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Quantitative Assessment of T Cell Repertoire during *Plasmodium falciparum* Infection

by

Augustina Frimpong (10357518)

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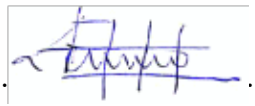
Declaration

I, Augustina Frimpong, declare that the work presented in this thesis is my own original work except where specified that the information has been derived from other sources and have been appropriately referenced in this thesis. This submitted thesis does not include any work which has been submitted to this institution or to any other institution for the award of a degree.



…… 10th August 2020

Augustina Frimpong Date

Supervisory Committee

11th August 2020

Prof. Wilfred Ndifon Date

11th August 2020

Dr Michael F Ofori Date

11th August 2020

Dr. Kwadwo Asamoah Kusi Date

Abstract

Malaria pathogenesis is an intricate process mediated by factors found in both the host and the parasite. The adaptive immune response to the most lethal malaria parasite, *Plasmodium falciparum*, remains to be fully comprehended. Meanwhile, T cells, which are critical for the host responses interact with antigens coupled with MHC molecules, through their membrane surface-expressed T cell receptor (TCR). These TCRs are produced by a process of imprecise gene rearrangement called V(D)J recombination, which is critical for antigen recognition. This may indicate that, the interaction between lymphocytes and pathogens alters the lymphocytes' frequencies and, hence, diversity of their antigen receptor repertoire, suggesting that both the repertoire and the diversity of the antigen receptors may differ at various disease stages. Therefore, the dynamics of T cells in children during *Plasmodium falciparum* infections were examined using peripheral blood mononuclear cells from children residing in a hyperendemic area in Ghana. The frequency of activated Tregs and T cell activation markers during *P. falciparum* infections, and the relationship between these markers and parasitaemia in children with acute *P. falciparum* infections were investigated. In addition, the expression profile of inhibitory and immune-senescence markers on peripheral T cells were examined. Lastly, the repertoire and diversity of the antigen receptor of T cells were determined.

Using flow cytometry, T cell phenotypes involved in the regulation and activation of the immune response were found to be significantly increased in children with symptomatic malaria compared to asymptomatic and healthy controls. In addition, levels of two regulatory and activation phenotypes could explain 68% of the variation in parasitaemia found in the asymptomatic and symptomatic groups. Also, the expression of T cell senescent and exhaustive markers were significantly upregulated in the symptomatic

malaria children compared to the asymptomatic and healthy children. Importantly, levels of CD4PD-1 positively correlated with parasitaemia and could predict inflammation.

Furthermore, applying high throughput sequencing on the T cell receptor β chain from healthy, asymptomatic, uncomplicated and severe malaria-infected children, the selective usage of specific Vβ and Jβ gene segments were observed in each group. Interestingly, the CDR3 nucleotide length in the severe malaria group was found to be significantly shortened, a characteristic shared with other autoimmune diseases. Also, increased repertoire diversity as well as significant sharing of TCR sequences was common in the asymptomatic cohort which seemed to be influenced by recombination events. Applying the GLIPH (grouping of lymphocyte interactions by paratope hotspots) algorithm, T cell receptors from the diseased groups could be clustered based on their antigenic specificity. Additionally, the binding motifs of TCR β chains were predicted.

The data presented here, shows that malaria infection drives T cell expansion. However, T cell clones that are publicly shared as observed in the asymptomatic cohort may have an added advantage of being cross reactive to various *P. falciparum* antigens. These antigen-specific clones maybe able to maintain low parasitaemia levels providing favourable outcome by alleviating disease symptoms.

In summary, studying T cells through quantitative approaches, accelerate the determination of T cell protective signatures and inform the choice of developing interventions.

Dedication

To Him, who uncovers for us to discover, my family, my dear husband (Dr. Danso), my lovely children (Gyamfuaa and Frimpomaa), parents and siblings. Also, to my lab mates and to all who are on the quest to finding an effective vaccine for malaria.

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Abbreviations

ACTs	Artemisinin-Based Combination Therapies
APCs	Antigen Presenting Cells
cDNA	Complementary Deoxyribonucleic Acid
CDR	Complementary Determining Region
CLIP	Class II-associated Ii Peptide
CSP	Circumsporozoite Protein
CTLA-4	Cytotoxic T-cell Lymphocyte antigen 4
CMV	Cytomegalovirus
DBL	Duffy Binding Domain
DN	Double Negative
DNA	Deoxyribonucleic Acid
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HSV	Herpes Simplex Virus
HTS	High-throughput sequencing
IFNγ	Interferon Gamma
Ii	Invariant chain
IL-10	Interleukin 10
iRBC	infected Red Blood Cells
KLD	Kullback-Leibler divergence

LAG-3	Lymphocyte-Activation Gene 3
MHC	Major histocompatibility complex
MPCR	Multiplex Polymerase Chain Reaction
mRNA	messenger Ribonucleic Acid
NGS	Next Generation Sequencing
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD-1	Programmed Cell Death-1
PfEMP1	*P. falciparum* Erythrocyte
Membrane Protein-1 RACE	Rapid
Amplification of cDNA Ends	
RAG	Recombination Activation Gene
RBC	Red Blood Cell(s)
RDT	Rapid Diagnostic Test
RNA	Ribonucleic Acid
RSS	Recombination Signal Sequences
SCID	Severe Combined Immune Deficiency
SIV	Simian Immunodeficiency Virus
TAP	Transport associated with Antigen Processing
TCR	T cell receptor
Tdt	Terminal Deoxynucleotidyl Transferase
TIM-3	T-Cell Immunoglobulin and Mucin Domain-3
TRAP	Thrombospondin-related adhesive protein
Tregs	Regulatory T Cells
TNFα	Tumour Necrosis Alpha

UMI	Unique Molecular Identifiers
uRBCs	uninfected Red Blood Cells

Chapter One

1.0 Introduction

1.1. Malaria: a disease of public health importance

Malaria is a parasitic disease caused by apicomplexans from the genus *Plasmodium.* According to the World Health Organization (WHO), more than half of the world's population remain at risk of developing the disease. In 2017, there were 219 million cases of malaria compared to 217 million cases in 2016 [1]**.** The reported morbidity was estimated as 435, 000 with sub-Saharan Africa accounting for 92% of all reported malaria cases and 93% of the associated morbidity. Among children under five years of age living in sub-Saharan Africa, the disease resulted in 266, 000 deaths in 2017 alone. In addition, pregnant women especially the primigravidae remain susceptible to malaria-associated morbidities such as anemia which may result in low birth weights, pre-term deliveries and susceptibility to other infections [1]**.**

1.1.1 Plasmodium falciparum, the malaria parasite

P. falciparum, P. vivax, P. ovale, P. malariae and *P. knowlesi* are the five main *Plasmodium species* responsible for causing human malaria. The deadliest form of the disease is caused by *P. falciparum* which is responsible for about 99% of malaria-associated mortality [1]. Malaria is endemic in sub-Saharan Africa, Asia and South America. However, the predominant species in Asia and South America is *P. vivax* which has the capability of causing relapsing fever by forming hypnozoites or dormant stages in the liver. The mode of transmission of the parasite is through the bite of an infected female *Anopheles* mosquito. It has been reported that, global distribution of the disease is influenced by climatic conditions that influence the survival of the transmission vector

(*Anopheles spp*) [2]**.**

P. falciparum has a very complex life cycle that involves an invertebrate host and a vertebrate host; the female *Anopheles* mosquito and the human host. The lifecycle in the human host involves two main stages, the pre-erythrocytic stage which is usually asymptomatic and the blood stage which may be either asymptomatic or symptomatic (**Figure 1.1**).

The infection begins when sporozoites are inoculated into the human host once a female *Anopheles* mosquito feeds on blood. These sporozoites circulate for a short time within the bloodstream and lymph nodes before invading liver cells (hepatocytes) to begin the pre-erythrocytic stage where they undergo asexual multiplication. In the hepatocytes, they develop into schizonts and mature within 10-14 days into merozoites, causing rupture of the infected liver cells to enter the bloodstream, initiating the asexual erythrocytic stage. The erythrocytic stage which occurs within 48 hours is mostly associated with the pathologic symptoms of the disease. This cycle begins when *P. falciparum* can invade a red blood cell (RBC). Interestingly, *P. falciparum* can infect RBCs of all developmental stages with no discrimination [3]**.**

In the RBCs, the merozoites develop to form trophozoites, which are single nucleated and contained in vacuoles made up of host RBC membrane. However, these host membranes are lost over the course of the 48hour cycle and replaced by membranes made by the parasite. These trophozoites -feed on host hemoglobin and mature into schizonts, which later rupture to release merozoites and other end products of hemoglobin digestion. The parasite can cause serious morbidity and mortality due to the possession of surface adhesive proteins at this stage.

