S/$AS01_E$ (pediatric formulation of $AS01_B$) tested in Kenyan and Tanzanian children aged 5-17 months demonstrated moderate efficacy with few adverse events, showing promise as a malaria vaccine candidate, leading the way to the phase 3 pediatric clinical trial (315).

4.1 Phase 3 Clinical Trial

The double-blind, randomized, controlled phase 3 trial (MAL055) was conducted between 2009 and 2014 at 11 research centers within seven countries in sub-Saharan Africa with a range of malaria transmission intensities, including Burkina Faso, Gabon, Ghana, Kenya, Malawi, Mozambique, and Tanzania and aimed to assess the efficacy and safety of RTS,S vaccination with or without a booster dose (260,316). The trial involved two age cohorts consisting of 6,537 infants aged 6-12 weeks and 8,922 children aged 5-17 months at the time of first vaccination and participants were randomized (1:1:1) to receive either three primary doses at month (M) M0, M1, and M2 of RTS,S plus a fourth dose (booster) 18 months later at M20 (R3R); three doses of RTS,S plus the comparator vaccine at M20 (R3C), or three doses of control vaccine plus a control vaccine at time of booster (C3C) (**Figure 8)**. Control vaccines consisted of rabies vaccine given to the children for the primary doses and meningococcal serogroup C conjugate vaccine given to the infants for the primary doses, and to both children and infants for the fourth dose (260). An ancillary immunology study (MAL067) involved seven African trial sites including Bagamoyo, Kintampo, Kombewa, Lambaréné, Manhiça, Nanoro, and Siaya and had two main study components, antibody and cellular immunology. All sites collected sera or plasma samples at M0, M3, M20, and M32, and three sites, Bagamoyo, Lambaréné, and Manhiça, collected PBMCs at M0, M3, M21 and M32 in a subset of study participants for cellular immunology studies.

Passive case detection was used to identify clinical malaria cases at local health clinics and a case was defined as presenting with a temperature of >37.5ºC and positive detection of parasitemia of >5000 parasites/uL (316). Estimating VE in the RTS,S field studies were carried out measuring time-to-event according to predefined study endpoints such as first episode of clinical malaria. Children and infants were followed for a median of 48 and 38 months, respectively (260). During the first year of follow-up post primary immunization during the phase 3 trial in children VE was 55.8% (95% CI 50.6-60.4) against clinical malaria (317) and 31.3% (97.5% CI 23.6-38.3) in infants (318). At study end (average 38-48 months since first dose), VE against clinical malaria ranged from

28.3-36.3% in children and 18.3-25.9% in infants, without and with a 4th booster dose given at M20, respectively (260).

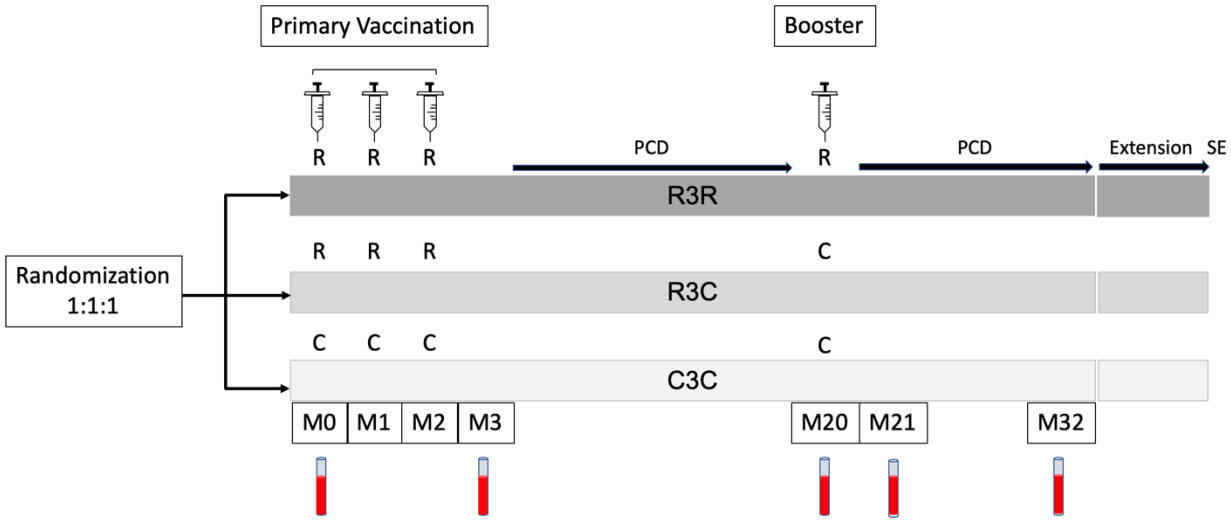

Figure 8: RTS,S phase 3 clinical trial design. R: RTS,S; C: comparator vaccine; R3R: 4 doses of RTS,S; R3C: 3 doses of RTS,S and 1 dose of comparator vaccine; C3C: 4 doses of comparator vaccine; PCD: passive case detection; M: month; SE: study end. Created with PowerPoint.

4.2 RTS,S Immunogenicity and Immune Correlates

Early RTS,S clinical trials in malaria-naïve adult volunteers and African field trials involving adults and children demonstrated that RTS,S induces strong anti-CSP antibody responses to B cell NANP epitopes of the central repeat region (3), as measured by an ELISA developed by GSK using synthetic R32LR antigen and expressed in ELISA Units per milliliter (EU/mL) (319). RTS,S field trials reported overall higher anti-CSP antibody response in children than adults (3,320) with the highest found in children aged 5-17 months old at first vaccination (321). Older children from Kenya and Tanzania (315) and Gabon (322) and adults (323) also exhibited lower antibody concentrations. The RTS,S phase 3 trial reported the highest anti-CSP in children aged 5-17 months old at first vaccination with an overall geometric mean titer (GMT) reaching 621 EU/mL (ranging from 348.4 to 787.1 EU/mL across study sites) and an overall lower GMT in infants at 210.5 EU/mL (ranging from 116.9 to 225.3 EU/mL across study sites) (324). Anti-CSP antibody titers correlated with protection, and while no antibody threshold has been determined, an antibody titer of 121 EU/mL has been shown to prevent 50% of infections

(325). Overall, RTS,S generates a strong antibody response but it decays quickly (325). During the phase 3 trial, the RTS,S booster dose recovered waning antibody levels, but remained lower following the booster dose than following peak post primary immunization (325,326).

Prior malaria exposure or concurrent infection has been proposed to impact RTS,S vaccination (325,327–329). RTS,S-vaccinated malaria-naïve adults have shown higher anti-CSP GMT (330) post-vaccination than malaria-experienced Kenyans (323), which could suggest immunological imprinting may be a factor (120). A combined analysis study of nine phase 2 trials including infants, children, and adults found higher pre-RTS,S vaccination anti-CSP titers associated with lower peak titers in infants, while children and adults had higher peak titers suggesting improved immunogenicity with pre-existing NAI (320). However, differences in trial characteristics such as study populations, MTI, adjuvant formulations, and co-administration of other vaccines during EPI can complicate the findings (320). An analysis of all participating RTS,S phase 3 trial sites found that higher baseline anti-CSP antibody levels were associated with higher anti-CSP level post-vaccination during the peak response in children aged 5-17 months, while the opposite effect was found for infants aged 6-12 weeks (325). Infants likely have a less developed immune capacity than children and data is suggesting that high maternal malaria exposure may further reduce immunogenicity in infants (324). A recent study used data from three study sites during the RTS,S phase 3 trial, and found antibody responses post primary vaccination to be higher in Ghana than in Malawi and Gabon, but antibody levels did not vary due to background incidence or parasitemia during vaccination (329).

Various mechanisms have been identified to understand how RTS,S-induced CSP repeat antibody may be acting against sporozoite infection. Antibodies can directly neutralize sporozoites by inhibiting gliding motility and cell traversal. Early studies performed CSP precipitin reactions by incubating *Plasmodium* sporozoites with immune sera demonstrating antibody cross-linking of surface CSP (331,332). Murine studies using intravital microscopy demonstrated antibody immobilization of sporozoites injected into the skin by mosquitoes (5). Recently, an assay was developed to quantitatively measure

anti-CSP antibody inhibitory activity of *P. falciparum* sporozoite motility in vitro and in human skin explant models (333). RTS,S-induced antibody bound to sporozoites can cross-link Fcγ receptors on the surface of neutrophils and monocytes, facilitating phagocytosis of opsonized sporozoites (334,335). Antibody-mediated engagement of FcγRIIIa and ADCC activity by NK cells has also been shown (335). RTS,S-induced cytophilic IgG1 and IgG3 have demonstrated protection in children (327). IgG1, IgG3, and IgM antibodies targeting *P. falciparum* sporozoites were found to activate complement and inhibit hepatic cell traversal and sporozoite killing in vitro (336). Higher avidity of IgG antibodies to the C-terminal region of CSP has been associated with protection following RTS,S vaccination in children (337) and may have a functional role in inhibiting sporozoites invasion of hepatocytes (338,339).

RTS,S-induced T-cell activation has been assessed using a variety of approaches, including in vitro assays such as T cell proliferation assays, cytokine secretion, intracellular cytokine staining (ICS) for flow cytometry, ELISpot and cytotoxicity assays (3). Variations of conditions and approaches used between trials have made comparison of results between trials challenging to interpret, and efforts to initiate an international standardization of T cell protocols have yet to be formulated (3).

In contrast to the strong anti-CSP antibody responses found with RTS,S vaccination, trials studying cellular responses in malaria-endemic regions reported relatively low-to-moderate T_H1-type responses, predominantly by $CD4^+$ T cells expressing IL-2, TNF, and IFN-γ (340–345). Two trials conducted at WRAIR in RTS,S-vaccinated malaria-naïve adults assessing ICS of stimulated PBMCs reported an association between T-cell responses and protection (330,346). A field trial in Mozambican infants reported a tendency of association between IL-2 levels (albeit low) in CSP-specific T cell supernatants with protection, yet it was not significant (340).

No study investigating cellular responses has consistently demonstrated a correlation with protection during RTS,S phase 2 field trials (343,344); however, some indications of synergistic interactions between vaccine-induced CSP-specific antibodies and

TNF^+CD4^+T cells induced by natural exposure were found (344). $CD4^+$ T are likely central to RTS,S vaccine-mediated protection by supporting B cells to produce and maintain neutralizing antibodies and innate cell populations involved in antibody-mediated mechanisms. CSP-specific $CD4^+$ T cells producing IL-2 have been shown to correlate with anti-CSP antibody levels (347). Modeling analysis of RTS,S-vaccinated malaria-naïve adults subjected to CHMI indicated that anti-CSP antibodies and $CD4^+$ T cells are essential for protection (348). Studies by our group of Mozambican children following the phase 3 primary immunizations found CSP-specific $CD4^+$ T cells producing IL-2, TNF and expressing CD154 (CD40L); however, frequencies of CSP-specific $CD4^+$ T cells producing these cytokines were low (349). This study included CD45RA and CCR7 markers to identify memory cell subsets, and RTS,S induced both central and effector memory compartments (349). Another study by our group focusing on cytokines secreted in culture supernatants after in vitro antigen stimulation, reported T_H1-type responses such as the production of IFN-γ, IL-15, and granulocyte-macrophage colony-stimulating factor (GM-CSF) associated with protection in the RTS,S phase 3 trial following primary vaccination and the T_H2 cytokine IL-5 and the chemokine RANTES (regulated on activation, normal T cell expressed and secreted) associated with risk (350). The predominant producers of IFN-γ appear to be NK cells following activation by IL-2-secreting CSP-specific $CD4^+$ T cells (345).

RTS,S-induced $CD8^+$ T cells producing IFN-γ have been reported in a study of Mozambican infants (340) and another in naïve adults (346). However, neither study found a direct association with protection. The study by our group of Mozambican children during the phase 3 trial post-primary vaccination detected increased frequencies of $CD8^+$ T cells expressing CD154 and IL-2 or TNF; however, significance was lost after correcting for background, suggesting that there may be non-specific effect of RTS,S vaccination (349).

No extensive assessment of T_{FH} cells, known to provide B cell support and development of memory and long-lived plasma cells, has been done in RTS,S field studies. Our previous study of Mozambican children also found HBsAg-specific $CD4^+$ T cell subsets

producing IL-2, TNF, CD154 plus IL-21 or IFN-γ, or IL-4, indicating the differentiation of T_{FH}, T_H1, and T_H2 effector cell subsets (349) Recently, T_{FH} cells have been found in malaria-naïve adults vaccinated with a delayed fractional dose of RTS,S (351). Additional studies would be needed to assess RTS,S-induced T_{FH} responses, and correlations with antibody responses' potential role in vaccine-induced protection.

RTS,S-induced cellular responses, and mechanisms of immune protection are still being investigated, and further research is needed to gain a fuller understanding of the contributions of the various cell subsets and their interactions in mediating protection against malaria.

4.3 Knowledge Gaps

Identifying immune correlates of protection is crucial for improving VE and longevity, key for eliminating malaria. Determining the immune mechanisms and specific factors leading to the decline of RTS,S-induced immunity is necessary to improve upon RTS,S, and existing vaccines that may be similarly affected and develop more effective and long-lasting second-generation vaccines.

A deeper understanding of RTS,S-specific cell-mediated immunity, and the factors affecting T-cell responses is essential as they support B cells in developing high quality and sustained antibody responses and provide help to effector cells. Why levels of antibodies to malaria antigens are so short-lived, whether from natural acquisition or vaccine-induced responses, remains uncertain. Further defining immune system dysregulation and dysfunction due to acute and chronic malaria exposure and the role of regulatory T and B cells could help inform improved vaccine design and strategies. Further studies on how T cell responses, particularly T_{FH} cells, can be enhanced to support B cells and the production of neutralizing antibodies are needed, as well as a better-defined optimal balance of IgG antibody subclasses and functional responses to determine how they associate with protection and risk. Understanding how to increase and support memory T cell induction contributes towards achieving more durable responses.

Similarly, characterizing B cell-mediated memory and the heterogeneity of antibody responses generated by NAI and vaccination would help shed light on waning antibody responses and VE. Additionally, further investigation is necessary into antibody binding specificity and the mechanisms of monoclonal antibody binding to junctional, minor, and major repeat epitopes of CSP and their interaction with sporozoite inhibition. Broader knowledge of parasite antigenic targets for antibodies that could be included in vaccines and how multi-antigen or multi-stage vaccine formulations may be optimized to induce neutralizing antibodies and CD4 and CD8 T cells is needed.

The impact of various factors such as age, prior or concurrent malaria exposures, geographic site differences, and health status on vaccine-induced responses must be further defined. Why do immune responses differ between infants and young children? How do maternal antibodies and exposures during pregnancy impact susceptibility to malaria or vaccine response, and how can vaccines be improved to protect these vulnerable age groups? Does partial vaccine protection interfere with the development of natural immunity, and how can vaccination and natural exposure be combined for better long-term protection?

Despite its limitations, the RTS,S vaccine represents a significant milestone as the first approved malaria vaccine for children living in medium-to-high MTI countries. Ongoing research, critical for the future of malaria vaccines, aims to address these knowledge gaps and strategies to improve RTS,S vaccine effectiveness and design of improved malaria vaccines and strategies and this thesis aims to cover some of these gaps.

Hypotheses

1. Antibodies to *P. falciparum* circumsporozoite protein epitopes are associated with protection against malaria, however fine specificity to distinct epitopes is associated with different functional sporozoite-neutralizing activity.

2. The RTS,S/AS01$_E$ vaccine booster elicits cellular immune responses that support protective antibody responses and their maintenance, and that are associated with protection against malaria. Epidemiological factors impact the booster cellular immunogenicity and the association of cellular responses with malaria protection.

3. Chronic malaria exposure leads to T cell “exhaustion”, resulting in increased expression of inhibitory and senescence markers, which correlates with alterations in the B cell compartment and can affect the immune response to natural infections and vaccination.

Objectives

This doctoral thesis aims to identify the humoral and cellular immune responses to the *P. falciparum* circumsporozoite protein induced by natural malaria exposure or vaccination, including the factors that may affect those responses.

Specifically:

1. To assess the fine specificity and sporozoite neutralizing capacity of polyclonal and monoclonal antibodies targeting circumsporozoite epitopes.

2. To characterize the cellular response elicited by the RTS,S/AS01$_E$ vaccine booster dose at the peak response and 11 months later and identify immune correlates of malaria protection or risk.

   2.1. To evaluate the effect of epidemiological factors on immunogenicity and protection.

   2.2 To assess the association between cytokine response and antibodies.

3. To characterize the immunophenotypic changes induced by chronic malaria exposure associated with T-cell exhaustion and its association with B cell subsets previously found to be altered by malaria exposure.

Material, methods, and results

Article 1

This article addresses the first objective to assess the fine specificity and sporozoite neutralizing capacity of polyclonal and monoclonal antibodies targeting circumsporozoite epitopes. The results of this research have been published in the following article:

J. Mauricio Calvo-Calle*, **Robert Mitchell***, Rita Altszuler, Caroline Othoro, and Elizabeth Nardin. ***Identification of a neutralizing epitope within minor repeat region of Plasmodium falciparum CS protein***. npj Vaccines. 2021 Jan 18;6(1):10. *co-first authors.

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Identification of a neutralizing epitope within minor repeat region of *Plasmodium falciparum* CS protein

J. Mauricio Calvo-Calle[1,2,4]✉, Robert Mitchell[1,3,4], Rita Altszuler[1], Caroline Othoro[1] and Elizabeth Nardin[1]

Malaria remains a major cause of morbidity and mortality worldwide with 219 million infections and 435,000 deaths predominantly in Africa. The infective *Plasmodium* sporozoite is the target of a potent humoral immune response that can protect murine, simian and human hosts against challenge by malaria-infected mosquitoes. Early murine studies demonstrated that sporozoites or subunit vaccines based on the sporozoite major surface antigen, the circumsporozoite (CS) protein, elicit antibodies that primarily target the central repeat region of the CS protein. In the current murine studies, using monoclonal antibodies and polyclonal sera obtained following immunization with *P. falciparum* sporozoites or synthetic repeat peptides, we demonstrate differences in the ability of these antibodies to recognize the major and minor repeats contained in the central repeat region. The biological relevance of these differences in fine specificity was explored using a transgenic *P. berghei* rodent parasite expressing the *P. falciparum* CS repeat region. In these in vitro and in vivo studies, we demonstrate that the minor repeat region, comprised of three copies of alternating NANP and NVDP tetramer repeats, contains an epitope recognized by sporozoite-neutralizing antibodies. In contrast, murine monoclonal antibodies specific for the major CS repeats $(NANP)_n$ could be isolated from peptide-immunized mice that had limited or no sporozoite-neutralizing activity. These studies highlight the importance of assessing the fine specificity and functions of antirepeat antibodies elicited by *P. falciparum* CS-based vaccines and suggest that the design of immunogens to increase antibody responses to minor CS repeats may enhance vaccine efficacy.

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INTRODUCTION

The repeat region of the circumsporozoite (CS) protein of all *Plasmodium* species contains a species-specific immunodominant B cell epitope that is recognized by sera of sporozoite-immunized rodents, monkeys and human volunteers, as well as by naturally infected individuals[1,2]. When sporozoites are targeted by anti-repeat antibodies, parasite motility is inhibited thus blocking egress from the site of the mosquito bite into the blood and invasion of host cell hepatocytes thereby preventing subsequent development of blood-stage infection and clinical disease[3–6].

Early studies demonstrated that monoclonal antibody (MAB) specific for CS repeats derived from sporozoite-immunized experimental hosts were protective following passive transfer to susceptible rodents or monkeys. As little as 10 µg of MAB specific for the CS repeats of rodent *P. berghei* sporozoites was shown to protect naïve rodents against sporozoite challenge and 2 mg of MAB specific for the *P. vivax* CS repeats protected Saimiri monkeys from homologous sporozoite challenge[7]. In recent studies, passive transfer of 100–300 µg of human MAB specific for *P. falciparum* CS repeats, derived from volunteers immunized with the CS-based RTS,S vaccine, protected mice against challenge with transgenic *P. berghei* parasites expressing full length *P. falciparum* CS protein[8].

The demonstration of antibody-mediated protection in experimental hosts has encouraged efforts to design CS-based vaccines that elicit high titer antirepeat antibodies to neutralize sporozoite infectivity. However, despite decades of vaccine research, a protective antirepeat antibody titer remains to be defined and there is limited information on the range of functional antirepeat antibodies elicited following immunization with sporozoites or CS subunit vaccines. The immunodominant repeat region of *P. falciparum* CS protein is comprised of major and minor tetramer repeat sequences that are conserved in all isolates. In the *P. falciparum* NF54 strain[9], used in the majority of human malaria challenge studies, the CS repeat region is comprised primarily of 37 NANP tetramers (Fig. 1). In addition, there is a minor repeat region comprised of three alternating NVDP and NANP tetramers in the 5′ repeat region, which is adjacent to a CS protein proteolytic cleavage site that plays a role in sporozoite invasion of host cells[10,11].

Early studies demonstrated that a major repeat peptide, comprised of three copies of the NANP repeat, $(NANP)_3$, could block binding of monoclonal and polyclonal antirepeat antibodies to *P. falciparum* sporozoites and *P. falciparum* CS protein[12]. The NANP repeats were used in the first CS-based subunit vaccine clinical trials, which tested safety and efficacy of a synthetic peptide-protein conjugate, $(NANP)_3$-TT[13], or a recombinant protein R32 containing 32 NANP repeats[14]. The Phase II trials demonstrated modest protective efficacy following challenge of a small number of immunized volunteers by bites of *P. falciparum*-infected mosquitoes.

Differences in fine specificity of polyclonal antirepeat antibodies elicited in NANP peptide-immunized simian and rodent hosts were noted in early studies based on peptide ELISA and IFA reactivity with *P. falciparum* sporozoites[15,16]. The functional relevance of the variations in fine specificity of antirepeat antibodies could not be defined in these early studies as *P. falciparum* sporozoites are infective only in human hosts or splenectomized chimpanzees. More recently, the development of transgenic rodent parasites expressing all or parts of the *P. falciparum* CS protein has provided rodent models to address

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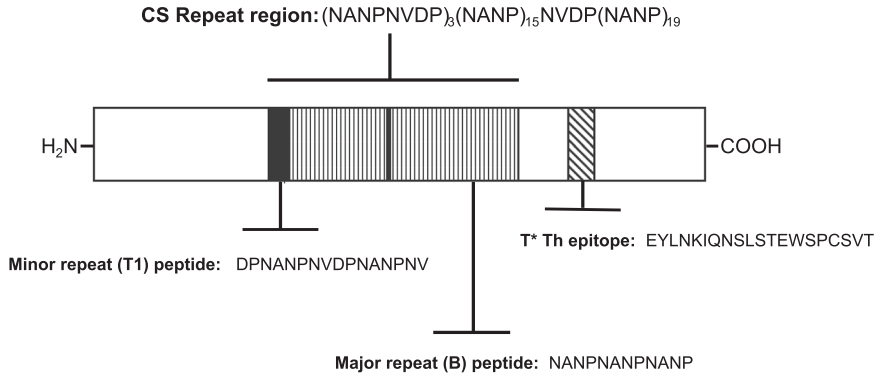

Fig. 1 Schematic of the *P. falciparum* CS proteins and peptides. *Illustration of P. falciparum* CS protein showing the B major (NANP) tetramer repeats (open bars) and the T1 minor repeat epitope comprised of alternating NVDP NANP repeats (solid bars). Synthetic tetrabranched peptides representing the major repeats NANPNANPNANP, or minor repeats DPNANPNVDPNANPNV, were used as antigens in the ELISA to measure fine specificity of polyclonal and monoclonal antirepeat antibody responses. The T* epitope (hatched bar) is a universal T helper cell epitope located in the C -terminus (aa 326–345 NF54 isolate) that is recognized by a broad range of human and murine class II HLA genotypes. Peptide immunogens were constructed as either linear or tetrabranched peptides comprised of major repeats combined with the T* universal T helper cell epitope, $(NANP)_3$EYLNKIQNSLSTEWSPCSVT (designated BT*), or minor repeats combined with T* universal T helper cell epitope $(DPNANPNV)_2$EYLNKIQNSLSTEWSPCSVT (designated T1T*).

the role of antibody fine specificity in neutralizing sporozoite infectivity.

In the current paper, we used transgenic *P. berghei* sporozoites expressing *P. falciparum* CS repeats[17] to examine neutralizing activity of MAB and polyclonal antirepeat antibodies derived from sporozoite- or peptide-immunized mice. We found that MAB specific for the minor repeats were protective in vitro and in vivo. In addition, several MABs elicited by immunization with major repeats could be isolated, which did not have neutralizing activity. These studies on fine specificity of sporozoite-neutralizing antibodies have important implications for vaccine design and analysis of functional humoral responses to the *P. falciparum* CS repeat region.

RESULTS

Fine specificity of MAB derived from mice immunized with *P. falciparum* sporozoites

CS-specific MABs derived from *P. falciparum* sporozoite-immunized mice[18] were analysed for fine specificity by ELISA using either the major repeats $(NANP)_3$ or minor repeats $(DPNANPNV)_2$ as coating antigen. MAB fine specificity varied, either cross-reacting with both the minor and major repeats, as exemplified by MAB 2A10, or skewing toward the minor repeat region with little or no reactivity with the major repeats as shown by MAB 2F9, 2C2 and 4H3 (Fig. 2a). All of the MABs specific for minor repeats also reacted with *P. falciparum* sporozoites by indirect immunofluorescence assay (IFA) (data not shown).

MABs recognizing only the minor repeats were compared to cross-reacting MAB 2A10 using a Transgenic Sporozoite Neutralizing Assay (TSNA) to measure ability of the MAB to inhibit invasion of PfPb sporozoites into hepatoma cells (Fig. 2b). The minor repeat-specific MABs 2C2 and 4H3 demonstrated dose-dependent sporozoite-neutralizing activity. These MAB gave >90% reduction in parasite rRNA copy number when tested at 25 and 12.5 µg/ml, similar to inhibition obtained with MAB 2A10 that cross-reacted with both major and minor repeats. The minor repeat-specific MABs were also inhibitory at 3.125 µg/ml, the lowest concentration tested. MAB 3D11, specific for *P. berghei* CS repeats, did not block PfPb sporozoite invasion of hepatoma cells. These results provide evidence that antibodies specific for the *P. falciparum* CS minor repeats can neutralize sporozoite infectivity.

To confirm the specificity of neutralizing activity, a peptide competition TSNA was carried out by preincubating minor repeat-specific MAB 2C2, or cross-reacting MAB 2A10, with either major or minor repeat peptides prior to addition to PfPb sporozoites (Fig. 2c). MAB 2C2 was inhibited by minor repeat peptide (closed squares), but not the major repeat peptide (open squares), confirming that antibody neutralizing activity was directed at the minor repeats. Interestingly, MAB 2A10, which recognizes both major and minor repeats in peptide ELISA, was primarily inhibited by the minor repeat peptide. Preincubation of MAB 2A10 with the major repeat peptide gave minimal inhibition of sporozoite neutralization. The peptide competition TSNA confirms that the CS minor repeats are targets of neutralizing antibodies.

Major or minor repeat peptides elicit antibodies that differ in fine specificity

To further investigate fine specificity of neutralizing antibodies, mice were immunized with tetrabranched peptides containing either the minor repeats $(T1T^*)_4$ or the major repeats $(BT^*)_4$. Previous studies had found that a related immunogen $(T1BT^*)_4$ elicited antirepeat antibodies in mice and human volunteers[19,20].

Mice were immunized subcutaneously (s.c.) with three doses of $(T1T^*)_4$ or $(BT^*)_4$ in QS21 adjuvant. Immune sera of C57BL/6 mice immunized with $(T1T^*)_4$ reacted equally with minor repeat peptide $(DPNANPNV)_2$ and with major repeat peptide $(NANP)_3$ in ELISA (Fig. 3a). In contrast, immune sera of mice immunized with major repeat peptide $(BT^*)_4$ recognized the major repeats with four-fold higher titer than the minor repeat peptide. Skewing of the fine specificity of the antirepeat antibody response was also observed in BALB/c mice immunized with $(T1T^*)_4$ or $(BT^*)_4$ (Supplementary Table 1). The BALB/c mice immunized with $(T1T^*)_4$ developed eight-fold higher titer against T1 minor repeats compared to major repeats while immunization with $(BT^*)_4$ elicited eight-fold higher titer against B major repeats.

To examine the function of the antirepeat antibodies elicited by minor repeat peptide, the serum from $(T1T^*)_4$ immunized C57BL/6 mice was tested for ability to neutralize PfPb sporozoite infectivity in vitro. Sera of BALB/c mice were not analyzed in TSNA due to nonspecific resistance to *P. berghei* that comprise the genetic backbone of the PfPb transgenic sporozoites[21,22].

In the TSNA, the sera of C57BL/6 mice immunized with $(T1T^*)_4$ had high levels of neutralizing activity, with a 1:10 serum dilution inhibiting >90% sporozoite invasion of hepatoma cells (Fig. 3b). Inhibition by the $(T1T^*)_4$ immune serum was comparable to that observed with 25 µg/ml MAB 2A10. The neutralizing activity in $(T1T^*)_4$ serum was specific for CS repeats as no inhibition was obtained with serum obtained prior to immunization (hatched

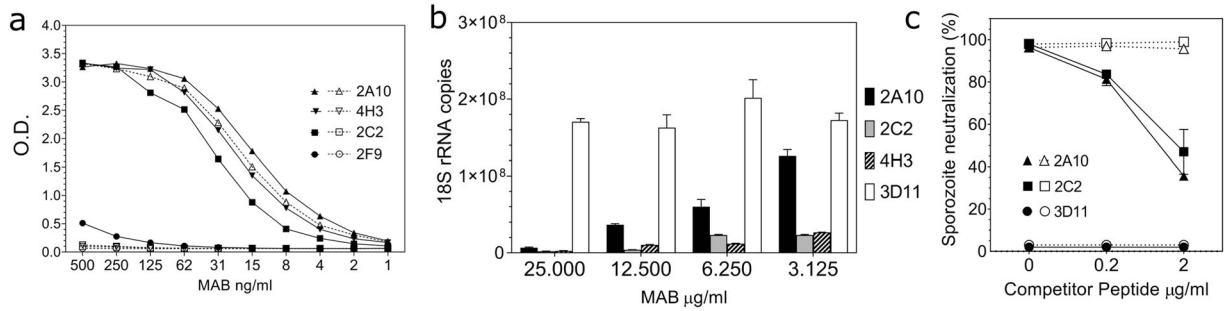

Fig. 2 Fine specificity and sporozoite-neutralizing activity of MAB derived from *P. falciparum* sporozoite-immunized mice. a MAB initially selected by *P. falciparum* sporozoite IFA were analyzed for fine specificity using CS repeat peptide ELISA. ELISA plates coated with either minor repeat peptide $(DPNANPNV)_2$ [closed symbols] or major repeat peptides $(NANP)_3$ [open symbols] were reacted with two-fold dilutions of MAB. **b** Sporozoite-neutralizing activity of MAB specific for minor repeats was determined by TSNA using transgenic *P. berghei* sporozoites expressing *P. falciparum* CS repeats (PfPb). Sporozoites were incubated with MAB 2C2 and 4H3, specific for *P. falciparum* CS minor repeats, or MAB 2A10 which cross-reacts with both the minor and major repeats. MAB 3D11 specific for *P. berghei* CS repeats served as negative control. Hepatoma cell extracts of duplicate wells obtained 48 h post sporozoite infection were pooled and tested in triplicate by qRT-PCR and results are shown as mean ± SD 18 S rRNA copy number. At all concentrations tested, the minor repeat-specific MABs 2C2 and 4H3, and MAB 2A10, significantly reduced *Plasmodium* rRNA copy numbers compared to negative control MAB 3D11 (one-way ANOVA with Dunnett's test, adjusted $P < 0.0001$). **c** A Peptide Competition TSNA was carried out by preincubating 25 µg/ml MAB 2C2 or MAB 2A10 with various concentration of minor repeat peptide $(DPNANPNV)_2$ [closed symbols] or major repeat peptide $(NANP)_3$ [open symbols]. MAB 3D11 served as negative control. Following incubation of MABs with competitor peptide for 1 h at 37 °C, PfPb sporozoites were added and TSNA was carried out per protocol using qRT-PCR. Results are shown as percent inhibition ± competitor peptide. For MAB 2A10 and MAB 2C2 there was a significant difference in rRNA copy number in presence of 2 µg/ml and 0.2 µg competitor minor repeat peptide versus no peptide (one-way ANOVA with Dunnett's test, adjusted $P < 0.0004$).

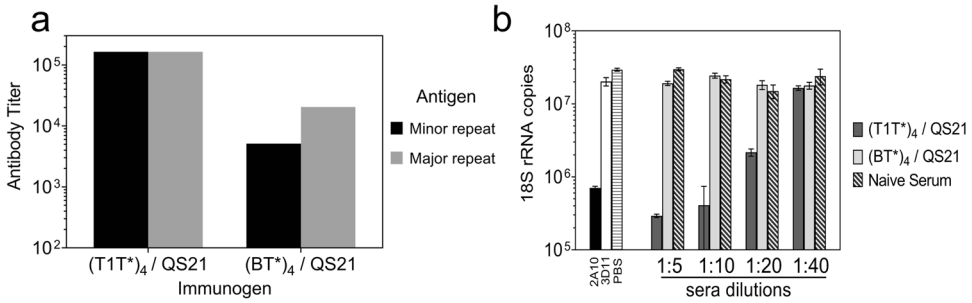

Fig. 3 Tetrabranched peptides containing major or minor repeats elicit antibodies that differ in sporozoite-neutralizing activity. C57BL/6 mice were immunized with three doses of tetrabranched peptide containing minor repeats, $(T1T^*)_4$, or major repeats, $(BT^*)_4$, using QS21 as adjuvant. **a** IgG ELISA titers against minor repeat peptide (black bars) or major repeat peptide (grey bars) were determined using serum obtained post 3rd dose. Endpoints were taken as final dilution of pooled serum giving OD in peptide-coated wells >3× OD of BSA-coated wells. **b** TSNA was carried out using 1:5–1:40 dilution of pooled serum from mice immunized with minor repeat peptides (black bars) or major repeat peptides (grey bars). MAB 2A10 and MAB 3D11 (25 µg/ml) served as positive and negative controls, respectively. Results are shown as 18 S rRNA copy number mean ± SD measured by qRT-PCR. Inhibition by $(T1T^*)_4$ immune sera was significantly greater than $(BT^*)_4$ antisera at 1:5, 1:10 or 1:20 dilution (one-way ANOVA with Tukey's test, adjusted $P = 0.0004$).

bars), which gave 18 S rRNA copy number similar to control wells without serum (PBS, horizontal hatched bar). In contrast, serum of C57BL/6 immunized with the major repeat peptide, $(BT^*)_4$, did not inhibit sporozoite infectivity at the lowest dilution tested (1:5). Since previous studies have demonstrated that high levels of antibodies elicited by (NANP)n constucts can inhibit *P. falciparum* sporozoites[12–14,23], the lack of neutralizing activity of $(BT^*)_4$ serum suggests that magnitude, as well as specificity of the antirepeat antibodies, is important in neutralizing sporozoite infectivity.

Linear peptide containing minor repeats also elicits sporozoite-neutralizing antibodies

To determine if differences in the fine specificity of the antirepeat antibodies were dependent on peptide configuration, mice were immunized with linear T1T* peptides using various adjuvant formulations. Previous murine studies had found that immunogenicity of related T1BT* sequence was comparable using either branched or linear peptides as immunogens[24]. Following three s.c. injections of linear T1T* peptide in various adjuvants in C57BL/6 and BALB/c mice, the linear T1T* peptide was found to elicit antirepeat ELISA titers comparable in magnitude to those elicited by the more complex tetrabranched $(T1T^*)_4$ (Fig. 3a, Supplementary Fig. 1). Linear peptides formulated in oil-in-water adjuvants, Montanide ISA 720 or ISA 51, or by the inclusion of the TLR 9 agonist, CpG, elicited ELISA titers that were an order of magnitude higher than peptide without adjuvant (PBS). With all adjuvant formulations, immune sera IFA titers with *P. falciparum* sporozoites were similar in magnitude to the repeat ELISA titer.

In a second experiment, a head-to-head comparison of tetrabranched $(T1T^*)_4$ peptide versus linear T1T* peptide formulated in ISA 720 adjuvant was shown to elicit comparable antirepeat antibodies responses with ELISA GMT 1.6×10^4 and 5.6×10^4, respectively. TSNA demonstrated that immune sera from C57BL/6 mice immunized with either linear T1T* peptide or with $(T1T^*)_4$ peptide significantly decreased parasite rRNA copy numbers when compared to Day 0 sera (adjusted $P = 0.0024$ and $P = 0.042$, respectively) (Supplementary Fig. 2).

The antirepeat antibodies elicited by immunization with the linear T1T* peptide were also functional in vivo (Fig. 4b). Challenge

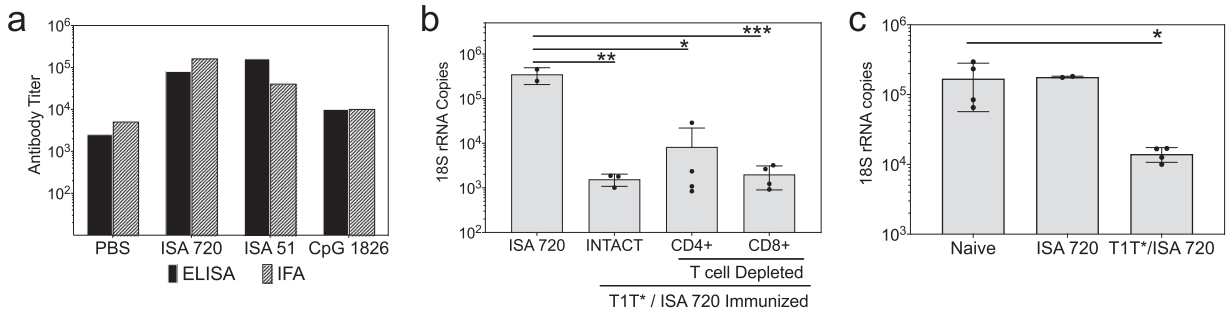

Fig. 4 Linear peptides containing minor repeats elicit sporozoite-neutralizing antibodies. a C57BL/6 mice were immunized with three doses of linear T1T* peptide without adjuvant (PBS), or with adjuvant comprised of oil-in-water emulsions (ISA 720, ISA 51) or a TLR 9 agonist CpG. Pooled sera were tested against repeat peptide by ELISA or against *P. falciparum* sporozoites by IFA. **b** Mice immunized with three doses of T1T*/ISA 720 were challenged by exposure to the bites of PfPb-infected mosquitoes. A subset of immunized mice were treated with MAB specific for CD4 + or CD8 + T cells prior to challenge. Parasite 18 S rRNA copy numbers in liver extracts obtained 40 h post-challenge were measured by qRT-PCR. Bars show 18 S rRNA copy number mean ± SD and individual mice are shown as circles. There was a statistically significant decrease in parasite 18 S rRNA copy numbers in all peptide-immunized groups compared to adjuvant only control (one-way ANOVA log-transformed values with Tukey's test, adjusted $^{**}P = 0.0012$, $^{*}P = 0.0019$, $^{***}P = 0.0010$). The difference in parasite rRNA copy number between intact and CD4 + or CD8 + T-cell-depleted mice was not statistically significant (one-way ANOVA log-transformed values with Tukey's test, adjusted $P = 0.8511$ and $P = 0.9967$, respectively. **c** Pooled immune serum obtained post 3rd dose of T1T*/ISA 720 was passively transferred by i.v. injection to naïve recipients prior to challenge by exposure to the bites of PfPb-infected mosquitoes. Control mice received pooled sera from naïve mice or mice immunized with ISA 720 adjuvant only. Due to limited supply, only two mice received serum from mice immunized with ISA 720 adjuvant only. Parasite 18 S rRNA copy numbers in liver extracts obtained 40 h post-challenge were measured by qRT-PCR. Results shown as 18 S rRNA copy number mean ± SD with individual mice shown as closed circles. Recipients of T1T*/ISA 720 immune serum had a statistically significant decrease in parasite 18 S rRNA copy numbers when compared to recipients of naïve serum (unpaired t-test in log-transformed values, $P = 0.0010$).

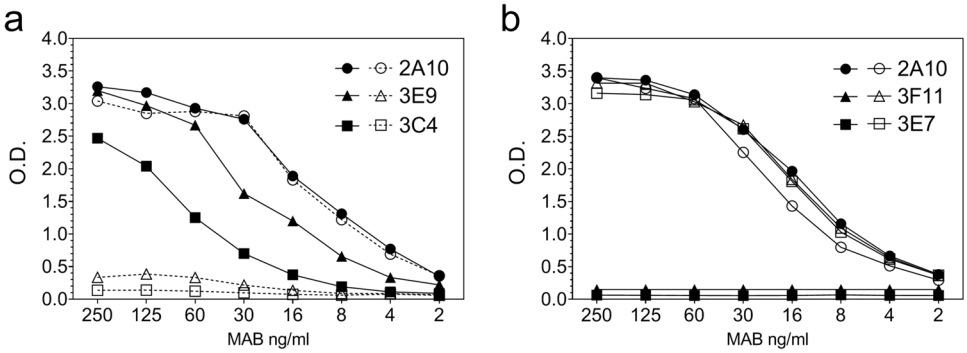

Fig. 5 Fine specificity of MAB derived from mice immunized with linear minor or major repeat peptides. a MABs 3E9 and 3C4 derived from mice immunized with (T1T*) minor repeat peptide, or **b** MABs 3F11 and 3E7 derived from mice immunized with the (BT*) major repeat peptide, were tested by ELISA against wells coated with minor repeat peptide (closed symbols) or major repeat peptide (open symbols).

of T1T* /ISA 720 immunized C57BL/6 mice by exposure to bites of mosquitoes infected with PfPb transgenic parasites demonstrated >90% reduction of liver parasite burden when compared to adjuvant only control (adjusted $^{*}P < 0.012$). Protection in the challenged mice was antibody mediated, as depletion of CD4 + or CD8 + T cells prior to challenge did not abrogate immune resistance. Similar to intact T1T* immunized mice, T-cell-depleted T1T* immunized mice had >90% reduction of parasite 18 S rRNA copy number when compared to mice immunized with ISA 720 adjuvant only. There was no significant difference in rRNA copy numbers in CD4- and CD8-depleted mice compared to intact mice (adjusted $P = 0.8317$ and $P = 0.7685$, respectively). The lack of a direct role for cell mediated immunity in resistance to challenge is consistent with our previous studies in repeat peptide-immunized mice[24].

To confirm that in vivo sporozoite-neutralizing activity was antibody mediated, serum of mice immunized with T1T* /ISA 720 was passively transferred by intravenous injection (i.v.) into naïve recipients prior to challenge by exposure to the bites of PfPb-infected mosquitoes (Fig. 4c). The recipients of T1T*/ISA 720 immune serum had >90% reduction of parasite density in liver compared to naïve mice ($^{*}P = 0.0010$), confirming that protection in the T1T* immunized mice was antibody mediated.