These proteins enable sequestration in various cells, tissues and organs to escape host defense mechanisms, to repeat the erythrocytic stage [4, 5] .

Also, some of the trophozoites may develop into male and female gametocytes, which are the sexual forms of the parasite. These gametocytes may be picked up by the transmission vector when taking a blood meal. In the mosquito midgut, gametes are formed and fertilized to produce a zygote. The zygote develops into an ookinete, which permeates the epithelial lining in the midgut of the mosquito and forms an oocyst. The oocysts contain sporozoites which mature, rupture and travels to the salivary glands of the mosquito, waiting to be transmitted when the mosquito feeds on a human host.

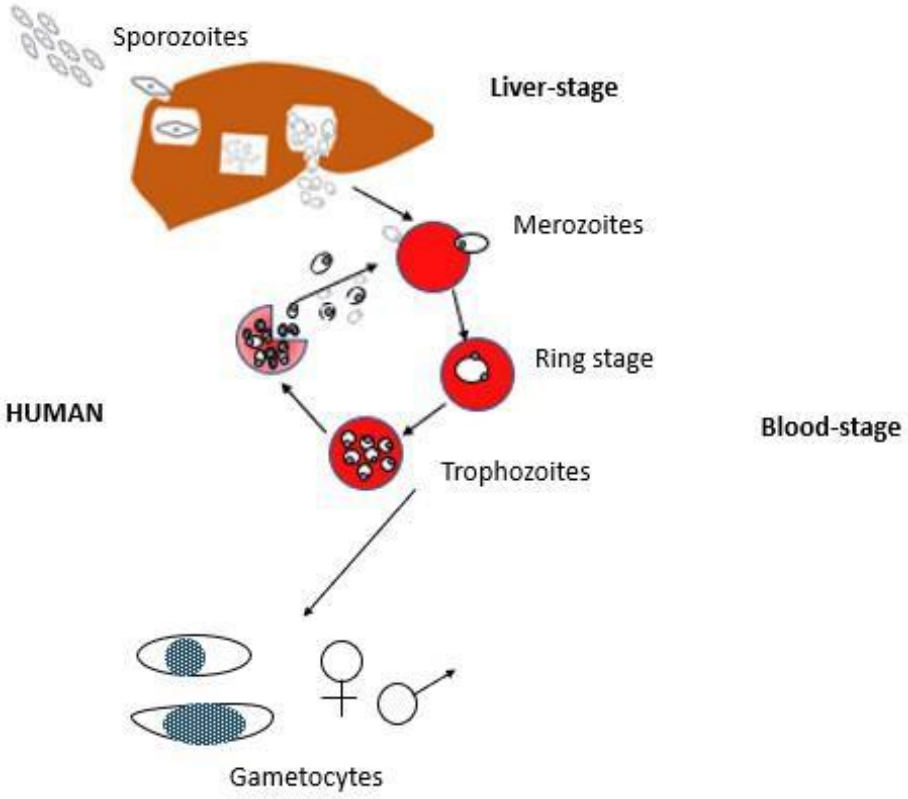

Figure 1.1: Schematic illustration of *P. falciparum* lifecycle in the human host. Infected mosquitoes inject sporozoites whilst taking a blood meal. The sporozoites invade the liver cells to produce merozoites. Merozoites exit the liver to invade red blood cells. The ring stage forms

(trophozoites) may develop into schizonts to make the blood stage infection cyclical or develop into gametocytes. Gametocytes may be later picked by a female *Anopheles* mosquito during a blood meal to initiate the sporogonic stage.

1.1.2 Clinical signs and symptoms of Plasmodium falciparum malaria

P. falciparum infections may result in asymptomatic, uncomplicated or severe forms of the disease including severe malarial anemia and cerebral malaria. The clinical manifestation of the disease may be absent in children with asymptomatic malaria or mild in those with uncomplicated malaria. However, most malaria-related cases are uncomplicated and can be treated effectively with the available approved anti-malarial drugs. There can also be severe forms of the disease such as cerebral malaria, which may be characterized by deep coma caused by neurological disturbances or severe malaria anaemia which is characterized by the loss/destruction of RBCs. The susceptibility to developing either asymptomatic, mild or severe forms of the disease may be affected by age, level of exposure to the parasite, genetic factors, *Plasmodium species,* and specifically the host immune status [6-8] .

In addition, metabolic symptoms such as lactic acidosis, pulmonary oedema and hypoglycaemia are exhibited during the blood stage of the parasite development. These occur when the parasite produces energy through the anaerobic glycolysis of glucose to lactic acid. Furthermore, the anaemia and renal failure associated with severe symptoms of the disease occur when there is haemolysis as a result of the breakdown of the infected red blood cells (iRBC) membrane. Also, the increased clearance of the parasite in the spleen by immune cells such as macropahges, similarly results in the breakdown of the iRBC [9].

1.2 The human adaptive immune system

The human body protects itself against invading pathogens using various mechanisms which may be germline-encoded or newly generated. The immune system is made up of two non-mutually exclusive units; the innate immune system and the adaptive immune system. The innate system, which is germline encoded is dominated by different cells that serve as the first defense against invading pathogens. It is non-specific, rapid and mostly targets conserved antigens. In contrast, the adaptive immune system responds to pathogens within a specific manner and it keeps a memory of previous encounters with pathogens to ensure faster responses upon subsequent encounter.

Unlike the innate system, the adaptive system can respond to the dynamic changes in a pathogen through differentiation, clonal expansion and proliferation or by affinity maturation. This is mostly attributed to the development of a large repertoire of receptor proteins that can interact with variant parasite proteins or antigens. Nonetheless, these two arms of the immune system respond to pathogens using a myriad of effector cells and molecules. Also, the adaptive system is not restricted to humans but it is also used by jawed vertebrates to distinguish between self-antigens and non-self [10, 11].

The major cells involved in the adaptive system are B and T lymphocytes. B cells target extracellular parasite proteins by secreting antibodies to interact with these parasite antigens whereas T cells target intracellular pathogens and release soluble immune factors to activate other immune cell subsets or mediate indirect killing of infected cells and/or intracellular pathogens.

1.2.1 T cell biology

T cells are important immune cells that play significant roles in the adaptive immune response and can also control the activity of other immune cell subsets. T cells are produced from the pluripotent stem cells of the bone marrow and develop in the thymus. In the thymus, the cells undergo differentiation and maturation into committed T cell lines (CD4+/CD8+). T cells have specialized receptors expressed on their extracellular membrane, which enable the identification of antigenic epitopes coupled to major histocompatibility complex (MHC) molecules. These antigenic receptors (T cell receptor or TCR) are very diverse with an estimated theoretical diversity of $10^{15} – 10^{20}$. However, because the human body can contain a limited number of T cells, only about 10^{8} -10^{11} different antigenic receptors are typically observed in a given individual [12, 13].

The TCR is heterodimeric and consists of two protein chains: an α or γ chain and a β or δ chain. T cells with α and β chains make up the majority (~95%) [14]. The γδ TCRs are restricted to the epithelial surfaces such as the skin and form part of the innate immune response [15]. The TCR chains are formed in an almost random process known as genetic recombination. The α chain is formed from the recombination of variable (V), joining (J) and a constant (C) gene segments, whereas the β chain is formed from variable (V), joining (J), diversity (D) and constant (C) gene segments (**Figure 1.2**). The β chain consists of the complementarity determining regions (CDR), CDR1, CDR2 and CDR3. Owing to its high sequence variability, the CDR3 determines much of the diversity in TCRs.

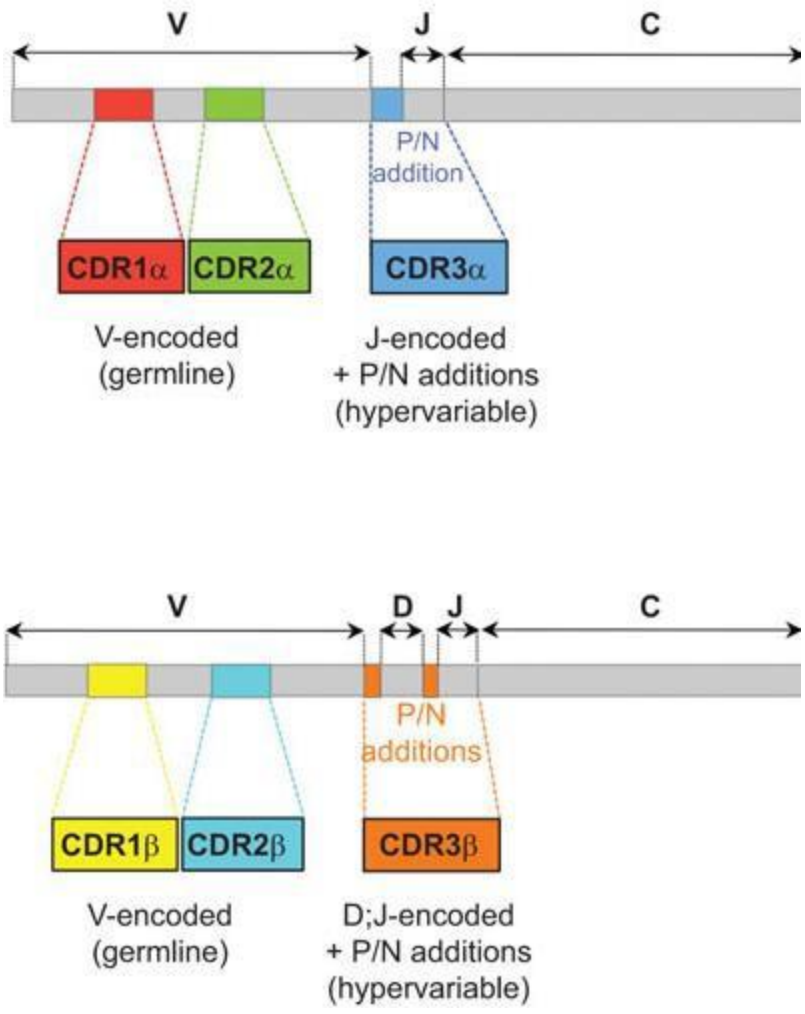

Figure 1.2: **The TCR gene structure [16]**. The CDR1 and CDR2 regions from both α and β chains are encoded in the germline V gene segments. CDR3α is encoded in the V-J junction and the J gene segments in the α chain whereas CDR3β is encoded within the junctions of the V-D and D-J genes and the D and J gene regions.

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1.2.2 T cell development and maturation

T progenitor cells emerge from the bone marrow and enter the thymus [17], to become double negative (DN) or triple negative immature thymocytes expressing neither CD3/CD4/CD8 nor any trans-membrane receptor [18]. The thymocytes are activated to differentiate by the thymic epithelial-stromal cells. They subsequently undergo different

stages of development involving, respectively, TCRαβ gene rearrangement, and positive and negative selection.

The DN thymocytes are distinguished from each other based on their expression of CD44 and CD25. Accordingly, there are CD44+/hi CD25- (DN1), CD44+CD25- (DN2), CD44-/lo CD25+ (DN3), CD44-CD25- (DN4) thymocytes. At the DN3 stage, the cells are selected for rearrangement of their TCR genes in a V(D)J recombination process mediated by the recombination activation gene (RAG) enzymes and terminal deoxynucleotidyl transferase (Tdt). Cells unable to undergo β gene rearrangement die due to either lack of signaling or apoptosis. Cells that have undergone β rearrangement are subsequently selected to undergo pre-TCRα chain rearrangement to form the CD3 molecule and express both CD4 and CD8, thus becoming double positive (DP) thymocytes. Productive rearrangement of both α and β chains leads to deletion of the γ and δ chains. Cells with such productive rearrangements move to the medullary cortex in the thymus, where they undergo positive and negative selection [18]. In humans, the expression levels of CD1a, CD34, and CD38 phenotypic markers are used in experiments for selection of such cells [19].

During positive selection, the DP thymocytes are presented with antigens by cortical epithelial cells to determine their affinity for self-peptide MHC complexes. DP thymocytes that bind appropriately with peptide-MHC Class 1 complexes downregulate CD4 and become CD8+ T cells, whereas those that interact with peptide-MHC Class 2 complexes become CD4+ T cells. In the negative selection process, thymocytes that bind either too strongly or too weakly with self-peptide-MHC complexes are eliminated. Cells that successfully go through the stages of maturation leave the thymus as immature naïve

T cells to periphery or home to other secondary lymphoid organs. The positive and negative selection processes ensure that these cells can react with peptides presented by self MHC molecules without causing autoimmune disease due to over-reactivity with self-antigens.

1.2.3 Somatic recombination in T cells

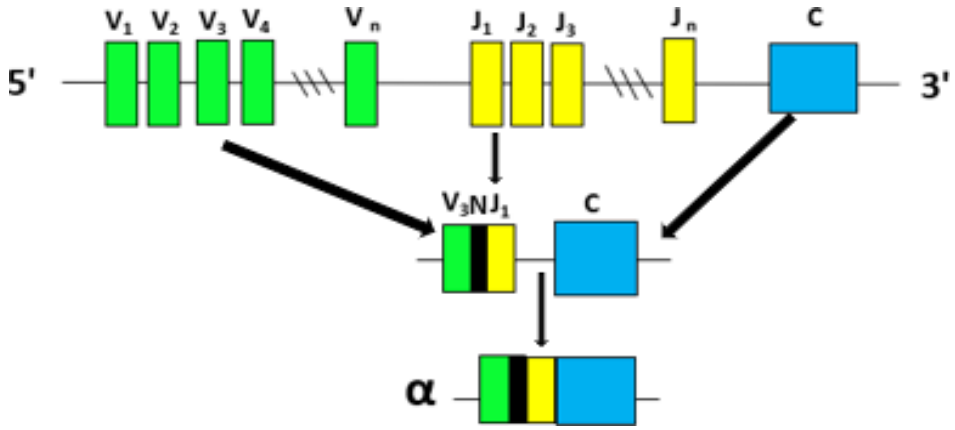
Diversity in TCR is initially generated by random somatic recombination of the V(D)J gene segments from germline encoded genes, subsequent insertion and deletion of nucleotides at the junctions and TCR α and β pairing [20] (**Figure. 1.2**). The somatic generation of antigen receptors in lymphocytes is different from germinal cells [21]. Here, the RAG complex binds and cleave the double strand DNA to break at the specific site of the recombination signal sequences (RSS) at the various V(D)J segments to create flanking regions. VDJ recombination is initiated by recombination-activating genes 1 and 2. For initiating, RAG-1 binds to the RSSs to cause a double strand break [20, 22]. The second stage is ligation of the broken DNA by RAG1 and RAG2 genes. The flanked genes then joined by the 12/23 rule (12RSS/23RSS) separately to conserved heptamer and nonamer. The Vβ segments bind to 5' 12RSS following the beyond 12/23 (B12/23) rule [23, 24]. During ligation, nucleotides are deleted and inserted by the Tdt.

This non-stochastic gene rearrangement is tightly regulated to ensure that the breaking of the DNA double strands occurs in lymphoid cells. It is noted that V(D)J genes are assembled in T cells for the TCR, where rearrangement occurs in double negative (DN; pro-T cells) for the β gene, and in double positive cells (pre-T cells) for the α gene. For instance, the rearrangement of the β segment is by the addition/joining of D-J gene segments, which is later joined to a V segment and a C region, whereas, the α chain is

formed by the joining of a V -J gene segments, then a C region (**Figure 1.3**). In αβ T cells, these chains form three complementarity determining regions (CDR). CDR1 (α and β) and CDR2(α and β) are encoded by germline V genes, are highly conserved, and determine the TCR's interaction with MHC molecules. Antigen interaction occurs in the cleft of the CDR3 (α and β) which is associated with generating diversity in recognition of a repertoire of antigenic peptides coupled to MHC molecules (**Figure 1.2**). Therefore, making CDR3 the most diversified among the CDRs since it is made up of junctional diversities and segments of the V and J genes.