MAB specific for major or minor repeats derived from peptide-immunized mice

To further define the fine specificity of the antirepeat antibody response, MAB were derived from the mice immunized with either the T1T* minor repeat or BT* major repeat peptides. When screened by ELISA, MAB specific for either minor or major repeats could be readily obtained from the mice immunized with each immunogen (Fig. 5). MABs 3E9 and 3C4, two representative MAB from T1T* immunized mice, reacted with minor repeat (closed symbols) but not major repeats (open symbols) (Fig. 5a). Conversely, two representative MABs from BT* immunized mice, MABs 3F11 and 3E7, gave high levels of reactivity with major, but not minor repeats (Fig. 5b). MAB 2A10, cross-reacted with both minor and major repeats and confirms comparable peptide coating in each of the peptide ELISA.

When tested in TSNA, the minor repeat-specific MAB 3C4 inhibited PfPb sporozoite invasion into hepatoma cells at levels comparable to or greater than MAB 2A10 derived from

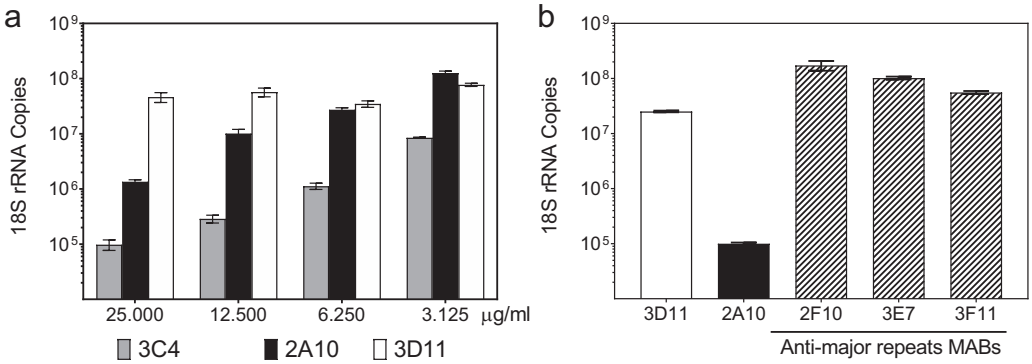

Fig. 6 Sporozoite-neutralizing activity of MAB derived from mice immunized with minor or major repeat peptides. a TSNA was carried out with varying concentrations of MAB 3C4, specific for minor repeats, and MAB 2A10 that cross-reacts with minor and major repeats. MAB 3D11 specific for *P. berghei* repeats served as negative control. Results are shown as 18 S rRNA copy number mean ± SD as measured by qRT-PCR. Incubation with MAB 3C4 resulted in statistically significant lower parasite rRNA copy number at all concentrations tested when compared to MAB 3D11 (One-way ANOVA with Dunnett's test. adjusted $P < 0.0001$). MAB 2A10 at 25 µg/ml and 12.5 µg/ml significantly decreased 18 S rRNA copy number (one-way ANOVA with Dunnett's test, adjusted $P < 0.0001$). **b** TSNA was carried out using 12.5 µg/ml of MAB specific for major repeats (2F10, 3E7, 3F11), MAB 2A10 or MAB 3D11. Results are shown as mean ± SD parasite 18 S rRNA copy number as measured by qRT-PCR. The parasite rRNA copy numbers in cultures containing major repeat MABs were not decreased compared to MAB 3D11. As expected, the positive control MAB 2A10 reduced 18 S rRNA copy number >90% when compared to MAB 3D11 (one-way ANOVA with Dunnett's test, adjusted $P < 0.0001$).

P. falciparum sporozoite-immunized mice (Fig. 6a). In contrast, several MAB derived from mice immunized with the major repeat peptide were not inhibitory at the highest concentration tested (12.5 µg/ml) (Fig. 6b). Therefore, as illustrated by these major repeat-specific MABs, high levels of antirepeat antibodies as measured by ELISA do not necessarily predict neutralizing activity against viable parasites.

DISCUSSION

A recent malaria vaccine milestone was reached with the WHO supported pilot implementation of a CS-based vaccine, RTS,S, in three African nations[25]. RTS,S vaccine efficacy was 30–50% in infants and children, respectively[26], and efforts to improve CS-based vaccines are ongoing in many laboratories. While evidence for protection mediated by antirepeat antibodies has been obtained in RTS,S clinical trials[23,26], a correlate of protective immunity for CS-based vaccines has not yet been defined. A better understanding of the antibody response to *P. falciparum* CS repeats is required to design more efficacious vaccines.

In testing of polyclonal and monoclonal antibodies, we found variation in both fine specificity and function of antibodies specific for *P. falciparum* CS repeats. MAB derived from mice immunized with *P. falciparum* sporozoites, such as MAB 2A10, reacted with both major and minor repeats, while other MAB skewed to recognition of the minor repeats (Fig. 2a). MAB specific for the minor repeats neutralized PfPb sporozoite infectivity in vitro similar to MAB 2A10 and preincubation with minor, but not major, repeat peptide decreased sporozoite-neutralizing activity (Fig. 2b, c).

Antibodies that recognized the minor CS repeats could also be elicited in C57BL/6 mice by immunization with peptide immunogens containing the T1 minor repeats (Figs. 3a, 4a). A similar skewing of antibodies to minor repeat could be elicited by T1T* peptide immunogens in BALB/c mice (Supplementary Table 1) indicating that ability to elicit antibodies to the minor repeats is not limited by genetic background.

The antibodies elicited by minor repeat peptide immunogens neutralized sporozoite infectivity in vitro in a dose-dependent manner (Fig. 3b). Consistent with in vitro results, protection in C57BL/6 mice immunized with a linear T1T* peptide was antibody mediated (Fig. 4b, c). T-cell-depleted T1T*-immunized mice had similar levels of protection as intact mice and passive transfer of immune serum from these mice protected naïve recipients following PfPb challenge. Minor repeat-specific MAB derived from T1T* immunized mice efficiently neutralized PfPb sporozoite infectivity in vitro (Fig. 6a). Of note, several MAB specific for the major repeats, isolated from mice immunized with BT* major repeat peptide, lacked neutralizing activity when tested in TSNA (Fig. 6b), illustrating that not all antirepeat antibodies are equally effective in neutralizing sporozoite infectivity.

The current findings demonstrate that the *P. falciparum* CS minor repeat region contains an important sporozoite-neutralizing epitope. The functional variations of the antirepeat antibody response could have potentially significant biological consequences. Vaccine efficacy would be lower if the majority of antibodies elicited by vaccines recognize only the more numerous major repeats and have minimal neutralizing capacity, as in Fig. 6b, in contrast to specifically targeting, or cross-reacting, with the minor repeats, as in Fig. 6a. The presence of protective and nonprotective epitopes within the repeats potentially provides an explanation for the paradox of why the parasite would express a highly immunogenic repeat region. As originally proposed by Anders for repeat epitopes of blood-stage antigens[27], the more numerous major repeats may potentially serve as an immunological "smoke screen" to divert responses away from the development of inhibitory antibody responses. The design of immunogens that contain only minor repeats in the absence of major repeats, as in the T1T* peptide immunogens, may drive responses toward a higher proportion of functional minor repeat-specific antibodies and away from major repeat antibodies that are potentially non-neutralizing. Analysis of a larger panel of MAB is required to determine if the frequency of neutralizing vs non-neutralizing antibodies is increased following immunization with minor repeat peptide as compared to major repeat peptides.

A mechanism for why antibodies to minor repeats may be more protective was suggested by recent studies using human MABs derived from sporozoite-immunized malaria-naïve or African adults[28,29]. These human MAB were dual specific and bound to a unique junctional epitope within the KQPADGNPDPNANP sequence preceding the CS repeat region, as well as to NANP repeats. Passive transfer of human MAB reactive with the junctional epitope derived from *P. falciparum* sporozoite-immunized Tanzanian adults protected humanized mice against *P. falciparum* sporozoite challenge[28]. The junctional epitope is located three amino acids upstream of a previously identified proteolytic CS cleavage site in Region 1[10]. A human MAB specific

for the junctional epitope blocked cleavage of *P. falciparum* CS protein[29]. MAB 2A10, which cross-reacts with both major and minor repeats, can also inhibit the proteolytic processing of CS protein[30].

The human MABs specific for the junctional epitope recognized two distinct minimal epitopes within the junctional region, DPNANP[28] and NPN[29]. BALB/c mice immunized with a $(NPDP)_{19}$ peptide containing the junctional epitope, elicited high titers of antibodies specific for the junctional epitope that were poorly reactive with repeats and did not have sporozoite-neutralizing activity[28]. Additional studies by Oyen et al. have shown that dual binding of antibody to both the junctional and repeat epitopes was required for neutralizing activity[31].

The minor repeat peptide DPNA**NPN**VDPNA**NPN**V, used as immunogen in the current studies (Fig. 1), contains two copies of the junctional epitopes identified by Kisalu et al.[29] (indicated in bold) and Tan et al. (underlined)[28]. Whether the antibodies specific for the minor repeats, or MAB that cross-react with minor repeats such as MAB 2A10, can also recognize the junctional epitope and thus show dual specificity of binding to CS remains to be determined. Studies to examine affinity of larger panels of murine anti-T1 MAB and to identify key residues in the T1 epitope by positional scanning and generation of peptide-antibody complexes are planned.

Recent immunoglobulin gene sequencing studies have also detected a range of fine specificity of human recombinant MAB specific for CS repeats derived from volunteers immunized by exposure to *P. falciparum* sporozoites under chloroquine prophylaxis[32]. The human MAB recognized multiple epitopes within the *P. falciparum* CS repeat region, including NVDP and NANPNVDP. The current studies in sporozoite- and peptide-immunized mice are consistent with the diversity of antirepeat antibodies found in the MAB from sporozoite-immunized human volunteers and support the use of the more experimentally amendable small rodent model for analysis of antibody responses to *P. falciparum* CS repeat region relevant to the design of more efficacious human malaria vaccines.

The variable fine specificity and function of the antirepeat antibodies detected in the current studies has significant implications for the design of subunit vaccines that elicit inhibitory, rather than nonfunctional, antirepeat antibodies. Epitope-focused vaccines based on the CS minor repeat, such as the T1T* immunogen, potentially can focus the immune responses on sporozoite-neutralizing epitopes and reduce immune response to more numerous but potentially less protective major repeats. Our current studies, showing that immunization with minor repeat peptides elicits neutralizing antibodies that inhibit sporozoite infectivity in vitro and in vivo, combined with recent findings with human MABs[28,29,32], provide a framework for the design of subunit vaccines that elicit optimal levels of neutralizing antibodies to more effectively target CS protein on *P. falciparum* sporozoites.

METHODS

Ethics statement

Studies in mice were approved by the Institutional Animal Care and Use Committee, NYU School of Medicine (Protocol 1501081) and conducted following recommendations specified in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Peptides

For the peptide constructs, the *P. falciparum* CS major repeats were represented by the $(NANP)_3$ sequence (designated "B" epitope) (Fig. 1). The 5′ minor repeat region, comprised of alternating NVDP and NANP repeats, was represented by the peptide sequence (DPNANPNVDPNANPNV). The minor repeat sequence was designated T1, as it also contains the first CS-specific CD4 + T helper cell epitope identified in sporozoite-immunized human volunteers[33]. The major or minor repeat sequences were synthesized in tandem with a malaria universal Th cell epitope, EYLNKIQNSLSTEWSPCSVT, designated T* (Fig. 1). The T* T helper cell epitope, located in the C-terminus of the *P. falciparum* CS protein (NF54 isolate), is recognized in the context of multiple murine and human class II molecules and was included in all constructs to overcome genetic restrictions[34–36]. The peptide immunogens were synthesized either as a tetrabranched construct containing the respective repeat and the T* sequences in each of four branches, or as a linear peptide using standard F-Boc peptide chemistry (AnaSpec, San Jose, CA).

Antibodies

Fine specificity and function of antirepeat antibodies was carried out using polyclonal sera elicited by peptide immunization of C57BL/6 ($H\text{-}2^b$) and BALB/c mice ($H\text{-}2^d$) mice (Jackson Labs, Bar Harbor Maine). BALB/c mice were included in the initial immunogenicity studies to examine genetic restriction of humoral immunity elicited by the peptide immunogens. Since BALB/c are nonresponders to CS repeats, the peptides were synthesized to contain T*, a universal T helper cell epitope[36–39] (Fig. 1). BALB/c are high responders to the T* T helper cell epitope, while C57BL/6 are high responders to CS repeats and the T* epitope[36,39].

Mice were immunized subcutaneously (s.c.) at 14 day intervals with three doses of 50 µg peptide with or without various adjuvants. Adjuvants included QS21, a purified saponin derivative (Antigenics Inc., Woburn, MA); water-in-oil adjuvants, either Montanide ISA 51, a mineral oil emulsion comparable to Freund's Incomplete Adjuvant, or ISA 720 a natural metabolizable nonmineral oil and mannide monooleate emulsifier (Seppic, Inc, Fairfield, NJ); or CpG 1826, a TLR 9 agonist comprised of synthetic oligodeoxynucleotide containing unmethylated CpG motifs (InvivoGen, San Diego, CA).

MAB 2A10, a monoclonal antibody specific for CS repeats (ATCC BEI MRA-183), generated in our laboratory from mice immunized with *P. falciparum* sporozoites[18], was included as a standard. MAB 2A10 has been studied extensively in the analysis and development of CS subunit vaccines[40–43]. MAB 3D11, specific for *P. berghei* CS repeats[44], was included as a negative control, as the PfPb transgenics express *P. falciparum* CS repeat region within the *P. berghei* CS protein[17]. MAB 2A10 neutralizes infectivity of PfPb sporozoites but not *P. berghei* sporozoites and, conversely, MAB 3D11 neutralizes *P. berghei*, but not PfPb sporozoites[17].

Serological assays

MAB fine specificity and antirepeat IgG endpoint titers in immune sera were determined by ELISA using wells coated with synthetic tetrabranched peptides representing either the major repeat $(NANP)_3$ peptide or minor repeat $(DPNANPNV)_2$ peptide. Two-fold dilutions of MAB or serum were tested and bound antibody was detected by reaction with HRP-labeled anti-mouse IgG (MP Biochemicals, Solon, OH) and TMB/H_2O_2 substrate (KPL, SeraCare; Gaithersburg, MD). Endpoints were determined as final dilution of serum giving OD > 3X BSA-coated wells. A >4-fold difference in antibody geometric mean titers was considered meaningful. Reactivity with *P. falciparum* sporozoites was determined by Indirect Immunofluorescence Assay (IFA), as in previous studies[39,45].

In vitro studies of antibody function used a Transgenic Sporozoite Neutralization Assay (TSNA), as previously described[46–48]. In the PfPb transgenic rodent parasites, the *P. berghei* CS repeat region has been replaced with the entire *P. falciparum* CS repeat region, $(NANPNVDP)_3(NANP)_{15}NVDP(NANP)_{19}$, as well as 26 aa of the N-terminus, NNEDNEKLRKPKHKKLKQPGDGNPDP preceding the repeat region[17]. For TSNA, MAB or polyclonal serum at various dilutions were incubated with 2×10^4 PfPb sporozoites for 40 min on ice prior to addition to confluent human hepatoma HepG2 cells (ATCC HB 8065) in cRPMI (RPMI 1640 supplemented with 10%FBS, 50U Penicillin/50 µg Streptomycin, sodium pyruvate, non-essential amino acids, all from Gibco, ThermoFisher, Waltham, MA). Plates were incubated at 5% CO_2 for 48 h, with media change at 24 h, followed by extraction of total RNA (PureLink, RNA Mini Kit, ThermoFisher, Waltham, MA). The amounts of parasite 18 S rRNA in each culture extract was quantitated by real-time PCR (qRT-PCR) using cDNA primers specific for 18 S ribosomal RNA (rRNA)[46,49]. The parasite 18 S rRNA copy number was calculated based on a standard curve generated with known amounts of plasmid 18 S cDNA. To assess the peptide specificity of antibodies mediating sporozoite neutralization, a peptide competition TSNA was carried out in which MAB (25 µg/ml) were pre-incubated with various concentrations of either minor repeat peptide $(DPNANPNV)_2$ or major repeat peptide $(NANP)_3$ for one hour prior to addition of PfPb sporozoites and performance of TSNA per protocol.

For in vivo studies, peptide-immunized and naive C57BL/6 mice were challenged by exposure to the bites of 10–15 PfPb-infected mosquitoes. BALB/c mice were not used in these studies due to nonspecific resistance to *P. berghei* sporozoites[21,22]. Following sporozoite challenge, total RNA was extracted from the livers 40-h post-challenge and levels of parasites in the liver quantitated by qRT- PCR, as for in vitro TSNA. In passive transfer studies, immune serum was passively transferred to naïve recipients by intravenous injection 24 h prior to exposure to the bites of PfPb-infected mosquitoes.

Previous studies have found that >90% reduction in PfPb parasite copy number measured by qRT-PCR is associated with delayed parasitemia or sterile immunity[24,47,48]. The >90% cutoff is consistent with early studies in which i.v. injection of known numbers of sporozoites of rodent and non-human primate Plasmodium species demonstrated that a log reduction in parasite numbers results in a delay in prepatent period or absence of infection[50,51].

Statistical analysis

Statistical analysis was carried out using GraphPad Prism version 8.4.2. Differences between control and experimental groups were determined significant by one-way ANOVA with post-hoc Dunnett's or Tukey's test to correct for multiple comparisons or unpaired *t*-test. Normal distribution and equal standard deviation were corroborated. Differences were considered statistically significant at $P < 0.05$. Adjusted P values for multiple comparison are reported.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

All data presented in the studies described here are included in this article or will be available upon request from the corresponding author.

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AUTHOR CONTRIBUTIONS

E.N., J.M.C.C. and R.M. planned experiments and wrote manuscript; J.M.C.C., R.M. and C.O. analyzed peptide immunogenicity and MAB fine specificity; C.O., R.A. and R.M. performed functional antibody assays using PfPb transgenic parasites. All authors reviewed and approved the manuscript. J.M.C.C. and R.M. contributed equally to work in this manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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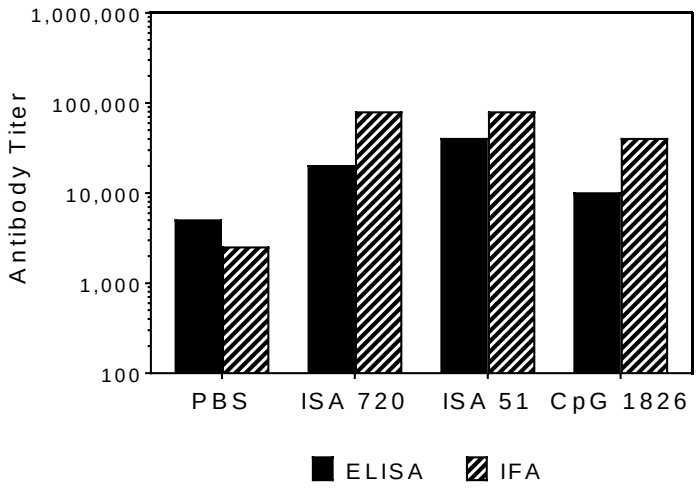
Supplementary Table and Figures

Supplementary Table 1: Fine specificity of anti-repeat antibodies elicited in BALB/c mice immunized with tetrabranched peptides containing CS major or minor repeats		
Immunogen[1]	**ELISA Titers[2]**	
	T1 Minor Repeats	B Major Repeats
$(T1T^*)_4$	81,920	10,240
$(BT^*)_4$	5,120	40,960

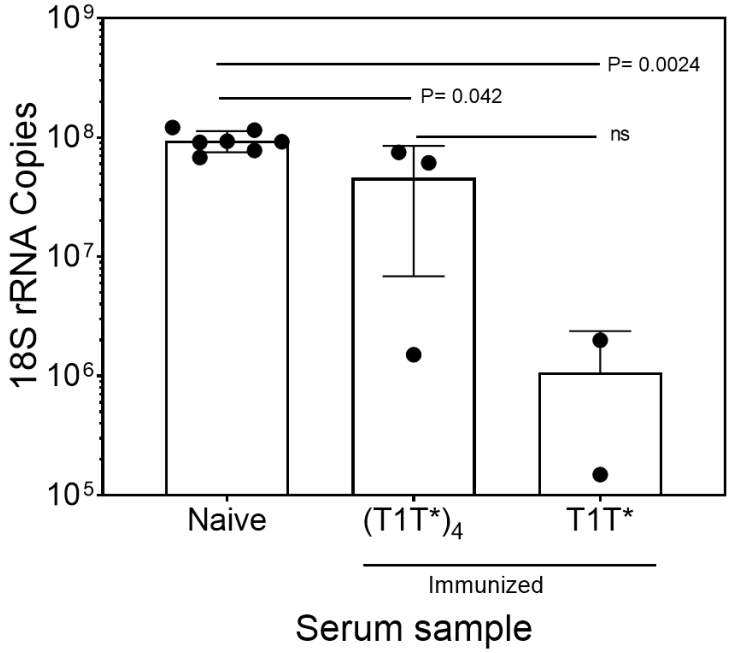
1. BALB/c mice were immunized s.c. with three doses of $(T1T^*)_4$ peptide containing minor repeats $(DPNANPNV)_2$, or $(BT^*)_4$ peptide containing major repeats $(NANP)_3$, synthesized in tandem with universal Th epitope T*.

2. IgG titers against minor or major repeat peptides measured in pooled serum obtained post 3rd dose. ELISA titers shown as highest dilution of serum giving OD against repeat peptide >3X OD BSA coated wells. A $\geq$ four-fold difference in titer was considered meaningful.



Supplementary Figure 1. Antibody responses in BALB/c mice immunized with linear peptide T1T*

BALB/c mice were immunized with three s.c. doses of linear T1T* peptide without adjuvant (PBS), or with adjuvant comprised of oil-in-water emulsions (ISA 720, ISA 51) or a TLR 9 agonist CpG. Two-fold dilutions of pooled sera were tested against tetrabranched $(T1B)_4$ repeat peptide by ELISA with endpoint titer taken as final serum dilution giving >3X OD of BSA coated wells, or by IFA using *P. falciparum* sporozoites.



Supplementary Figure 2: Sporozoite neutralizing activity of serum derived from mice immunized with tetrabranched $(T1T^*)_4$ or linear T1T* peptides.

TSNA was used to measure neutralizing antibody elicited by tetrabranched peptide $(T1T^*)_4$ versus linear peptide T1T* formulated in ISA 720. Results shown as mean +/- SD of 18S rRNA measured by qRT-PCR. Immune sera of C57BL/6 mice immunized with either tetrabranched or linear T1T* was significantly lower when compared to naive sera (one-way ANOVA with Tukey's test, adjusted P values).

Article 2

This article addresses the second objective to characterize the cellular responses elicited by the RTS,S/AS01$_E$ vaccine booster dose at the peak response and 11 months later and identify immune correlates of malaria protection or risk. The sub-objectives to evaluate the effect of epidemiological factors on immunogenicity and protection, and to assess associations between cytokine responses and antibodies were also addressed. The results of this research have been accepted in the following article:

Robert A. Mitchell, Dídac Macià*, Chenjerai Jairoce*, Maxmillian Mpina, Akshayata Naidu, Ana Chopo-Pizarro, Miquel Vázquez-Santiago, Joseph J. Campo, Pedro Aide, Ruth Aguilar, Claudia Daubenberger, Carlota Dobaño, Gemma Moncunill. ***Effect of RTS,S/AS01$_E$ vaccine booster dose on cellular immune responses in African infants and children.*** *Accepted by npj Vaccines.*

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Running title: Cellular immune response elicited by RTS,S/AS01E booster

Effect of RTS,S/AS01E vaccine booster dose on cellular immune responses in African infants and children

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ABSTRACT

RTS,S/AS01E, the first approved malaria vaccine, demonstrated moderate efficacy during the phase 3 pediatric trial. We previously investigated cell-mediated immune (CMI) responses following the primary 3-dose immunizations and now report responses to the booster dose given 18 months later. Thirty CMI markers were measured by Luminex in lymphocytes from 709 children and infants following RTS,S/AS01E antigen stimulation, which were assessed for associations with malaria risk and antibodies one month post-booster and one year later. IL-2, IFN-γ, IL-17, IL-5, and IL-13 were associated with RTS,S/AS01E vaccination after booster, and IL-2 responses to circumsporozoite protein (CSP) remained higher after one year. IL-2 was associated with reduced malaria risk in one cohort, and IL-10 was associated with increased risk in infants. Anti-CSP IgG and IL-2 were moderately correlated one year after booster. This study highlights the moderate cell-mediated immunogenicity of the RTS,S/AS01E booster dose that aligns with partial recovery of RTS,S/AS01E vaccine efficacy.

KEYWORDS

malaria; vaccine; booster; cytokines; antibodies; cellular immune responses; immunity

INTRODUCTION

The decades-long effort to develop a malaria vaccine reached a historic achievement in 2021, when RTS,S/AS01E (RTS,S), the first malaria vaccine to pass a pediatric phase 3 clinical trial, was recommended by the World Health Organization for use in children living in regions with moderate-to-high malaria transmission [1]. The pre-erythrocytic RTS,S vaccine contains a portion of the central repeating amino acid sequence NANP and C-terminal region of the P. falciparum circumsporozoite protein (CSP), fused to and co-expressed with unfused hepatitis B surface antigen (HBsAg) and formulated with AS01E, a liposomal-based adjuvant containing Monophosphoryl lipid A and QS-21 [2]. RTS,S was designed to generate strong humoral and cell-mediated immunity [3]; nevertheless, identifying RTS,S' immune correlates of protection has been an ongoing challenge [4].

The phase 3 trial consisted of a 3-dose primary immunization regimen (month [M] 0, M1, and M2) followed by a booster dose at M20. The antibody type and titer level generated by RTS,S primary vaccination towards CSP have demonstrated a correlation with protection in children during the phase 3 trial [5–7]. While the booster dose restored vaccine efficacy [8] from 18.3 to 25.9% in infants and 28.3 to 36.3% in children, unexpectedly, IgG antibody titers to the NANP repeat region of CSP post-booster were lower compared to the response post-primary vaccination [5,9]. B cell responses are likely supported by CD4+ T cells; therefore, understanding the role of RTS,S-induced cellular responses in generating and maintaining antibodies is essential.

RTS,S studies using controlled human malaria infection (CHMI) of malaria-naïve adults [10–12] or in field studies with children [13–16] have made some advances toward understanding RTS,S-induced cellular responses. However, its association and mechanisms of malaria protection remain unclear [17]. CSP-specific CD4+ T cells appear to be consistently associated with RTS,S, specifically with the expression of interleukin (IL)-2 and tumor necrosis factor (TNF) identified during RTS,S phase 2 trials [11,13–15,18]. In children following primary vaccination during the RTS,S phase 3 trial, we detected higher frequencies of CSP- and HBsAg-specific central memory and effector memory CD4+ T cells co-expressing IL-2,

TNF and co-stimulatory molecule CD40L, as well as, HBsAg-specific CD4+ T cells producing interferon (IFN)-γ and IL-17, and effector memory CD4+ T cells producing IL-21, a T follicular helper (TFH) cytokine, to both vaccine antigens [19]. In a CHMI study in malaria naïve adults, RTS,S also induced TFH cells producing IL-21, which were associated with protection [20]. In the RTS,S phase 3 clinical trial, following in vitro PBMCs vaccine antigen stimulations, we found primary RTS,S vaccination to be associated with CSP specific IL-2 and IL-5 secretion. TH1 cytokines, particularly IFN-γ, IL-15, and granulocyte-macrophage colony-stimulating factor (GM-CSF) correlated with malaria protection, while TH2 cytokines IL-5 and regulated on activation normal T cell expressed and secreted (RANTES), were associated with risk [16].

No data exists for RTS,S-induced cellular immune responses following the booster dose during the pediatric phase 3 trial. Our study aimed to assess the RTS,S-induced cellular responses following the booster dose at M21 and the maintenance one year later at M32 from two African sites, which is an essential step towards filling this gap. We evaluated associations between markers and the risk of malaria 11 months later, and considered the effect of epidemiological and clinical factors and previous malaria episodes on the response to the booster dose. Additionally, we explored associations of cytokine responses with RTS,S-induced antibodies after the booster dose. Identifying factors associated with RTS,S immunogenicity, and protective cellular responses is critical to designing effective, longer-lasting, second-generation malaria vaccines.

RESULTS

Study population

At M21, a total of 709 infants and children from Bagamoyo, Tanzania, and Manhiça, Mozambique, were analyzed between the three vaccine groups that either received the RTS,S booster (R3R) or comparator vaccine (R3C and C3C) at M20 (Table 1; Supplementary Figure 1). The median age was 26.7 months, and approximately 52% of participants were female. Previous malaria cases within 1-year before M20 occurred in 6.6% of participants; hemoglobin levels measured at M20 showed that 29.5% of the participants had anemia (<10 g/dL), and 6.2% were malnourished according to weigh-for-age Z-scores (WAZ score <-2).

Immunogenicity of the RTS,S booster dose at M21 and M32

To identify markers of immunogenicity elicited by RTS,S booster dose, we compared RTS,S antigen cytokine concentration ratios from R3R (RTS,S-boosted) versus R3C (RTS,S-unboosted) and R3R versus C3C (Comparator) at M21 and M32. At M21, CSP and HBsAg ratios were significantly higher for IL-2 in R3R group compared to C3C **(Figure 1a)** whereas the increase relative to the R3C was not statistically significant. CSP-stimulated IL-13 ratios were also higher in the R3R compared to the C3C group but not statistically significant after adjustment for multiple testing (P=0.007, Padj=0.07). In addition, HBsAg-stimulated cytokine ratios were also significantly higher for IFN-γ, IL-5, and IL-13 in R3R than in C3C and the booster dose was found to significantly increase IL-17 in the R3R group compared to both R3C and C3C **(Figure 1a)**. At M32, CSP responses were maintained for IL-2 which were significantly higher in R3R compared to both R3C and C3C; and HBsAg ratios were higher for IL-2, IFN-immunogenicity markers (IL-2, IL-13, IFN-γ, and IL-5 in the R3R compared to C3C **(Figure 1a)**.

Box plots displaying individual data points for the primary immunogenicity markers IL-2, IL-13, IFN-γ, IL-17 and IL-5 show an increasing trend for R3C (RTS,S-unboosted) and R3R (RTS,S-boosted) over C3C (Comparator) for both CSP and HBsAg ratios at M21 and M32 **(Figure 1b)**. Analysis of crude concentrations shows a similar pattern of significantly elevated cytokines following CSP and HBsAg stimulation as was found for the antigen ratios **(Supplementary Figure 2)**. Correlations between levels of CSP-stimulated versus HBsAg-stimulated primary immunogenicity markers assessed at M21 showed low to moderate positive correlations between the two vaccine antigens in all three vaccine groups **(Supplementary Figure 3)**.

Factors affecting RTS,S immunogenicity at M21 and M32

To evaluate if there were clinical or demographic factors affecting the above RTS,S immunogenicity markers (IL-2, IL-13, IFN-γ, IL-17 or IL-5), we analyzed the effect modification of age, sex, site, previous malaria exposure, anemia, and WAZ scores on the cytokine increases in R3R (RTS,S-unboosted) versus C3C (Comparator) group (Figure 2). At M21, no statistically signifi-

cant effect modification by any condition on immunogenicity markers was observed (interaction Padj>0.05). However, unadjusted analysis showed age and site were effect modifiers of vaccination immunogenicity, specifically for CSP-stimulated IL-5 for age (interaction P=0.02; Padj=0.32) and IFN-γ (interaction P=0.005; Padj=0.14) and IL-17 (interaction P=0.02; Padj=0.32) for site (Figure 2). In stratified analyses, CSP-specific IL-13, IFN-γ, IL-17 were associated with RTS,S booster for children (P=0.03, P=0.006, P=0.02, respectively) but not for infants (P=0.78, P=0.9, P=0.33, respectively), who had increased CSP-specific IL-5 by vaccination (P=0.02) (Figure 2). For HBsAg stimulation, age was an effect modifier of vaccination on IL-17 and IL-13 responses (RTS,S booster induced IL-17 and IL-13 in children but not in infants), although the interaction was not statistically significant after adjustment for multiple testing (interaction P=0.003; Padj=0.14 and P=0.006; Padj=0.14, respectively) **(Figure 2)**.

At M32, no immunogenicity effect modifiers were identified for CSP stimulations (Supplementary Figure 4). However, age and site were associated with significantly different RTS,S-induced HBsAg IL-2 responses (Supplementary Figure 4), though significance was lost after adjusting for multiple comparisons. RTS,S booster dose induced higher IL-2 concentrations in infants than children at M32 (interaction P=0.006, Padj=0.16) and higher in Bagamoyo than Manhiça (interaction P=0.007; Padj=0.17).

Malaria exposure before M21 was not associated with CSP-specific responses in non-vaccinated participants (C3C), suggesting absence of naturally acquired immunity for CSP cellular responses **(Supplementary Figure 5)**.

Correlates of malaria risk

The association of the main immunogenicity markers at M21 with the risk of malaria during the following 11 months was assessed through cox models including the interaction with vaccination group **(Figure 3a)**. None of the CSP and HBsAg primary immunogenicity markers were associated with risk of malaria between the different vaccine groups (interaction), nor was there a direct association within any of these groups (stratified association) **(Figure 3a; Supplementary Figure 6)**. For the other markers, CSP-stimulated RANTES showed a tendency towards increasing malaria risk with more RTS,S vaccination (interaction P=0.02, Padj=0.5) (Supplementary Figure 6). No association was found for any marker with malaria regardless of the vaccine group (main effect).

For HBsAg responses, increased IL-10, macrophage inflammatory protein (MIP-1α) and TNF levels in R3R (RTS,S-boosted) showed tendencies for lower risk of malaria (P=0.045 and P=0.040 and P=0.072, respectively) but were not significant after adjustment. HBsAg monocyte chemoattractant protein (MCP-1) was significantly associated with lower risk of malaria for main effect (regardless of vaccine group), though significance was lost after adjustment (P=0.01; Padj=0.160) **(Supplementary Figure 6)**.

Factors affecting correlates of malaria risk

In stratified analyses by age cohort (Figure 3b and c; Supplementary Figures 7a and b), in infants and the R3C (RTS,S-unboosted) group, CSP IL-2 was associated with risk of malaria (Hazard Ratio (HR)=3.3, confidence intervals (CI) [1.3, 8.2]), but this was not significant when adjusting for multiple comparisons (P=0.009, Padj=0.5) and CI were very wide **(Figure 3c)**. Likewise, in R3C for HBsAg, IL-2 and IL-13 were associated with increased risk of malaria, but significance was lost after adjustment (P=0.01, Padj=0.5; P=0.02, Padj=0.6, respectively) **(Figure 3c)**. For infants, IL-10 was significantly associated with a higher risk of malaria regardless of vaccination (main effect P=0.0005, Padj=0.02), an effect that also reached unadjusted significance in the R3R (RTS,S-boosted) group (P=0.02, Padj=0.5) **(Supplementary Figure 7b)**.

Stratifying the correlates analysis by site, showed a significant effect modification of CSP-stimulated IL-2 on the risk of malaria by the different vaccination groups in Manhiça before adjusting for multiple comparisons (interaction P=0.02, Padj=0.8), with a stronger association with lower risk in the R3R (RTS,S-boosted) group, but not in Bagamoyo **(Supplementary Figure 8a and b)**. In line with these observations, CSP-specific IL-2 levels were higher in controls than malaria cases in the R3R group in Manhiça (unadjusted P=0.0356) **(Supplementary Figure 8c)**, whereas there were no differences in Bagamoyo (P=0.597). No other primary immunogenicity markers were found to have significant associations with malaria risk for either vaccine antigen in any of the two sites **(Supplementary Figure 8a and b)**.

Correlation between RTS,S-induced cytokines and antibody responses at M21 and M32

For total anti-CSP NANP IgG antibody levels measured by ELISA, no statistically significant correlations were found with any of the primary immunogenicity markers at M21 **(Figure 4a)**; however, a significant inverse correlation of RANTES with anti-CSP IgG was observed **(Figure 4b)**. Total anti-CSP NANP IgG had a significant moderate positive correlation with IL-2 at M32 **(Figure 4c)**.

Further correlations were performed using anti-CSP full length (CSP FL), anti-NANP, and anti-C-terminus (Cterm) IgG subclasses measured by Luminex in a previous study showing that the booster dose induced IgG, IgG1, IgG3, and IgG4, but not IgG2, which had relatively unchanged levels following the primary vaccination out to M32. At M21, for the primary immunogenicity markers, we only observed a significant moderate positive correlation for IL-17 with anti-NANP IgG2 antibody (Supplementary Figure 9a). IL-15 had a moderate significant inverse correlation with anti-CSP FL IgG4 at M21 **(Supplementary Figure 9b)**. At M32 a significant moderate inverse correlation was found for IL-2 and IL-13 with anti-NANP IgG2, and IL-2 and IL-5 with IgG4 **(Supplementary Figure 9c and d)**. For the remaining primary markers and IgG subclasses against CSP antigens, there were no significant correlations **(Supplementary Figure 10)**. RANTES had moderate significant positive correlations with anti-CSP FL and Cterm IgG and IgG1 and anti-NANP IgG4 at M32 **(Supplementary Figure 11)**.

Regarding HBsAg responses, at M21, IL-17 had significant moderate positive correlations with HBsAg IgG1, and IL-2, IL-5, and IL-13 with IgG2 **(Supplementary Figure 12)**. At M32, positive correlations were found between IL-17, IL-5, and IL-13 with total anti-HBsAg IgG and IgG1, and IL-17 and IL-5 with IgG2, and IL-5 with IgG4 **(Supplementary Figure 13)**.

Additionally, some HBsAg-specific cytokine responses correlated positively with CSP antibodies at M21, mainly involving IL-17 and IL-2 responses correlating with protective IgG1 and IgG3 against the CSP FL and NANP **(Supplementary Figures 14-15)** and IL-17, IL-5 and IL-13 correlating with Cterm IgG3, and IL-17 with Cterm IgG1 **(Supplementary Figures 16)**. At M32, fewer significant correlations between HBsAg-specific responses and CSP antibodies were found **(Supplementary Figure 17)**.

DISCUSSION

The RTS,S fourth dose in the pediatric phase 3 trial significantly increased levels of CSP-specific IL-2 one month post-booster and, importantly, it remained elevated one year after. However, significant differences in cytokine levels one month post-booster were only detected when compared with comparator vaccinees and not with RTS,S vaccinees without RTS,S booster, suggesting a modest cellular response may have been generated from the booster dose. This is in line with the limited recovery of vaccine efficacy and limited antibody induction by the booster dose. HBsAg-specific responses included more cytokines besides IL-2 (TH1), encompassing different functions: TH1 (IFN-γ), TH17 (IL-17), and TH2 (IL-13, and IL-5). IL-17 was the sole cytokine significantly increased as a result of the booster dose compared to RTS,S vaccinated children without the RTS,S booster. Of those responses, IL-2 and IL-5 were still detected one year after the booster, indicating maintenance of some T cell memory response. This confirms our previous results after primary vaccination that the HBsAg component of RTS,S may result in higher cellular immunogenicity than CSP, perhaps from the higher proportion of HBsAg than CSP antigen in RTS,S or due to previous hepatitis B vaccination included in the expanded immunization program.

Despite RTS,S induction of IL-2, no clear association of this cytokine with malaria risk was detected in overall RTS,S vaccinees. However, stratifying by site, CSP-specific IL-2 in the RTS,S-boosted group tended to lower the risk of malaria in Manhiça, but not Bagamoyo, suggesting that site may affect the immune response to RTS,S vaccination and its role in protection. A previous study that compared children from Tanzania and Mozambique during the phase 3 trial found differences in immune development between sites, with individuals from Tanzania having significantly lower frequencies of circulating innate and adaptive cell subsets, including T cell subsets, which are the producers of IL-2, and effector cells which could be involved in RTS,S protective mechanisms (NK cells, monocytes, memory B and T cells) [21]. In contrast, stratifying the correlates analysis by age cohort, infants showed tendencies for CSP IL-2 to be associated with a higher risk of malaria in the RTS,S-unboosted group. Nevertheless, the infant cohort had fewer

overall participants in this study. Additionally, the infant cohort tended to produce lower levels of cytokines, which resulted in many markers below one after correcting for DMSO background and yielded wide confidence intervals.

CSP-specific IL-2 has been highlighted in past field studies of RTS,S as a key immunogenicity marker [13–16,18,19,22,23]. Still, it has only been demonstrated as having a role in protective immunity in a study of vaccinated malaria-naïve adults [12]. Our previous studies following primary immunization at M3 found increased frequencies of CSP-specific IL-2 expressing CD4+ T cells by flow cytometry, and IL-2 measured in PBMC culture supernatants by Luminex, however, association with malaria protection was not observed either [16,19]. Yet, IL-2 may still contribute to RTS,S-induced protection, as it can stimulate proliferation, augment effector functions of T cells, and support memory CD4+ T cell populations [24,25]. NK cells producing IFN-γ via activation by CSP-specific CD4+ T expressing IL-2 has also been demonstrated in RTS,S vaccinated children in Tanzania [23].

Furthermore, IL-2 plays a key role in supporting CD4+ T cell and B cell responses, which help support antibody production [25]. IL-2 has been demonstrated to be associated with B cell differentiation into plasma cells and the maintenance of antibody titers [26,27]. Here, we found that IL-2 had a low positive correlation with anti-CSP IgG one month post-booster in RTS,S vaccinated children, with and without booster dose. However, positive correlation strength and significance increased at M32. Interestingly, there were significant moderate inverse correlations of IL-2 with anti-NANP IgG2 and IgG4. Our group previously showed IgG2 and IgG4 responses to vaccine antigens at M3 to associate with malaria risk over the one-year follow-up post-primary immunization [6]. Of note, IgG4 (and IgG1 and 3) but not IgG2 was induced by the booster dose [9].

Demographic factors can impact RTS,S immunogenicity at primary vaccination, including age, geographic location, previous exposure to malaria, anemia, and health status [5,6,16,21]. Here, no clear-cut effect modifications were detected, although there were some tendencies at M21 of children responding with higher antigen-specific cytokines to booster vaccination (mainly IL-17, but also IL-13 and IFN-γ). Instead, infants had increased IL-5 responses after booster vaccination compared to children. Age cohort differences could be due to differences on immune composition and response according to age but also could be a consequence of differences in primary vaccine responses.

Interestingly, the correlates analysis for infants showed IL-10, an immunoregulatory cytokine, to be significantly associated with an increased risk of malaria independently of vaccination. Elevated IL-10 has been shown in a previous study of Tanzanian children to be associated with a lower capacity to clear P. falciparum parasites [28]. An earlier prospective study by our group involving Mozambican mothers and their children demonstrated that higher IL-10 production was associated with a greater risk of clinical malaria, and furthermore, the increased risk could be inherited [29].