The regulation of V(D)J recombination is determined by the expression levels and accessibility of the RAG genes (RAG 1 and 2) as well as the RSSs [25, 26]. Mutations in RAG genes have been associated with the development of fatal syndromes in infants (from 1 to 6 months) known as Omenn syndrome which is associated with recurrent infections [27-29]. Also, a deficiency of the RAG leads to the development of immune deficiencies such as severe combined immune deficiency (SCID), a rare disease with fatal consequences [27, 30]. After successful recombination, a constant (C) gene segment is added to V(D)J rearranged β chain. Then the heterodimeric T cell antigen receptor is expressed on the cell surface to undergo clonal selection to cull out deleterious T cells, a thymic selection process that decreases diversity.

a.



b.

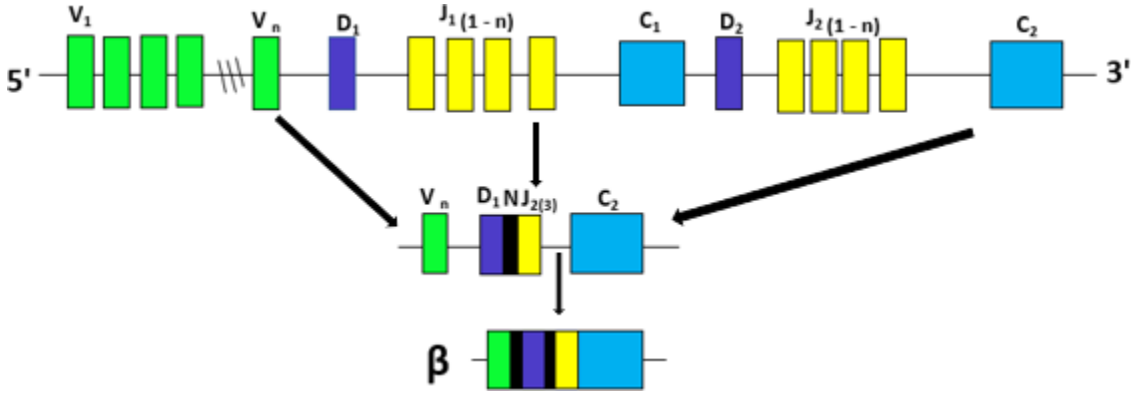


Figure 1.3 Schematic illustration of somatic recombination in the (a) α and (b) β chain of the T cell receptor. During somatic recombination, the various gene segments are randomly selected and joined together. Nucleotide are added and deleted at the joining regions to increase diversity.

1.2.4 MHC I and II associated antigen processing and presentation to T cells

1.2.4.1 MHC I-associated antigen presentation

During infection of cells with intracellular pathogens, antigens from the invading pathogen are taking up. For antigens requiring a CD8+ T cell response including *P. falciparum* antigens, the antigenic peptides are presented using the MHC Class1 molecule for which the peptides are generated through the lysosomal pathway. The processing of MHC Class 1 for antigen presentation involves the use of the antigen presentation machinery (the proteasome, transport associated with antigen processing (TAP), the endoplasmic reticulum (ER) and its chaperones (calnexin, calreticulin. ERp57 and tapasin) as well as the Golgi apparatus [31, 32]. The presentation of antigenic peptides using the MHC Class 1 complex involves four main stages; the generation of the peptides, peptide transport, assembling of the MHC Class 1 complex, antigen presentation [31, 33]. During pathogen invasion of an infected cell, cytosolic proteins undergo proteolysis in the proteasome where these proteins are degraded into peptide fragments of about 2-25amino acids in length and then released into the cytosol. The amino acid sequence and length of the generated peptides depends on the kind of proteasome or immunoproteasome used [31, 32]. These fragmented peptides are transported into the endoplasmic reticulum using the TAP1 and TAP2. They are trimmed by aminopeptidases into about 8-10 amino acids in length [34]. The MHC Class 1 complex consisting of the heterodimeric domain and the ER chaperones help in loading the fragmented peptides into the peptide binding groove of the nascent MHC Class 1 molecule. The MHC Class 1-peptide complex is released to the cell surface through the Golgi apparatus where it can be presented to the CD8+ T cells for cytotoxic activity (**Figure 1.4a**) [32, 33, 35, 36].

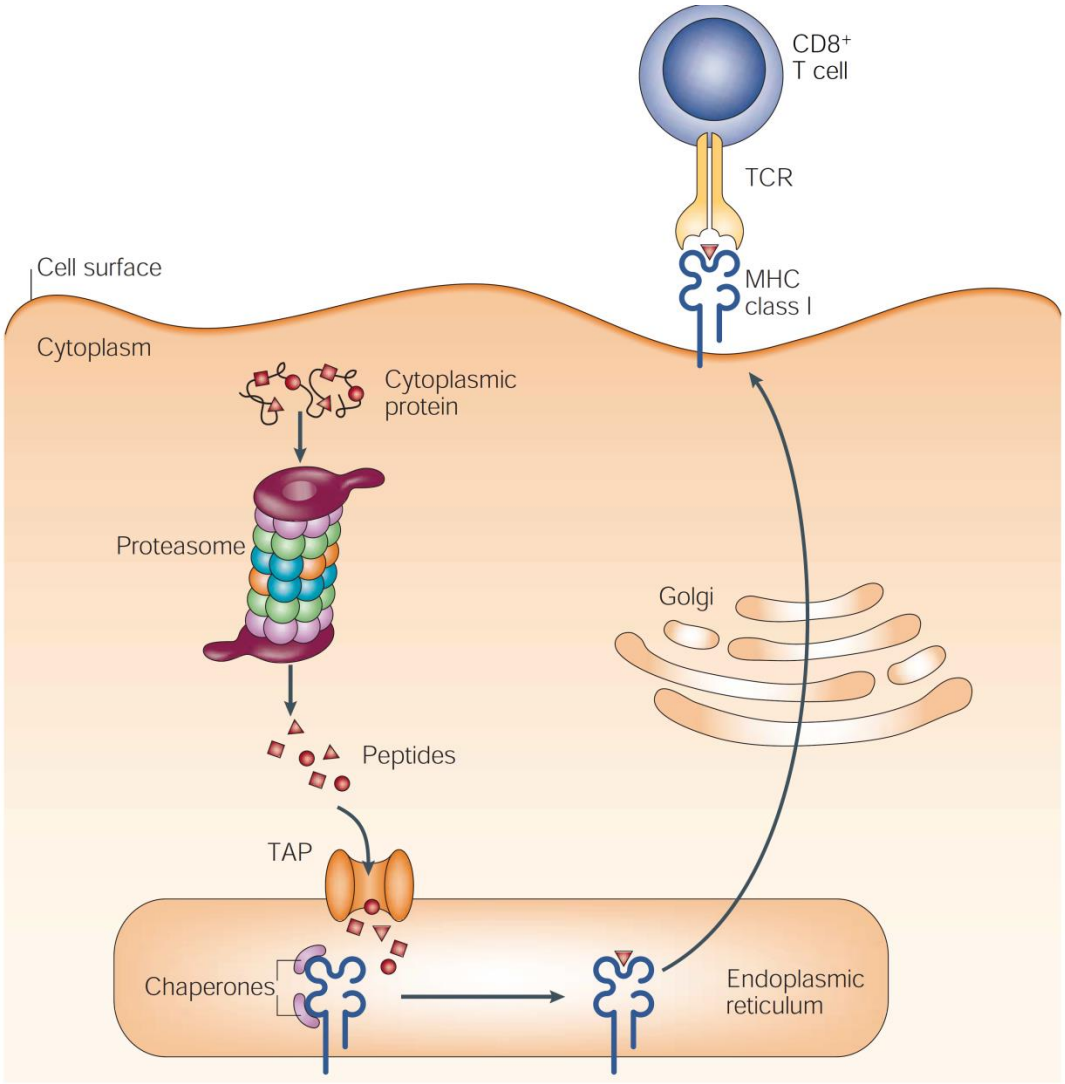


Figure 1.4a: **The MHC Class I Antigen Presentation[36]**. Cytosolic proteins are degraded into peptides. These peptides transported by the TAP into the endoplasmic reticulum where MHC Class I molecule is assembled. Peptide with specificity to the MHC molecule binds to the peptide groove. The peptide MHC complexes are released and transported through the Golgi apparatus to the cell surface to await recognition by a CD8+ T cell to initiate cytotoxic activity.

1.2.4.2 MHC Class II antigen presentation

MHC Class II antigen processing and presentation is restrictive to only professional antigen presentation cells (APCs); dendritic cells, macrophages and B cells. These APCs may engulf pathogens via phagocytosis, micropinocytosis or endocytosis based on the cell type [37]. For MHC Class II molecules, the classic pathway for antigen processing is through the endocytic pathway. For *P. falciparum* antigens that may require a CD4+ T cell response, the antigens are processed and processed through the endocytic pathway. The organelles involved in MHC Class II processing include the endoplasmic reticulum, the ribosome, Golgi apparatus and lysosome which contain acids. the pathogen is broken down into fragments or antigenic peptides in an acid environment provided by the fusion of the lysosome and phagosome to become the phagolysosome. The endoplasmic reticulum, the ribosomes synthesize and assemble the MHC Class II molecule which is made up of two α domains and two β domains. In the ER, a chaperone, the invariant chain (Ii) prevents ER proteins from binding to the MHC peptide groove [38]. The Ii and MHC Class II complex are transported outside the ER through the Golgi apparatus into the MHC Class II compartment which has a slightly acidic environment. In the acidified compartment. In the compartment, the Ii protein is degraded by cathepsin S and cathepsin L proteases leaving only a residual known as the class II-associated Ii peptide (CLIP) that binds to the peptide groove and inhibits other proteins from interacting with the peptide binding groove. Following this, another chaperone known as HLA-DM (HLA-DO for B cells) interacts with the MHC Class II molecule to release the CLIP [39, 40]. The fused phagolysosome then fuses with the MHC Class II compartment to allow the fragmented peptides or antigens into the endosome where the antigenic peptide specific to MHC Class II molecule, then binds to the peptide binding groove. The APC then transports the MHC Class II

molecule coupled with the antigenic peptide to its cell surface to await interaction with a CD4+ T cell (**Figure 1.4b**).

Notwithstanding, both MHC Class I and II molecules can undergo cross presentation, where exogenous antigens may be presented by MHC Class I molecules and endogenous antigen can undergo MHC Class II presentation [41].

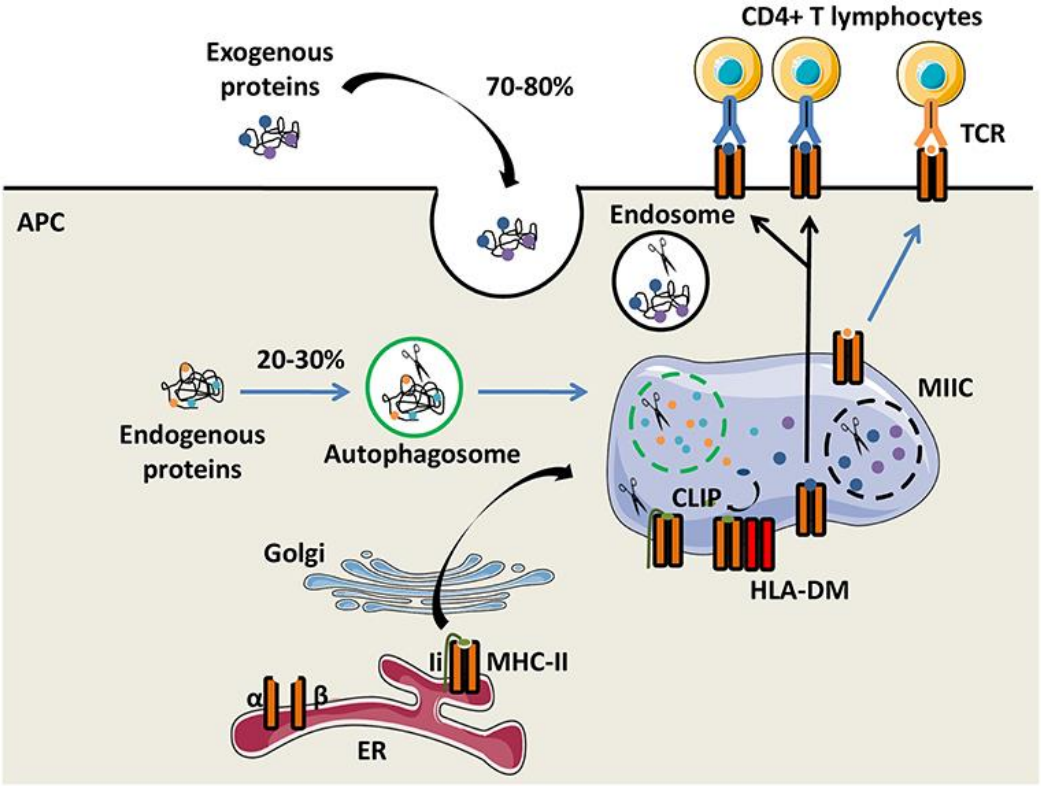


Figure 1.4b: **The MHC Class II Antigen Presentation [42]**. Exogenous proteins are degraded into peptides. The degraded fragments and assembled MHC Class II complex including the Ii protein are transported through the Golgi apparatus to the MHC Class II antigen processing compartment. The Ii protein is degraded leaving a residual CLIP binding to the peptide binding groove. The interaction of chaperone HLA-DM with the MHC complex releases the CLIP to allow a peptide with specificity to the MHC molecule to bind to the binding groove. The peptide-MHC complex is transported to the cell surface to await interaction with a T cell receptor of a CD4+ T cell.

1.2.5 T cell recognition and activation

T cells are mainly divided into two types based on the glycoprotein receptors they preferentially express during their development in the thymus, namely CD4 and CD8 T cells. Upon activation, CD4 T helper cells function by the secretion of cytokines, whereas CD8 function by direct cell cytotoxicity to eliminate infectious pathogens or cells. T cell activation requires several different signals, most notably an antigen signal, which is recognized by the TCR, and a co-stimulation signal [43]. The αβ TCRs cannot recognize antigens in their native form but require the processing and presentation of the antigens by other cells [43]. The TCRs are MHC restricted and therefore can identify antigens only when they are coupled to an MHC molecule; MHC I for CD8 T cells, and MHC II for CD4 T cells. The antigen source determines the MHC molecule that is used for presentation. For instance, endogenous antigens are presented in association with MHC I whereas exogenous antigens are presented in association with MHC II.

The MHC molecule may be polygenic and polymorphic, and a large number of variants is observed in populations. This determines the peptides that participate in thymic selection and, hence, shapes an individual's TCR repertoire [44, 45]. TCR recognition of an antigen complexed with MHC molecules initiates a cascade of signaling pathways that may lead to the upregulation or expression of surface markers associated with T cell activation (**Figure 1.4**). Adequate activation requires a productive co-stimulatory interaction between the CD28 surface molecule found on T cells and the B7 proteins found on antigen presenting cells. The early activation marker CD69 may be upregulated to allow for the differentiation and proliferation (clonal selection) of T cells with specificity for the antigen-MHC complex. This enables the secretion of cytokines which may serve an

autocrine or a paracrine function by promoting differentiation of the T cells into various subsets [46].

Once the source of antigen causing T cell activation has been eliminated, effector T cells die by apoptosis, leaving a small subset called memory T cells for more effective responses to future exposures. The dynamics of the T cell repertoire are thus brought to homeostasis. However, for pathogens causing chronic infections such as HIV, HBV and HCV, the cells may express higher levels of molecules such as PD1, CTLA4 which have been described as exhaustive or inhibitory markers [47, 48]. These negative inhibitory markers may downregulate the activity of the T cells leading to inefficient immune responses and persistence of invading pathogens.