In our previous study on RTS,S post-primary immunization [16], RANTES was also associated with malaria risk. RANTES, produced by memory T cells, monocytes, and macrophages in PBMCs [30,31], can polarize cellular responses towards a TH1 profile, leading to lower antigen-specific humoral responses [32], which may explain the inverse correlation that we observed between RANTES and anti-CSP IgG at M21. Acute malaria has also been shown to drive TH1 cytokine responses, resulting in a less functional TH1-polarized subset of TFH cells [33]. These TH1-type TFH cells were associated with impaired B cells producing suboptimal antibody responses [33]

A limitation of the study is that we cannot ascertain the cell origin of the RTS,S induced cytokines or if these are due to an increase in numbers of stimulated cells producing cytokines or rather the result of few primed cells producing higher levels of cytokines.. However, previous flow cytometry studies can allow us to infer on what cells may be producing these cytokines. For instance, CSP-specific CD4+ T cells produce weak or no IFN-γ, while HBsAg-stimulated CD4+ T cells did [14,19], and other cells do produce IFN-γ after bystander stimulation [23]. Furthermore, IL-2 is induced mainly in activated T cells and we know from previous studies that RTS,S increases frequencies of CD4+ T cells producing IL-2 [13,14,18,19,22]. Characterization of T cell-specific responses by intracellular cytokine staining and flow cytometry experiments will be explored in future studies. These studies will inform upon the effect of the booster dose on memory and

TFH cells among other cell subsets, which are critical for germinal center responses and supporting B cells and the production of high-affinity antibodies, as well as the role of RANTES on TFH cells. Another limitation inherent to malaria field trials is that participants without clinical malaria may not have been necessarily protected as they may not have been exposed (bitten by an infectious mosquito), or they may have been infected but asymptomatic or not gone to a health post even if sick. In addition, we tested all available samples, yet the sample size still limited the power of our analyses of immune correlates of malaria risk. However, a number of cytokines were associated with malaria risk in exploratory and unadjusted analyses, which may very well be chance findings. Despite controlling for FDR (<5%), our exploratory study contains numerous comparisons, opening the possibility of false positves and therefore unadjusted associations with malaria risk have not been highlighted. Unfortunately, antibody data was only available in a subset of participants, limiting correlation analyses between antibodies and cytokines. Furthermore, the distribution of age cohorts between sites was unequal. Manhiça consisted of children and infants, while Bagamoyo only had children, which reduced the power of the effect modification analysis since we limited the site analysis to only children and age analysis to participants only from Manhiça. Despite controlling for FDR (<5%), our exploratory study contains numerous comparisons, opening the possibility of false positives. We have highlighted those results that align with previous studies or with theory. New results should help generate new hypotheses but also require further independent validation.

In summary, our results further support IL-2 as a key RTS,S-induced CSP-specific immunogenicity marker that tends to associate with reduced risk of malaria and to correlate positively with anti-CSP total IgG antibody and negatively with IgG subclasses associated with increased risk. The stronger association of IL-2 with reduced risk of malaria in Manhiça compared to Bagamoyo suggests that there may be site-specific differences in RTS,S-protective responses. CSP-specific IL-10 was significantly associated with an increased risk of malaria in the infants, suggesting that this immunoregulatory cytokine should be analyzed in malaria vaccine studies. HBsAg-specific responses were more numerous than CSP, and several tendencies were found for lower risk of malaria, highlighting the need to better understand the role of HBsAg in RTS,S vaccination. Our findings align with previous studies indicating that a strong vaccine-induced IL-2 response likely provides the support necessary for a robust humoral and cellular response to control a pre-erythrocytic infection. However, RTS,S induced a relatively low IL-2 response overall that was undetected in many participants. Therefore, modifying the vaccine design and strategy to elicit a higher memory T cell response may increase vaccine durability and efficacy.

MATERIALS AND METHODS

Ethics

Parents or guardians of the study participants provided written informed consent. The following ethics review boards and regulatory authorities from participating countries approved the study protocol: The Ethical Committee of the Hospital Clínic in Barcelona (CEIC, Spain), National Health and Bioethics Committee (CNBS, Mozambique), Ethikkommission Beider Basel (EKBB, Switzerland), National Institutional Review Board (NIMR, Tanzania), Ifakara Health Institute IRB (IHIIRB, Tanzania), and the Research Ethics Committee (REC, USA).

Study Design

This study included a subset of vaccinees who were enrolled for the cellular component of the immunology study MAL067, an ancillary study to the RTS,S/AS01E phase 3 trial (Malaria-055, ClinicalTrials.gov, NCT00866619) which has been previously described [8,16]. Study participants included children ages 5-17 months (age at first dose) and infants ages 6-12 weeks who were vaccinated with three primary doses at one-month intervals of either RTS,S or a comparator vaccine, which was meningococcal C conjugate vaccine (Menjugate™, Novartis) for infants and rabies vaccine (VeroRab™, Sanofi Pasteur) for children. At M20, a booster dose was given to the three trial groups as follows: (i) three primary doses of RTS,S followed by an RTS,S booster (R3R); (ii) three primary doses of RTS,S followed by a comparator dose (R3C), and (iii) three primary comparator vaccine doses followed by a comparator vaccine boost (C3C). Both age cohorts received the Menjugate™ vaccine as comparator at M20. Malaria cases were participants presenting clinical malaria (fever > 37.5ºC and parasite positive blood smear) over an 11-month follow-up period between M21 and M32, and controls were participants without malaria. Prior exposure to

malaria was assessed for the one year leading up to the booster dose at M20. Demographic factors such as hemoglobin levels and WAZ were measured at M20. Manhiça Health Research Center, Fundação Manhiça in Mozambique, and Ifakara Health Institute and Bagamoyo Research and Training Centre in Tanzania participated in this study and both sites had low-medium malaria transmission intensity (MTI) [8,34,35]. Infants and children were recruited in Manhiça, whereas Bagamoyo only included children.

PBMC Collection and Stimulation

PMBCs were collected at M21 and M32; isolated and stimulated onsite as previously described [16]. Briefly, blood was collected from infants and children using BD Vacutainer® heparin tubes and BD Vacutainer® CPT™ sodium citrate tubes, respectively. PBMCs were isolated by density gradient centrifugation, quantified with a Countess automated cell counter (Life Technologies), and then resuspended in culture medium (RPMI-1640, Gibco, SKU# 42401-042) supplemented with 10% heat-inactivated human AB serum (Patricell, L82263), and penicillin-streptomycin-L-glutamine (Gibco, SKU#10378-016). Cells were plated in a 96-well, U-bottom culture plates (Costar, Cultek Cat No. CLS3799-50EA) at a concentration of 4x105 cells per well in duplicate and allowed to rest for 12 hours in a 37ºC, 5 % CO2 incubator. Stimulations were performed in duplicate with peptide pools from CSP antigen (31 peptides) and HBsAg (53 peptides) consisting of 15-mers overlapping by 11 amino acids, with individual peptides tested at a final concentration of 1 µg/ml (Biosyntan, Germany) [14]. PHA-P (Sigma-Aldrich, L1668) was the positive control, and DMSO, the peptides solvent (Sigma-Aldrich, D2650), was the negative background control. Following stimulation for 12 hours at 37ºC, 5% CO2, cells were centrifuged at 250 x g for 5 minutes at room temperature, then 180µL of supernatant was collected from each replicate and stored at -80°C in 96-well V-bottomed plates (Kisker, AttendBio, G096-VB) sealed with adhesive foil (Kisker, AttendBio G071-P) until analyzed. All available samples were analyzed for this study.

Multiplex Bead Array Assay

The 30-Plex Human Cytokine Magnetic Panel for Luminex® (Thermo Fisher Scientific) was used to quantify the following cytokines, chemokines, and growth factors present in collected culture supernatants: G-CSF, GM-CSF, IFN-α, IFN-γ, IL-1β, IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40/p70), IL-13, IL-15, IL-17, TNF, Eotaxin, IFN-γ induced protein (IP-10), MCP-1, monokine induced by IFN-γ (MIG), MIP-1α, MIP-1β, RANTES, epidermal growth factor (EGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and vascular epidermal growth factor (VEGF). A modified version of the manufacturer's protocol was followed in which half of the volume of all kit reagents were used except for the assay wash buffer [16], and 25 µL of supernatant from each sample were tested. Plates were read on a Luminex® 100/200. A randomized sample distribution was used according to the site, age cohort, vaccination, and case/control to ensure balanced testing across assay plates. Each assay plate included a standard with known concentrations of each analyte (provided by the kit) that was used to prepare a curve with serial dilutions, as well as, two blank controls and three reference sample positive controls prepared with the standard and with low, medium, and high analytes' concentrations tested in duplicate for quality assurance/quality control (QA/QC).

QA/QC was performed using drLumi, an R package developed by our group [36], which fitted the standard curves based on four- or five–parameter log-logistic models. Limits of quantification (LOQ) were based on a cutoff value (30%) of the coefficient of variation (CV) for the standard curve of each analyte. The analytes IL–6, IL–8, Eotaxin, HGF, IFN–α and IL–7 had more than 30% of measurements below or above the LOQ, and therefore were excluded from the study. For all other markers, concentrations that could not be estimated for being outside the quantification range were imputed according to the following boundaries: i) for median fluorescence intensity (MFI) values under the lower LOQ or flagged as not quantified, the lower LOQ and half of the LOQ were used as boundaries for a random imputation; ii) for MFI over the upper LOQ or flagged as not quantified, the upper LOQ and double of the upper LOQ were used as boundaries for a random imputation.

Antibody data

Antibody data against vaccine antigens analyzed for correlations with cytokine responses were obtained in previous studies and were available only for participants from Manhiça. Specifically, IgG titers (EU/mL) against NANP measured by ELISA were obtained from the MAL055 clinical trial (CEVAC ELISA [5,37]. IgG and IgG subclasses (quantified as MFI) against P. falciparum CSP antigens: CSP CSP FL, NANP, and the Cterm were measured by

Luminex at ISGlobal [9]. Due to low participant overlap between cytokine and antibody studies (N= 39 for ELISA data and 31 for Luminex data) and since we observed minor differences for cellular immunogenicity markers elicited by the booster dose, R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) were analyzed together for correlations with antibody levels.

Statistical analyses

Similarly to our previous study [16], the main analysis was based on ratios between analytes concentrations after stimulations with the corresponding antigen of interest (CSP or HBsAg) and background (DMSO) to assess antigen-specific responses. Analysis of antigen-specific responses through antigen/background ratios proved more reliable and had lower variability across plates than background subtractions. Cytokine ratios analyses are complemented with crude concentration analyses, and in both cases, data was log10 transformed. As some substantial outliers resulted in skewing the data distribution, data from all markers were truncated based on the 99.75 percentile. Fold differences and their 95% CI in geometric means between groups were estimated by regressing log-transformed concentrations on the group variable and exponentiating back the regression coefficients. We also used a Wilcoxon rank-sum tests to compare the distribution of log-transformed concentration ratios between groups. Effect modifiers of immunogenicity were evaluated through interactions with the vaccination group in regression models. Only participants from Manhiça were analyzed for the effect modification of age and only children for the effect modification of site. Association of cytokine responses with the risk of clinical malaria were assessed through cox regression. Cytokine concentrations were scaled to make sure that the estimated HR could be interpreted as comparing hazards of infection when log-transformed cytokine levels were increased by one standard deviation. Confidence intervals and significance for HR were based on Wald-tests on the corresponding Cox regression coefficient. We conducted a number of different HR estimations and tests for each cytokine of interest:

1. HRs within each vaccination group (stratified analysis)
2. A test on the effect modification of the vaccination group. This was assessed as an interaction in models including all three vaccination groups. Significance was tested with a log-likelihood ratio test comparing models with and without the interaction terms.
3. HRs within all vaccination groups together, that is, the main effect of cytokine levels independent of any effect modification by the vaccination group. This was obtained from the same regression models as in 2), using an effect-coded regressor design matrix, which made the regression coefficient for the cytokine levels and its associated HR to be thus interpretable in the presence of interaction terms.

Tests were two-tailed and p-values were considered statistically significant to a 0.05 α-level and asterisks were used to indicate results that remained significant after adjustment to keep a false discovery rate (FDR) <5% by applying the Benjamini-Hochberg method. Spearman correlation was used to measure correlation coefficients and statistical significance between cytokines and antibody responses. Due to the exploratory nature of this antibody-cytokine correlation analysis, p-values were not adjusted for multiple testing. All analyses were performed using R, and Cox regression analyses were fitted with the survival package (version 3.2-7) [38].

Data availability

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Code availability

The code used for this study will be made available to qualified researchers upon reasonable request from the corresponding author.

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Author contributions

RM wrote the first draft of the manuscript. DM, CDo, and GM contributed to the writing of the manuscript. DM and RM analyzed the study data and interpreted results with CDo and GM. GM, CDo, and CDa conceived and designed the cellular immunology experiments. MM, CDa, CDo, CJ, JJC, and GM processed the samples and performed the stimulations. CDo and GM led the development of the experiments. CJ, RM, AN, AC, and RA performed the Luminex assays. RA managed Luminex plate design and coordinated Luminex assays. RM, CJ, DM, RA, CDo, and GM developed the analytical plans. MV, RA and GM performed Luminex data QA/QC and MV preformed data preprocessing. CDa and CDo were site PIs for the immunology study. PA was the CISM PI for the MAL055 study. All authors agree with the manuscript results and conclusions.

Competing interests

JJC is employed by Antigen Discovery Inc., Irvine, CA, United States, but declares no financial or non-financial competing interests. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be seen as a potential conflict of interest.

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Table 1. Characteristics of study participants

Month 20 Booster dose	R3R (RTS,S-boosted)	R3C (RTS,S-unboosted)	C3C (Comparator)	Total
Total (infants and children)	241	237	231	709
Manhiça, n	138	133	128	399
Bagamoyo, n	103	104	103	310
Infants, n	59	54	53	166
Children, n	182	183	178	543
Month 21, n	174	183	170	527
Month 32, n	177	175	177	529
M21/M32 intersection	110	121	116	347
Age[a], median months (IQR)	27 (2.78)	26 (2.44)	27 (2.84)	26.7 (2.69)
Female, n (%)	110 (45.6)	128 (54.0)	130 (56.3)	368 (51.9)
Anemia[b], n (%)	67 (27.8)	79 (33.3)	63 (27.3)	209 (29.5)
Malnourishment[c], n (%)	17 (7.0)	14 (5.9)	13 (5.6)	44 (6.2)
Previous malaria[d] n (%)	18 (7.5)	11 (4.6)	18 (7.8)	47 (6.6)

s
a Age at M21

b Hemoglobin <10 g/dL, measured at M20

c WAZ < -2, measured at M20

d Previous case of malaria within 1 year prior to M20

Proportions presented as n (%)

Figure 1

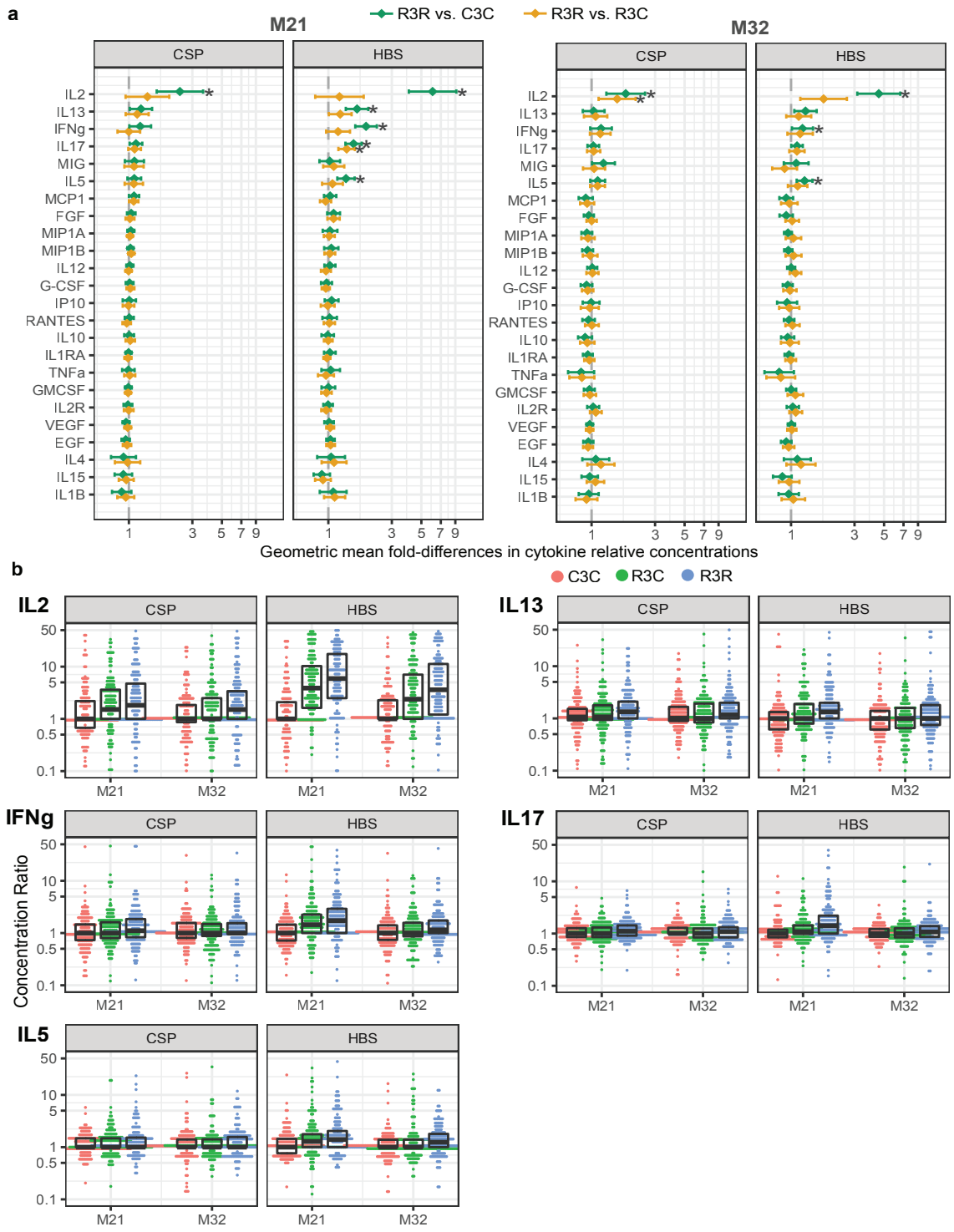

Figure 1. Comparison of CSP and HBsAg-specific cytokine responses (relative concentrations expressed as ratios of vaccine antigen over background) in antigen stimulated PBMC between R3R (RTS,S-boosted), R3C (RTS,S-unboosted), and C3C (Comparator) vaccine groups. a) Forest plots illustrating fold differences in geometric mean relative concentrations between vaccine groups for all cytokines, one month following the booster at M21 (left) and one year later at M32 (right). Point estimates and whiskers indicate 95% CI; asterisks (*) indicate significant differences in distributions according to two Wilcoxon tests after Benjamini-Hochberg adjustment. b) Boxplots showing individual levels and comparing vaccination groups for the five markers that were significantly increased upon vaccination for either CSP- and/or HBsAg-stimulated PBMC samples. The central line in the box represents the median of the relative concentrations in each vaccination group whilst the box limits indicate the quartiles.

Figure 2

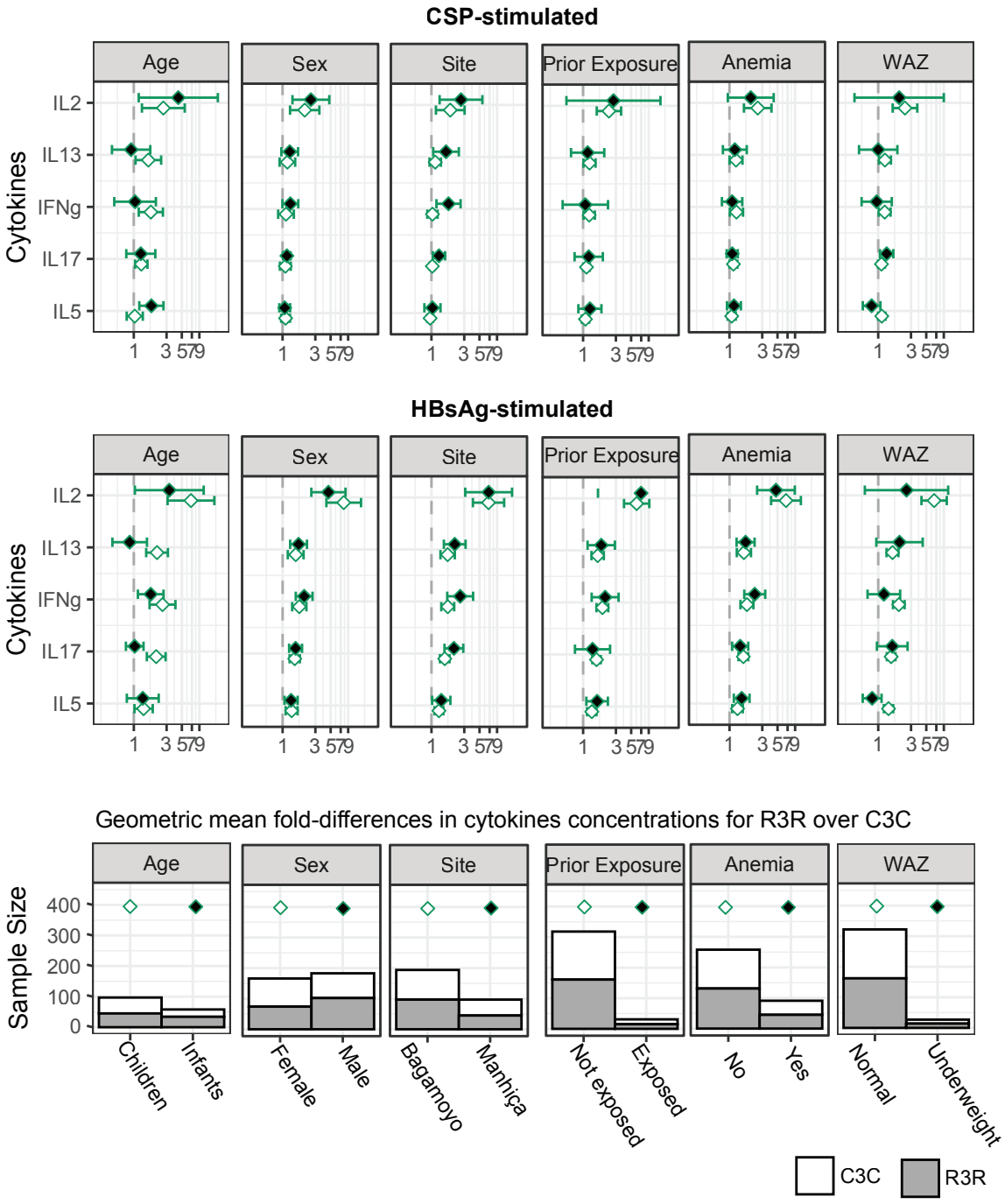

Figure 2. Effect modification of clinical and demographic variables on RTS,S-induced immunogenicity markers following the booster dose at M21. Forest plots illustrating fold differences in geometric mean relative concentrations of CSP- and HBsAg-stimulated cytokine responses between R3R (RTS,S-boosted) and C3C (Comparator) groups stratified by age, sex, site, prior malaria exposure (1-year before M20), anemia, and WAZ scores, for the primary RTS,S immunogenicity markers (IL-2, IL-13, IFN- , IL-17 or IL-5). Open and closed diamonds representing conditions within each factor are defined in the bottom panel showing sample size. Point estimates and whiskers indicate 95% CI. No significant effect modification (interaction) in a regression model was found following Benjamini-Hochberg adjustment. Bar plots representing numbers of study participants within each group are shown at the bottom.

Figure 3

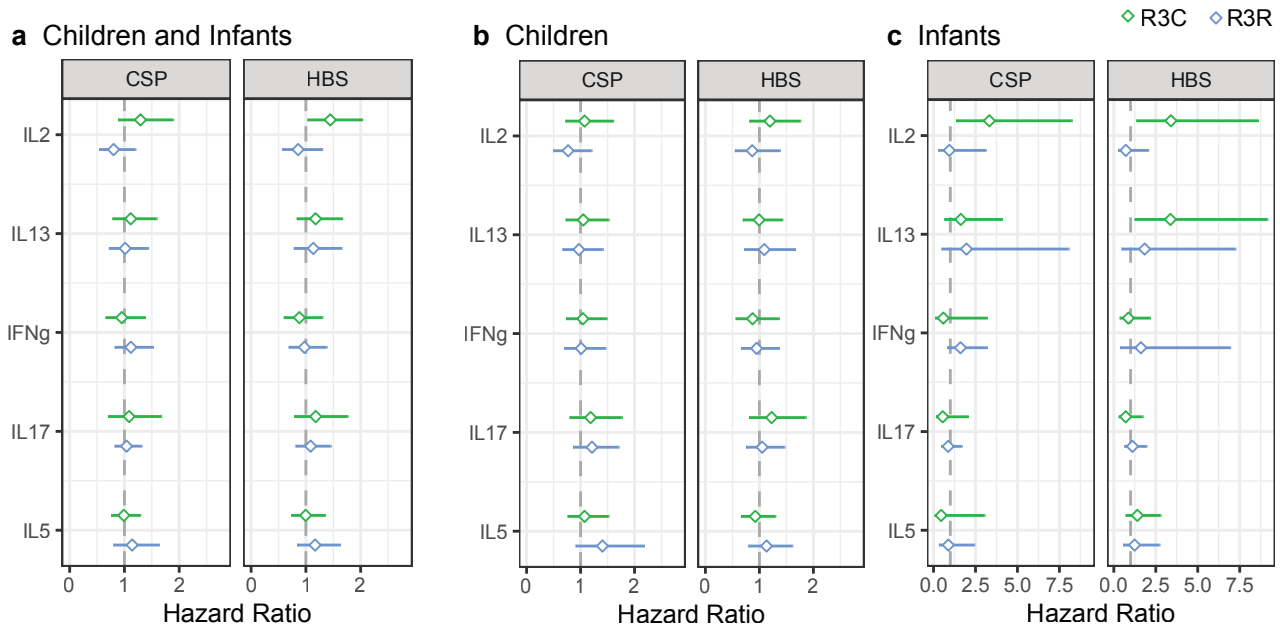

Figure 3. Association of the primary immunogenicity markers levels at M21 and hazard of malaria infection during the following 11 months in the R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) vaccine groups. Hazard ratios indicate the factor by which the hazard is estimated to increase or decrease for one standard deviation change in the cytokine relative concentrations (ratio). Association of the primary immunogenicity markers with hazard of infection in a) children and infants together; b) children only; and c) infants only. Significance was tested with a Wald test on the corresponding Cox regression coefficient used to obtain hazard ratios. No significant differences were found following Benjamini-Hochberg adjustment.

Figure 4

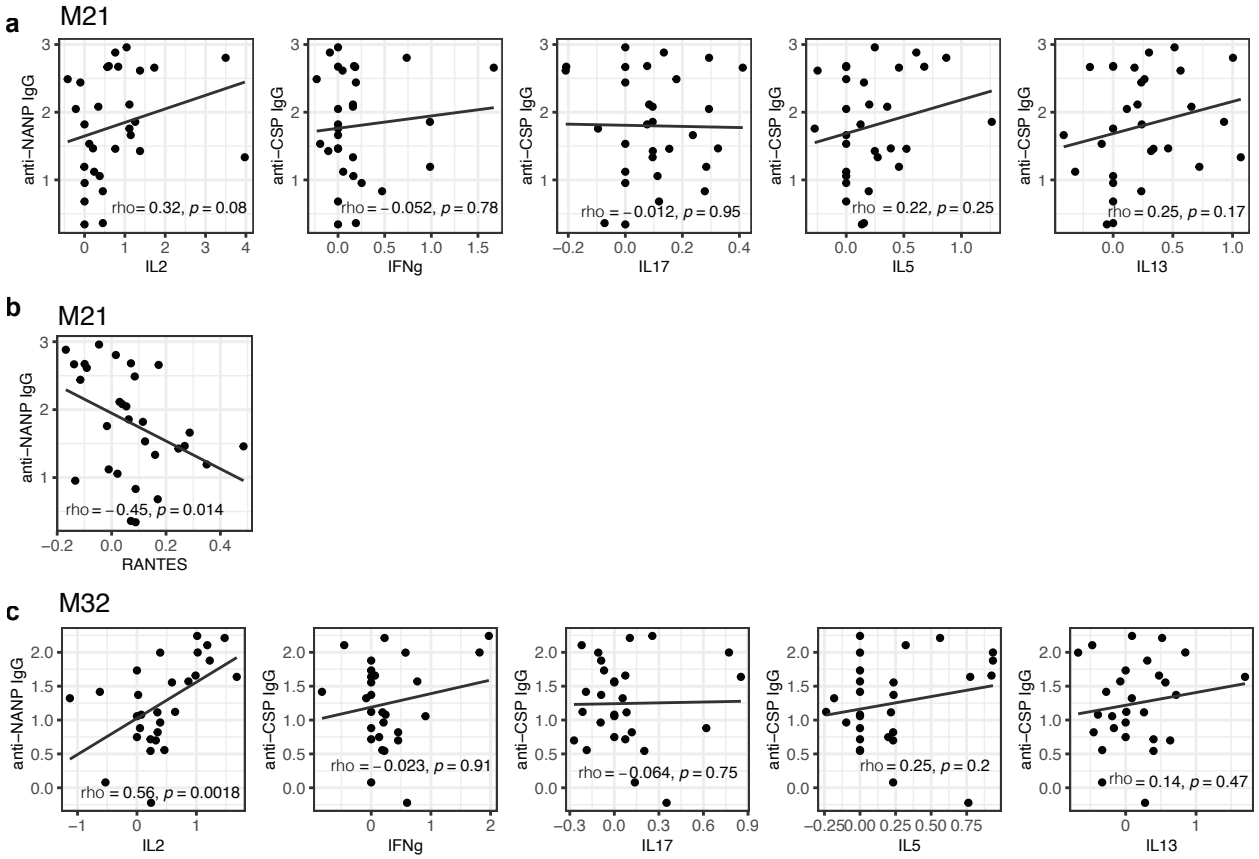

Figure 4. Correlations of anti-NANP IgG titers with CSP-stimulated cytokines in RTS,S vaccinees. a) Primary immunogenicity markers measured at M21, b) RANTES measured at M21, and c) primary markers measured at M32. Antibody titers shown as EU/mL and cytokines as log10 ratios. Spearman rho and P-values shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled, n=30 at M21 and n =28 at M32.

Supplementary Material

Effect of RTS,S/AS01$_E$ vaccine booster dose on cellular immune responses in African infants and children

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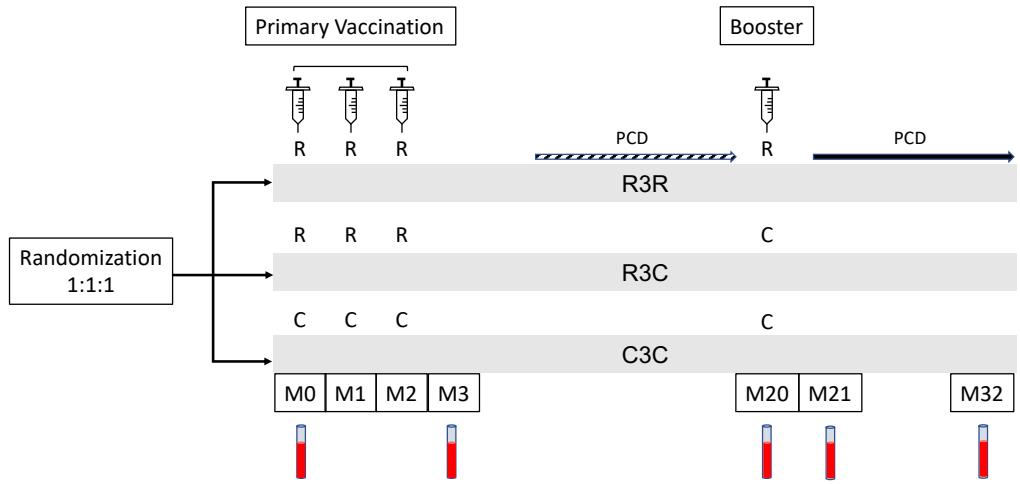
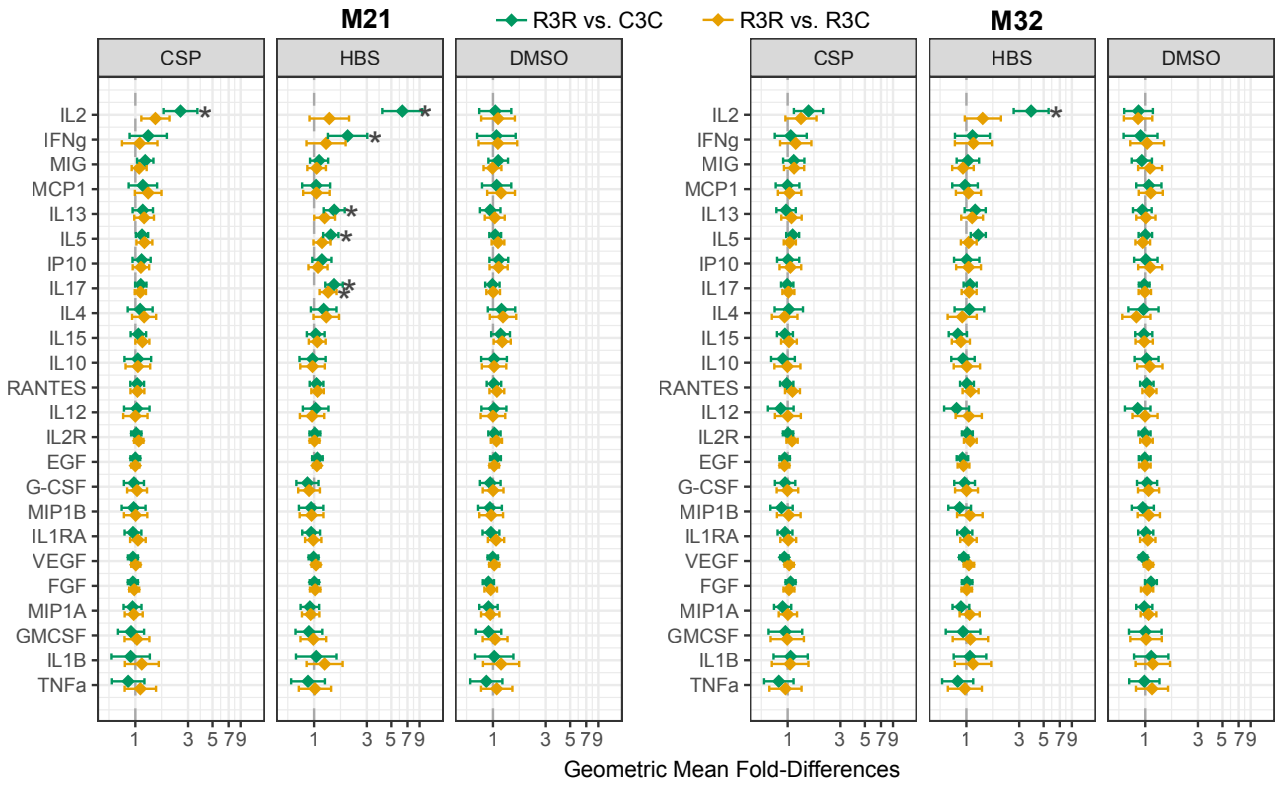
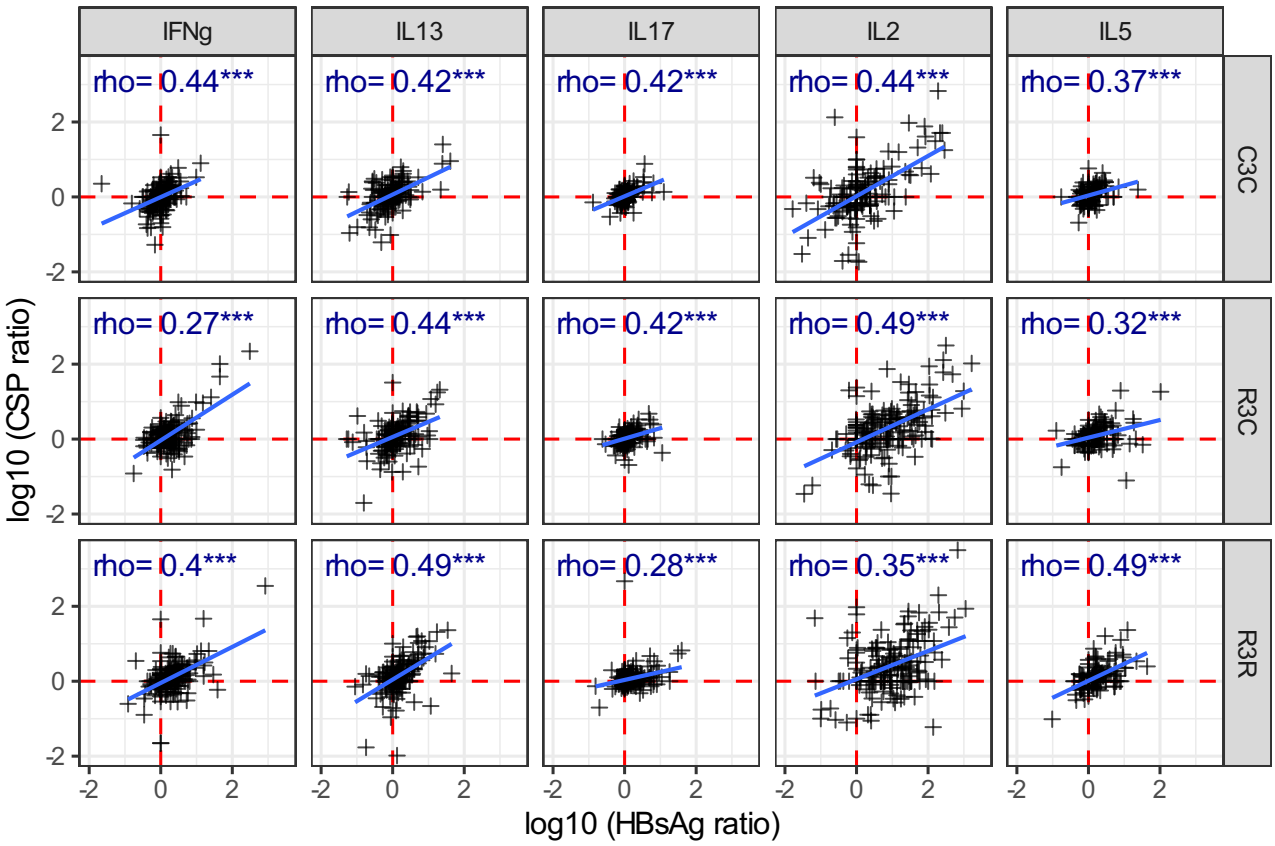


R: Immunization with RTS,S/AS01$_E$ vaccine
C: Immunization with Comparator control vaccine
M: Month
PCD: Passive case detection for clinical malaria 1 year prior to M20 M21-M32

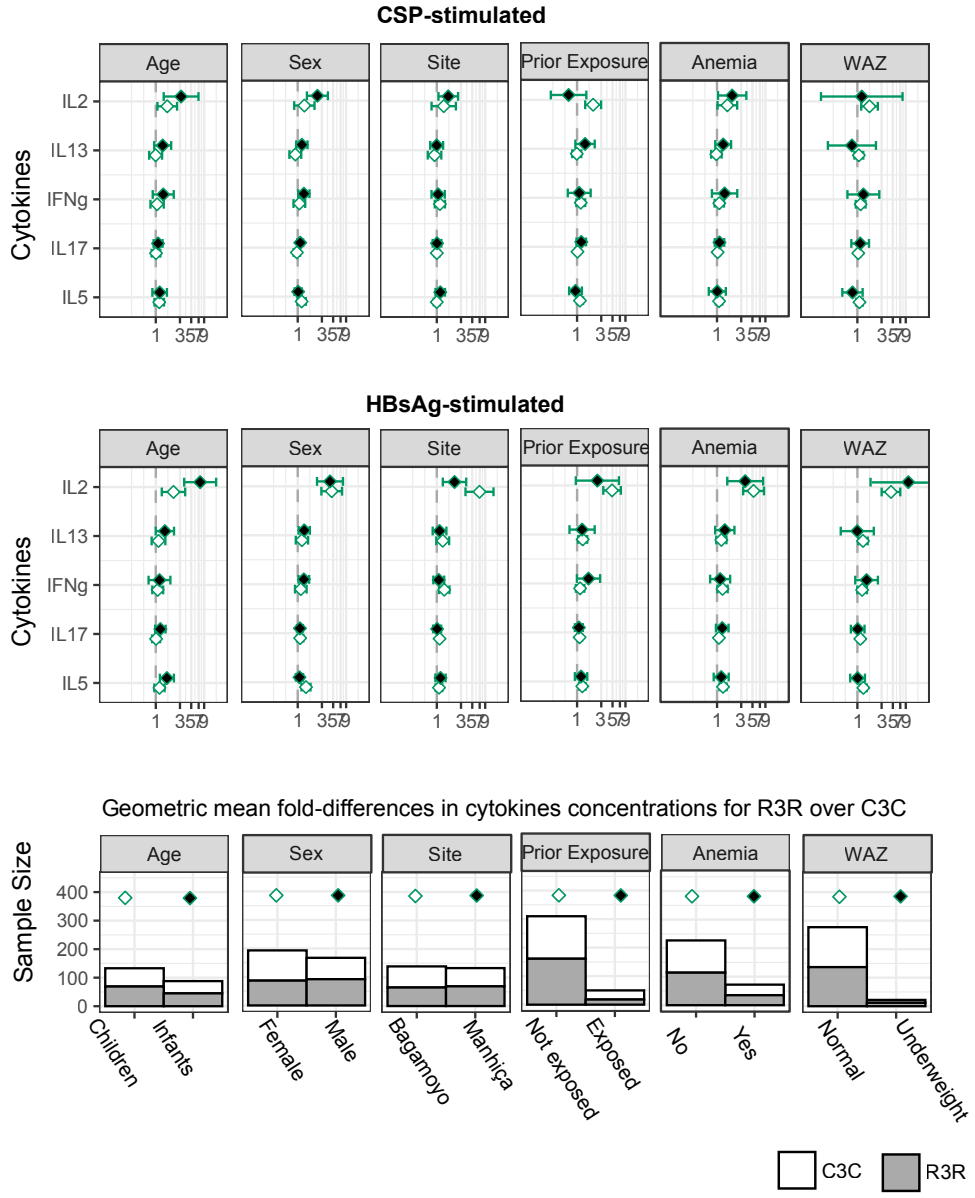
Supplementary Figure 1. Study design. The R3R vaccine group received RTS,S/AS01$_E$ at M0, M1, M2, and a booster dose at M20 (RTS,S-boosted). The R3C group received RTS,S/AS01$_E$ at M0, M1, M2, and a comparator control vaccine dose at M20 (RTS,S-unboosted). The C3C group (Comparator) received a comparator vaccine at M0, M1, M2, and M20. PBMC samples collected at M21 (one-month post booster) and M32 (one-year post booster) were analyzed in the current study and passive case detection for clinical malaria was used for analysis of exposure before the booster dose at M20 and after M21.