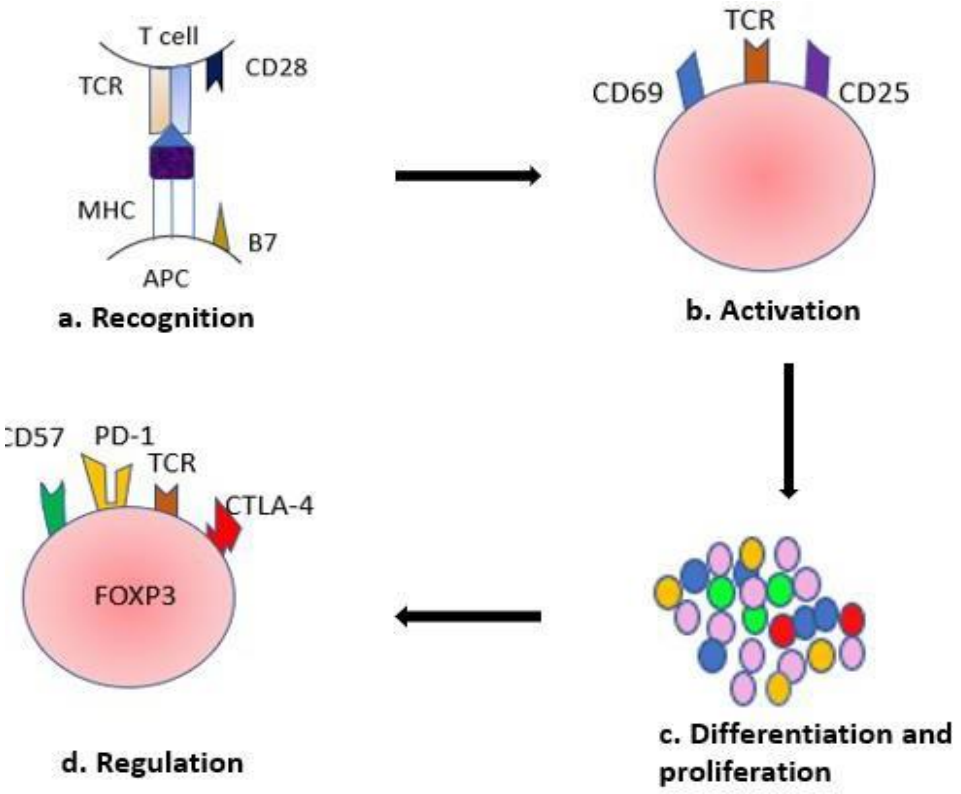


Figure 1.5: T cell recognition, activation and regulation. T cell **(a)** recognition is

initiated by MHC binding to antigen and interacting with the TCR. **(b)** Activation is associated with the release of soluble factors and upregulation of activation markers such as CD25 that leads to the **(c)**differentiation and proliferation of selected T cell antigen receptors. **(d)** During effector state, the activity of the immune response is regulated to prevent immune pathology.

1.2.6 Significance of TCR diversity in disease

T cells orchestrate adaptive immunity against pathogens, by recognizing pathogen-derived antigens using their TCR. The strength of the T cell response to a pathogen depends crucially on the number of functionally competent T cells with TCRs that recognize antigens derived from that pathogen [49, 50]. Because there is a large diversity of possible antigens, the TCR diversity must be correspondingly large to ensure effective adaptive immune responses. Indeed, the rearrangement of the TCR genes ensures that a T cell can respond to a vast array of antigens and can sustain protective immunity. There are multiple V, D, J gene segments in the T cell loci that allow for the combinatorial diversity that is observed during recombination. Diversity is further increased by the addition and deletion of nucleotides at the junctions between gene segments (V and J, V and D, D and J)

Interestingly, it has been observed that improper V(D)J recombination can result in major diseases such as type I diabetes and tumorigenesis [51-53]. However, the composition of an individual's TCR repertoire is affected by age. In addition, the specificity of the repertoire is shaped by an individual's antigenic history and past exposures [48, 50], implying that the changes in TCR repertoire during a disease state may serve in identifying and tracking TCR clonotypes (a unique CDR3 nucleotide sequence generated during gene rearrangement for a specific TCR) associated with disease. There are about 25 x 10^6 clonotypes recorded in humans of which some are shared (public) within

population and others are restrictive (private) to an individual [54-56]. These clonotypes enable the understanding of host immune responses to disease.

Also, increase diversity in the composition of the repertoire is associated with protection or good outcome from a disease. Of note, infections with common pathogens skews the TCR repertoire due to the expansion of antigen-specific T cells and may result in shared TCRs which are mostly associated with favourable outcome. For instance, it was observed that in mice with HSV-1 infection [57] and macaques with Simian immunodeficiency virus (SIV) infection [58], the expansion of shared clonotypes were associated with positive disease outcome. These public clonotypes were observed to be inversely related to viral load [58]. This may imply that the quantitative assessment of such shared clonotypes may act as predictors of disease outcome or help in the development of diagnostic tools [59]. This is similarly observed in humans and associated with diseases like cancer [60, 61]and some viral infections , where the expansion of specific clonotypes has been used as a surrogate of disease status.

1.2.7 Analysis of T cell repertoires

1.2.7.1 Experimental approaches

The T cell repertoire can be studied using various approaches. For instance, studying the nature of cells using monoclonal antibodies directed to the cellular receptors is one very popular approach. Flow cytometry provides facile and quantitative access to the TCR Vβ profile of different T cell subsets. In this approach, T cells are intracellularly stained with specific Vβ monoclonal antibodies targeting the Vβ domains. The observed expression levels or frequency of the Vβ domains are then quantified in a defined T cell population

[62, 63]. Here, the assumption is that a decrease in the frequency of clonotypes corresponds to increased diversity [13]. Moreover, the degree of heterogeneity found in the Vβ segment frequencies can indicate the TCR diversity in the specified population [64]. In addition, using Gini TCR skewing index, (a diversity index used to study the distribution of income among people in economics), the distribution of Vβ segments can be analyzed to detect differences that might be associated with either protection or susceptibility to an infection [65]. However, this approach does not provide any information about the polymorphisms that may occur in the TCR genes nor about the junctional diversity found in the CDR3 regions. Furthermore, not all TCR genes have monoclonal antibodies that can be used to specifically target them, inadvertently preventing the identification of novel clonotypes or gene segments usage during infection or in health [12, 63].

Apart from flow cytometry, the advent of polymerase chain reaction (PCR) technology has been adopted to help understand quantitatively the expression of the various V gene sub-families through amplification. A PCR-based technology employed in T cell repertoire profiling is spectratyping. In this approach, mRNA is reverse transcribed to cDNA. The resulting cDNA spanning the CDR3 region is amplified. The PCR products, which are CDR3 fragment lengths are assessed using gel electrophoresis. Spectratyping analyzes these CDR3 lengths by determining patterns of their distribution and how they deviate from the Gaussian-like distribution in the TRBV gene segments [66-68]. It can also be used to assess the clonality of the T cell population(s) under study [69, 70]. Here, measurement of diversity can be determined from the frequency distribution of the CDR3 length [71, 72]. This method provides moderate or better resolution of the TCR assessment in comparison to flow cytometry. This technique is relatively quick and cheap.

Next-generation sequencing (NGS) offers high throughput and high-resolution data. The use of NGS platforms such as Illumina Miseq, Illumina HiSeq or Roche 454 provides a wholistic overview of characterizing the TCR repertoire. It analyses the nucleotide sequence information for over thousands of clones in a single experiment. In addition, the usage of these platforms may be complemented with molecular techniques that enrich the VDJ or CDR3 sequences such as Rapid Amplification of cDNA Ends (5'RACE) and multiplex PCR (MPCR) [73]. In multiplex PCR, recombination genes are targeted by a pool of primers which may probably be associated with amplification bias [74]. Also, due to the specific use of primers, MPCR may not be able to identify new gene segments [75]. The 5'RACE on the other hand is targeted by a single primer to the C region and a general primer that uses the Tdt to add nucleotides to the 3' end of the cDNA, thereby producing non-significant amplification bias [74]. Also, relying on a template-switch that contains oligo nucleotides, cDNA strands containing the 5' end of the mRNA are synthesized, enabling the full length of the mRNA to be captured. This ensures that all variants of TCR within the sample are synthesized, which also facilitates the identification of novel alleles [74, 75]. With NGS, the CDR3 nucleotide length, clonotype frequency and β rearrangements can be determined [51, 73]. Despite the fact that NGS is costly, labour and time intensive, it provides very high-resolution data in comparison to flow cytometry and spectratyping. Such data are crucial for accurately quantifying TCR diversity in health and disease.