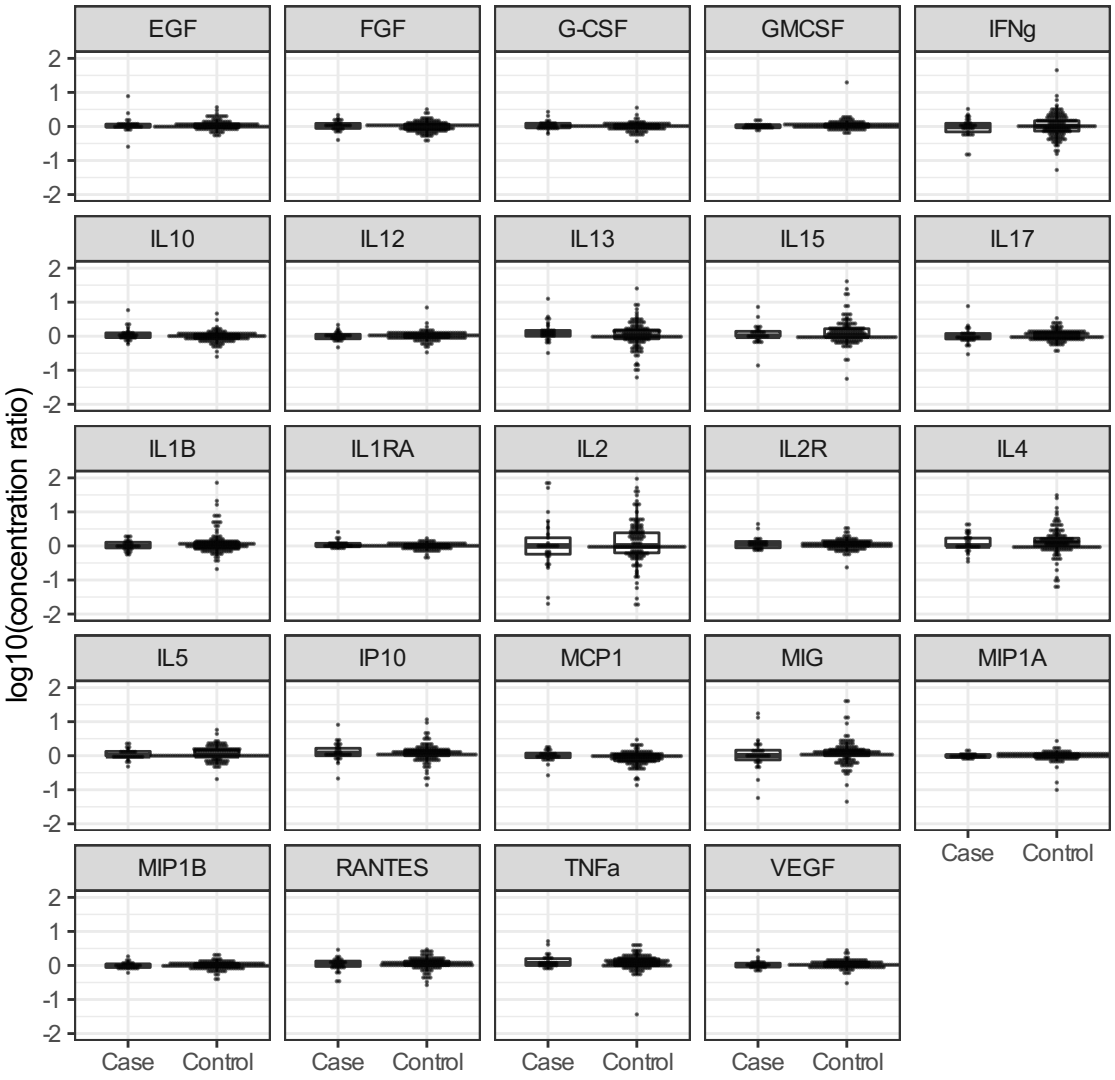
Supplementary Figure 2. Comparison of cytokine crude concentrations in antigen stimulated PBMC samples between R3R (RTS,S-boosted), R3C (RTS,S-unboosted), and C3C (Comparator) vaccine groups. Forest plots illustrating fold differences in geometric mean concentrations between vaccine groups for all markers analyzed, one month following the booster at M21 (left) and one year following the booster at M32 (right). Point estimates and whiskers indicate 95% CI; asterisks (*) indicate significant differences in distributions according to two Wilcoxon tests after Benjamini-Hochberg adjustment.



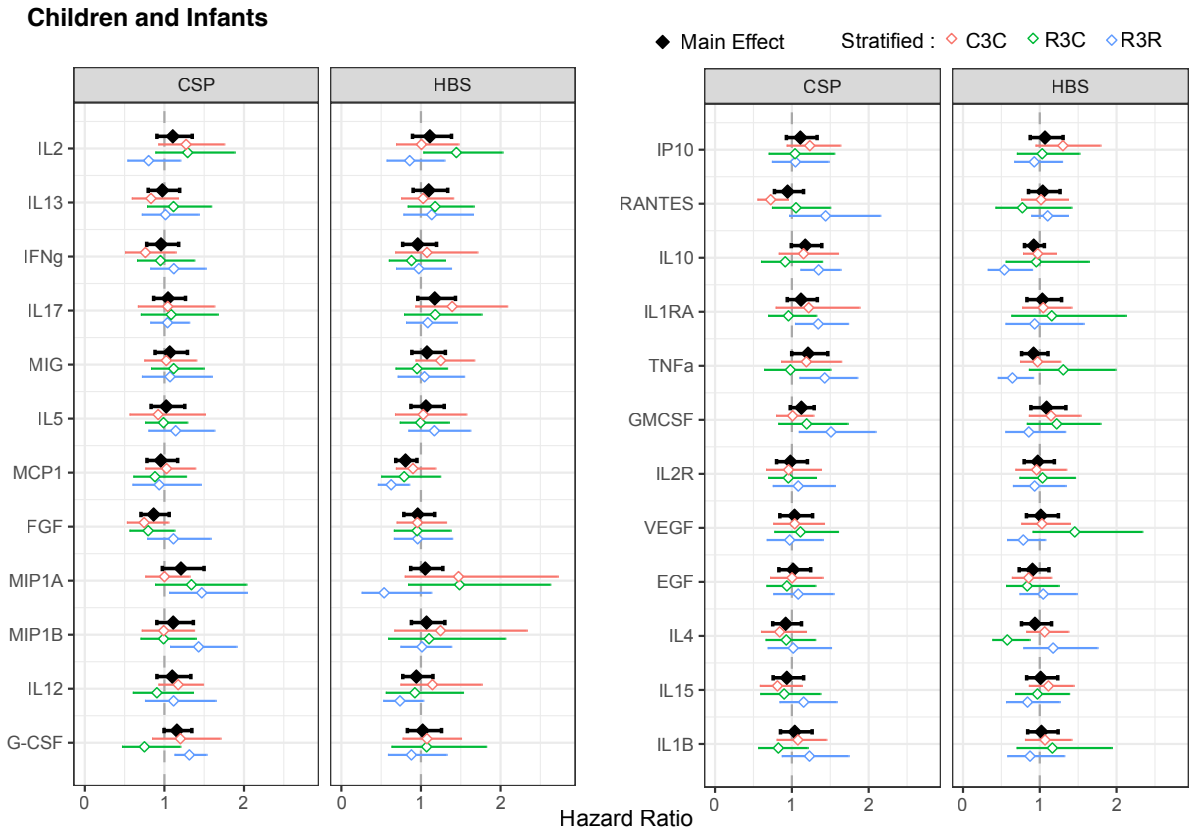
Supplementary Figure 3. Correlations for CSP- versus HBsAg-stimulated primary immunogenicity markers in each vaccine group R3R (RTS,S-boosted), R3C (RTS,S-unboosted), and C3C (Comparator) at M21. Spearman rho and significance following Benjamini-Hochberg adjustment represented by asterisk (* <0.05; ** <0.005; *** <0.0005) are shown in the plot.



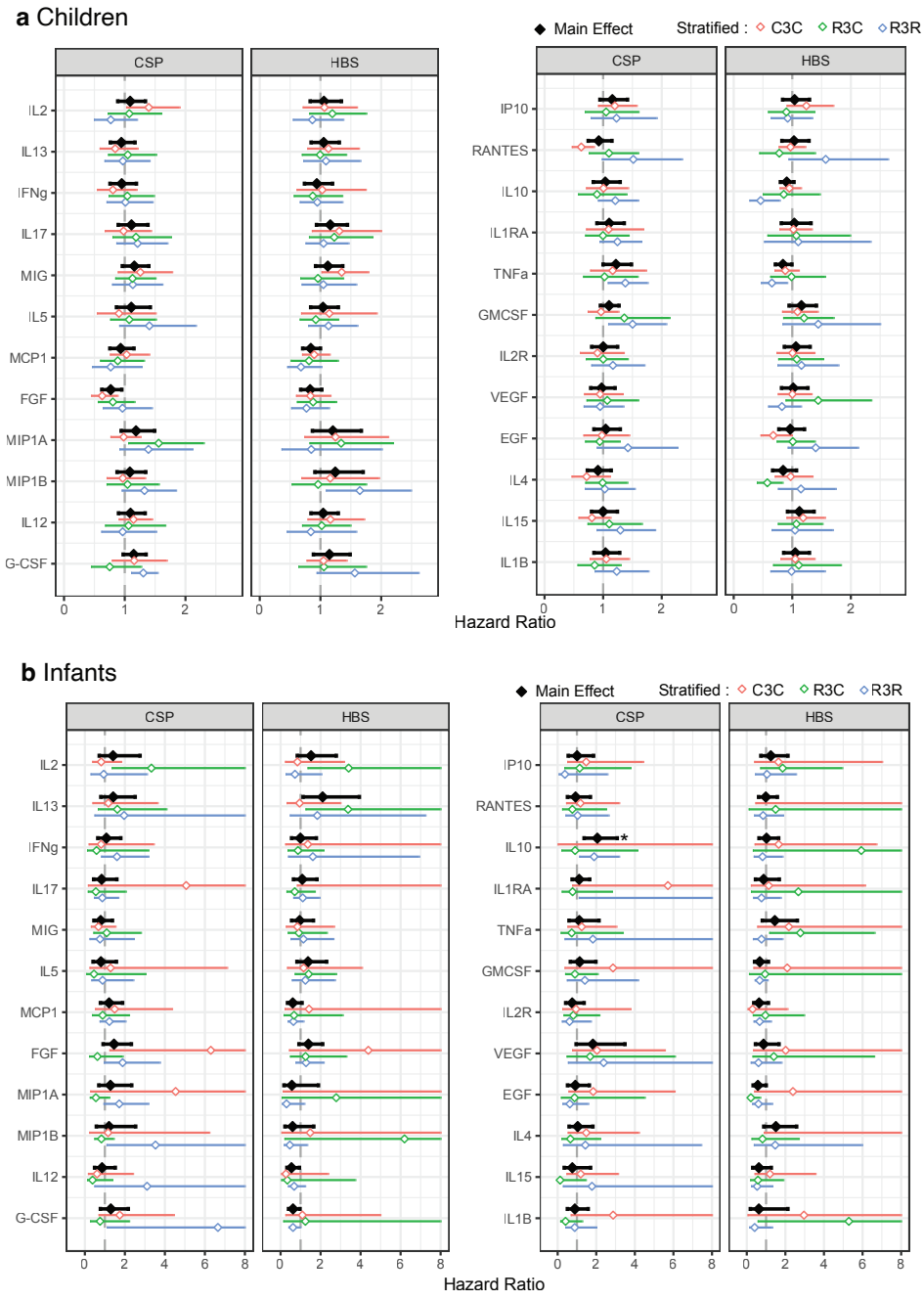
Supplementary Figure 4. Effect modification of clinical and demographic variables on RTS,S-induced immunogenicity markers following the booster dose at M32. Forest plots illustrating fold differences in geometric mean relative concentrations of CSP- and HBsAg-stimulated cytokine responses between R3R (RTS,S-boosted) and C3C (Comparator) groups stratified by age, sex, site, prior malaria exposure (1-year before M20), anemia, and WAZ scores, for the primary RTS,S immunogenicity markers (IL-2, IL-13, IFN-γ, IL-17 or IL-5). Open and closed diamonds representing conditions within each factor are defined in the bottom panel showing sample size. Point estimates and whiskers indicate 95% CI. No significant effect modification (interaction) in a regression model was found following Benjamini-Hochberg adjustment. Bar plots representing numbers of study participants within each group are shown at the bottom.



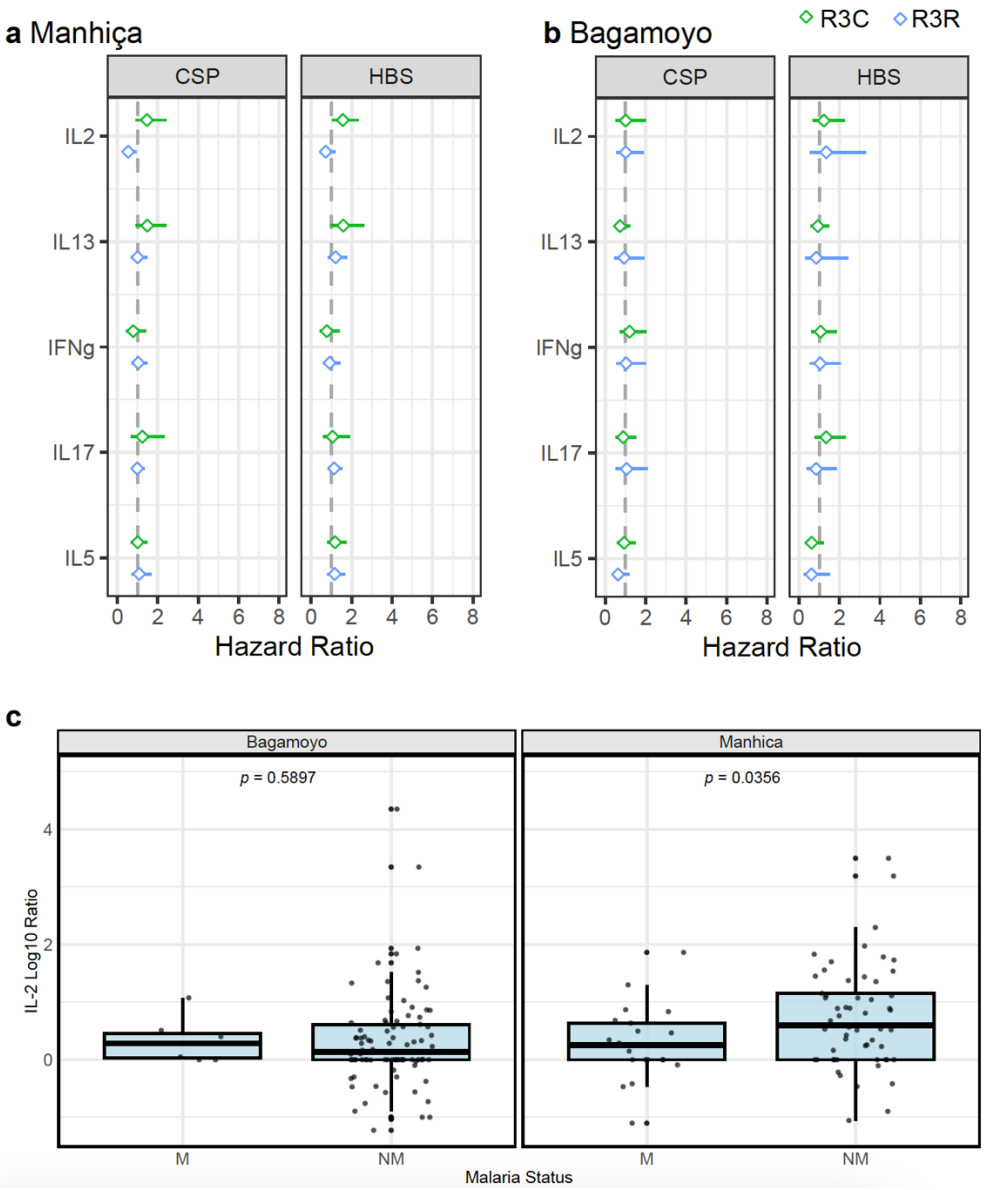
Supplementary Figure 5. Effect of previous malaria exposure on response to CSP vaccine antigen in the C3C (Comparator) group at M21. Individual boxplots for all study markers following CSP-stimulation in the C3C group at M21 comparing cases (individuals who experienced a malaria case during 1 year prior to M21) versus controls (no malaria cases). The plots show a central line representing the median and diamonds for geometric mean, the 25th and 75th quartiles, and whiskers display the 1.5 interquartile ranges. No significant differences were found following Benjamini-Hochberg adjustment.



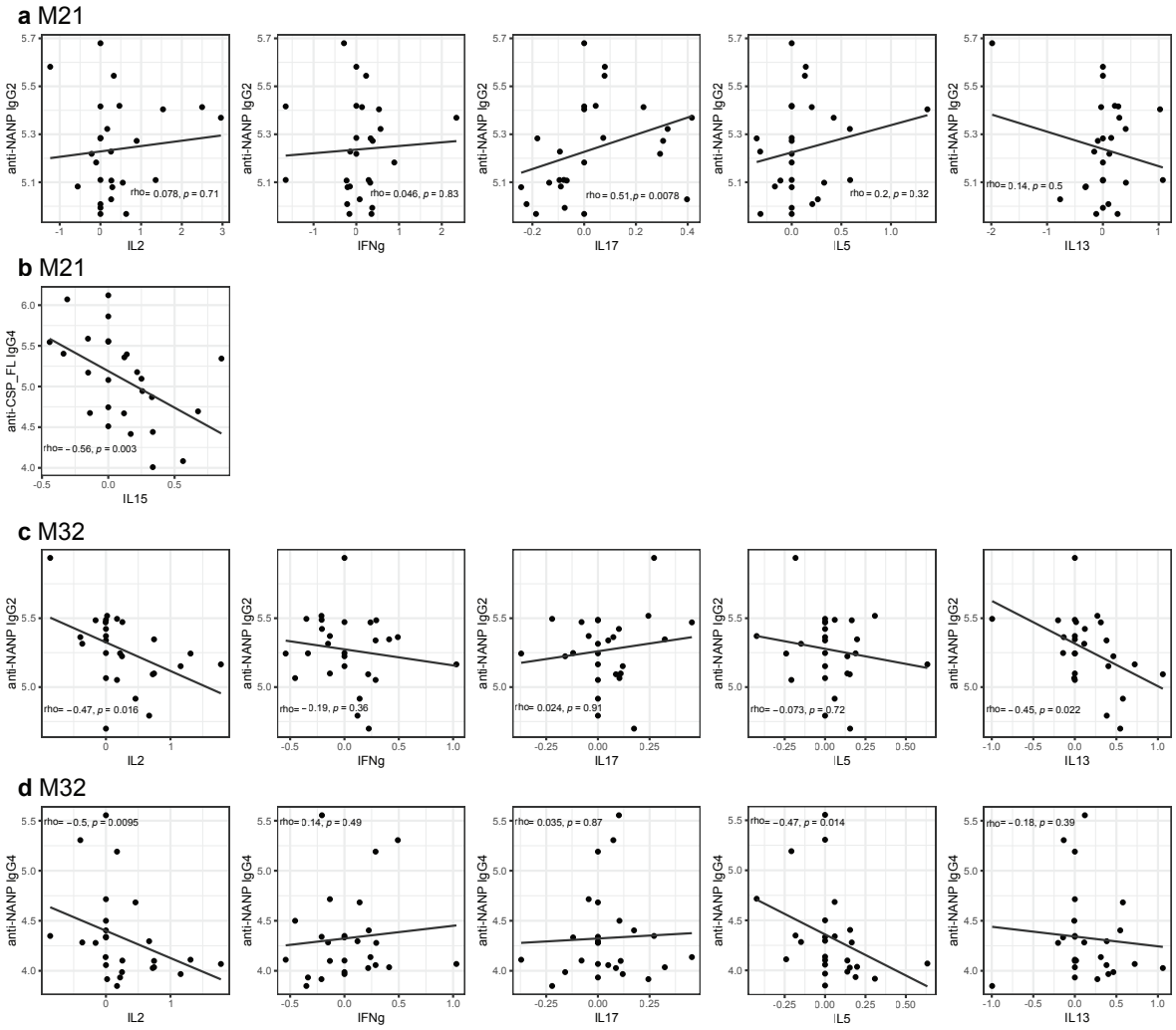
Supplementary Figure 6. Association of cytokine levels at M21 and hazard of malaria infection during the following year in R3R (RTS,S-boosted), R3C (RTS,S-unboosted), and C3C (Comparator) vaccine groups for children and infants together, testing main effect and interaction. Hazard ratios indicate the factor by which the hazard is estimated to increase or decrease for one standard deviation change in the cytokine levels. Black corresponds to the main effect association, that is, all participants included adjusting by vaccination group (no significant results following Benjamini-Hochberg adjustment). Colors correspond to stratified analyses by vaccination group. Effect modification of vaccination group on the association, whether the estimated associations within each vaccination group are different, was tested by comparing models with or without interaction terms (no significant result following Benjamini-Hochberg adjustment).



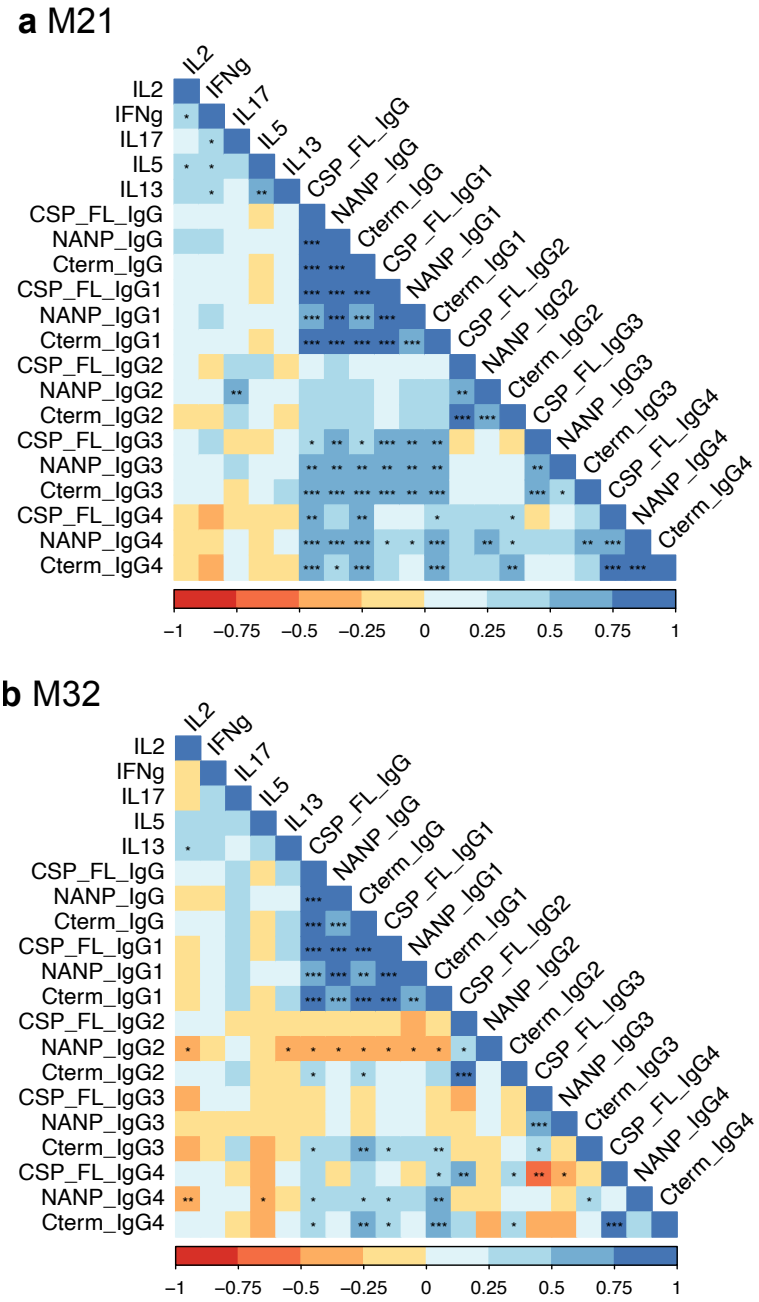
Supplementary Figure 7. Hazard Ratios showing main effect and stratification by R3R (RTS,S-boosted), R3C (RTS,S-unboosted), and C3C (Comparator) for all markers in a) children and b) infants. Hazard ratios indicate the factor by which the hazard is estimated to increase or decrease for one standard deviation change in the cytokine levels. Black corresponds to the main effect association, that is, all participants included adjusting by vaccination group (no significant results following Benjamini-Hochberg adjustment). Colors correspond to stratified analyses by vaccination group. Effect modification of vaccination group on the association, whether the estimated associations within each vaccination group are different, was tested by comparing models with or without interaction terms (no significant result following Benjamini-Hochberg adjustment).



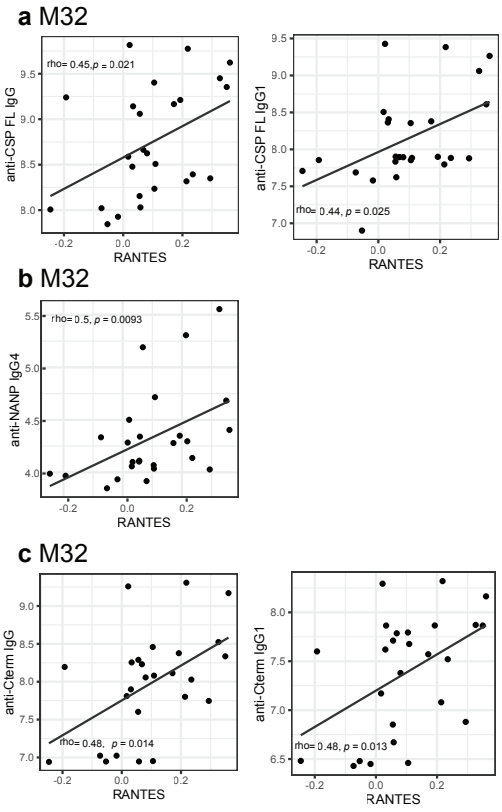
Supplementary Figure 8. Association of cytokine levels at M21 and hazard of infection during the following year in the R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) vaccine groups stratified by site. Association of the primary immunogenicity markers levels with hazard of infection in **a)** Manhiça and **b)** Bagamoyo. For A and B, significance was tested with a Wald test on the corresponding Cox regression coefficient used to obtain hazard ratios. No significant differences were found after Benjamini-Hochberg adjustment. **c)** Boxplots showing CSP-stimulated IL-2 Log_{10} ratio levels in cases (M) versus controls (NM) during the 1-year follow-up post booster in the R3R group between Manhiça and Bagamoyo. Line represents the median in the boxplot with quartiles and whiskers showing 1.5 times interquartile range from upper and lower quartiles. Mann-Whitney test was used to compare IL-2 levels between cases (M) or controls (NM) for each study site with unadjusted p-values shown in the plot (panel c).



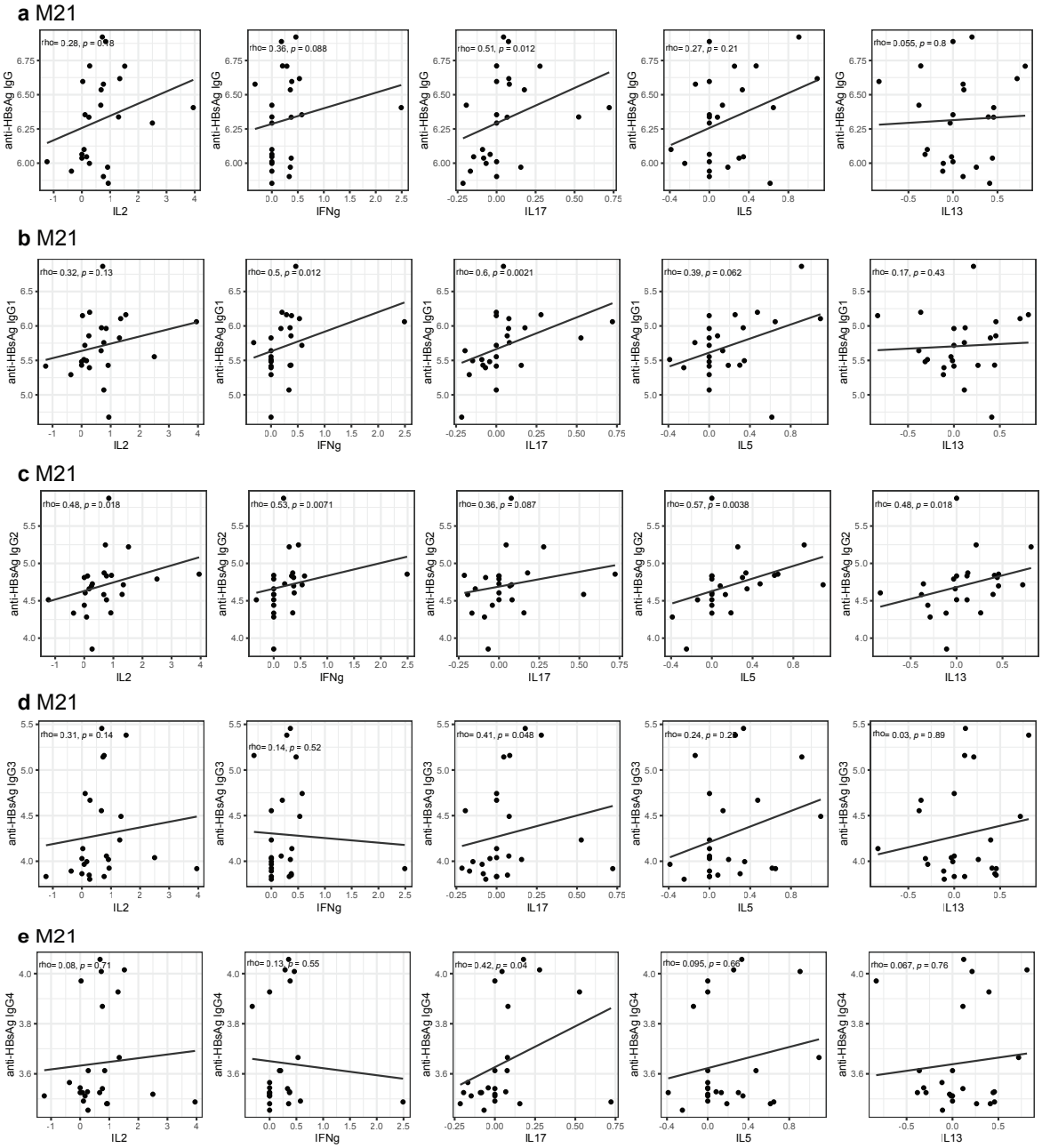
Supplementary Figure 9. Correlations of anti-CSP IgG subclasses with CSP-stimulated cytokines in RTS,S vaccines . a) Primary immunogenicity markers versus anti-NANP IgG2 at M21 and **b)** IL-15 versus anti-CSP FL IgG4 at M21; **c)** Primary immunogenicity markers versus anti-NANP IgG2 at M32 and **d)** Primary immunogenicity markers versus anti-NANP IgG4 at M32. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled, n =26 at M21 and n =26 at M32.



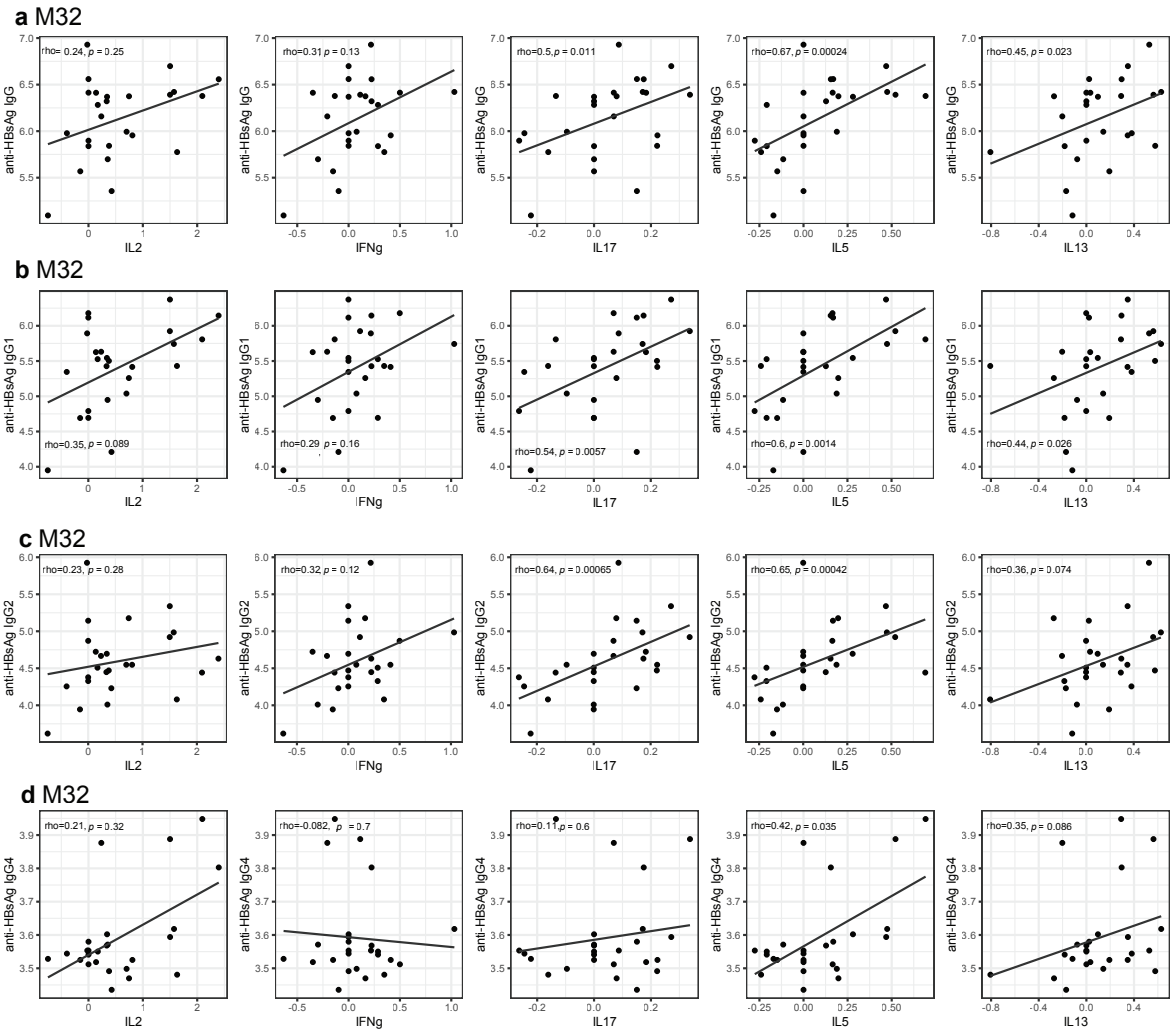
Supplementary Figure 10. Correlations of anti-CSP IgG subclasses with CSP-stimulated cytokines in RTS,S vaccinees. Primary immunogenicity markers (log_{10} ratios) versus anti-CSP FL, anti-NANP, and anti-Cterm IgG subclasses (Log_{10} MFI) at **a)** M21 and **b)** M32. Colored bar ranging from red -1 (negative correlation) to blue 1 (positive correlation) showing Spearman correlation coefficient and asterisks show significance levels (unadjusted) *P<0.05, **P<0.01, ***P<0.001. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled, *n* =26 at M21 and *n* =26 at M32.



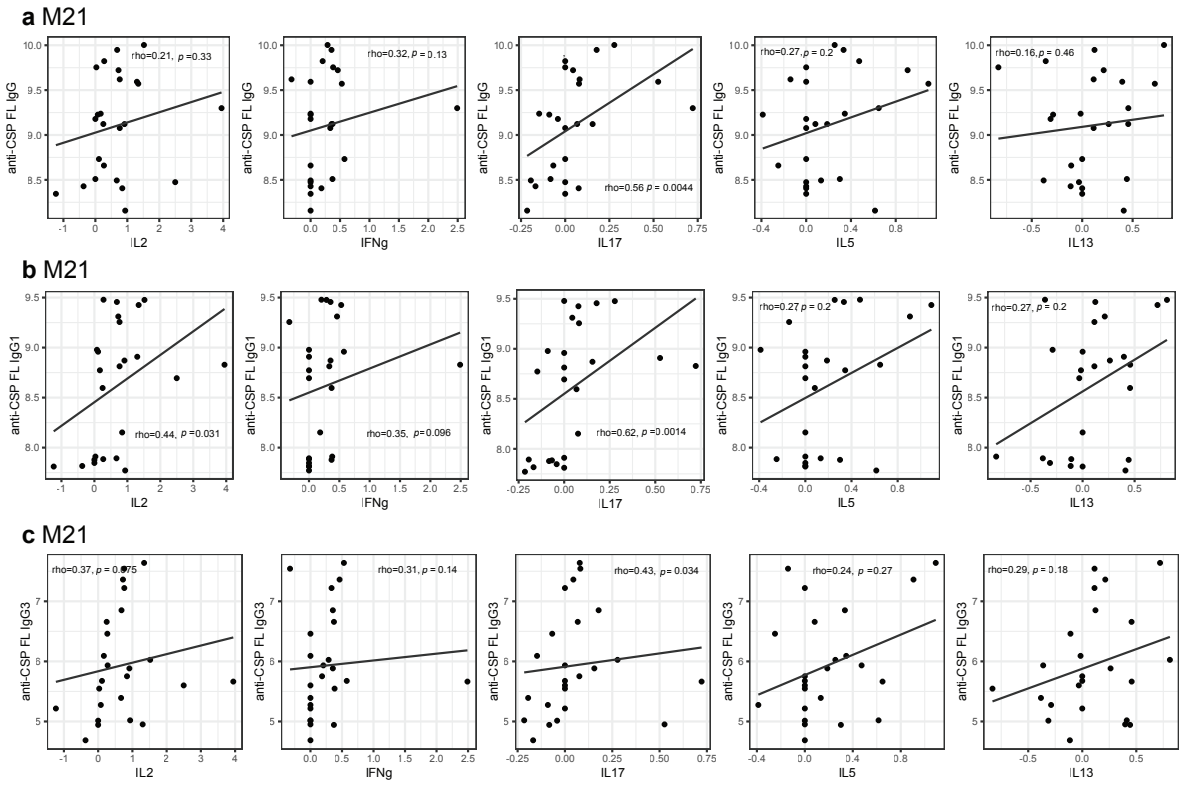
Supplementary Figure 11. Correlations of anti-CSP IgG subclasses with CSP-stimulated RANTES levels at M32 in RTS,S vaccinees. a) Anti-CSP FL IgG and IgG1 **b)** anti-NANP IgG4; and **c)** anti-Cterm IgG and IgG1. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled *n* =26.



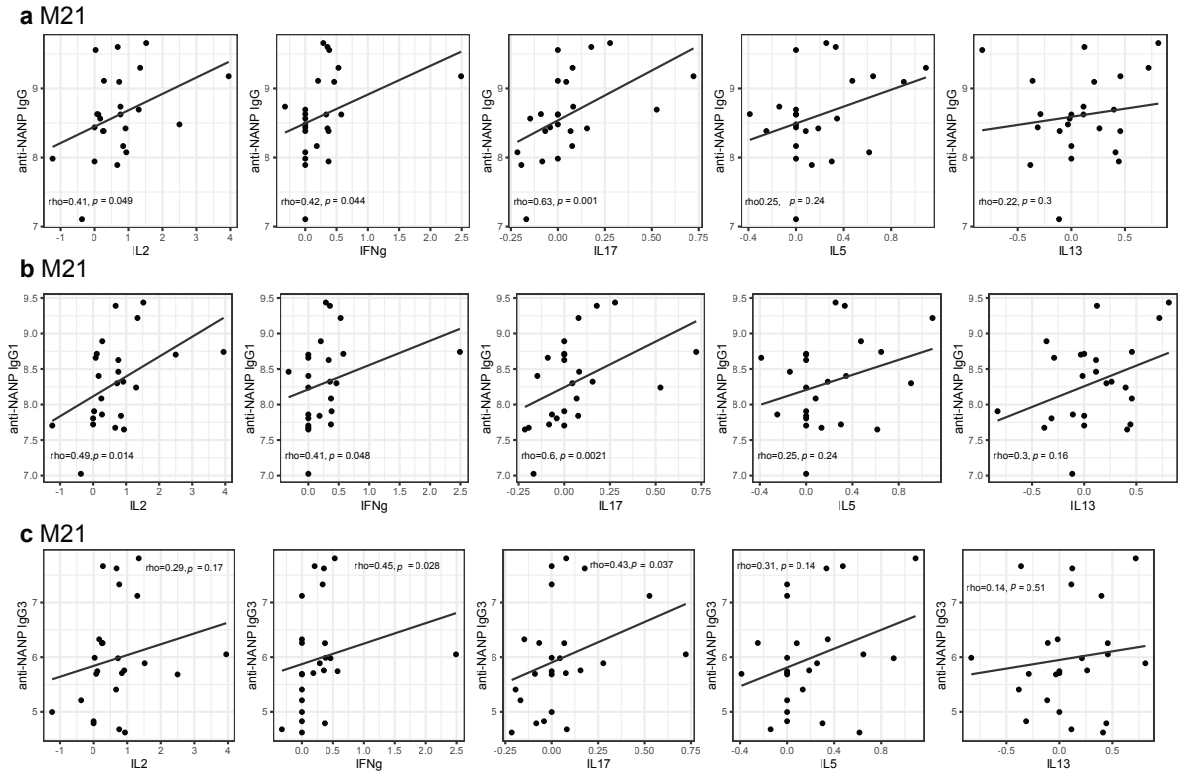
Supplementary Figure 12. Correlations of anti-HBsAg IgG and IgG1-4 subclasses with HBsAg-stimulated primary immunogenicity markers at M21 in RTS,S vaccinees. a) Anti-HBsAg IgG; **b)** Anti-HBsAg IgG1; **c)** Anti-HBsAg IgG2; **d)** Anti-HBsAg IgG3; **e)** Anti-HBsAg IgG4. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled *n* =24.



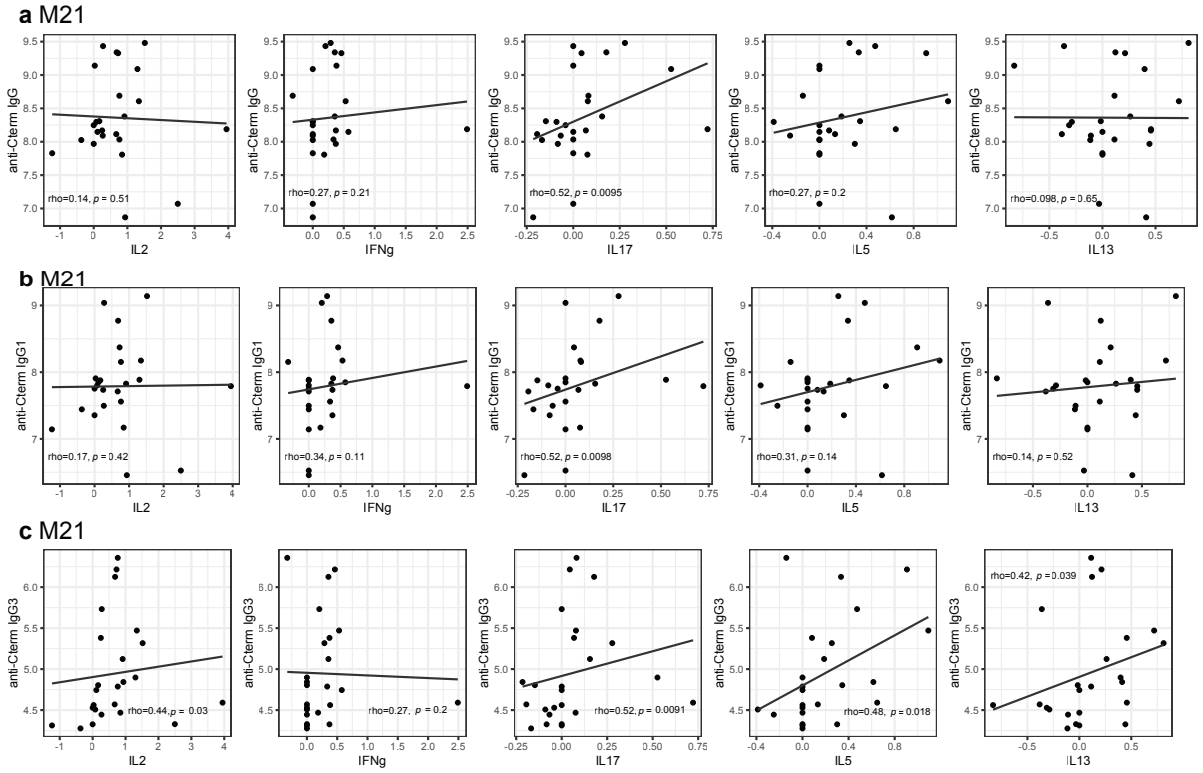
Supplementary Figure 13. Correlations of anti-HBsAg IgG and IgG1, IgG2, and IgG4 subclasses with HBsAg-stimulated primary immunogenicity markers at M32 in RTS,S vaccinees. a) Anti-HBsAg IgG; **b)** Anti-HBsAg IgG1; **c)** Anti-HBsAg IgG2; **d)** Anti-HBsAg IgG4. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled n =25.



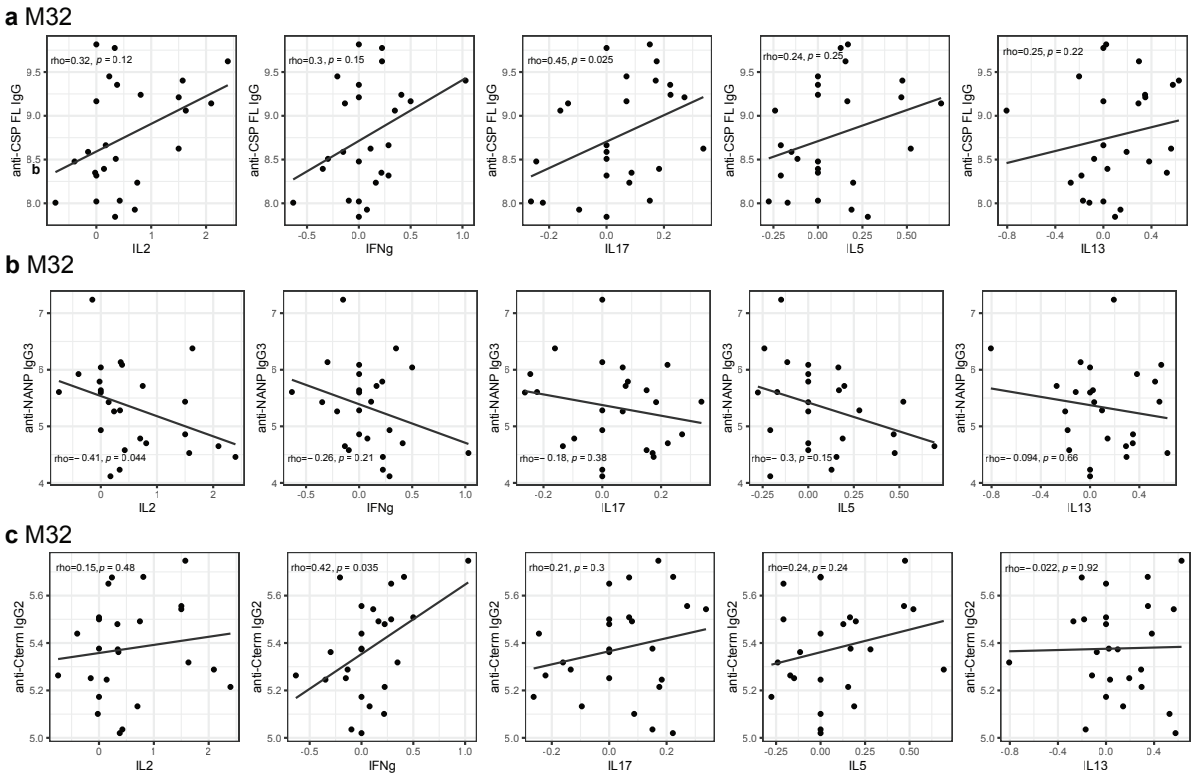
Supplementary Figure 14. Correlations of anti-CSP FL IgG and IgG1, and IgG3 subclasses with HBsAg-stimulated primary immunogenicity markers at M21 in RTS,S vaccinees. a) Anti-CSP FL IgG; **b)** Anti-CSP FL IgG1; **c)** Anti-CSP FL IgG3. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled *n* =24.



Supplementary Figure 15. Correlations of anti-NANP IgG and IgG1, and IgG3 subclasses with HBsAg-stimulated primary immunogenicity markers at M21 in RTS,S vaccinees. a) Anti-NANP IgG; **b)** Anti-NANP IgG1; **c)** Anti-NANP IgG3. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled n =24.



Supplementary Figure 16. Correlations of anti-Cterm FL IgG and IgG1, and IgG3 subclasses with HBsAg-stimulated primary immunogenicity markers at M21 in RTS,S vaccinees. a) Anti-Cterm IgG; **b)** Anti-Cterm IgG1; **c)** Anti-Cterm IgG3. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled n =24.



Supplementary Figure 17. Correlations of antibodies to anti-CSP antigens with HBsAg-stimulated primary immunogenicity markers at M32 in RTS,S vaccinees. a) Anti-CSP FL IgG; **b)** anti-NANP IgG3; and **c)** anti-Cterm IgG2. Log_{10} MFI antibody levels and cytokine log_{10} ratios are shown. Spearman rho and P-values are shown in the plots with a linear regression trend line. R3R (RTS,S-boosted) and R3C (RTS,S-unboosted) have been pooled n =25.

Article 3

This article addresses the third objective to characterize the immunophenotypic changes induced by chronic malaria exposure associated with T-cell exhaustion and its association with B cell subsets previously found to be altered by malaria exposure. The results of this research have been published in the following article:

Robert A. Mitchell, Itziar Ubillos, Pilar Requena**,** Joseph J. Campo, Maria Ome-Kaius, Sarah Hanieh, Alexandra Umbers, Paula Samol, Diana Barrios, Alfons Jiménez, Azucena Bardají, Ivo Mueller, Clara Menéndez, Stephen Rogerson, Carlota Dobaño, and Gemma Moncunill**.** ***Chronic malaria exposure is associated with inhibitory markers on T cells that correlate with atypical memory and marginal zone-like B cells.*** Clinical and Experimental Immunology. 2024 Apr 23;216(2):172-191.

Impact factor (2023): 3.4 Quartile: 2. Category: Immunology

Research Article

Chronic malaria exposure is associated with inhibitory markers on T cells that correlate with atypical memory and marginal zone-like B cells

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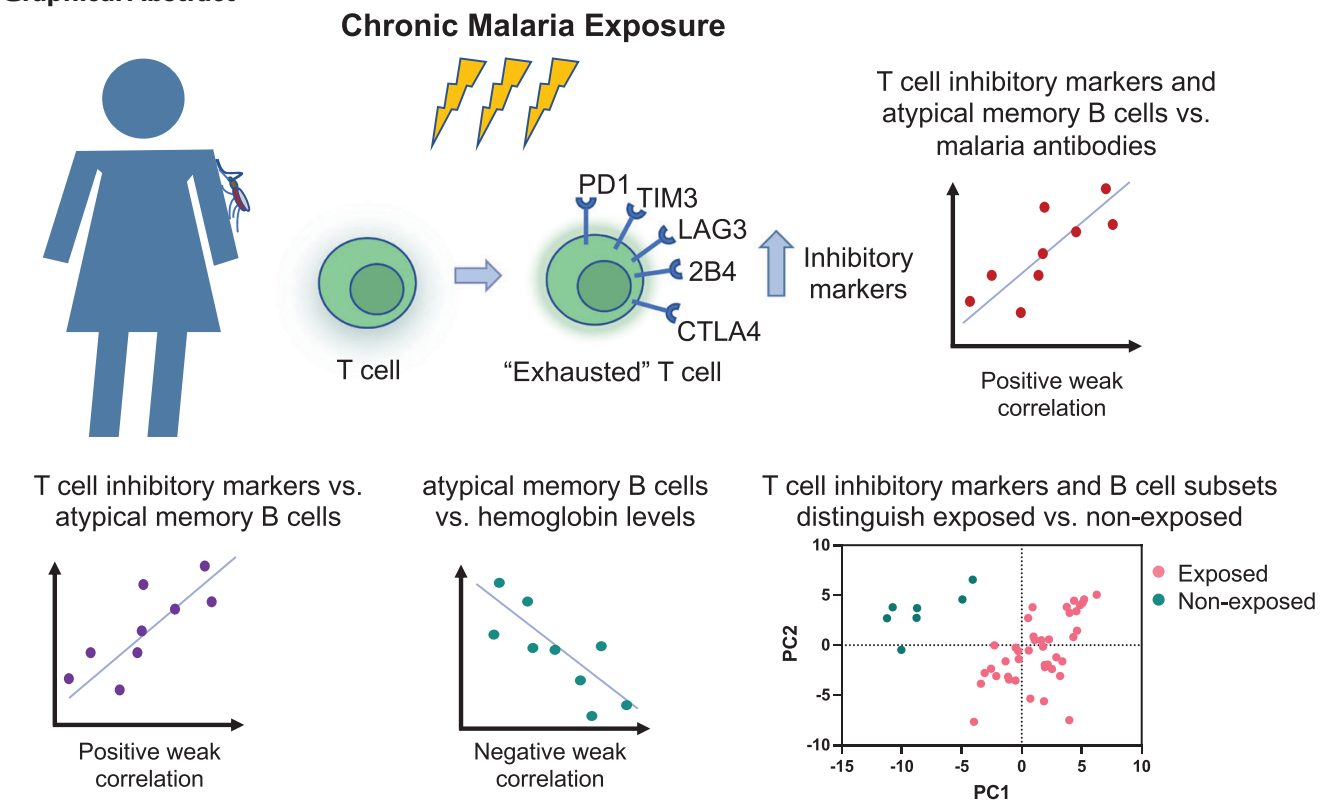
[‡]These authors share senior authorship.

Abstract

Chronic immune activation from persistent malaria infections can induce immunophenotypic changes associated with T-cell exhaustion. However, associations between T and B cells during chronic exposure remain undefined. We analyzed peripheral blood mononuclear cells from malaria-exposed pregnant women from Papua New Guinea and Spanish malaria-naïve individuals using flow cytometry to profile T-cell exhaustion markers phenotypically. T-cell lineage (CD3, CD4, and CD8), inhibitory (PD1, TIM3, LAG3, CTLA4, and 2B4), and senescence ($CD28^-$) markers were assessed. Dimensionality reduction methods revealed increased PD1, TIM3, and LAG3 expression in malaria-exposed individuals. Manual gating confirmed significantly higher frequencies of $PD1^+CD4^+$ and $CD4^+$, $CD8^+$, and double-negative (DN) T cells expressing TIM3 in malaria-exposed individuals. Increased frequencies of T cells co-expressing multiple markers were also found in malaria-exposed individuals. T-cell data were analyzed with B-cell populations from a previous study where we reported an alteration of B-cell subsets, including increased frequencies of atypical memory B cells (aMBC) and reduction in marginal zone (MZ-like) B cells during malaria exposure. Frequencies of aMBC subsets and MZ-like B cells expressing $CD95^+$ had significant positive correlations with $CD28^+PD1^+TIM3^+CD4^+$ and DN T cells and $CD28^+TIM3^+2B4^+CD8^+$ T cells. Frequencies of aMBC, known to associate with malaria anemia, were inversely correlated with hemoglobin levels in malaria-exposed women. Similarly, inverse correlations with hemoglobin levels were found for $TIM3^+CD8^+$ and $CD28^+PD1^+TIM3^+CD4^+$ T cells. Our findings provide further insights into the effects of chronic malaria exposure on circulating B- and T-cell populations, which could impact immunity and responses to vaccination.

Graphical Abstract



Keywords: chronic malaria, exhaustion, tolerance, T cell, B cell

Abbreviations: AMA1: apical membrane antigen-1; aMBC: atypical memory B cells; CD: cluster of differentiation; CTLA4: cytotoxic T-lymphocyte-associated antigen-4; DBL: Duffy binding like; DBP: Duffy binding protein; DN: double negative; EBA: erythrocyte binding antigen; FMO: fluorescence minus one; FSC: forward scatter; g/dL: grams/deciliter; IFN: interferon; LAG3: lymphocyte activation gene-3; MFI: mean fluorescence intensity; MSP: merozoite surface protein; MZ: marginal zone; PBMC: peripheral blood mononuclear cells; PC: principal components; PCA: principal components analysis; PD1: programmed cell death-1; PDL1: programmed cell death ligand-1; Pf: *Plasmodium falciparum*; PfSPZ: *Plasmodium falciparum* sporozoite; PNG: Papua New Guinea; Pv: *Plasmodium vivax*; SLE: systemic lupus erythematosus; SSC: side scatter; TACI: trans-membrane activator calcium modulator and cyclophilin ligand interactor; TCR: T-cell receptor; TFH: T follicular helper; TIM3: T-cell immunoglobulin and mucin domain-3; UMAP: Uniform Manifold Approximation and Projection.

Introduction

Chronic immune activation resulting from cancer [1], autoimmune disorders such as systemic lupus erythematosus (SLE) [2], and persistent infections, including viral hepatitis, HIV, and malaria [3, 4], can induce immunophenotypic and functional changes associated with immune dysregulation, often termed "exhaustion". The exhausted phenotype typically results in the expression of inhibitory receptors, loss of function of T cells, reduced production of proinflammatory cytokines, impaired B-cell proliferation, presence of autoantibodies, and diminished production of neutralizing antibodies [5].

In malaria, naturally acquired immunity to clinical symptoms develops slowly with age and repeated exposure, yet sterile protection is never fully achieved [6]. Studies have proposed that chronic malaria exposure leads to functional exhaustion of lymphocytes, potentially impeding control of infection and naturally acquired immunity and ultimately leading to a state of immune tolerance [7–9].

During acute infections and chronic malaria exposure, upregulation of inhibitory markers on T cells, such as CD279 [programmed cell death-1 (PD1)], CD223 [lymphocyte-activation gene-3 (LAG3)], and CD366 [T-cell immunoglobulin and mucin domain-3 (TIM3)], has been reported in both murine models and humans [10–13]. In addition, studies of children living in Ghana comparing complicated versus uncomplicated or asymptomatic malaria showed varying expression patterns of co-inhibitory molecules on $CD4^+$ T cells, with higher frequencies of $CD152^+$ [cytotoxic T-lymphocyte-associated antigen-4 (CTLA4)] or $PD1^+$ $CD4^+$ T cells in children with complicated malaria than in those with uncomplicated malaria [14]. The inhibitory molecule CD244 (2B4) has been reported to be upregulated on $CD8^+$ T cells undergoing functional exhaustion during chronic malaria infection in mice [10]; however, its role in chronic human malaria has yet to be fully defined. Besides, the loss of costimulatory molecule CD28 expression on T cells, known to be a sign of immune senescence, has been shown to occur along with the co-expression of inhibitory markers PD1 and CTLA4 during symptomatic *Plasmodium falciparum* (*Pf*) infection [15].

Atypical memory B cells (aMBCs), a cell subset that has been associated with chronic infections and autoimmune diseases [16], have been reported to increase in frequency during chronic malaria exposure [17]. The expansion of circulating aMBCs may be accompanied by the upregulation of inhibitory receptors FcRL4 [18], FcRL3, and FcRL5 [19], known modulators of B-cell function associated with exhaustion. Moreover, a study of children living in Kenya found that chronic *Pf* exposure was associated with an expansion of PD1-expressing and PD1/LAG3-co-expressing $CD4^+$ T cells that were inversely correlated with activated and classical MBCs,

as well as an increased frequency of aMBC [20]. However, not all studies have pointed to an association of aMBC with immune exhaustion. A previous study of aMBC from asymptomatic, semi-immune adults from Gabon showed the ability of this subset to produce protective neutralizing antibodies against *Pf* blood-stage parasites [21]. A recent study of chronic malaria infection in Malian donors demonstrated that aMBCs are not hyporesponsive to malaria antigen, but rather the high expression of inhibitory receptors on these cells permits a selective response to membrane-bound antigens (not soluble antigens) and are capable of BCR signaling, antigen internalization, and differentiation towards plasma cells [16, 22]. Another recent study has shown that aMBCs are part of a broader alternative B-cell lineage and may be more common in healthy individuals than was previously thought [23]; aMBCs were present in both malaria-exposed and non-exposed healthy donors. Importantly, when malaria-naïve individuals were vaccinated with whole *Pf* sporozoites (PfSPZ), aMBCs were found to increase following the primary dose and could be recalled upon subsequent boosts, suggesting that chronic stimulation may not be required to elicit this alternative B-cell lineage [23]. However, the authors noted that the aMBC defined by $CD21^-$ and $CD27^-$ were more abundant following successive boosts, which aligns with earlier studies showing that aMBC develops following repeated antigen exposure [18, 20, 23].

Previous studies by our group involving pregnant women from malaria-endemic Papua New Guinea (PNG), demonstrated that chronic malaria exposure altered frequencies and phenotypes of B-cell subsets [24, 25]. Specifically, an increase in the frequencies of resting and active aMBCs expressing inhibitory PD1, CD95, and costimulatory CD80 makers were found in the malaria-exposed individuals [25]. Furthermore, a reduction in frequencies of circulating marginal zone (MZ)-like B cells with upregulated CD95 and decreased expression of the trans-membrane activator calcium modulator and cyclophilin ligand interactor (TACI) were also found in malaria-exposed women [25]. These findings suggest mechanisms that may permit B-cell activation to control malaria infections while exhibiting a level of impairment allowing tolerance [25].

Here, peripheral blood mononuclear cells (PBMC) from the same PNG cohort were analyzed by flow cytometry to phenotypically profile inhibitory (PD1, LAG3, TIM3, CTLA4, and 2B4) and senescence $CD28^-$ markers on T cells associated with chronic malaria exposure. We further assessed the correlations of T-cell frequencies expressing inhibitory and senescence markers with the B-cell subsets previously characterized by our group [25]. Results provide further insight into the heterogeneity of co-inhibitory markers expressed during chronic malaria exposure and their association with B-cell subsets that could help guide therapeutic studies and vaccine development efforts in malaria-endemic populations.

Materials and methods

Study participants and ethical approval

This study involved 46 malaria-exposed pregnant women from the Madang Province in PNG, a region known for year-round high malaria transmission, who were enrolled during their first antenatal clinic visit between 2008 and 2010 (Table 1) as part of a larger multi-center PregVax project designed to assess the burden of *P. vivax* (*Pv*) malaria during pregnancy in five endemic countries [26]. In addition, eight male malaria-naïve donors from Barcelona, Spain, who had never traveled to a malaria-endemic country, were included in the study. All volunteers provided written informed consent, and ethical approval for this study was granted by the Medical Research Advisory Committee in PNG (MRAC 08.02) and Hospital Clinic of Barcelona Ethics Review Committee (Comitè Ètic d´Investigació Clínica) in Spain.

Sample processing and PBMC isolation

Ten milliliters of heparinized blood were collected from each volunteer in PNG, and samples were processed as previously described [24, 25]. Briefly, plasma was separated from the cellular fraction of blood by centrifugation at 600×*g* for 10 minutes at room temperature, then aliquoted and stored at – 80°C. A density gradient (Histopaque-1077, Sigma-Aldrich) was used to fractionate blood cells and obtain PBMCs, which were then frozen in fetal calf serum and 10%

Table 1. Characteristics of study participants by malaria exposure.

	Non-exposed (*n* = 8)	Low-exposure (*n* = 21)	High-exposure (*n* = 25)
Age[a]	48 (34–53)	23 (19–34)	25 (18–38)
Gestational age (weeks)[a]	NA	24 (18–29)	24 (19–31)
Number of pregnancies (including current) [a]	NA	2 (1–4)	2 (1–7)
Proportion of previous spontaneous abortions	NA	1 (4.76)	0 (0)
Proportion with moderate anemia (<11 g/dL)	-	20 (95.23)	21 (84)
Proportion with severe anemia (<7 g/dL)	0 (0)	0 (0)	2 (8)
Proportion with fever in the last 24 hours[b]	0 (0)	2 (9.5)	1 (4)
Proportion with active malaria infection[c]	NA	7 (33.3)	4 (16)

[a]Self-reported data presented as medians (interquartile range).
[b]Self-reported.
[c]Confirmed by PCR, either *Pv* or *Pf*.
Proportions presented as *n* (%).
-, data not available.
NA, not applicable.
g/dL, grams/deciliter.

dimethyl sulfoxide, followed by storage in liquid nitrogen. PBMCs were shipped in liquid nitrogen to the Barcelona Institute for Global Health for further processing and analysis. Control PBMCs from Spain were collected and processed following the same protocol used in PNG.

Classification of malaria exposure

An earlier study from our group investigated the antibody responses to malaria from 93 pregnant women living in PNG [24], followed by a comprehensive study of B cells in women with chronic malaria exposure [25] involving a subset of 55 out of the 93 malaria-exposed individuals. Prevalence of malaria in the PregVax cohort measured by PCR of a subset of samples was 13.9% (36/258) for *Pv*, 11.6% for *Pf*, and 2.2% (26/1165) for mixed *Pf-Pv* infection [26]. Classification of intensity levels of exposure has been described previously [25]. Briefly, Luminex technology was used to measure median fluorescence intensity (MFI) levels of IgG antibody responses to *Pf* apical membrane antigen 1 (PfAMA1), merozoite surface protein 1 (PfMSP-1_{19}), region II of the 175 kDa erythrocyte binding protein (PfEBA175-II) and the VAR2CSA DBL domains PfDBL3x, PfDBL5_{ε}, PfDBL6_{ε} associated with placental malaria; as well as *Pv* merozoite surface protein 1 (PvMSP-1_{19}) and Duffy-binding protein (PvDBP). Malaria exposure was classified as low or high according to the breadth and magnitude of antibody levels based on cumulative exposure to *Plasmodium* [25]. The magnitude of IgG responses to the five antigens was distributed into tertiles and ranked from 1 (low exposure) to 3 (high-exposure), and breadth was defined by combining the responses with a total ranking from 5 to 9 and 10 to 15 as low- and high-exposure, respectively. Antibody levels to malaria antigens were generally high in those participants, supporting a high cumulative exposure to both *Pf* and *Pv* that was expected given that the study participants lived in an area of high malaria transmission. Although not all participants were infected at the time of sample collection, they may have been recently infected or exposed but protected. Therefore, we consider that the participants suffered chronic exposure.

Phenotyping and gating strategy

The panel, staining procedure, and gating strategy for the B-cell study were previously described [25], and the same staining protocol was used for the T-cell exhaustion marker panel. Briefly, PBMCs were thawed, and their viability was checked on a Guava® Cytometer using Guava ViaCount Reagent (Millipore, Madrid, Spain). All samples with viabilities >60% were stained for flow cytometry assays. The T-cell exhaustion panel staining was performed if there were enough cells after B-cell staining; 46 out of 55 samples were stained here. BD Comp Beads were used for compensation controls. One million PBMCs per sample were used for staining, and comp beads and cells were acquired on a BD LSR II Fortessa cytometer. Samples were stained and acquired in batches of 6 to 11, and each batch consisted of a mix of non-exposed controls and low- and high-exposure individuals. PBMC were stained for T-cell lineage, inhibitory, and senescence markers and acquired during the same period as the samples analyzed in the previously published B-cell study [25]. Cell suspensions were stained with LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen, Madrid, Spain), washed, and blocked with PBS-BSA 0.05%. Cells were stained in a multiparametric panel including the following antibodies: anti-CD14 v500/AmCyaN (clone M5E2; BD Biosciences, Spain), anti-CD19 v500/AmCyaN (clone HIB19; BD), anti-CD3 AF700 (clone UCHT1; BD), anti-CD4 APC/H7 (clone SK7; BD), anti-CD8 PerCP (clone SK1; BD), anti-TIM3 BV421/Pacific Blue (clone F38-2E2; Biolegend), anti-LAG3 (CD223) FITC (clone 17B4, Enzo), anti-CTLA-4 (CD152) APC (clone BN13; BD), anti-PD1 (CD279) BV605 (clone EH12.2H7; Biolegend), anti-2B4 (CD244) PE (clone C1.7; Biolegend), and anti-CD28 PECy7 (clone CD28.2; BD). One experimental batch (six individuals) did not have anti-LAG3 antibody in the staining mix, so those individuals were not included in the analysis reporting LAG3 based on manual gating.

Gating (Supplementary Fig. S1) and data analysis were performed using FlowJo version 10.8.2 (BD). The gating of positive events was defined using fluorescence minus one (FMO). Cleanup gating was performed in the time gate and FSC-A versus FSC-H and SSC-A versus SSC-H for singlets. $CD14^+$ and $CD19^+$ cells and dead cells were excluded from the analysis via a dump channel. Lymphocytes were gated using FSC-A and SSC-A. Within the lymphocytes gate, $CD3^+$ cells were identified, followed by gating of $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ (double-negative [DN]) T cells. Surface expression levels of CTLA4, LAG3, CD28, 2B4, TIM3, and PD1 were examined within the $CD4^+$, $CD8^+$, and DN T-cell subsets. A gate was created for each marker separately, without considering the co-expression of markers. Boolean gates were then created based on these single marker positive gates to identify cells expressing all possible combinations of markers.

Dimensionality reduction of flow cytometry data was performed by Uniform Manifold Approximation and Projection (UMAP) in FlowJo using the UMAP plugin (version 3.1). Samples were first down-sampled using the FlowJo plugin DownSample (version 3.3) so that non-exposed and exposed (low- and high-exposure groups pooled) had equal numbers of events within the $CD4^+$ and $CD8^+$ gates. FlowJo plugins FlowSOM (version 2.1.7) and ClusterExplorer (version 1.7.4) were used to measure the relative expression of exhaustion and senescence markers expressed within the $CD4^+$ and $CD8^+$ gates. One experimental batch was acquired using a logarithmic scale, while the others were in a linear scale on the cytometer; therefore, those six individuals were omitted from the UMAP and MFI analyses. Additional dimensionality reduction methods were performed to assess the relationships between frequencies of T-cell markers and B-cell subsets across the three exposure groups and infected individuals by principal components analysis (PCA). The selection of principal components (PC) was based on a parallel analysis that performed Monte-Carlo simulations of random data of equal dimensions to our study data. Eigenvalues of PCs from the study data were compared to the mean and confidence interval of the eigenvalues from the simulation data. Study PCs with eigenvalues greater than a percentile level of 95% compared to the simulations were selected.

Statistical analysis

Percent frequencies of T-cell subsets and marker expression were compared between the three exposure groups (non-exposed, low-, and high-exposure) and between the individuals with active *Plasmodium* infection versus non-infected exposed individuals using the Kruskal–Wallis test with

Dunn's test for correction for multiple comparisons. Initial comparison between non-exposed and exposed (low- and high-exposure pooled) individuals for Boolean analysis was done by the Mann–Whitney test with the Holm-Šídák test to correct for multiple comparisons. For any of the comparisons in the multiple Mann–Whitney test that showed a significant *P*-value (<0.05) before adjustment, the three exposure groups were then compared by Kruskal–Wallis test with Dunn's test to assess any differences between the non-exposed, low-, and high-exposure. Spearman correlation was used to measure correlation coefficients and statistical significance between cell populations and cell populations with hemoglobin levels and malaria antibody responses. All tests were two-tailed, and *P*-values were considered statistically significant at $P < 0.05$. Benjamini-Hochberg method with a 5% false discovery rate was used to correct for multiple testing in all correlations (R software version 4.2.2). Statistical analyses were performed, and figures were created in GraphPad Prism version 9.5.1 (La Jolla, CA, USA) except for Spearman correlations and Benjamini-Hochberg adjustments and correlograms, which were performed in R software version 4.2.2.

Results

Study population

Study population characteristics at the time of blood collection are described in Table 1. This analysis involved 46 malaria-exposed pregnant women from PNG and eight malaria-naïve controls from Spain (non-exposed). Malaria exposure level (low and high) was defined based on antibody magnitude and breadth to malaria antigens, and active malaria infections were confirmed by PCR performed during a previous study [24]. The low- and high-exposure groups had 21 and 25 individuals, respectively, and 11 individuals (23.9%) within the malaria-exposed groups had active *Plasmodium* infections at the time of sample donation.

Malaria exposure is associated with higher relative expression of inhibitory markers in T cells

Initial visualization of the T-cell marker expression using UMAP dimensionality reduction provided an overview of the relative intensity and clustering of elevated markers within malaria-exposed versus non-exposed groups (Fig. 1). Inhibitory markers PD1, LAG3, and TIM3 showed a more elevated expression in the malaria-exposed compared to non-exposed controls for both $CD4^+$ and $CD8^+$ T cells. Overlap of regions with elevated expression could be observed particularly for PD1 and TIM3 in the $CD4^+$ T cells and TIM3 and LAG3 in $CD8^+$ T cells for malaria-exposed individuals. No substantial difference was found for CTLA4 in $CD4^+$ and $CD8^+$ T cells, although a tendency for higher expression could be observed in the non-exposed individuals. 2B4 expression was higher in $CD8^+$ than in $CD4^+$ T cells for exposed and non-exposed individuals (Supplementary Fig. S2). Interestingly, the highest expression of 2B4 was found localized to T cells that were $CD28^-$ or had low CD28 expression.

To confirm the surface expression of inhibitory markers demonstrated by UMAP, we turned to conventional manual gating of the single marker cell frequencies within the $CD4^+$, $CD8^+$, and DN T-cell populations (Supplementary Fig. S1). Frequencies of $CD4^+$ T cells expressing PD1 and TIM3, and $CD8^+$ T cells and DN T cells expressing TIM3, but not LAG3 in any subset, were significantly elevated in the malaria-exposed compared to non-exposed individuals (Fig. 2). For frequencies of $CTLA4^+$, $2B4^+$, and $CD28^+$ T cells, no significant differences were found between malaria-exposed and non-exposed individuals; however, there appeared to be a tendency for lower $2B4^+$ T-cell frequencies in the exposed groups (Supplementary Fig. S3). Analysis of MFI showed significantly elevated levels of PD1 in $CD4^+$ and 2B4 in $CD8^+$ for the malaria-exposed individuals (Supplementary Fig. S4). We did not find significant differences between low- and high-exposure individuals in single marker T-cell frequencies nor MFI. Since there were individuals with active infection in both exposure groups, we checked if infection was associated with increased inhibitory markers. We found no significant differences between infected and non-infected malaria-exposed individuals; however, when those with active infection were separated from the high- and low-exposure groups, $PD1^+CD4^+$ T cells, $TIM3^+CD8^+$ T cells, and $TIM3^+$ DN T cells maintained significantly higher frequencies in the high-exposure group over non-exposed controls (Supplementary Fig. S5).

After observing the overlapping regions with high relative markers expression in the UMAP of malaria-exposed $CD4^+$ and $CD8^+$ T-cell subsets, we turned to Boolean gating to assess the frequencies of T cells co-expressing multiple inhibitory markers. These markers could be differentially expressed on $CD28^+$ T cells than on $CD28^-$ T cells that may be undergoing senescence: therefore, we assessed the frequencies of markers in $CD28^{+/-}$ T cells separately. Frequencies of several subsets of $PD1^+CD28^+CD4^+$ T cells with different combinations of TIM3, LAG3, and 2B4 were significantly elevated in malaria-exposed compared to malaria-naïve individuals (Fig. 3A). In contrast, no $CD28^-CD4^+$ T-cell subset differences were observed between the two comparison groups (Fig. 3B). Next, we compared the frequencies of T-cell subsets showing significant differences or tendencies ($P < 0.1$) between individuals with no, low-, and high-exposure to malaria (Fig. 3C). As expected, all individuals, irrespective of the degree of exposure or presence of infection, had a higher percentage of $CD28^+CD4^+$ T-cell subsets co-expressing PD1 plus several other biomarkers than malaria-naïve individuals. However, no differences were found when comparing high versus low-exposure groups (Fig. 3C). Moreover, within the $CD28^-$ subset, the only significant difference was between the highly malaria-exposed and non-exposed group with an increased frequency of $PD1^+LAG3^+2B4^+CD4^+$ T cells (Fig. 3C).

No significant differences were observed in the $CD28^+CD8^+$ T-cell subset after pooling individuals in the high- and low- malaria exposure groups and comparing them to non-exposed individuals (Fig. 4A). However, $TIM3^+$ and multi-marker $TIM3^+CTLA4^+2B4^+$ significantly differed in the $CD28^-CD8^+$ T-cell subset (Fig. 4B). Separating the exposed individuals into their respective low- and high-exposure groups showed a significant increase in the frequency of $CD28^+TIM3^+2B4^+CD8^+$ T cells in the high-exposure group compared to the non-exposed (Fig. 4C). Interestingly, none of the $CD8^+$ T-cell subsets with differences between malaria-exposed and naïve individuals included co-expression of PD1; instead, the malaria-exposure-associated marker was TIM3 (Fig. 4B and C).

As was observed with $CD4^+$ T cells, pooling the low and high-exposure groups, significant differences in frequencies were only found in the $CD28^+$ and not in the $CD28^-$ DN T-cell subsets compared to non-exposed individuals

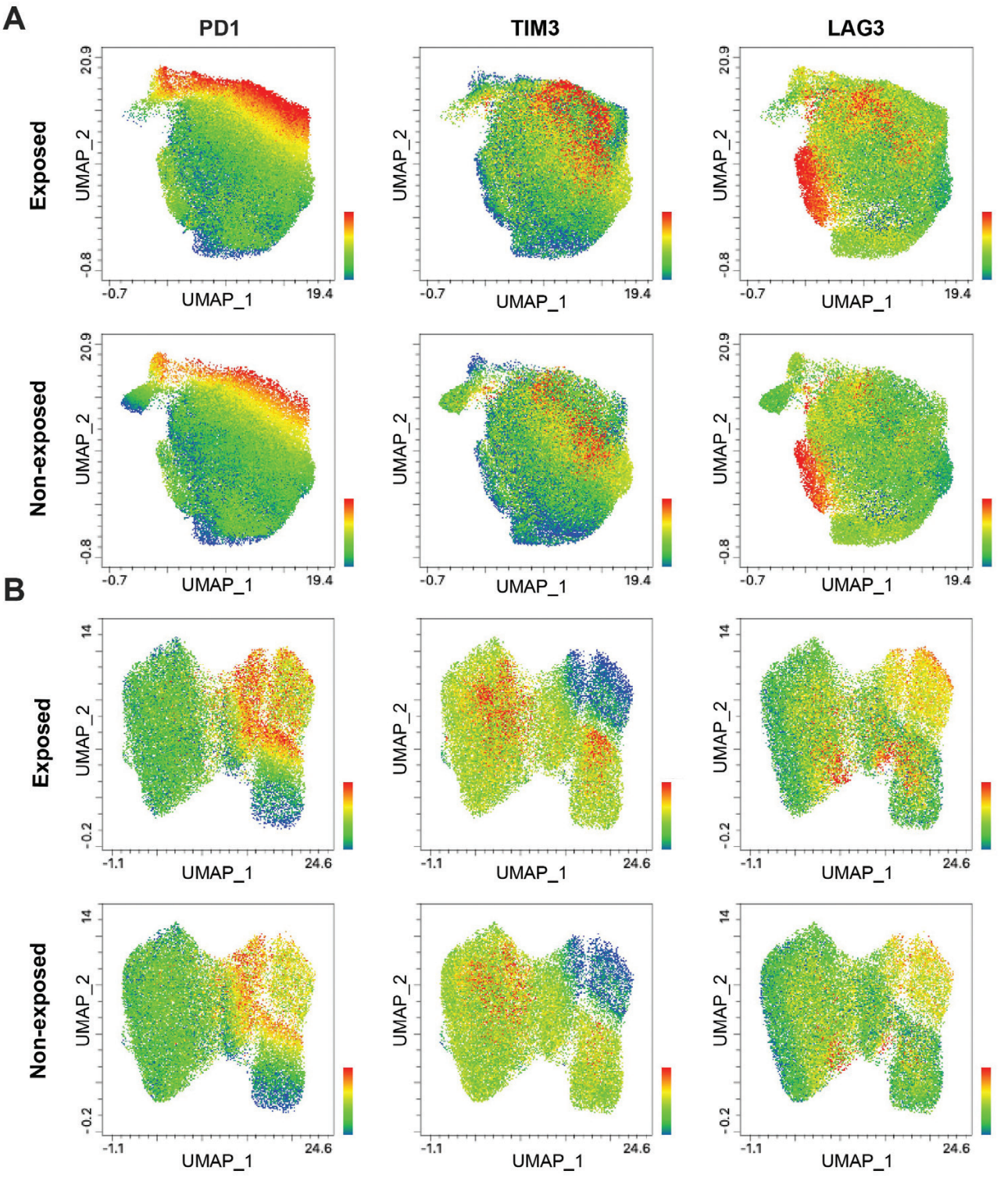


Figure 1. Malaria exposure and surface expression of inhibitory markers on T cells. UMAP plots of the expression of inhibitory markers PD1, LAG3, and TIM3 in exposed versus non-exposed individuals on (**A**) $CD4^+$ T cells and (**B**) $CD8^+$ T cells. Exposed ($n = 41$) and non-exposed ($n = 7$). Individuals were down-sampled within their respective groups, exposed and non-exposed, so that each contributed an equal number of events toward $CD4^+$ T cells (91 000 events) and $CD8^+$ T cells (35 000 events).

(Fig. 5A and B). DN T cells had higher frequencies of cells expressing PD1 and TIM3 marker combinations in the malaria-exposed group for the $CD28^+$ and $CD28^-$ subsets (Fig. 5C). In contrast, within the DN $CD28^+$ T-cell subset, frequencies of cells with single marker expression 2B4 and co-expression of CTLA4 and 2B4 were significantly lower in the malaria-exposed individuals compared to the non-exposed (Fig. 5C).

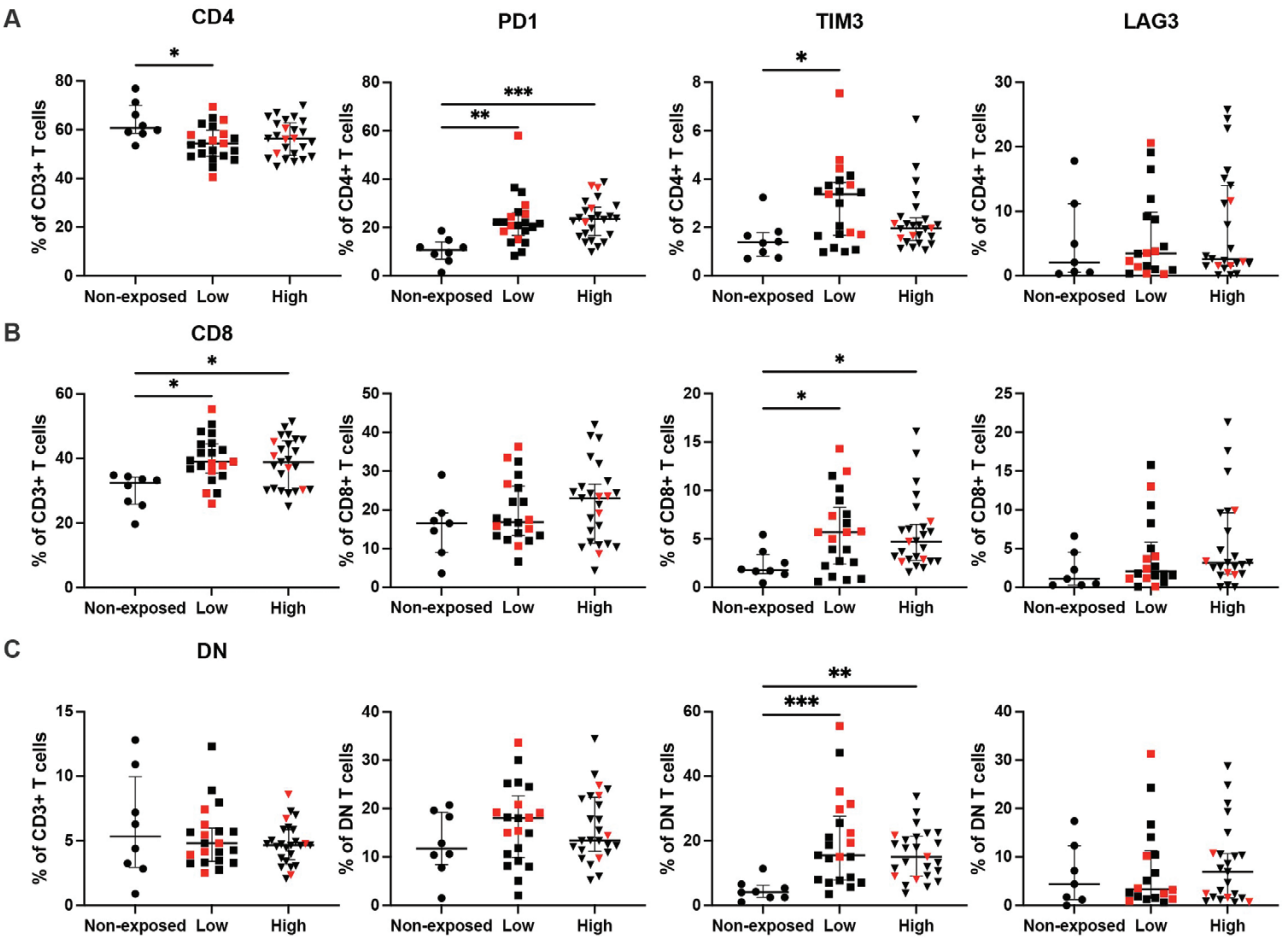


Figure 2. Malaria exposure and frequencies of T-cell subsets expressing inhibitory markers. Scatter plots of frequencies of PD1, TIM3, and LAG3 expression on (**A**) CD4⁺ T cells, (**B**) CD8⁺ T cells, and (**C**) DN T cells, categorized by malaria exposure. Non-exposed ($n = 8$), low ($n = 21$), and high ($n = 25$). LAG3⁺ T cells: non-exposed ($n = 7$), low ($n = 18$), and high ($n = 23$). Individuals with active malaria infection have non-black symbols. Lines and whiskers represent median and interquartile range. Differences were assessed with Kruskal–Wallis test with Dunn's post hoc test comparing each exposure category (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

T cells expressing PD1 and CTLA4 markers and B-cell subsets correlate with malaria antibodies

We further explored the association of T-cell inhibitory markers subsets with *Plasmodium* exposure, analyzing correlations with antibody levels to malaria blood-stage antigens detected in exposed individuals (Fig. 6A and Supplementary Fig. S6A). We found that CTLA4⁺CD4⁺ T cells had a significant low/moderate positive correlation with antibodies for malaria antigens PfDBL3x, PfDBL5$_e$, and PfEBA175-II, and PD1⁺CD8⁺ T cells had a positive low correlation with antibodies to PvMSP1$_{19}$ ($\rho = \sim 0.3$, $P < 0.05$); however, P-values were not significant (NS) after adjusting for multiple testing.