1.2.7.2 Computational approaches

Diversity in ecological terms refers to the number of unique species (richness) and frequency of observed species (evenness) in a community. TCR diversity is the measure of

T cell clones and the relative abundance of the clones in a giving sample. In immunology, diversity index enables the comparison of immune repertoire and its relation to an individual's immunological status. Diversity index have the possible application of identifying and diagnosing perturbations in the immune response to disease or vaccination. There are various single diversity indices, however, they do not yield similar results based on the assumptions or parameters estimated. Renyi's entropy, which is often described as the $\log_2$ or ln of "true diversity", provides a continuum of diversity indices profile. It normalizes the frequency distribution of TCR clones in a giving sample. It is defined as:

$$H_\alpha(X) = \frac{1}{1-\alpha} \log\left(\sum_x (f_x)^\alpha\right)$$

Where $H_\alpha(x)$ is the Renyi's entropy, α is the discrete random variable with possible outcomes, f is the clonal frequency distribution, and x being the total number of all clones. When α is equal to 0, $H_\alpha(x)$ assumes the exponent of Shannon's entropy which is the species richness. As α approaches 1, $H_\alpha(x)$ it tends to Shannon's entropy which quantifies the species richness and evenness. Mathematically, it is defined:

$$H = -\sum_i Pi \log_b Pi$$

where H is the Shannon's entropy, $\log_b$ is the logarithmic base, Pi represents the frequency of clone (i) in the giving sample.

Also, as α approaches 2, Renyi's entropy assumes the Simpson's diversity index. Simpson's diversity index is used to characterize the richness and evenness of TCR diversity of the T cell receptor in a given sample. It measures the probability that two

TCRs that are randomly sampled from the same individual will be identical, in terms of their CDR3 binding motifs. For an individual, if the reciprocal of Simpson's index equals D, then the diversity of that individual's TCR repertoire is equivalent to that of someone whose repertoire contains only D equally abundant TCRs. In principle, the larger the value of D the larger the diversity of antigens to which an individual's TCR repertoire can respond. The equation may be represented as:

$$D = 1 - \left(\frac{\sum n(n-1)}{N(N-1)} \right)$$

n = the number of copies of ith clonotype, N = total number of TCR clonotypes sequenced. However, as α approaches ∞, the Renyi's entropy gives more weight to the abundant clones in the sample.

Another method for determining the repertoire of TCR CDR3 sequences is the Kullback-Leiber (KL) divergence. KLD originated from information theory and helps to quantify the degree of uncertainty in a data or given distribution. KL divergence (KLD) measures the probability distribution between 2 populations; g(v) and h(v). It makes the initial assumption that the distribution between the 2 populations are the same, where KLD = 0, and then compares the similarity between the 2 distributions. KLD computes the similarities in distribution by determining how much information is lost in g(v), when h(v) is used to estimate g. In addition, the information provided on h by g is not the same as the information provided by h on g, making KLD non-symmetric. Hence, it cannot be used to measure the distance between the 2 distributions. Combining the distributions of g and h, modifies KLD to be symmetrical and produce a finite value. A normalized KLD is known as the Jensen-Shannon divergence. Mathematically, the KLD of 2 distributions, g and h is

expressed as:

$$\mathrm{KLD}(g||h) = \sum_{v} g(v)\log\left(\frac{g(v)}{h(v)}\right)$$

Where g is the probability distribution of g(v) and h is the probability distribution of h(v)

1.2.8 Role of T cells in malaria infection

T cells have been shown to play a crucial role in mediating immune responses to malaria and understanding their mechanisms have significant impact on the development of an efficacious vaccine. The interaction of the TCR with an antigen may result in activation, differentiation, proliferation into subsets, secretion of cytokines or direct cell-to-cell cytotoxicity.

Few infectious sporozoites are inoculated into the liver during *P. falciparum* infection [76]. The presence of T cells in the liver has been reported three days after inoculation. Majority of these T cells are believed to migrate from the lymph nodes where they are presented with malarial antigens from the liver by dendritic cells [77, 78] whereas, some are also activated in the liver [79, 80]. However, activation of T cells in the liver has been associated with tolerance to the infection [81, 82].

T helper cells are significant sources of IFN-γ and TNFα. Studies done in both human and murine models show early sources of IFN-γ to be associated with positive outcome of disease and with the development of protective immunity against malaria [83]. However, antigen specific T cell responses identified in response to pre-erythrocytic antigens such as the circumsporozoite protein (CSP) and thrombospondin-related adhesive protein (TRAP) in minority of individuals in endemic areas, have been observed to be very

low [84, 85]. Additionally, CSP specific T cell responses were not associated with protection [86, 87]. Nonetheless, recent evidence suggests that correlates of immune protection in individuals immunized with radiation-attenuated sporozoites were mediated mostly by T cells [88-91].

Also, during blood-stage infections, the effector and regulatory role of T cells has been described [92]. Recent studies have identified the significant role of T cells in differentiating asymptomatic from symptomatic children. It was observed that specific type of T cell subsets (Th1 self-regulatory T cells) that co-produce IFN-γ and IL-10 were observed to be associated with anti-disease immunity [93, 94]. Likewise, it was reported that regulatory T cells expressing CTLA-4 were associated with the lack of developing sterile immunity [95]. Also, antigen specific responses such as the CD4+IL-4+ T cells elicited by the homologous DBLα domain of PfEMP1 during acute malaria in children has been associated with the absence of clinical symptoms in a longitudinal study [96].

Also, at the gametocyte stage, it has been observed that proinflammatory cytokines (IFNγ and TNFα) levels correlate with levels of gametocytes. In addition, it is believed that during mosquito infectivity, cytokines may be ingested alongside the gametocytes which have the potential to hinder further development in the mosquito midgut [97, 98].

In addition, the significance of T cells in malaria may be affected by other diseases such as co-infection with other diseases such as HIV/AIDS [99], Hepatitis [100], Schistosomiasis [101], and Burkitt Lymphoma [102]. For instance, co-morbidity with HIV/AIDS, have been reported to have both a bidirectional and synergistic effect on T cell immunity. Malaria has been associated with a high CD4+ T cell response as well as upregulating the levels of pro-inflammatory cytokines and activating memory T cells

enhancing the spread of HIV through its target cells, CD4+ T cells [103]. Also, HIV infection elevates levels of Tregs, which are likely to increase the parasite density with malaria infection by providing a suppressive environment[104]. Likewise, the loss of CD4+ T cells caused by HIV infection has been reported to increase the risk of developing severe malaria [105].

1.3 Rationale

T cells which are a subset of lymphocytes are major immune cells which play significant roles ranging from modulating immune responses against cancer, infections, allergic reactions, transplant rejection and in autoimmune diseases.

Circulatory T cells are mostly located within the secondary lymphoid organs and the blood. T cells migrate to the lymph nodes to interact with antigen presenting cells to get activated. Here, they encounter antigenic peptides coupled to MHC molecules corresponding to their cognate receptor [106]. Upon encountering an antigen, the repertoire of the T cell increases to a sufficient amount to eradicate the infection. With a disease like malaria which has a complex life cycle and an intricate process involving the host, studying the repertoire of the T cells is critical in identifying and understanding the immune correlates of protection. Since this will aid in effective drug and vaccine design through the identification of T cell biomarkers that may be associated with protection or disease pathogenesis [107, 108].

Severe malaria has been associated with *P. falciparum* infections with mortality around 90% among infants under five years in sub-Saharan Africa [1]. This high fatality rate has been attributed to the virulent nature of the parasite and its ability to cytoadhere to

the host endothelium to avoid splenic clearance. Sequestration is mediated by PfEMP1, a family of adhesion ligands with the ability to modulate immune responses [109]. For instance, in studies performed by Sampiano et al., it was observed that parasites expressing PfEMP1 can alter type 1 IFN response [110], decreased NFκB transcription factor in macrophages as well as decreased the release of chemokines and cytokines compared to parasites lacking PfEMP1 expression [110, 111]. These may imply that PfEMP1 may serve as immunomodulatory molecules dampening host immune response to parasites by suppressing immune activation and the release of cytokines and chemokines

In addition, the immune response associated with malaria has been described to be partial and not sterile. As a result of this, there is the need to provide information on the host immune factors during malaria infections. For instance. the role of T cells in malaria has been described to be protective and both pathogenic [112]. These T cell responses have been described to either aid in effective parasite control or exacerbate the degree of infection. In addition, previous studies have shown the involvement of αβT cells in the pathogenesis of cerebral malaria in animal models by sequestering to the endothelium and interacting with endothelial cells and platelets [113]. These T cells classified as pathogenic T cells have also been found adhering in the brain tissues in Malawian children who died of cerebral malaria [114].