In contrast, many significant moderate correlations were found for B-cell subsets (Fig. 6B; Supplementary Fig. S6B), similar to what we previously reported [24]. As expected, frequencies of active aMBC, which were expanded with malaria exposure, were positively correlated with antibodies against several antigens, including good markers of exposure like MSP-1$_{19}$ ($\rho = 0.3$–0.5, $P < 0.01$, adjusted $P < 0.05$) [27].

Frequencies of T cells expressing inhibitory markers correlate with aMBC and MZ-like B cells

To assess the possible relationship between the expression of T-cell inhibitory markers and B-cell subsets, we performed correlation analyses within the malaria-exposed individuals (Table 2, Fig. 7, and Supplementary Fig. S7). Among the statistically significant correlations identified with the primary B-cell subsets, albeit none significant after adjustment for multiple testing, we highlight the positive correlation of PD1⁺CD4⁺ T cells with active aMBC ($\rho = 0.341$, $P = 0.020$) and an inverse correlation with plasma cells (rho = –0.298, $P = 0.044$; Fig. 7A). Unexpectedly, some positive correlations were found for T-cell subsets associated with exposure and MZ-like B cells, which were decreased in malaria-exposed individuals. For instance, a positive correlation was found between frequencies of CD4⁺ and CD8⁺ T cells expressing TIM3⁺ and MZ-like B cells (rho = 0.389 and 0.302, $P = 0.008$ and 0.041, respectively; NS after adjustment). Similarly, TIM3⁺ DN T cells positively correlated with MZ-like B cells (rho = 0.332 $P = 0.024$, NS after adjustment). Also, CTLA4⁺CD8⁺ T cells, which were not associated with malaria exposure, had a significant inverse correlation with active aMBC and active classical MBC that were expanded in exposed individuals (rho = –0.307 and –0.398, $P = 0.038$ and 0.006, respectively; NS after adjustment; Fig. 7D).

Our previous B-cell study found higher frequencies of PD1⁺, CD95⁺, and CD80⁺ resting and active aMBC, and CD71⁺ and CD40⁺ active aMBC in the malaria-exposed individuals

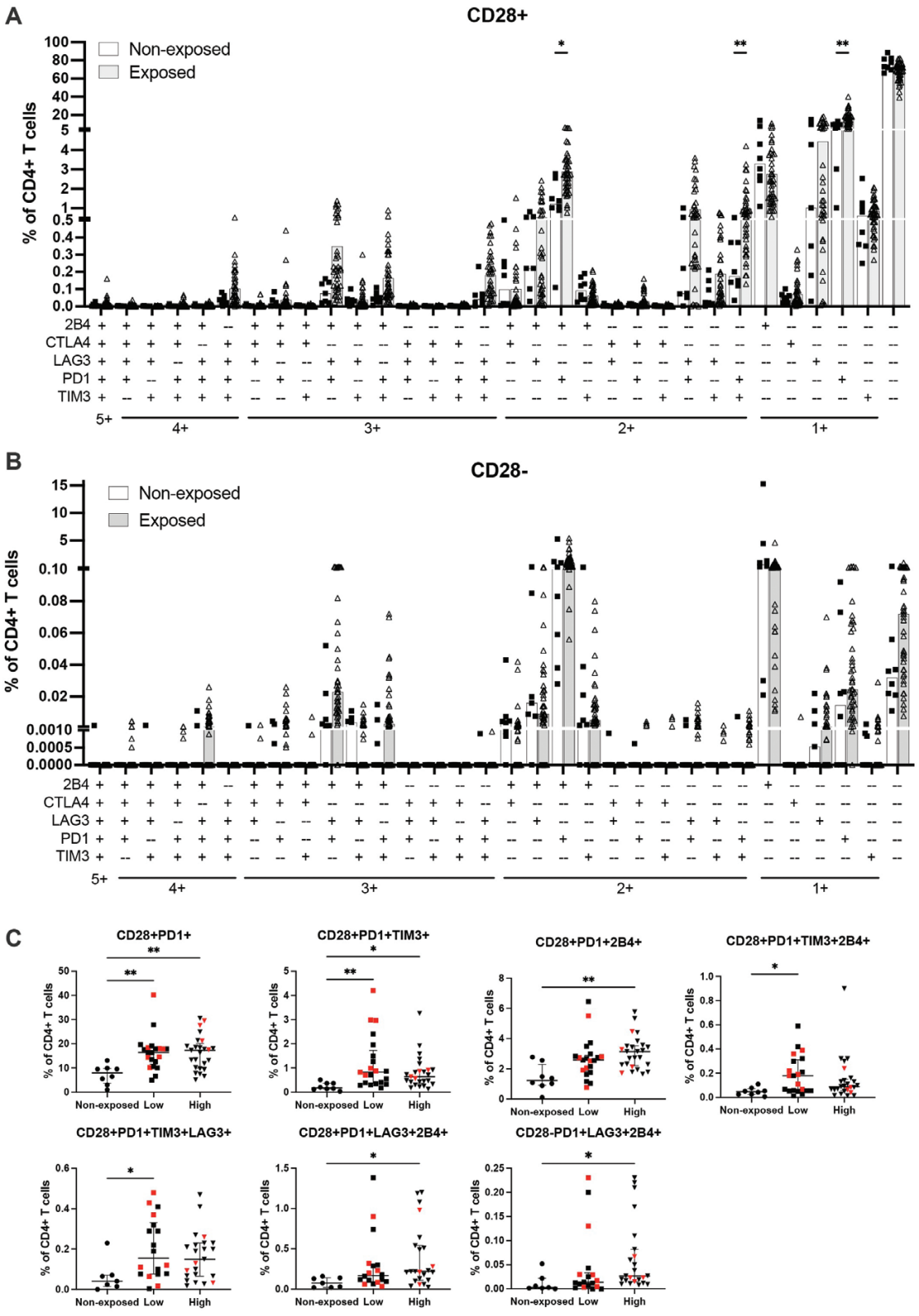


Figure 3. Association of malaria exposure with changes in frequencies of CD4⁺ T-cell subsets co-expressing multiple inhibitory markers. Scatter plots showing frequencies of (**A**) CD28⁺CD4⁺ T cells and (**B**) CD28⁻CD4⁺ T-cell subsets co-expressing multiple markers in exposed versus non-exposed individuals. For A and B, non-exposed ($n = 8$) and exposed ($n = 46$) are shown with the bar representing the median. For A and B, LAG3⁺ T cells: non-exposed ($n = 7$) and exposed ($n = 41$). Differences between non-exposed and exposed were assessed by multiple Mann–Whitney tests with Holm-Šídák test. (**C**) Frequencies of CD28$^{+/-}$CD4⁺ T cells co-expressing multiple markers in high- and low-malaria-exposed versus non-exposed individuals. Non-exposed ($n = 8$), low ($n = 21$), and high ($n = 25$). LAG3⁺ T cells: non-exposed ($n = 7$), Low ($n = 18$), and High ($n = 23$). Individuals with active malaria infection have non-black symbols. Lines and whiskers represent the median and interquartile range. Differences were assessed by Kruskal–Wallis test with Dunn's post hoc test comparing each exposure category (*$P < 0.05$, **$P < 0.01$).

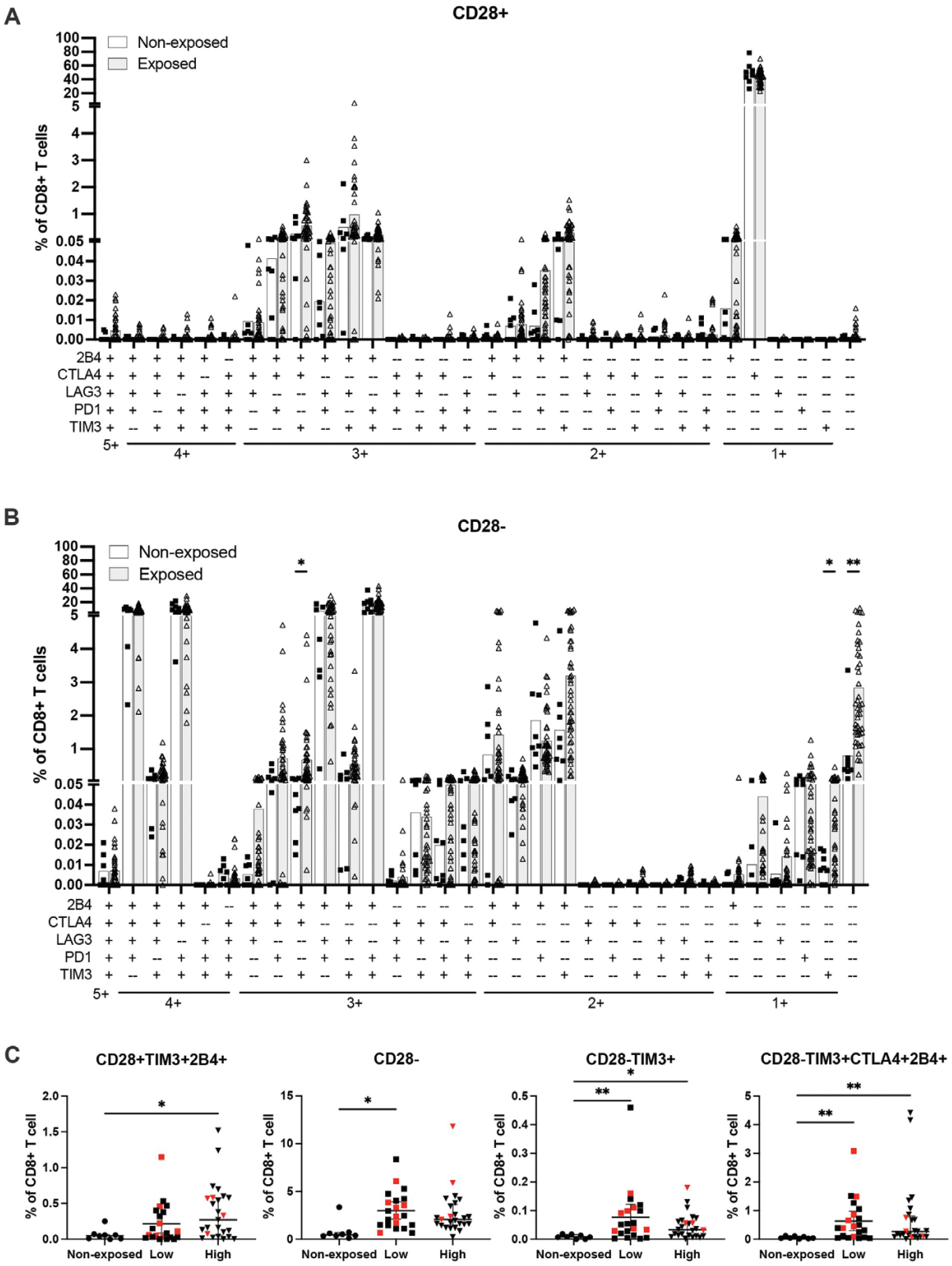


Figure 4. Association of malaria exposure with changes in frequencies of the CD8⁺ T-cell subsets co-expressing multiple inhibitory markers. Scatter plots showing frequencies of (**A**) CD28⁺CD8⁺ T cells and (**B**) CD28⁻CD8⁺ T-cell subsets co-expressing multiple markers in exposed versus non-exposed individuals. For A and B, non-exposed ($n = 8$) and exposed ($n = 46$) are shown with the bar representing the median. For A and B, LAG3 + T cells: non-exposed ($n = 7$) and exposed ($n = 41$). Differences between on-exposed and exposed were assessed by multiple Mann–Whitney tests with Holm-Šídák test. (**C**) Frequencies of CD28$^{+/-}$CD8⁺ T cells expressing multiple markers in high- and low- malaria-exposed versus non-exposed individuals. Non-exposed ($n = 8$), low ($n = 21$), and high ($n = 25$). Individuals with active malaria infection have non-black symbols. Lines and whiskers represent the median and interquartile range. Differences were assessed by Kruskal–Wallis test with Dunn's post hoc test comparing each exposure category (*$P < 0.05$, **$P < 0.01$).

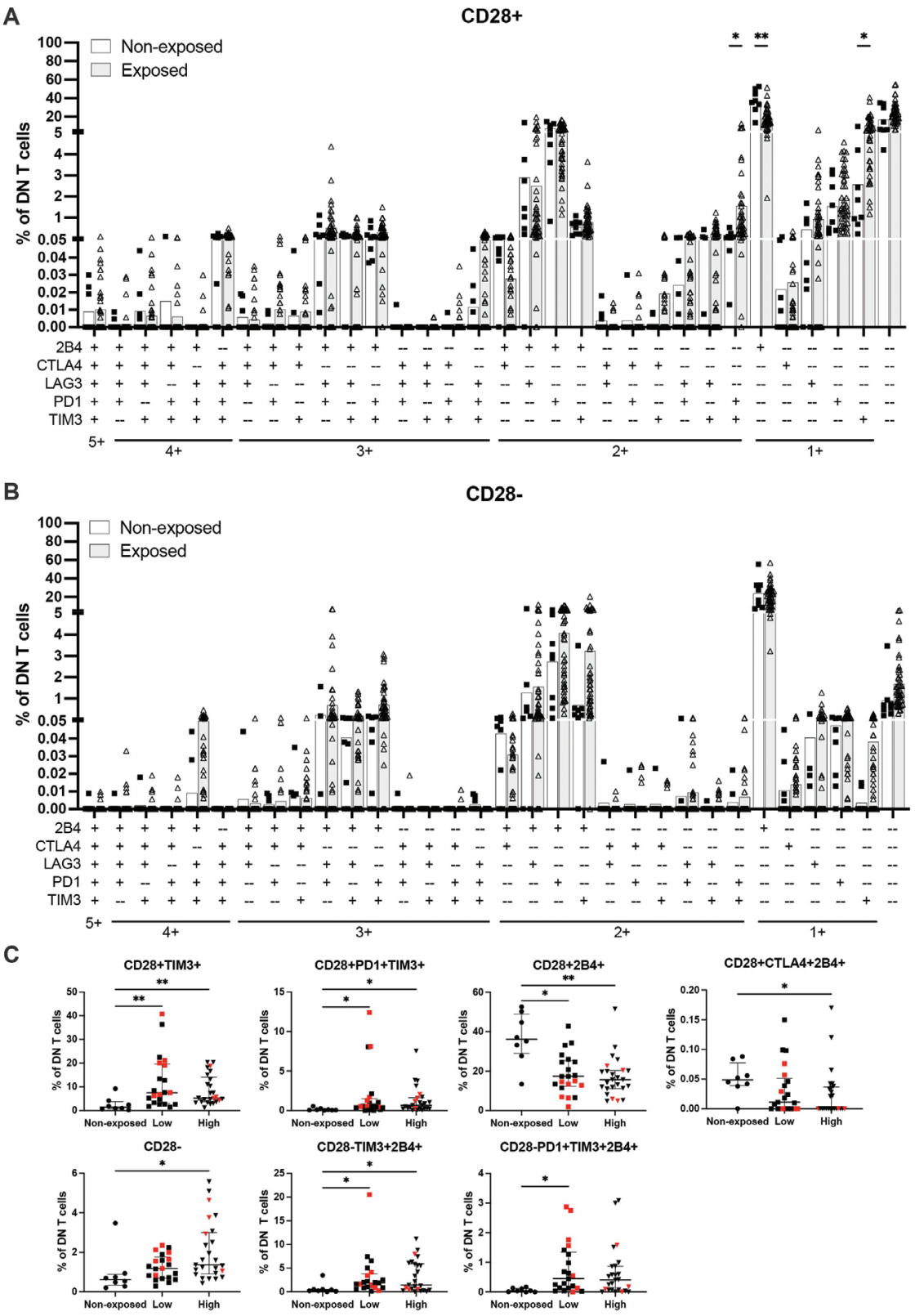


Figure 5. Association of malaria exposure with changes in frequencies of the DN T-cell subsets co-expressing multiple inhibitory markers. Scatter plots showing frequencies of multi-marker expression of exposed versus non-exposed individuals for (**A**) $CD28^{+}$DN T cells and (**B**) $CD28^{-}$DN T cells. For A and B, non-exposed ($n = 8$) and exposed ($n = 46$) are shown with the bar representing the median. For A and B, $LAG3^{+}$ T cells: Non-exposed ($n = 7$) and exposed ($n = 41$). Differences between Non-exposed and Exposed were assessed by multiple Mann–Whitney tests with Holm-Šídák test. (**C**) Frequencies of multi-marker expression in $CD28^{+/-}$DN T cells in high- and low- malaria-exposed versus non-exposed individuals. Non-exposed ($n = 8$), Low ($n = 21$), and High ($n = 25$). Individuals with active malaria infection have non-black symbols. Lines and whiskers represent the median and interquartile range. Differences were assessed by Kruskal–Wallis test with Dunn's post hoc test comparing each exposure category (*$P < 0.05$, **$P < 0.01$).

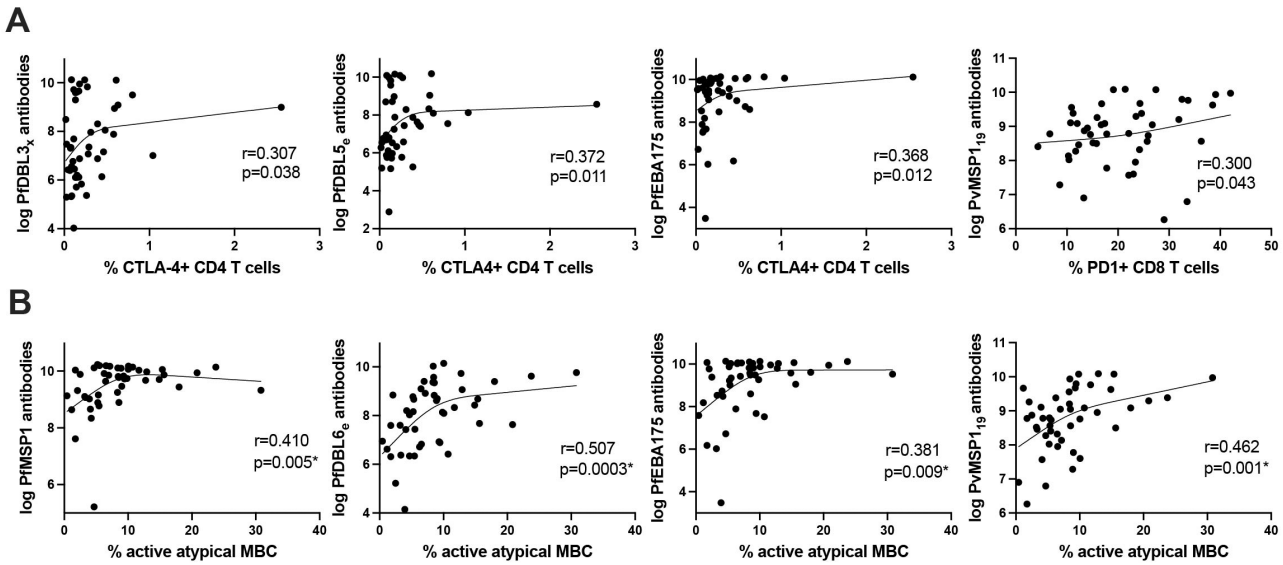


Figure 6. Correlations between frequencies of T and B-cell subsets and IgG against malaria antigens. Correlation of (**A**) CTLA4$^+$ CD4$^+$ T cells with IgG against PfDBL3x, PfDBL5$_e$, PfEBA175, and PD1$^+$ CD8$^+$ T cells with PvMSP1$_{19}$ IgG, (**B**) active atypical memory B cells (MBC) with IgG against PfMSP1, PfDBL6$_e$, PfEBA175, and PvMSP1$_{19}$ (*n = 46*). Spearman correlation coefficients, unadjusted *P*-values, and spline-fit trend lines are shown in the plots. Asterisk indicates *P*-values that remain significant (*P*-adjusted < 0.05) after Benjamini-Hochberg adjustment for multiple testing.

Table 2. Correlations between frequencies of T cells and B-cell subsets expressing inhibitory markers.

	Active aMBC	Active aMBC CD80$^+$	Active aMBC CD95$^+$	Resting aMBC	Resting aMBC CD80$^+$	Resting aMBC CD95$^+$	MZ-like	MZ-like CD80$^+$	MZ-like CD95$^+$
PD1$^+$CD4	*0.341*	0.057	0.248	0.236	0.182	*0.323*	−0.026	0.093	0.282
TIM3$^+$CD4	0.223	0.102	0.196	0.033	0.061	0.196	*0.389*	0.093	0.138
LAG3$^+$CD4	−0.176	−0.109	−0.158	0.146	−0.188	−0.149	0.000	0.134	−0.160
CTLA4$^+$CD4	−0.045	0.284	0.069	0.084	*0.332*	0.202	0.105	0.242	0.061
2B4$^+$CD4	−0.158	0.051	−0.193	0.153	−0.100	−0.247	0.062	0.200	−0.194
CD28$^+$CD4	0.015	−0.111	0.163	−0.206	0.173	0.159	−0.027	0.001	0.275
PD1$^+$CD8	0.238	−0.099	*0.326*	−0.008	0.133	*0.329*	−0.104	−0.236	*0.362*
TIM3$^+$CD8	0.252	0.150	0.094	0.012	−0.110	0.089	*0.302*	0.177	0.215
LAG3$^+$CD8	−0.062	−0.246	0.081	0.276	−0.140	0.101	−0.124	0.131	0.175
CTLA4$^+$CD8	*−0.307*	0.219	−0.196	−0.038	0.106	−0.198	−0.015	*0.347*	−0.209
2B4$^+$CD8	−0.239	−0.276	0.067	0.004	−0.105	0.085	−0.144	*−0.302*	0.075
CD28$^+$CD8	−0.048	0.013	−0.120	−0.010	−0.144	−0.172	−0.035	0.241	−0.175
PD1$^+$DN	0.247	0.299	0.095	−0.104	0.265	0.153	0.112	0.165	0.152
TIM3$^+$DN	0.237	*0.321*	*0.381*	0.168	0.166	*0.349*	*0.332*	−0.096	*0.348*
LAG3$^+$DN	−0.019	0.002	−0.119	0.093	−0.084	−0.208	−0.096	0.076	−0.243
CTLA4$^+$DN	−0.106	0.167	−0.060	−0.096	0.093	−0.131	0.110	0.236	−0.051
2B4$^+$DN	−0.075	−0.256	−0.278	−0.093	*−0.322*	*−0.342*	0.085	0.032	*−0.382*
CD28$^+$DN	−0.222	*0.302*	0.100	−0.122	*0.340*	0.120	−0.076	−0.193	0.237

Spearman's correlation test. Italic values indicate unadjusted $P < 0.05$. No *P*-values remained significant after correcting for multiple tests by Benjamini-Hochberg.

compared to the non-exposed individuals. Tendencies for increased frequencies of PD1$^+$, CD95$^+$, B220$^+$ and decreased TACI$^+$ MZ-like B cells were also observed [25]. Here, we found many correlations within the malaria-exposed women between these B and T-cell subsets, mostly from CD8$^+$ and DN T-cells (Table 2, Fig. 7, and Supplementary Fig. S7). IgG$^+$ resting and active aMBC had a significant positive correlation with TIM3$^+$CD4$^+$ and TIM3$^+$CD8$^+$ T cells (rho = 0.4–0.6, and $P < 0.0002$, adjusted $P < 0.05$; Fig. 7B). Frequencies of several B-cell subsets expressing costimulatory receptor CD80, which has a key role in signaling and immune regulation, were found to correlate positively with frequencies of CD4$^+$ T cells expressing CTLA4, a known ligand of CD80. Specifically, frequencies of CD80$^+$ resting aMBC, naïve, activated naïve, and immature B cells had significant positive correlations with CTLA4$^+$CD4$^+$ T cells (rho = 0.3–0.4, $P < 0.05$, NS after adjustment; Fig. 7C). Frequencies of the inhibitory and proapoptotic marker CD95 (Fas) expressed on resting and active aMBC and MZ-like B cells had significant low positive correlations with PD1$^+$CD8$^+$ T cells and TIM3$^+$ DN T

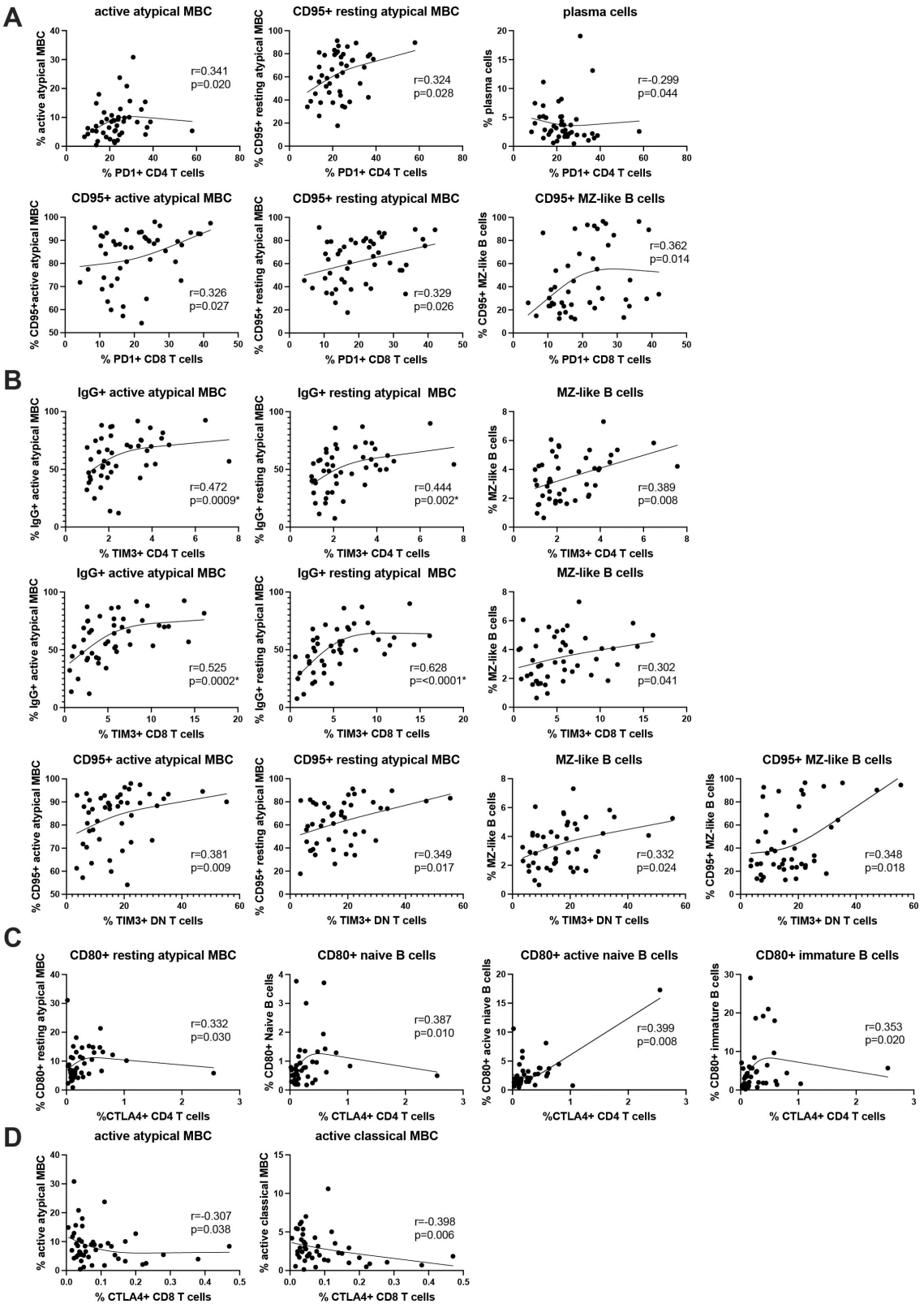


Figure 7. Correlations of frequencies of T-cell subsets expressing inhibitory markers with B-cell subsets. Correlation of (**A**) PD1$^+$ CD4$^+$ with active atypical memory B cells (MBC), CD95$^+$ resting atypical MBC, and plasma cells; and or CD8$^+$ T cells with CD95$^+$ active atypical MBC, CD95$^+$ resting atypical MBC, and CD95$^+$ MZ-like B cells. (**B**) TIM3$^+$ CD4$^+$ or TIM3$^+$ CD8$^+$ T cells with IgG$^+$ active atypical MBC, IgG$^+$ resting atypical MBC, and MZ-like B cells; and TIM3$^+$ DN T cells with CD95$^+$active atypical MBC, CD95$^+$ resting atypical MBC, MZ-like B cells and CD95$^+$ MZ-like B cells. (**C**) CTLA4$^+$ CD4$^+$ T cells with CD80$^+$ resting atypical MBC, CD80$^+$ naïve B cells, CD80$^+$ active naïve B cells, and CD80$^+$ Immature B cells. (**D**) CTLA4$^+$ CD8$^+$ T cells with active atypical MBC and active classical MBC. (n = 46). CD80 and CD86 which had missing data values, n = 43. Spearman correlation coefficients, unadjusted P-values, and spline-fit trend lines are shown in the plots. Asterisk indicates P-values that remain significant (P-adjusted < 0.05) after Benjamini-Hochberg adjustment for multiple testing.

cells (rho = ∼–0.3, $P < 0.05$, NS after adjustment; Fig. 7A and B). Frequencies of PD1⁺CD4⁺ T cells had a low positive correlation with CD95⁺ resting aMBC (rho = 0.324, $P = 0.028$, NS after adjustment; Fig. 7A).

Further correlation analyses were performed between frequencies of the B-cell subsets and the multi-marker T cells, whose combinations were identified to be significantly different between malaria-exposed and non-exposed individuals (Table 3, Fig. 8, and Supplementary Fig. S8). There were significant positive correlations between frequencies of CD95⁺ resting and active aMBC, and CD95⁺ MZ-like B cells with frequencies of CD4⁺ T cells (rho = 0.3–0.4, $P < 0.05$, only CD95⁺ resting aMBC adjusted $P < 0.05$; Fig. 8A) and DN T cells (rho = ∼0.5, $P < 0.0004$, adjusted $P < 0.01$; Fig. 8C) co-expressing the three markers CD28, PD1, and TIM3, as well as with CD28⁺2B4⁺TIM3⁺CD8⁺ T cells (rho = 0.5–0.7, $P < 0.0004$, adjusted $P < 0.006$; Fig. 8B). Interestingly, CD28⁺2B4⁺ DN T cells that were found to have significantly lower frequencies in malaria-exposed than non-exposed individuals (Fig. 5C) had significant inverse correlations with frequencies of CD95⁺ resting and active aMBC, and CD95⁺ MZ-like B cells (rho = ∼–0.5, $P < 0.0007$, adjusted $P < 0.05$; Fig. 8D).

Frequencies of TIM3⁺ T cells, resting atypical MBC, and CD95⁺ naïve and active naïve B cells correlate with decreased hemoglobin levels

aMBC have been associated with anemia during malaria [28, 29]; therefore, we evaluated the relationships between T-cell inhibitory marker expression or B-cell subsets and hemoglobin levels in malaria-exposed individuals (Supplementary Fig. S9). Resting aMBC had a significant inverse correlation with hemoglobin levels (rho = –0.359, $P = 0.015$, NS after adjustment; Supplementary Fig. S9A). We then assessed relationships between B-cell subsets expressing inhibitory markers and hemoglobin levels and found that CD95⁺ naïve, active naïve, and immature B cells, as well as TACI⁺ immature B cells, had significant inverse correlations with hemoglobin concentrations (rho = –0.3 to –0.4, $P < 0.02$), although only CD95⁺ naïve and immature B cells maintained significance after adjustment (adjusted $P < 0.05$; Supplementary Fig. S9A).

Regarding T-cell subsets, significant inverse correlations were found between hemoglobin concentrations and frequencies of TIM3⁺ CD8⁺ T cells and multi-marker CD28⁺PD1⁺TIM3⁺CD4⁺ T cells (rho = ∼–0.3, $P < 0.05$, NS after adjustment; Supplementary Fig. S9B).

T cells with inhibitory markers and B-cell subsets distinguish malaria-exposed from non-exposed individuals

Dimensionality reduction analysis by PCA, using the frequencies of T cells expressing single inhibitory markers and B-cell subsets as features, differentiated well the malaria-exposed from non-exposed individuals (Fig. 9A). PC1 loadings are shown in Fig. 9B. B-cell subtypes with high (loading > 0.5) positive PC1 loadings (so associated positively with malaria exposure) were IgG⁺ resting aMBC, IgM⁺ MZ-like, and IgG⁺ resting and active classical MBC. Many CD95⁺ B-cell subsets had high positive loadings, including resting and active aMBC, MZ-like B cells, resting and classical MBC, and immature, naïve, and active naïve B cells. Several B-cell subtypes had a high (loading < –0.5) negative loading (so negatively associated with non-malaria exposure) for PC1, including MZ-like and MZ-like expressing IgG, B220, PD1, and other subsets expressing these same markers. T cells predominantly expressing individual inhibitory markers had positive loadings for PC1, while there were fewer subsets with high loadings than B-cell subsets. The highest loadings for T cells were observed for PD1⁺ CD4⁺ T cells, TIM3⁺ CD4⁺ T cells, TIM3⁺

Table 3. Correlations between frequencies of T cells and B-cell subsets expressing multiple inhibitory markers.

	Active aMBC	Active aMBC CD95⁺	Resting aMBC	Resting aMBC CD95⁺	MZ-like	MZ-like CD95⁺
CD28⁺PD1⁺CD4	*0.328*	0.233	0.143	*0.359*	–0.084	*0.311*
CD28⁺PD1⁺TIM3⁺CD4	*0.433**	*0.386*	0.102	*0.422**	*0.352*	*0.315*
CD28⁺PD1⁺2B4⁺CD4	0.087	0.203	0.134	0.148	–0.040	0.147
CD28⁺PD1⁺TIM3⁺2B4⁺CD4	*0.255*	0.101	0.072	0.122	*0.296*	0.147
CD28⁺PD1⁺LAG3⁺2B4⁺CD4	–0.002	0.126	0.166	0.112	0.000	0.147
CD28⁺PD1⁺TIM3⁺LAG3⁺CD4	–0.019	0.024	0.136	0.027	0.135	–0.081
CD28⁻2B4⁺LAG3⁺PD1⁺CD4	–0.069	0.288	*0.378*	0.205	–0.031	0.245
CD28⁻CD8	0.267	0.093	–0.058	0.193	–0.020	0.219
CD28⁻TIM3⁺CD8	0.194	0.019	–0.082	0.125	0.115	0.270
CD28⁺TIM3⁺2B4⁺CD8	0.084	*0.508**	*0.318*	*0.516**	–0.244	*0.708**
CD28⁻TIM3⁺CTLA4⁺2B4⁺CD8	*0.384*	0.219	0.088	0.254	0.233	*0.294*
CD28⁻DN	*0.337*	0.144	0.177	0.287	–0.229	0.197
CD28⁺TIM3⁺DN	0.110	*0.365*	0.029	*0.327*	0.183	*0.439**
CD28⁺2B4⁺DN	*–0.313*	*–0.484**	*–0.350*	*–0.519**	–0.112	*–0.543**
CD28⁺PD1⁺TIM3⁺DN	*0.319*	*0.501**	0.209	*0.544**	–0.014	*0.517**
CD28⁺CTLA4⁺2B4⁺DN	–0.067	–0.065	–0.206	–0.173	0.063	–0.186
CD28⁻TIM3⁺2B4⁺DN	0.131	–0.162	0.058	–0.184	*0.297*	*–0.308*
CD28⁻PD1⁺TIM3⁺2B4⁺DN	0.178	–0.085	0.056	–0.064	0.218	–0.153

Spearman's correlation test. Italic values indicate raw P-value < 0.05. *P-values remained significant after correction for multiple testing by Benjamini-Hochberg, P-adjusted < 0.05.

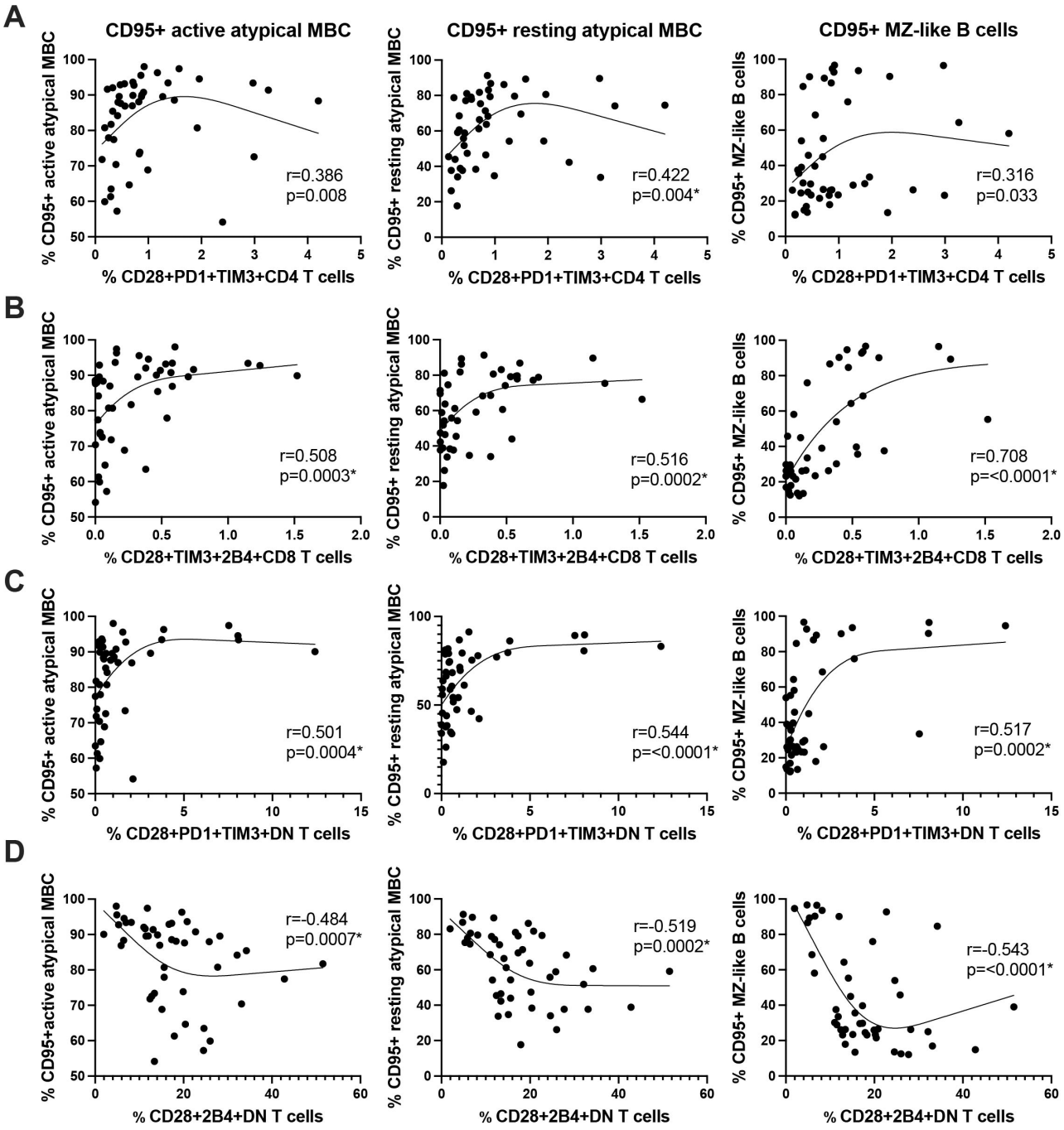


Figure 8. Correlations between frequencies of T-cell subsets expressing multiple inhibitory markers and B-cell subsets. Correlations with CD95⁺active atypical memory B cells (MBC), CD95⁺ resting atypical MBC, MZ-like B cells and CD95⁺ with (**A**) CD28⁺PD1⁺TIM3⁺ CD4⁺ T cells, (**B**) CD28⁺TIM3⁺2B4⁺ CD8⁺ T cells, (**C**) CD28⁺PD1⁺TIM3⁺ DN T cells, and (**D**) CD28⁺2B4⁺ DN T cells. ($n = 46$). Spearman correlation coefficients, unadjusted *P*-values, and spline-fit trend lines are shown in the plots. Asterisk indicates *P*-values that remain significant (*P*-adjusted < 0.05) after Benjamini-Hochberg adjustment for multiple testing.

CD8⁺ T cells, and TIM3⁺ DN T cells (loadings of 0.621, 0.391, 0.480, and 0.630, respectively). Only 2B4⁺ DN T cells had a considerable negative loading (–0.536).

A PCA using as features only the frequencies of T-cell subsets expressing combinations of markers that we found to be significantly different between malaria-exposed and non-exposed (Figs. 3, 4, and 5) showed some cluster separation with some overlap between malaria-exposed and non-exposed (Supplementary Fig. S10) along PC1. However, the infected appear more localized in the positive PC1 and PC2 scores quadrant. A high negative loading (–0.664) was found for DN CD28⁺2B4⁺ for PC1, where most of the PC scores for the non-exposed were positioned.

Discussion

Our study confirms that chronic malaria exposure in a cohort of pregnant women in PNG increased T-cell frequencies expressing certain inhibitory markers. Moreover, we

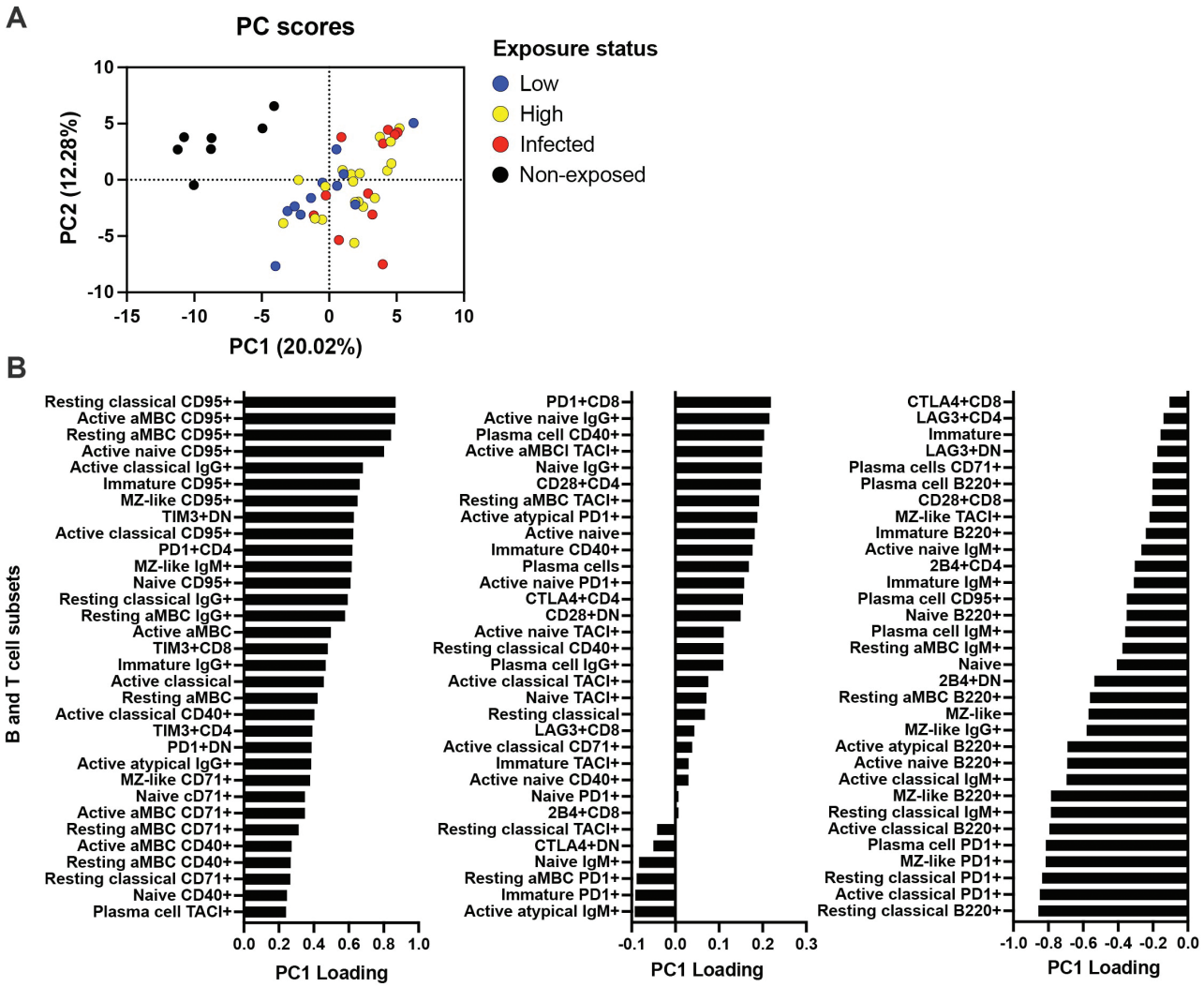


Figure 9. T cells with inhibitory markers and B-cell subsets differentiated malaria-exposed from non-exposed individuals. PCA analysis of frequencies of inhibitory markers expressed on T-cell subsets and B-cell subsets showing (**A**) PC scores plot (**B**) PC1 loadings for T-cell inhibitory markers and B-cell subsets. Individuals from the batch that were missing LAG3 antibody staining were excluded from the PCA. Non-exposed (n = 7), low (n = 11), high (n = 19), and Infected (n = 11). CD80 and CD86, which had missing data values, were not included in the PCA.

found that upregulation of these inhibitory markers correlated with frequencies of aMBC and MZ-like B cells, which had previously been confirmed to be subsets associated with the alteration of the B-cell compartment during chronic exposure.

We observed a significant increase of PD1$^+$ CD4$^+$ T cells and TIM3$^+$ for CD4$^+$, CD8$^+$, and DN T-cell subsets in the malaria-exposed individuals. Additional combinations of co-expressed inhibitory markers were observed within each T-cell subset. Studies of chronic viral infections have established that increased upregulation in the number of co-expressed inhibitory markers typically indicates the level of exhaustion [5, 30]. However, the context of the disease or state of immune activation needs to be considered, as studies have found that inhibitory marker expression alone may not necessarily indicate functional exhaustion, at least during acute infection, but instead may signify suppressive measures as homeostatic regulatory mechanisms [31, 32]. The presence of inhibitory molecules on T effector cells during acute malaria has been demonstrated to be a mechanism of immune regulation, as was reported in a study that blocked PD1 and CTLA4 *in vitro* on malaria-specific CD4$^+$ T cells, resulting in an enhancement of their effector response [33].

Studies involving malaria infection and therapeutic strategies to address malaria exhaustion have demonstrated that simultaneous blockade of multiple inhibitory receptors can synergistically reverse the state of T-cell exhaustion in mice [34]. Indeed, combined antibody blockade of PDL1 (the ligand for PD1) and LAG3 during mouse malaria infection accelerated clearance of parasitemia and improved CD4$^+$ follicular T helper cell (TFH) numbers, which correlated with enhanced antibody-mediated immunity [10]. A study of acute *Pv* infection in humans showed that blockade of CTLA4, PD1, and TIM3 restored cytokine production by antigen-specific T cells *in vitro* [35].

Phenotypic differences of co-expressed inhibitory markers were observed between CD4$^+$ and CD8$^+$ T-cell subsets. There were fewer CD8$^+$ T-cell subsets with combinations of inhibitory markers significantly different associated with malaria exposure than CD4$^+$ T-cell subsets. The above-mentioned *Pv* study of acute infection that looked at PD1, TIM3, LAG3, and CTLA4 also found differences in expression between

$CD4^+$ and $CD8^+$ T cells, with higher frequencies of $CD4^+$ T cells expressing more than one inhibitory receptor than $CD8^+$ T cells [35]. Interestingly, in our study, none of the combinations within the $CD8^+$ subset included co-expression of PD1 as was found for $CD4^+$, but instead, malaria exposure induced higher frequencies of $TIM3^+$ CD8 T cells. Additionally, combinations of LAG3 with other inhibitory markers were solely found on $CD4^+$ and not the $CD8^+$ subsets associated with malaria exposure. CTLA4 was not observed in any significant subset of $CD4^+$ T cells; however, it was found to be co-expressed with $TIM3^+2B4^+$ in the $CD28^-CD8^+$ subset associated with exposure. Murine studies of chronic lymphocytic choriomeningitis virus (LCMV) found differences in the inhibitory receptor profiles between exhausted $CD4^+$ and $CD8^+$ T cells [5, 36] with expression of 2B4 and LAG3 in $CD8^+$ T cells and PD1 and CTLA4 in $CD4^+$ T cells [36].

Many differences were found in DN T cells, which are a unique and rare subset of T cells that lack the CD4 and CD8 co-receptors and only express the T-cell receptor (TCR) alpha-beta (αβ) or gamma-delta (γδ) [37]. The exact origin of αβDN T cells is not entirely understood. Although it has been shown that they can develop in the thymus, recent studies suggest they can also develop in the periphery [38]. Furthermore, DN T cells can be derived from activated $CD4^+$ and $CD8^+$ T cells during chronic immune stimulation through the down-regulation of CD4 and CD8 molecules [38–40]. A previous study conducted on $CD8^+$ T cells expressing 2B4 in SLE patients found that they had significantly fewer $2B4^+CD8^+$ T cells than healthy individuals, and further observed that these $2B4^+$ cells can lose expression of CD8 and become DN T cells [41]. γδ T cells are expanded by malaria exposure and may have diverse immunogenic roles in function and tolerance immunity during malaria [7, 42]. Several studies have reported upregulation of TIM3 expression on γδ T cells in children under chronic malaria exposure, including a cohort living in PNG [43] and another from Uganda [44], resulting in reduced proinflammatory cytokine production and associated with asymptomatic infection, which may suggest a tolerogenic mechanism. The study from PNG also demonstrated that differences between *Pf* and *Pv* (or mixed malaria) can affect frequencies of inhibitory receptor expression with *Pf* or mixed malaria infection resulting in higher frequencies of $TIM3^+$ DN T cells compared to *Pv* infected [43]. In our study, the selection of individuals was based on antibody levels, which showed evidence of both *Pf* and *Pv* exposures, and not on current infection status. The elevated frequencies of $TIM3^+$ DN T cells that we found during chronic malaria exposure align with the observations reported in the studies mentioned above [43, 44]. Additionally, we found that the $CD28^-$ DN T-cell subset shared similar expression patterns of co-inhibitory markers that were exhibited by both the $CD4^+$ and $CD8^+$ T-cell subsets, including higher frequency of cells with combinations of co-expressed PD1 and 2B4 along with TIM3 expression in the malaria-exposed group. Interestingly, within the $CD28^+$ DN subset, the expression of 2B4 and co-expression of CTLA4 with 2B4 was significantly lower in the malaria-exposed individuals compared to the non-exposed. While it is not certain why there is a lower frequency of $2B4^+$ DN T cells during chronic exposure, this could be related to an increasing level of impairment, as was found to occur in the study mentioned above on $CD8^+$ T cells in SLE patients [41].

Few studies have characterized B-cell subsets alongside the expression of inhibitory receptors on T cells in the context of chronic malaria exposure. An earlier study in Kenya found an expansion of aMBC and an increased frequency of $CD4^+$ T cells expressing PD1 alone or in combination with LAG3 in children chronically exposed to malaria [20]. Here, we observed that a particular profile of co-inhibitory markers correlated with active aMBC and MZ-like B cells. We found positive correlations between resting and active aMBC with $PD1^+CD4^+$ T cells. Counterintuitively, all three T-cell subsets with individual expression of TIM3 positively correlated with MZ-like B cells.

Activation of B cells involves signaling through the interactions of CD80 and CD86 binding to CD28 on T cells. CTLA4 is known to compete with CD28 for binding CD80 and with higher affinity [45]. The binding of CTLA4 to CD80 inhibits B-cell proliferation and IgG secretion, and its engagement with CTLA4 on T cells downregulates T-cell activity and proliferation [46, 47]. In our previous study, the costimulatory molecule CD80 was significantly increased in resting aMBC and active aMBCs [25]. Here, we found frequencies of $CTLA4^+CD4^+$ and $CTLA4^+CD8^+$ T cells positively correlating with $CD80^+$ activated naïve and MZ-like B cells, respectively, suggesting negative signaling for proliferation and differentiation and possible modulation of IgG secretion. Interestingly, the primary T-cell inhibitory marker we found correlating with levels of antibodies to malaria blood-stage antigens was $CTLA4^+$ $CD4^+$ T cells, which may indicate a regulatory feedback mechanism to dampen the immune response and suppress antibody production.

Our study found an association between frequencies of CD95 expression on resting and active aMBC and MZ-like B cells with frequencies of $PD1^+CD8^+$ T cells and $TIM3^+$ DN T cells and an association between $CD95^+$ resting aMBC and frequencies of $PD1^+CD4^+$ T cells. Associations between frequencies of these B-cell subsets expressing CD95 were found to increase in significance and correlation strength with the co-expression of additional inhibitory markers such as TIM3 on $CD4^+$ T cells, 2B4 on $CD8^+$ T cells, and PD1 on DN T cells. Moreover, we found inverse correlations for certain $CD95^+$ B-cell subsets and $TIM3^+$ T-cell subsets with hemoglobin levels in the malaria-exposed individuals, suggesting a possible role for these particular markers expressed on B and T cells and malaria-induced anemia. CD95 receptor signaling has a role in immune homeostasis and apoptosis. It has been shown *in vitro* to induce phosphatidylserine externalization, which can lead to phagocyte recognition of apoptotic cells [48]. Phosphatidylserine expressed on infected and uninfected red blood cells is targeted by autoimmune anti-phosphatidylserine antibodies produced by aMBC, which have been shown to be associated with anemia [28, 29]. Phosphatidylserine is one of several known ligands for T-cell immunoglobulin-domain and mucin-domain-containing molecules, including TIM3 [49]. TIM3 has been demonstrated to be stimulated by phosphatidylserine binding using a Jurkat cell *in vitro* model [50], and antibody blocking of TIM3 has been demonstrated to interfere with phosphatidylserine, as well as CEACAM1 [51]. While associations between CD95 expression on B-cell subsets and TIM3 expression on T cells during chronic malaria exposure were found, with a possible connection to malaria-induced anemia, further studies would be required to elucidate these relationships fully. In addition,

it would be interesting to assess the antigen-specificity of the TIM3$^+$ T cells and confirm that they are malaria-specific as suggested by the increased trend in frequencies of PD1 and TIM3 with an increase in malaria exposure level or infection. This is in line with other studies reporting increases in these inhibitory markers, with either exposure to malaria or experiments using *in vitro* infected red blood cell stimulation [10, 33, 35].

A consequence of an exhausted phenotype is that protective responses may be inhibited. Recently, it has been demonstrated that CD8$^+$ T cells can directly kill malaria-infected RBC [52]. In addition, there has been evidence in murine studies showing the cooperation of CD8$^+$ T cells and macrophages to clear infected RBCs via TIM receptors and phosphatidylserine [53]. However, whether or not TIM3 facilitates the direct killing of malaria-infected RBC remains to be explored. CD8$^+$ T cells may also impact the killing of *Pv*-infected reticulocytes. Interestingly, some recent studies in mice demonstrated that LAG3$^+$CD8$^+$ T-cell splenocytes remained functional during acute malaria and capable of producing IFN-γ, granzyme B, and perforin [31, 54].

We did not assess other immune cell types such as monocytes and macrophages, but these populations play an important role in the immune response to malaria and also express inhibitory receptors. As mentioned above, a recent murine study showed that the cytotoxic activity of CD8$^+$ T cells induced phosphatidylserine externalization on infected RBCs, which enhanced the phagocytic activity of TIM4$^+$ macrophages [53]. TIM3$^+$ CD4$^+$ T cells have been shown to facilitate killing of intracellular pathogens phagocytosed by macrophages expressing galectin-9, a ligand for TIM3 [55]. TIM3$^+$ is known to mediate phagocytosis and has been demonstrated to clear apoptotic cells by mouse macrophages, as well as phagocytosis and cross-presentation by CD8$^+$ dendritic cells [56]. However, a recent study concluded that TIM3 expression on monocytes and macrophages restricts their activity and demonstrated that blocking TIM3 signaling with antibodies enhanced phagocytosis by murine splenic macrophages during malaria infection [57].

The main limitation of our study is that exposed individuals were pregnant women, whereas non-exposed individuals were non-pregnant men, and we cannot rule out that differences in the sex of the donors and pregnancy could have an effect on the expression of inhibitory markers. While there is no strong evidence of sex on T-cell subsets with inhibitory markers, during pregnancy, immunotolerance mechanisms are required to protect the allogeneic fetus, particularly at the maternal-fetal interface within the decidua [58]. Recent studies performing phenotypic assessments of CD4$^+$ and CD8$^+$ T cells in the maternal decidua and peripheral blood have found differential distributions of T cells expressing markers of inhibition or senescence that tend to be localized to the decidua [59–65]. Specifically, PD1 expression by CD4$^+$ and CD8$^+$ T cells was significantly higher in decidua than in PBMCs during the first trimester of normal pregnancy, and peripheral PD1$^+$CD4$^+$ and PD1$^+$CD8$^+$ T cells were significantly lower in frequency than in non-pregnant controls [59]. Another study found no difference in the proportion of PD1^{high} cells among clonally expanded CD8$^+$ effector memory cells from PBMC and decidua during the first trimester of normal pregnancy and levels from these cell populations were comparable to those from non-pregnant control PBMC [60]. In other studies, frequencies of CD8$^+$ and CD4$^+$ T cells co-expressing TIM3 and PD1, and CD8$^+$ T cells co-expressing TIM3 and CTLA4, were significantly higher in decidua than maternal blood during the first trimester [62–64]. Regarding the senescence marker CD28, no differences were found in the frequency of CD28$^-$CD8$^+$ T cells between non-pregnant control PBMC and PBMC during early pregnancy, and CD28 MFI levels on CD8$^+$ T cells were significantly lower for early pregnancy PBMC compared to non-pregnant controls [65]. Therefore, it is unlikely that differences found in peripheral cells in our study are due to the pregnancy status of the exposed women. Other studies analyzing T-cell inhibitory marker expression during acute malaria infection in mixed male and non-pregnant female patients found significant differences over healthy controls [33, 66]. Studies of children living in malaria-endemic countries have also demonstrated elevated frequencies of inhibitory markers as compared to healthy adult controls from non-malaria-endemic countries [10, 43]. In addition, the correlations observed with malaria antibodies from our study suggest that the differences were not related (or not only related) to the pregnancy status or sex. Other factors, such as concurrent helminth infections, exposure to other pathogens, nutritional status, and lifestyle differences, could also influence the observed phenotypes. HIV is not prevalent in the area, and there was no screening for coinfections when sampling was performed. Another limitation of the study is that we only assessed the phenotypic profile of T cells and could not infer the functionality of the cell subsets identified (exhausted versus activated). While we observed many biologically relevant associations, some lost significance when adjusting for multiple testing, indicating potential false positives. It is important to interpret the findings cautiously due to the exploratory nature of the study, the small sample size, and the lack of functional assays, which would be fundamental for elucidating the mechanistic roles of the inhibitory markers expressed by T cells and the relationship to aMBC and MZ-like B cells during chronic malaria infection; therefore, these associations would need to be confirmed in future studies.

In conclusion, our study has demonstrated that chronic malaria exposure results in the expression of inhibitory markers on T cells that differ between CD4$^+$, CD8$^+$, and DN T-cell subsets. These markers correlate with active aMBC and MZ-like B cells, which had previously been shown to have altered frequencies and phenotypes due to exposure, suggesting orchestrated alterations affecting both arms of the adaptive immune system. The findings presented here help to provide further insights into the impact of chronic malaria on circulating B- and T-cell populations. The impact of chronic exposure leading to persistent inflammation and possible tolerance, along with impaired immunity, has implications leading to possible increased susceptibility to reinfections with malaria or other diseases in addition to alterations in responses to vaccination.

Supplementary Data

Supplementary data is available at *Clinical and Experimental Immunology* online.

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Ethical Approval

All volunteers provided written informed consent, and ethical approval for this study was granted by the Medical Research Advisory Committee in PNG (MRAC 08.02) and Hospital Clinic of Barcelona Ethics Review Committee (Comitè Ètic d´Investigació Clínica) in Spain.

Conflict of Interests

Joseph J. Campo is employed by Antigen Discovery Inc., Irvine, CA, USA. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data Availability

The raw data supporting the conclusions of this article will be made available by the authors upon request.

Author Contributions

RM analyzed the data and wrote the first draft of the manuscript. IU performed the experiments. PR obtained the preliminary data. JC and GM designed the flow cytometry panels and conceived the experimental design. MO, SH, and AU recruited and followed-up with the volunteers. PS, PR, DB, and AJ processed lab samples. DB and AJ helped with performing the experiments. AB, IM, CM, SR, and CD contributed to the epidemiological study design and/or conduct (PregVax study). PR and CD were responsible for the immunological studies within PregVax. CM, SJR, and CD were responsible for funding acquisition. CD and GM supervised the experimental design. RM and GM interpreted the data and GM supervised data analysis. GM participated in the writing of the manuscript. All authors reviewed the manuscript.

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Supplementary Material

Chronic malaria exposure is associated with inhibitory markers on T cells that correlate with atypical memory and marginal zone-like B cells

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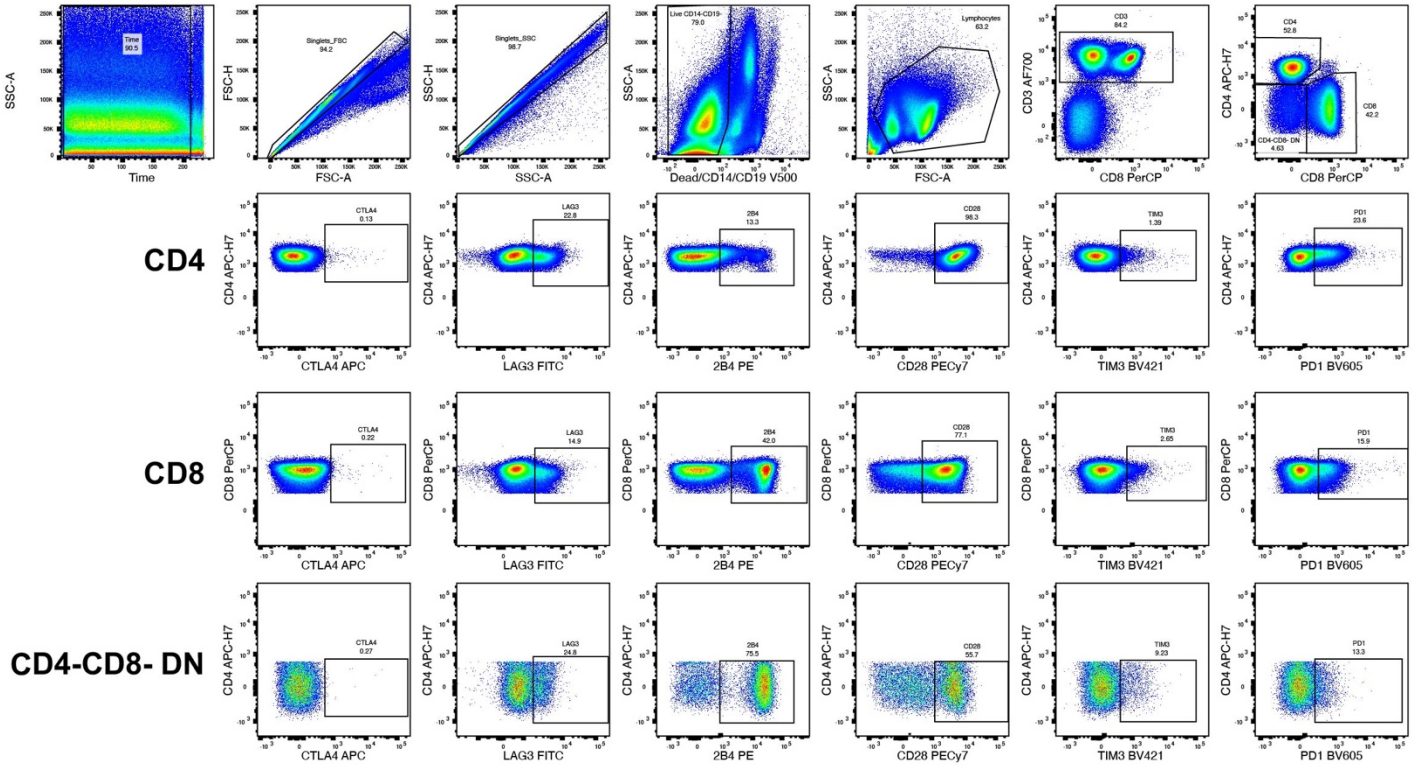

Figure S1. Gating strategy and data analysis performed using FlowJo (BD). Cleanup gating was performed in the time gate and singlets were gated with FSC-A vs. FSC-H and SSC-A vs. SSC-H. $CD14^+$, $CD19^+$, and dead cells were excluded from the analysis. Lymphocytes were gated using FSC-A and SSC-A, and $CD3^+$ cells were identified within the lymphocytes, followed by gating of $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ DN T cells. Positive surface expression of CTLA4, LAG3, CD28, 2B4, TIM3, and PD1 were gated within the $CD4^+$, $CD8^+$, and DN T cell subsets. Boolean gates were then created based on the single marker positive gates to identify cells expressing all possible combinations of inhibitory and senescence markers.

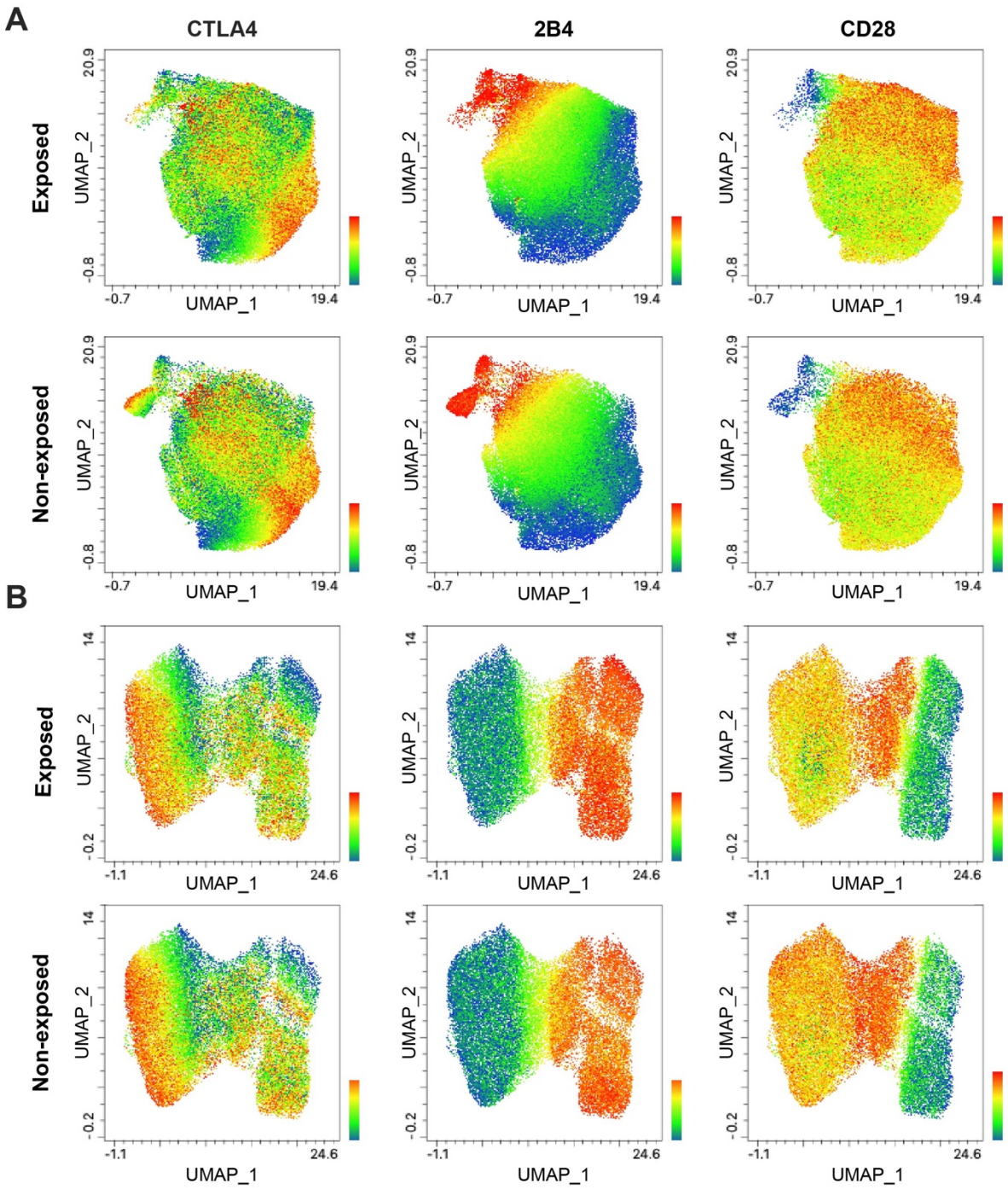

Figure S2. Malaria exposure and surface expression of inhibitory markers on T cells. UMAP plots of the expression of inhibitory markers CTLA4, 2B4, and senescence marker $CD28^-$ in exposed versus non-exposed individuals on **(A)** $CD4^+$ T cells and **(B)** $CD8^+$ T cells. Exposed (n = 41) and non-exposed (n = 7). Individuals were down sampled within their respective groups, exposed and non-exposed, so that each contributed an equal number of events towards $CD4_+$ T cells (91,000 events total) and $CD8^+$ T cells (35,000 events total).

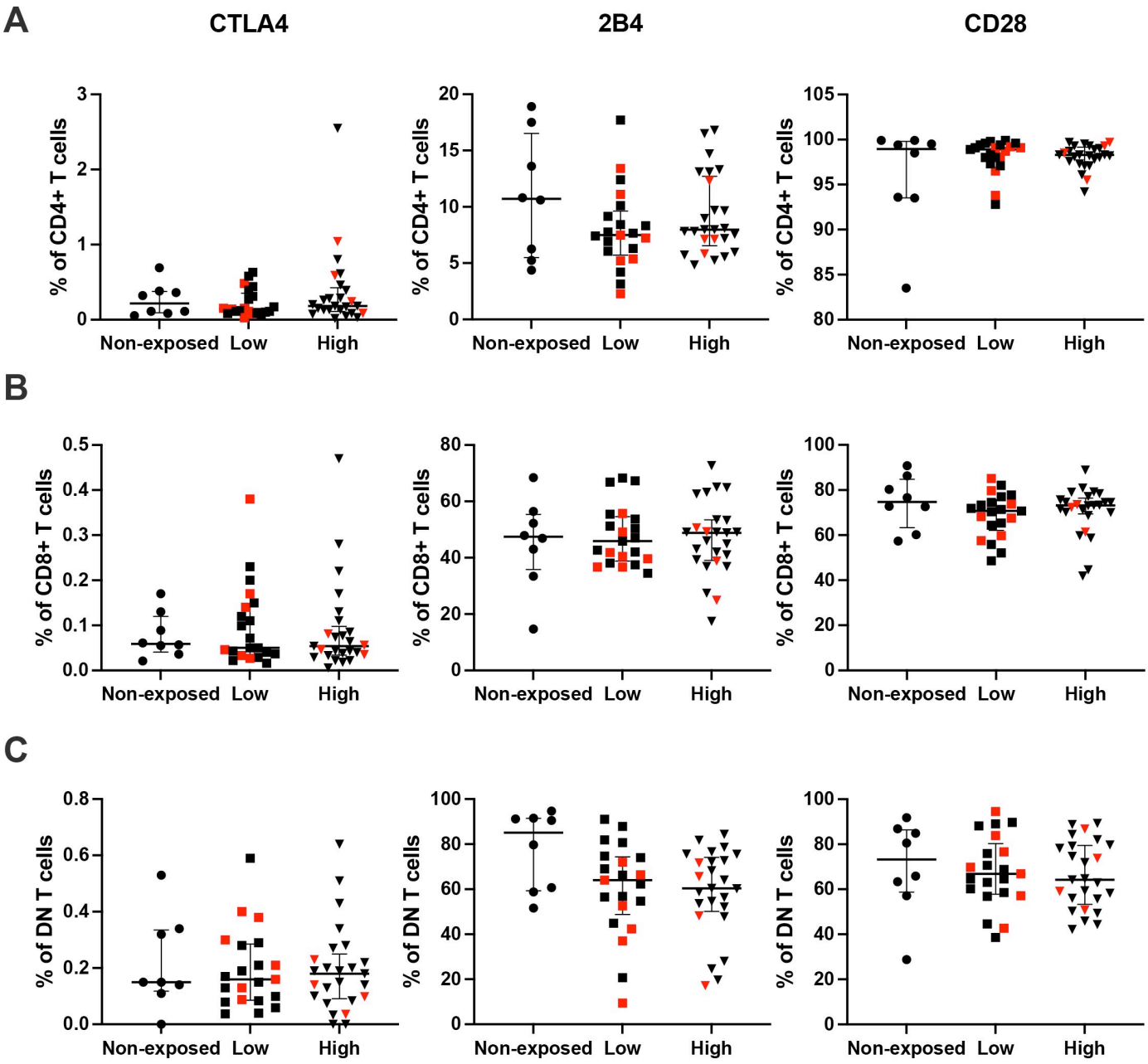

Figure S3. Malaria exposure and frequencies of T cell subset expressing inhibitory and senescence markers. Scatter plots of frequencies of **(A)** $CD4^+$ T cells, **(B)** $CD8^+$ T cells, and **(C)** DN T cells expressing CTLA4, 2B4, and CD28, categorized by malaria exposure. Non-exposed (n = 8), Low (n = 21), and High (n = 25). Individuals with active malaria infection have symbols colored red. Lines and whiskers represent median and interquartile range. Differences were assessed with Kruskal-Wallis test with Dunn's *post hoc* test comparing each exposure category and no significant difference were identified.

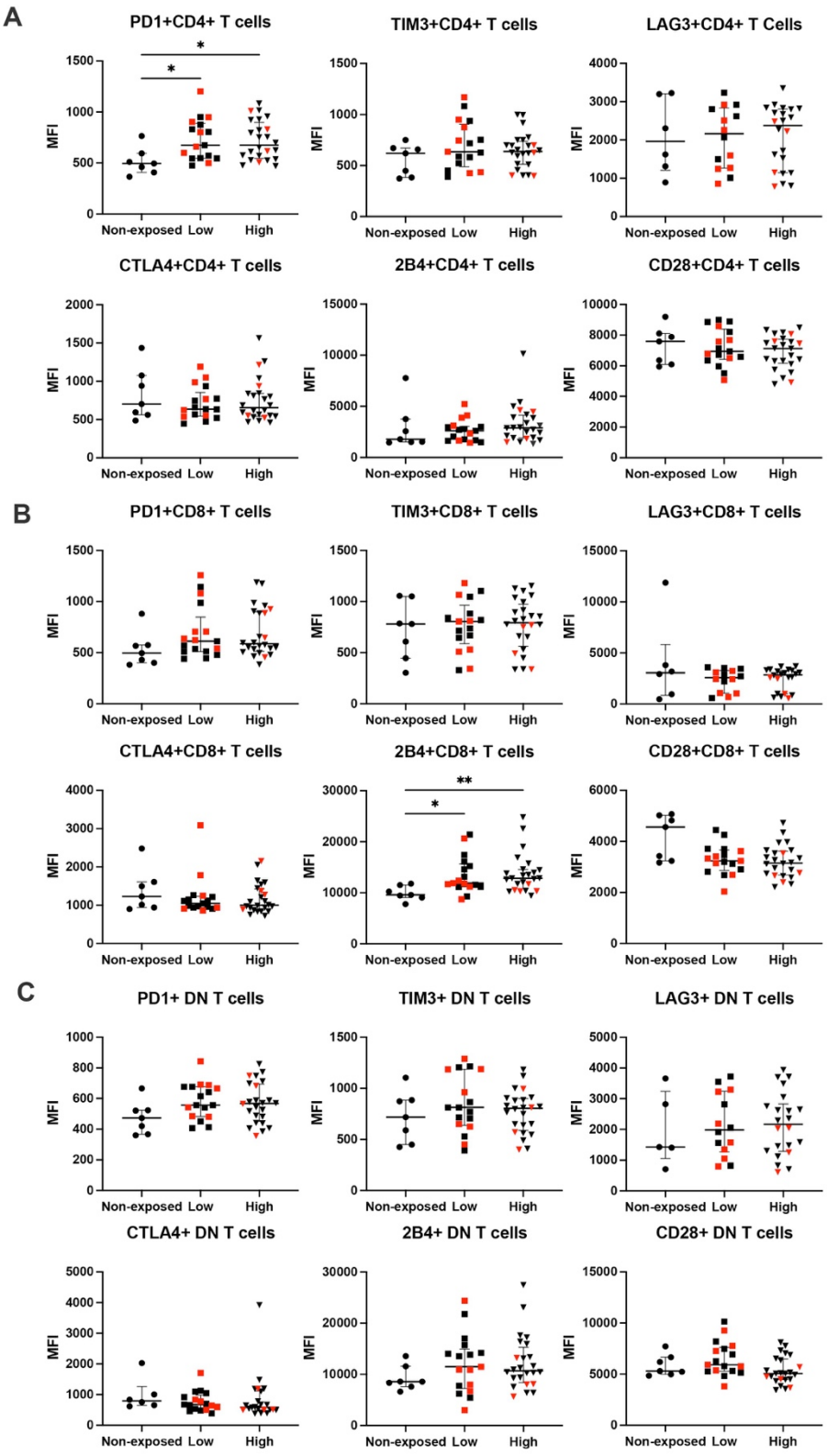

Figure S4. MFI levels for individual T cell inhibitory and senescence markers expressed on T cell subsets. Scatter plots of MFI levels of markers PD1, TIM3, LAG3, CTLA4, 2B4, and CD28 expressed on **(A)** $CD4^+$ T cells, **(B)** $CD8^+$ T cells, and **(C)** DN T cells, categorized by malaria exposure. Non-exposed (n = 7), Low (n = 17), and High (n = 24). $LAG3^+$ T cells: Non-exposed (n = 6), Low (n = 14), and High (n = 22). Individuals with active malaria infection have symbols colored red. Lines and whiskers represent median and interquartile range. Differences were assessed with Kruskal-Wallis test with Dunn's *post hoc* test comparing each exposure category (*p< 0.05, **p<0.01).

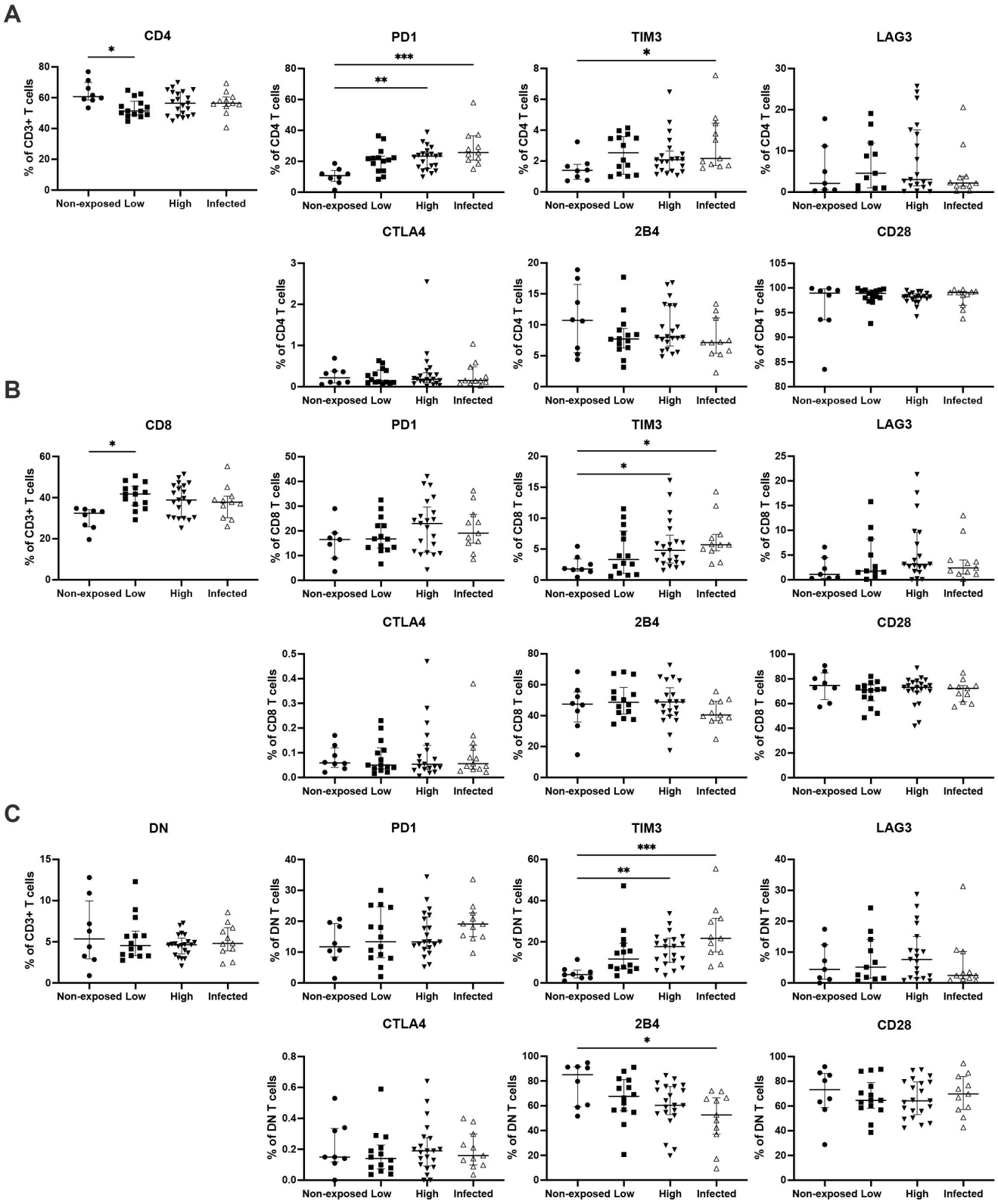

Figure S5. Malaria exposure and active infection and frequencies of T cell subsets expressing inhibitory and senescence markers. Scatter plots of frequencies of **(A)** $CD4^+$ T cells, **(B)** $CD8^+$ T cells, and **(C)** DN T cells expressing PD1, TIM3, CTLA4, 2B4, and CD28, by malaria exposure and active infection. Non-exposed (n = 8), Low (n = 14), High (n = 21), Infected (n = 11). LAG3: Non-exposed (n = 7), Low (n = 11), High (n = 19), and Infected (n = 11). Lines and whiskers represent median and interquartile range. Differences were assessed with Kruskal-Wallis test with Dunn's *post hoc* test comparing each exposure category (*p< 0.05, **p<0.01, ***p<0.001).

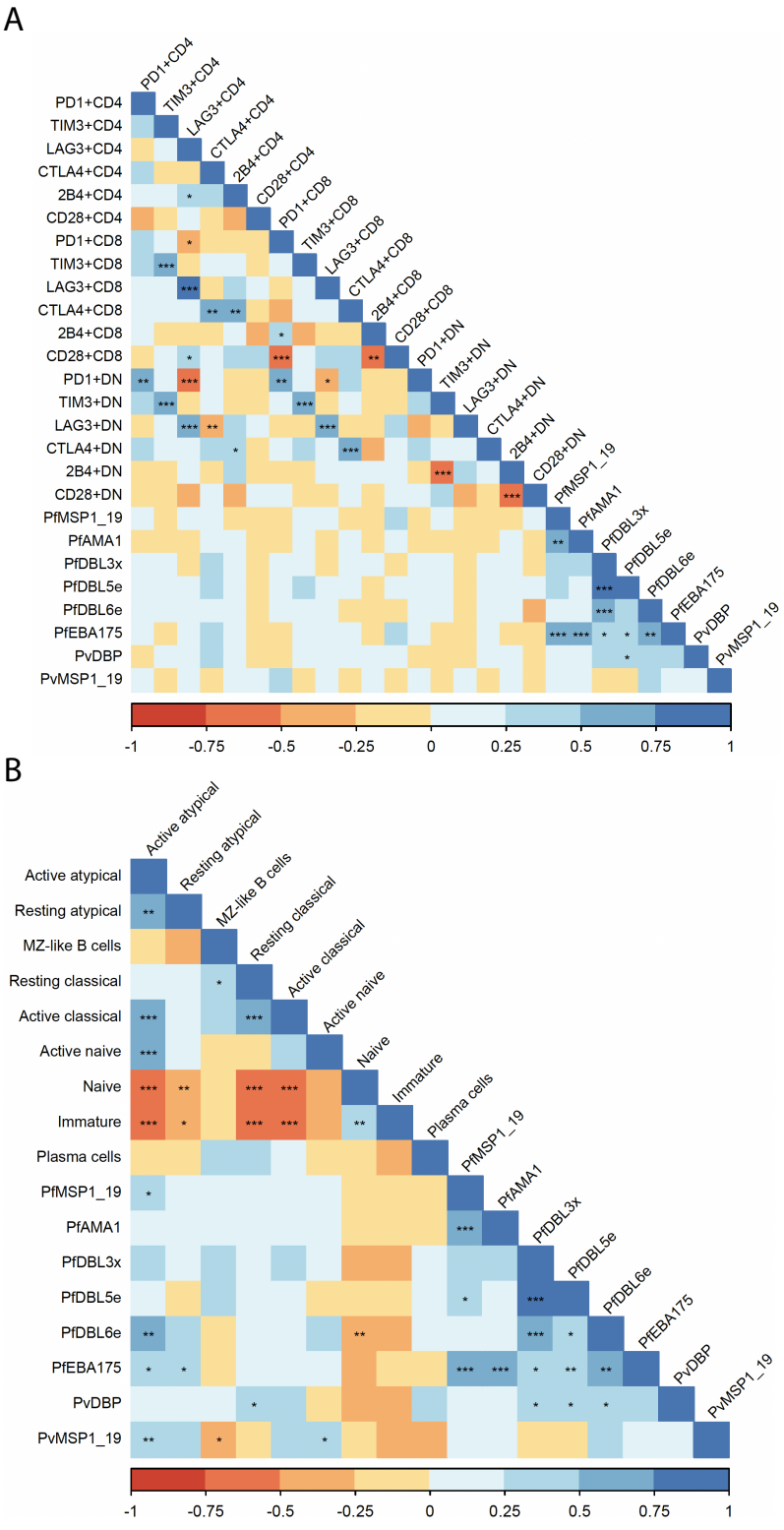

Figure S6. Correlation of frequencies of T cells expressing inhibitory markers and B cell subsets with antibodies to malaria antigens. Spearman correlation matrix of **(A)** frequencies of $CD4^+$, $CD8^+$, and DN T cells expressing inhibitory markers versus antibodies to malaria antigens (n = 46 for all markers except for LAG3, n = 41) and **(B)** frequencies of B cell subsets to antibodies to malaria antigens. Color-coded bar ranging from -1 (red, negative correlation) to 1 (blue, positive correlation) showing the Spearman correlation coefficient and asterisks indicate significance levels (Benjamini-Hochberg adjusted p-values are shown *p< 0.05, **p<0.01, ***p<0.001).

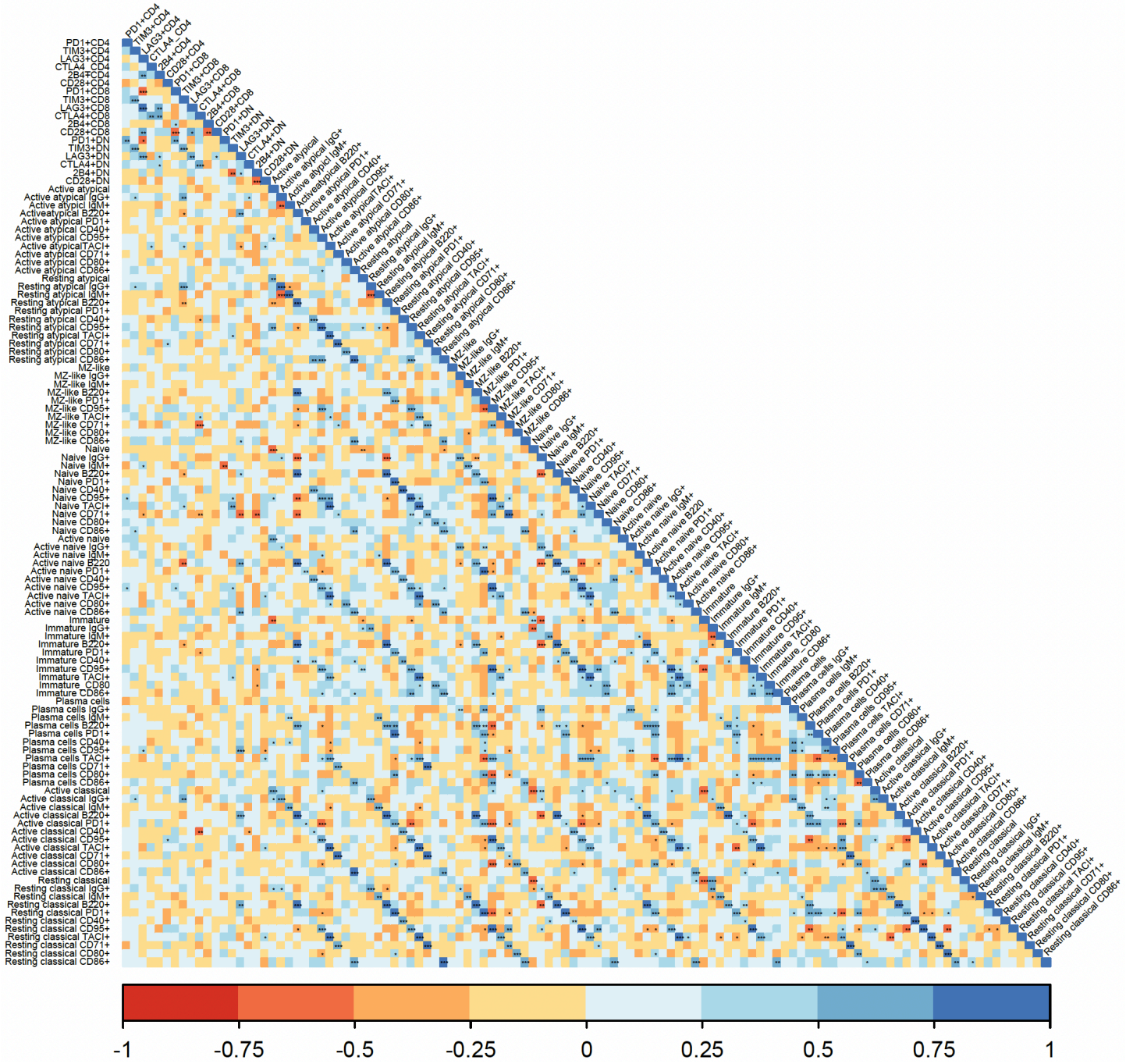

Figure S7. Spearman correlations of frequencies of T cell subsets expressing individual inhibitory markers with B cell subsets in the malaria-exposed individuals. Spearman correlation matrix of frequencies of $CD4^+$, $CD8^+$, and DN T cells expressing individual inhibitory markers and frequencies of active and resting atypical MBC, MZ-like B cells, active and resting classical MBC, plasma cells, immature, active, and naïve B cell subsets expressing IgG, IgM, B220, CD40, CD71, CD95, TACI, CD80 and CD86 in the malaria-exposed individuals (n = 46 for all variables except for LAG3, n = 41; and CD80 and CD86 which had missing data values, n = 43). Color-coded bar ranging from -1 (red, negative correlation) to 1 (blue, positive correlation) shows the Spearman correlation coefficient and asterisks indicate significance levels (Benjamini-Hochberg adjusted p-values are shown *p< 0.05, **p<0.01, ***p<0.001).

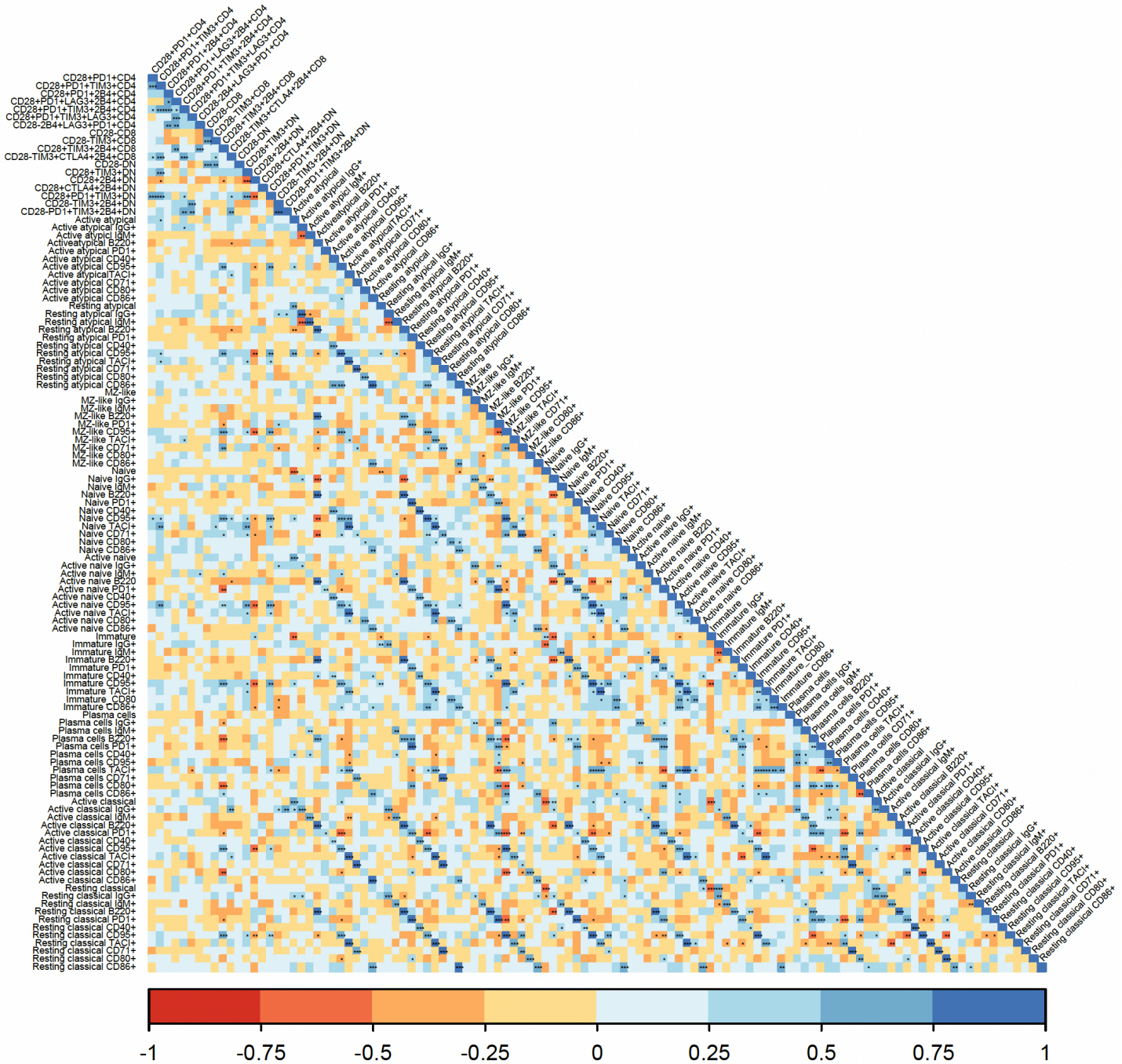

Figure S8. Spearman correlations of frequencies of T cell subsets expressing multiple inhibitory markers versus B cell subsets in the malaria-exposed individuals. Correlation matrix of frequencies of $CD4^+$, $CD8^+$, and DN T cells expressing multiple inhibitory markers and frequencies of active and resting atypical MBC, MZ-like B cells, active and resting classical MBC, plasma cells, immature, active, and naïve B cell subsets expressing IgG, IgM, B220, CD40, CD71, CD95, TACI, CD80 and CD86 in the malaria-exposed (n = 46 for all variables except for LAG3, n = 41; and CD80 and CD86 which had missing data values, n = 43). Color-coded bar ranging from -1 (red, negative correlation) to 1 (blue, positive correlation) shows the Spearman correlation coefficient and asterisks indicate significance levels (Benjamini-Hochberg adjusted p-values are shown *p< 0.05, **p<0.01, ***p<0.001).

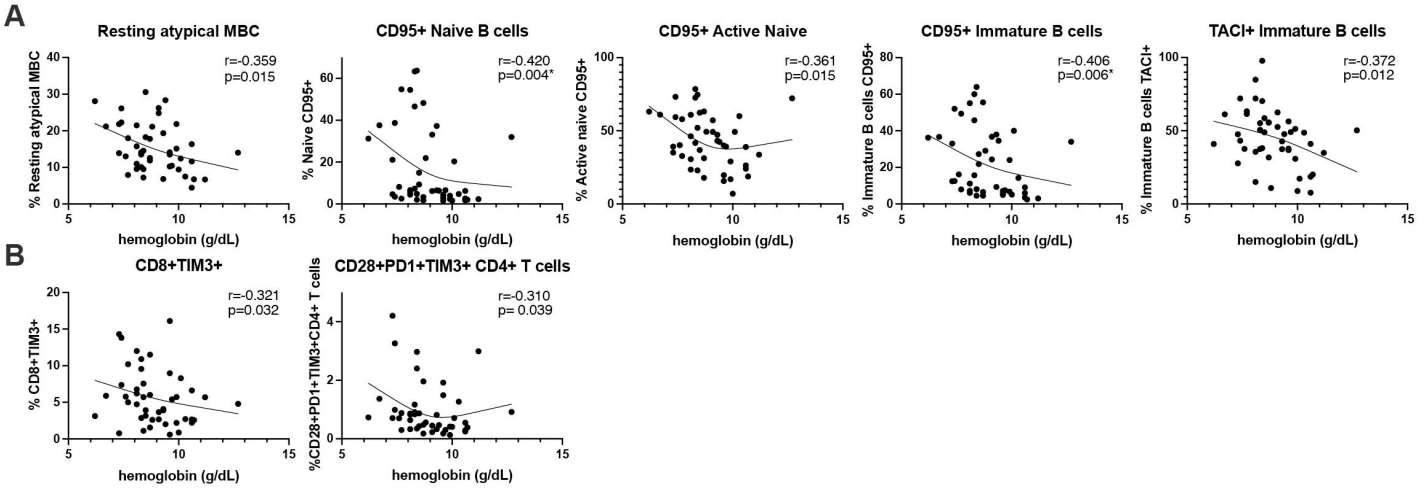

Figure S9. Correlations of frequencies of TIM3$^+$ T cells, resting atypical MBC and CD95$^+$ naïve, active naïve and immature B cells with decreased Hb levels. Correlation of **(A)** B cell subsets and **(B)** T cell subsets expressing inhibitory markers with hemoglobin concentrations in the malaria-exposed individuals (n = 46). Spearman correlation coefficients and p-values are shown in the plots. Asterisks indicate p-values that remain significant after Benjamini-Hochberg adjustment for multiple testing. Spline fit trend lines are shown in the plots.

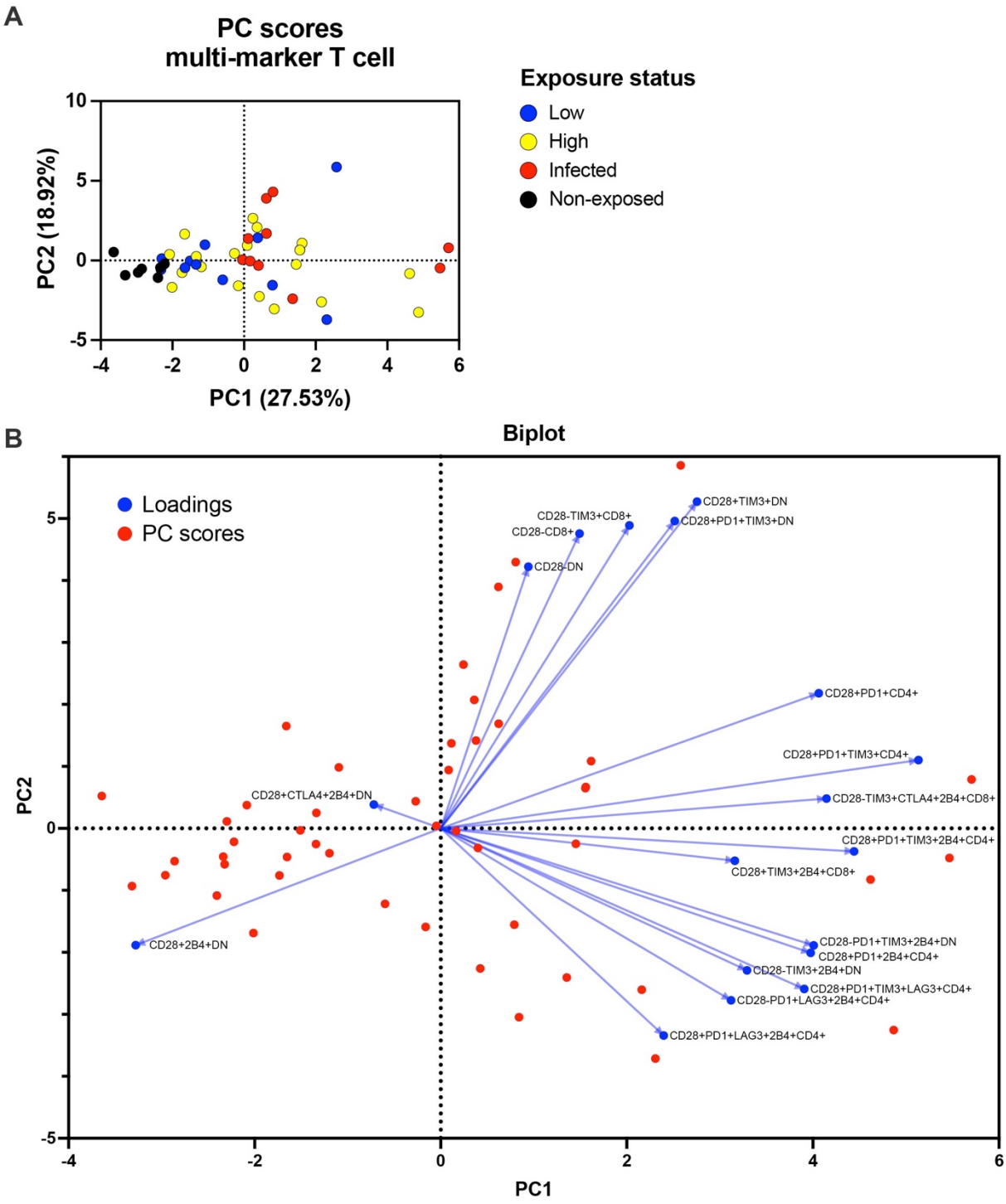

Figure S10. Frequencies of T cells expressing multiple inhibitory markers discerning malaria-exposed from non-exposed individuals. PCA analysis of frequencies of T cell subsets expressing multiple inhibitory markers **(A)** PC scores plot and **(B)** Biplot showing PC1 versus PC2 loadings and PC scores. Individuals from the batch that were missing LAG3 antibody staining were excluded from the PCA. Non-exposed (n = 7), Low (n = 11), High (n = 19), and Infected (n = 11). When looking at the contribution of T cell subsets to the PC, three clusters of T cell subsets with positive loadings (> 0.3) were observed. One cluster that had high positive PC1 and PC2 loadings consisted of $CD8^+$ and DN T cells expressing $TIM3^+$ ($CD28^+TIM3^+$ DN T cells, $CD28^+PD1^+TIM3^+$ DN T cells, and $CD28^-TIM3^+$ $CD8^+$ T cells). A second cluster with high positive PC1 loadings consisted of $CD4^+$ and $CD8^+$ T cell subsets with varying co-expression of markers, including PD1, TIM3, and 2B4. The third cluster contained only $CD4^+$ and DN T cell subsets with high positive PC1 and negative PC2 loadings. $CD28^{+/-}$ $PD1^+LAG3^+2B4^+$ $CD4^+$ T cells, $CD28^+PD1^+$ $CD4^+$ T cells, and either $TIM3^+$ or $2B4^+$ were localized here, as well as $CD28^-TIM3^+2B4^+$ DN T cells with or without $PD1^+$ expression.

Discussion

A significant milestone was achieved with the approval of RTS,S as the first malaria vaccine for children in 2021. While RTS,S demonstrated modest efficacy, it will save thousands of lives every year. Nevertheless, second-generation vaccines are needed with improved efficacy and durability. Alternative dosing strategies have already been tested, such as a delayed fractional dose with RTS,S in a phase 2b trial in children aged 5-17 months; however, results were similar to efficacy levels found for RTS,S at one year post the regular primary schedule (352). Still, a delayed fractional dose performed better when tested in malaria-naïve adults, which could be due to numerous reasons (353). Improved RTS,S vaccine efficacy has been shown recently by co-administering it with seasonal malaria chemoprevention (261,354). The results of this PhD thesis contribute to understanding RTS,S-induced immune mechanisms and identifying correlates of protection, different parasite targets, and epidemiological factors that can impact vaccine responses, including population demographics and chronic malaria exposure, with the final goal to help improve RTS,S, and next-generation vaccine design and immunization strategies.

We have shown that the *P. falciparum* CSP minor repeat peptide, which is not included in the RTS,S vaccine formulation, can elicit antibodies with sporozoite-neutralizing activity. We have also demonstrated that mAbs specific to the major CSP repeats included in RTS,S can have little or no sporozoite-neutralizing effect. The minor repeat peptide used as an immunogen contained two copies of the junctional epitope that was previously identified (299,355) and which was shown to be protective (300,301). Recent studies of mAbs generated from RTS,S vaccinated individuals are helping to further define antibody responses to the immunodominant NANP repeat region. While anti-NANP antibodies elicited by RTS,S vaccination have been shown to be necessary for protection, the cellular immune response has not been fully defined (246). Here, we reported the cell-mediated immune responses to the RTS,S booster dose during the phase 3 trial for the first time. We identified IL-2 as a key RTS,S-induced CSP-specific immunogenicity marker that tended to associate with reduced risk of malaria and correlated positively with anti-NANP total IgG antibody while inversely with IgG subclasses previously shown to

associate with risk. Our findings align with other RTS,S studies that have identified IL-2 response as a potential correlate of protection (246). We have also shown that chronic malaria exposure can alter the phenotypes of T cells, including increased expression of inhibitory markers. These phenotypes are associated with modified B cell subsets, including increased frequencies of aMBCs, which have been shown to produce autoantibodies and diminished neutralizing antibodies (241,356). These alterations in T and B cells could affect the immunogenicity and efficacy of vaccine responses, including those induced by RTS,S.

The *Plasmodium* sporozoite injected by the transmitting mosquito bite is a target for antibody-mediated immune responses that have been shown to protect against malaria challenge. CSP was identified as the major surface antigen of sporozoites, with the central repeat region being the dominant epitope and thus prime target for subunit vaccine design (3). The RTS,S vaccine contains a portion of the central repeat region comprising 19 copies of NANP repeats and the T cell epitopes towards the C-terminus (3). Using mAbs and polyclonal sera generated from mice following immunizations with *P. falciparum* sporozoites or synthetic CSP repeat peptides, we showed that the CSP minor repeat region, contains an epitope recognized by sporozoite-neutralizing antibodies, which is aligned with other studies that identified mAbs recognizing this epitope and junctional epitope (298–300). A proposed mechanism behind the protection of these antibodies is that they may inhibit the proteolytic processing of CSP, which is needed for sporozoite invasion (298,299,339,357,358).

Moreover, we found murine mAbs specific to the NANP major repeats with limited or no neutralizing activity. It has been suggested that the numerous NANP repeats may act as an “immunological decoy” that averts the generation of more protective inhibitory antibodies (308,359,360). This diversion of protective antibody responses towards immunodominant unprotective epitopes has been proposed to occur during both blood-stage and pre-erythrocytic stages as a potential immune evasion mechanism (361–364). Several studies have isolated NANP-specific human mAbs from RTS,S-immunized individuals that were found to protect mice against challenges with transgenic *P. berghei*

sporozoites expressing *P. falciparum* CSP (296,297). Designing subunit vaccines that target the minor repeats and junctional epitope may drive a higher proportion of antibodies away from potentially less protective major repeats. In a recent study screening a large panel of mAbs generated from RTS,S-immunized volunteers found a heterogeneity of anti-NANP neutralizing activity with many antibodies that were insufficient at protecting against malaria challenge (308). It was suggested that protective RTS,S-induced NANP-specific antibodies likely undergo affinity maturation to short NANP repeat epitopes (308). The short PNANPN sequence contained within both the major repeats included in RTS,S and the minor repeat and junctional epitope that is not part of RTS,S, can result in some cross-reactivity as found for the mAb MAM01 that has entered clinical trials (308).

In addition to changes in target epitopes included in the RTS,S immunogen, vaccine efficacy may be improved if the quality of the antibody response is improved. Besides antibody titer (anti-NANP IgG), antibody subclass or other isotype have been associated RTS,S-induced protection (325,327). Curiously, anti-NANP IgG antibody titers post RTS,S booster dose were lower than the response following primary immunization in the phase 3 trial (325,326), indicating that the booster response did not induce a robust humoral response which is in line with the modest recovery of vaccine efficacy by the fourth dose. B cells and antibody responses are supported by helper $CD4^+$ T cells and are impacted by the cytokines they produce. Our study of cell-mediated immunogenicity and protection elicited by the **RTS,S booster dose during the** phase 3 trial identified IL-2, IFN-γ, IL-17, IL-5, and IL-13 to be associated with RTS,S vaccination. We found increased levels of CSP-specific IL-2 one-month post-booster at M20, and it remained elevated 12 months later at M32. Significant differences in cytokine levels one month post-booster were only detected in RTS,S booster dose over the comparator control vaccine and not RTS,S without the booster, indicating that the booster induced a moderate cellular response, which may indicate why the antibody response post booster dose was relatively low. Various factors have been known to impact RTS,S responses, however, in our study, we detected no clear significant effect modifications associated with age, sex, site, previous malaria exposure, anemia, or health status and booster vaccination. IL-2 was associated with protection in the children from Manhiça but not in

Bagamoyo, which suggests that some site-specific differences may affect vaccine-induced protection.

IL-2 is an important T_H1 cytokine that supports $CD4^+$ T cell and B cell responses including plasma cell differentiation and antibody production (365–367). Accordingly, we found a low positive correlation of IL-2 with anti-NANP total IgG antibodies at M21 and a significant positive moderate correlation of IL-2 measured at M32 associated with RTS,S vaccination. Our group has previously shown that RTS,S predominantly induces IgG1 and IgG3, as well as IgG2 and IgG4 subclass responses (327). During the 1-year follow-up post-primary vaccination, cytophilic IgG1 and IgG3 were associated with protection, while IgG2 and IgG4 responses were associated with malaria risk (327). IgG1 and IgG3 can fix complement and enable interactions with Fcγ-receptors on phagocytic cells (123). We previously found the RTS,S booster dose to induce total IgG, IgG1, IgG3, and IgG4, but not IgG2 (326). Further assessing correlations between anti-CSP IgG subclasses showed significant inverse correlations between IL-2 and anti-NANP IgG2 and IgG4. Protection by RTS,S may be supported by inducing IL-2 and cytophilic antibodies and producing lower amounts of IgG2 and IgG4, which are less protective. Therefore, strategies to increase the induction of memory $CD4^+$ T cells should be sought for increasing the magnitude and duration of protective antibody responses.

Previous studies by our group found associations between HBsAg-specific antibodies and reduced malaria risk, which may be a result of HBsAg-specific T cells possibly supporting B cell anti-CSP antibody responses (hapten-carrier effect) (327,337). HBsAg-specific IL-2 and IL-5, observed post-primary immunization, have remained prominent markers at M21, and IL-17 and IL-13. Still, we did not detect a direct association of those responses with malaria risk or protection post-booster. Positive correlations between HBsAg-stimulated IL-17, IL-5, and IL-13 and antibodies to vaccine antigens, were observed at M21, and correlation strength and significance increased at M32. Apart from T_H2 cytokines IL-5 and IL-13 supporting B cell development, T_H17 cells, and proinflammatory IL-17 have been demonstrated to promote the formation of germinal centers and support B cell and isotype class switching to IgG2a (IgG1 in humans) and IgG3 in murine studies

(368). The role of HBsAg and anti-HBsAg immune responses in relation to the vaccine immunogenicity and protection should be clarified since HBsAg inclusion or proportion in the vaccine formulation could be modified. Indeed, the recently approved R21 vaccine, which has a similar design as RTS,S but with a lower proportion of HBsAg and formulated with a different adjuvant, induces comparable immune responses; however, no side-by-side comparison has been done to evaluate differences in efficacy. Likely, the same factors that affect RTS,S in malaria-endemic settings will also affect R21.

We also found a significant positive association of IL-10 with the risk of malaria in the infant cohort that was independent of the vaccine group. Immunoregulatory IL-10 is produced by multiple cell types, including monocytes, DCs, and T and B cells, with pleiotropic functions that have been shown to dampen proinflammatory responses and support humoral immunity. IL-10 has been shown to promote germinal center B cell responses and anti-*Plasmodium* antibody responses in mouse models (369). A previous study by our group investigating malaria during pregnancy found both proinflammatory responses and increased IL-10 production by T cells correlated with protection against malaria (218). However, IL-10 has been shown also to be associated with the reduced capacity to clear *P. falciparum* parasites in a study of Tanzanian children (215). A longitudinal study conducted by our group of a cohort of Mozambican mothers and their children found IL-10 to be associated with an increased risk of clinical malaria (370). The high capacity to produce IL-10 in children was inherited from their parents, resulting in reduced protection and increased risk of malaria (370). A study of *P. falciparum* placenta-positive neonates from Gabon reported elevated antigen-specific IL-10 producing $CD4_+$ T cells and reduced IFN-γ^+ T cells in cord blood mononuclear cells indicating newborns to mothers exposed to malaria at delivery may have increased susceptibility to infection later (219). Therefore, optimizing a pediatric vaccine that can drive the immune responses towards a more T_H1-type and away from an immunoregulatory profile may be valuable.

Increased IL-10 production and immunoregulatory responses by immune cells may allow parasite persistence, lead to chronic infections, and impact NAI and vaccine efficacy. Chronic malaria exposures have been shown to alter B and T cell populations

(235,241,371). Therefore, individuals living in malaria-endemic regions may experience changes in the state of these cell populations that could affect naturally acquired and vaccine-induced immunity. A previous study by our group characterized B cell populations in individuals living in a high MTI setting in PNG and found higher frequencies of aMBC expressing activation and inhibitory markers, including PD1, CD80, and CD95, among others in the malaria exposed compared to malaria non-exposed controls and proposed that these cells were being maintained in an anergic state rather than undergoing apoptosis to reduce immune activation (241). As with tolerogenic T cells, this suggests that the B cells are maintained enough to control infection but at a level that allows persistence (241). Few studies have investigated B cell subsets along with the expression of inhibitory markers on T cells during chronic malaria (235). Therefore, to complement the B cell study during chronic malaria, we characterized the T-cell subsets from the same cohort using a panel containing T-cell lineage and inhibitory markers, including PD1, TIM3, LAG3, CTLA4, 2B4, and CD28 as a marker for senescence and investigated associations between B and T cells. We confirmed that in addition to the altered B cell populations observed during chronic malaria exposure, frequencies of T cells expressing specific inhibitory markers also increased and correlated with frequencies of aMBCs and marginal zone-like B cells.

Increased frequencies of T cells expressing inhibitory markers have been described during acute and chronic malaria exposure (372–374). Studies reporting T cells expressing inhibitory markers during acute malaria suggest that suppressive immune conditions may be occurring to maintain homeostasis rather than exhaustion. Moreover, recent murine studies have demonstrated that $LAG3^{+}CD8^{+}$ splenocyte T cells maintained their functional capacity by producing proinflammatory IFN-γ and the cytotoxic molecules perforin and granzyme B, and the degranulation marker CD107a (238,239). The increasing expression of LAG-3 correlated with increasing co-expression of co-inhibitory receptors, including PD1, TIM3, and CTLA4 (238). Our study found frequencies of $CD95^{+}$ B-cell subsets correlating with frequencies of T-cell subsets co-expressing TIM3 with other inhibitory markers. Furthermore, we found inverse correlations between $CD95^{+}$ B-cell subsets and $TIM3^{+}$ T-cell subsets with hemoglobin levels in the malaria-exposed

individuals. CD95 receptor has been shown to induce phosphatidylserine (PS) externalization leading to clearance by phagocytes (375), and PS expressed on infected and uninfected RBC can be recognized by autoimmune antibodies produced by aMBC, which has been connected to anemia (356,376). However, no clear evidence of direct cytotoxic $CD8^+$ T cell activity targeting PS on infected RBC via TIM interactions has been reported. Nevertheless, a murine study demonstrated that $CD8^+$ T cells induce PS externalization on *Plasmodium*-infected erythroblasts in a cell-to-cell contact manner and then TIM-expressing macrophages clear the infected RBCs (377). The role of cytotoxic $CD8^+$ T cells against *Plasmodium spp*. blood-stage and malaria immunopathology should be clarified considering our results and that RBC do not express HLA-class I. Instead, $CD8^+$ T cells have recently been shown to kill *P. vivax*-infected reticulocytes which do express HLA-class I (378).

The impact of acute or chronic malaria exposure, whether leading to immune dysregulation or homeostatic conditions via regulatory mechanisms is important to understand as it could also impact vaccine responses. During malaria, the immune system has to balance the excessive inflammation that can cause disease pathology while controlling the infection. Immunoregulatory responses have been described in children that control malaria-induced inflammation, particularly during *P. falciparum* re-exposure to mitigate pathogenic inflammation while maintaining anti-parasitic effector mechanisms (76). This would explain why individuals exposed repeatedly while living in malaria endemic areas are often afebrile and typically have low levels of parasitemia. A study of PBMCs collected from Malian children after experiencing febrile malaria found that re-exposing immune cells to parasites after fever reduced expression of proinflammatory cytokines that cause fever and enhanced anti-inflammatory IL-10 and TGF-β, and an increase in *P. falciparum*-specific $CD4^+Foxp3^-$ T cells co-producing IL-10, TNF, and IFN-γ (76). Studies have demonstrated $CD4^+$ and $CD8^+$ T cells that suppress the activity of naïve T cells that may regulate the amount of T cell activation during acute malaria (239,372). Therefore, it is likely that these responses induced by chronic exposure affect vaccine responses in an antigen specific or in a non-specific manner.

Study Limitations

While our study of mAbs targeting CSP repeat epitopes found differences in neutralizing activity between minor and major repeat-specific antibodies, which are in line with findings from other groups, a larger panel of mAbs would be required to confirm if the frequency of neutralizing versus non-neutralizing antibodies is increased after immunizing with minor repeat peptide compared to major repeats.

A limitation of our study focusing on cellular immune responses to the RTS,S booster dose is that we cannot determine which cells produce the vaccine-induced cytokines. Moreover, we cannot confirm if cytokine levels increase due to an expansion in the proportion of antigen-specific T cells or increased secretion by each stimulated cell. However, we can deduct from previous flow cytometry studies which cells may produce specific cytokines. For example, a previous study by our group reporting on cellular responses following primary vaccination showed that IL-2 is induced mainly in activated $CD4^+$ T cells (349). Also, HBsAg-specific $CD4^+$ T cells produced IFN-γ, while CSP-specific $CD4^+$ T cells produced weak or no IFN-γ (349). Future studies will be needed to define the $CD4^+$ T cells including any bystander cells that may be producing the cytokines induced by the RTS,S booster dose, and assessment of inhibitory markers could help inform upon any role malaria exposure may have on altered vaccine-induced immunity. Furthermore, the sample size limited the power of our analyses of immune correlates of malaria risk, and only a small subset of participants had antibody data for the correlation analysis with cytokines. Additionally, the power of the effect modification analysis was limited by the unequal distribution of age cohorts between sites. Manhiça consisted of children and infants, while Bagamoyo only had children; therefore, we limited the site analysis to only children and age analysis to participants only from Manhiça. Although we controlled for less than 5% false discovery rate, our analysis contained many comparisons, increasing the possibility of false positives. While, we have emphasized results that are consistent with previous studies or proposed hypotheses, further confirmation would be needed.

Our study covering T cell exhaustion during chronic malaria exposure in PNG was limited by the small sample size of pregnant women and the fact that malaria non-exposed controls were unavailable from the study site. Pregnancy could have an effect on inhibitory marker expression, however many studies report elevated marker expression centralized to the decidua rather than maternal blood (379–381). Moreover, our findings aligned with other studies showing increases in inhibitory markers in T cells resulting from malaria exposure. It would also have been informative to have functional and cytokine data to assess if levels of proinflammatory or immunoregulatory markers were altered or associated with expression of exhaustion markers. While not the same cohort, our group previously reported on cellular responses in pregnant women in PNG during malaria and found increased frequencies of TR1 and T_{REG}-IFN-γ^+ production during active *P. falciparum* infections (218). T_{REGS} and TR1 cells also express inhibitory markers (203,382,383), and TR1 cells that co-produce IL-10 and IFN-γ can maintain their suppressor functionality (211,383,384). Indeed, functional assays would also be required to confirm if the expression of inhibitory markers translates to hypo-responsiveness to infection and its association with acquired or vaccine-induced immunity. The questions left by the study limitations leave room for future experiments to explore.

Concluding remarks and future perspectives

A more complete view of pre-erythrocytic antigenic targets and a better understanding of the mechanisms of antibodies and cellular responses are needed for rational vaccine design to achieve vaccine-induced sterile protection. Second-generation vaccines are needed that can induce long-lived antibody and cellular responses. Chemoattenuated whole sporozoite malaria vaccines have recently shown promising results demonstrating sterile hepatic immunity and have highlighted vaccine-induced antibody responses and expansion of Vδ2 γδ T cells (274). Incorporating additional antigens into current subunit vaccines may improve efficacy and increase the breadth of antibody response, such as the junctional and minor repeat epitopes not included in RTS,S and R21 (298–301,303). The addition of other non-CSP antigens to the subunit vaccine design may also provide broader and enhanced protection. Despite not demonstrating protective efficacy when delivered alone, a study of anti-TRAP mAbs showed they may enhance CSP-based

protection (385). LSA-1 can elicit antibodies and memory $CD8^+$ T cells, and its incorporation into multi-antigen malaria vaccines, including combining with CSP-based vaccines, could produce synergistic protective effects (386). Combining multi-stage antigenic targets using viral vector vaccines such as CSP and AMA1 induced antibodies and $CD8^+$ T cells, but did not show protection against CHMI (387). A multi-stage phase 1 clinical trial assessing co-delivery of R21 with RH5.2-VLP is currently underway.

New vaccine platforms leveraging the mRNA technology that was optimized during COVID-19 vaccine development are being used, such as the mRNA-LNP vaccine candidate expressing *P. falciparum* CSP (290) and multi-stage CSP and Pfs25 candidates (292) currently being evaluated in preclinical studies in mice. RTS,S and R21 vaccines produce protective antibodies and $CD4^+$ T cells, not $CD8^+$ T cells (or at least are not detectable in peripheral blood). Multi-epitope platforms that can induce antibodies and protective $CD8^+$ T cells, such as viral vector vaccines, are an approach to generating $CD8^+$ T cells targeting liver-stage infection (388). A Tobacco Mosaic Virus (TMV) platform is being used to create VLPs containing (NPNA)n repeats and the junctional epitope (389). Qβ-VLP platform is being used to explore potential VLP-peptide vaccines for preclinical screening of epitopes to permit the efficient and inexpensive production of new vaccines for murine malaria vaccine studies (390). Qβ-VLP targeting the junctional epitope reported high and long-lasting anti-CSP antibody responses (391). A SpyTag-SpyCatcher-mi3 approach was used to produce a multivalent CSP-based nanoparticle vaccine that included minor repeat and junctional epitopes and a truncated NANP region epitope (392). Previous studies have evaluated the responses of subunit peptide vaccines containing the CSP minor and major repeat epitopes that demonstrated high antibody titers in murine, non-human primates, and humans (393–395).

Recent studies have more closely investigated the immunodominant central repeat region of *P. falciparum* CSP B cell epitopes (308,359,396). Recall antibody responses following PfSPZ vaccination were shown to be inhibited by antibody feedback, which may mask immunodominant CSP epitopes and drive responses to potentially protective subdominant epitopes, suggesting that malaria vaccination would benefit by targeting

multiple antigens (396). A CSP C-terminal mAb derived from RTS,S immunized volunteers has also been identified (304). A study by our group also reported on protective effect of antibodies to different CSP epitopes and found C-terminus-specific antibodies associated with protection (337) However, another study showed that avid binding of B cells to *P. falciparum* CSP induced greater responses to immunodominant repeat epitopes instead of protective subdominant epitopes and suggest truncated CSP peptides carrying all regions of the antigen may be a more effective approach for next-generation vaccines (359).

RTS,S and other vaccine candidates have often performed worse in malaria endemic setting compare to trials involving malaria naïve volunteers in non-endemic areas. This could be due to a number of reasons including chronic exposure from repeated malaria infections. Chronic exposure resulting in immune dysregulation and disease tolerance is of concern for its effect on immune responses to natural infection and potentially to vaccine responses. Further studies are needed to investigate whether chronic exposure impacts vaccine-induced responses and durability. Additional research needs to be done on the effect of immune imprinting on innate cells, and antigen-specific B or T cells may have modified immune responses (120). Imprinting is a hypothesis whereby the adaptive immune response is shaped by the first exposure to an infection or vaccination (120). Studies have found potential antibody-negative feedback responses following malaria vaccination with high binding avidity to immunodominant CSP repeat epitopes (359,396). Additionally, it is important to understand how chronic inflammation and antigen persistence, either through repeat or sustained infections, leads to immune exhaustion or regulation and its impact on the plasticity of *Plasmodium*-specific memory $CD4^+$ T cells and T_{FH} cells (127).

The application of mAbs used as checkpoint inhibitors to block inhibitory receptors such as PD1 and CTLA4 and reverse immune exhaustion has been a significant focus in the cancer field. Studies in humans using mAbs for cancer immunotherapy have been ongoing, with some mAbs reaching approval; however, concern exists about adverse side effects resulting from their use (397,398). Studies of chronic malaria in mice have

demonstrated that blocking multiple inhibitory receptors can reverse the state of T-cell exhaustion (399) and enhance support of $CD4^+$ T_{FH} cells and parasite clearance (373). Whether mAb therapy could reverse the hyporesponsive immune state associated with chronic malaria exposure and benefit vaccine responses in humans remains to be studied.

Incorporating the MAL067 ancillary study into the phase 3 clinical trial demonstrated the importance of including immunological studies with clinical trials to better understand the humoral and cellular responses elicited by RTS,S vaccination and their role in vaccine efficacy. The multicenter RTS,S phase 3 trial and nested immunological studies demonstrated that factors including age, previous exposure to malaria, geographical location, health status, and anemia, can result in differences in vaccine responses (325,327,350,400,401); making clear the need to conduct more studies evaluating immunological responses in different field settings with varying demographics of population. Our results from studies within MAL067 have identified protective responses that can be induced or enhanced through vaccination and detrimental immune responses that should be avoided, leading to new hypotheses that our group is currently exploring.

Conclusions

1. The *P. falciparum* circumsporozoite protein minor repeat region, comprised of three copies of alternating NANP and NVDP tetramer repeats, contains an epitope recognized by sporozoite-neutralizing antibodies and, in contrast, monoclonal antibodies specific for the major circumsporozoite protein repeats (NANP)n that were isolated from peptide-immunized mice demonstrated reduced or no sporozoite-neutralizing activity. These findings indicate the importance of evaluating the fine specificity and functions of anti-repeat antibodies induced by *P. falciparum* circumsporozoite-based vaccines and suggest that the design of immunogens to increase antibody responses to minor repeats may enhance vaccine efficacy.

2. The RTS,S/AS01$_E$ vaccine booster dose induced circumsporozoite protein-specific IL-2 at relatively low concentrations but still detected 12 months later. IL-2 correlated positively with anti-circumsporozoite protein IgG and inversely with non-protective IgG2 and IgG4, and tended to associate with reduced malaria risk in children from Manhiça. These findings suggest that modifying vaccine design to induce higher memory T cell responses that can support B cells may increase vaccine efficacy and durability, while site differences should be further studied and considered for vaccination strategy.

3. An increased risk of malaria was associated with IL-10 in infants independently from RTS,S/AS01$_E$ vaccination, indicating that age-specific immunoregulatory responses may affect susceptibility to malaria.

4. Chronic malaria exposure increased the frequencies of T cell subsets expressing inhibitory markers associated with immune exhaustion that correlated with atypical memory B cells and increased anemia. These altered phenotypes could impact naturally acquired immunity in populations living in malaria-endemic settings and affect responses to vaccination.

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