However, the immune response elicited by these αβT cells are determined by the clonal diversity of the T cell receptors formed during somatic V(D)J recombination. Several studies including spectratyping and high throughput sequencing (HTS) have been employed in understanding the T cell receptor clonotypes. Among these, HTS has been accepted as the most effective tool in understanding T cell clonotype diversity and

responses during infections even though spectratyping has made remarkable discoveries. For instance, in Menezes et al., [115] study which involved the spectratyping technique, they were able to identify clonotype subsets of T cells involved in driving multiple sclerosis.

Nonetheless, since the TCR repertoire of an individual is constantly under evolution, it is high throughput sequencing coupled with computational analysis that can determine the repertoire correlations, mutations, sequence comparisons and diversity estimates among individual populations. Furthermore, high throughput sequencing of the T cell receptor has enabled the understanding of the immune repertoires in autoimmune diseases such as systemic lupus erythematosus and multiple sclerosis [116-118]. Immune repertoire analysis has also enabled better classification and prognosis of hepatocellular carcinoma by analyzing tumor-infiltrating lymphocytes [119].

Therefore, studying T cell repertoire through hypothesis testing provides better understanding of host-pathogen interactions and aid in the design of effective interventions such as vaccines. Moreover, testing the hypothesis through mathematical modeling and computational analysis will aid in generating predictive outcomes that can accelerate malaria vaccine development.

1.3.1 Specific Aims

Understanding the immunity resulting from *P. falciparum* infections remains the interest of vaccine developers. It is well noted that acquiring natural immunity to *P falciparum* malaria develops slowly. However, recent evidence suggests that T cell immunity may dictate the level of protective immunity acquired to the disease. However, whether there is skewness or expansion of specific lineage of T cells in malaria patients remain unknown.

In this study, using experimental, high-throughput sequencing, and mathematical approaches, we characterize the T-cell diversity and functional phenotypes from children with malaria (asymptomatic, uncomplicated and severe malaria). We also determine whether and how these important parameters of the T cell repertoire correlate with clinical outcome. The specific aims are:

1. To characterize phenotypic changes in the peripheral T cell repertoire during *P. falciparum* malaria infections in children with symptomatic malaria, asymptomatic malaria and aparasitemic controls.
    a. To determine the frequency of activated Tregs and T cell activation markers during *P. falciparum* infections, and the relationship between these markers and parasitaemia in children with acute *P. falciparum* infections.

    b. To determine the expression profile of inhibitory and immune-senescence markers on peripheral T cells in children with symptomatic malaria, asymptomatic malaria and aparasitemic controls.

2. To characterize the T cell antigen receptor repertoire and diversity in *P. falciparum*-infected children.
    a. To compare the diversity of the TCR repertoires in asymptomatic, uncomplicated, severe malaria and aparasitemic controls.

    b. To determine whether there are biases in gene segment use by T cells

responding to malarial parasites by comparing TCR V/J gene segment usage across the groups.

c. To determine the dominance of the clonotype variants that may be associated with disease pathogenicity, severity and persistence in the infected groups.

1.4 Thesis outline

The rest of the thesis is organized into 4 chapters:

Chapter 2 presents the output of the specific aim 1a. Here, the manuscript entitled, "Characterization of T cell activation and regulation in children with asymptomatic malaria", is presented. This is a comprehensive study that tries to explain the relationship between cellular activation, regulation and parasite control. The manuscript has been published in Malaria Journal (https://doi.org/10.1186/s12936-018-2410-6).

Chapter 3 is a manuscript entitled, "Phenotypic evidence of T cell exhaustion and senescence during symptomatic *Plasmodium falciparum* malaria". In this chapter, we present the evidence on T cell exhaustion and senescence during malaria infection and how these markers relate with inflammation and parasitaemia. The manuscript has been published in Frontiers in Immunology (https://www.frontiersin.org/articles/10.3389/fimmu.2019.01345/full).

Chapter 4 provides a comprehensive overview for the first time on the impact of malaria infection on T cell antigen receptor repertoire in children with falciparum malaria infection. Here, we discuss the frequency, diversity, TCR convergence and clonotype

specificity during malaria infections. A manuscript is being developed.

Chapter 5 is a summary of general discussion and conclusion of the results generated that are presented in this thesis. We also discuss measures that can be improved in future studies

Appendix is a list of supplementary data from Chapters 2, 3, 4 and a review manuscript on Novel strategies for malaria vaccine design in Frontiers in Immunology (https://doi.org/10.3389/fimmu.2018.02769).

Chapter Two

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Malaria Journal

RESEARCH Open Access

Characterization of T cell activation and regulation in children with asymptomatic *Plasmodium falciparum* infection

Augustina Frimpong[1,2,3*], Kwadwo Asamoah Kusi[1,2], Bernard Tornyigah[2], Michael Fokuo Ofori[1,2] and Wilfred Ndifon[3,4*]

Abstract

Background: Asymptomatic *Plasmodium* infections are characterized by the absence of clinical disease and the ability to restrict parasite replication. Increasing levels of regulatory T cells (Tregs) in *Plasmodium falciparum* infections have been associated with the risk of developing clinical disease, suggesting that individuals with asymptomatic infections may have reduced Treg frequency. However, the relationship between Tregs, cellular activation and parasite control in asymptomatic malaria remains unclear.

Methods: In a cross-sectional study, the levels of Tregs and other T cell activation phenotypes were compared using flow cytometry in symptomatic, asymptomatic and uninfected children before and after stimulation with infected red blood cell lysates (iRBCs). In addition, the association between these T cell phenotypes and parasitaemia were investigated.

Results: In children with asymptomatic infections, levels of Tregs and activated T cells were comparable to those in healthy controls but significantly lower than those in symptomatic children. After iRBC stimulation, levels of Tregs remained lower for asymptomatic versus symptomatic children. In contrast, levels of activated T cells were higher for asymptomatic children. Strikingly, the pre-stimulation levels of two T cell activation phenotypes (CD8+CD69+ and CD8+CD25+CD69+) and the post-stimulation levels of two regulatory phenotypes (CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+) were significantly positively correlated with and explained 68% of the individual variation in parasitaemia. A machine-learning model based on levels of these four phenotypes accurately distinguished between asymptomatic and symptomatic children (sensitivity = 86%, specificity = 94%), suggesting that these phenotypes govern the observed variation in disease status.

Conclusion: Compared to symptomatic *P. falciparum* infections, in children asymptomatic infections are characterized by lower levels of Tregs and activated T cells, which are associated with lower parasitaemia. The results indicate that T cell regulatory and activation phenotypes govern both parasitaemia and disease status in paediatric malaria in the studied sub-Saharan African population.

Keywords: Malaria, Regulatory T-cells, T-cell activation, Asymptomatic, Symptomatic, Children, *falciparum*, Immunity

*Correspondence: tinafrimp@gmail.com; wndifon@aims.ac.za
[1] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, P. O. Box LG 54, Accra, Ghana
[4] African Institute for Mathematical Sciences, University of Stellenbosch, 7 Melrose Rd, Muizenberg, Cape Town 7945, South Africa
Full list of author information is available at the end of the article

2.0 Characterization of T cell activation and regulation in children with asymptomatic *Plasmodium falciparum* infection

Abstract

Asymptomatic *Plasmodium* infections are characterized by the absence of clinical disease and the ability to restrict parasite replication. Increasing levels of regulatory T cells (Tregs) in *Plasmodium falciparum* infections have been associated with the risk of developing clinical disease, suggesting that individuals with asymptomatic infections may have reduced Treg frequency. However, the relationship between Tregs, cellular activation and parasite control in asymptomatic malaria remains unclear.

In a cross-sectional study, the levels of Tregs and other T cell activation phenotypes were compared using flow cytometry in symptomatic, asymptomatic and uninfected children before and after stimulation with infected red blood cell lysates (iRBCs). In addition, the association between these T cell phenotypes and parasitaemia were investigated.

In children with asymptomatic infections, levels of Tregs and activated T cells were comparable to those in healthy controls but significantly lower than those in symptomatic children. After iRBC stimulation, levels of Tregs remained lower for asymptomatic versus symptomatic children. In contrast, levels of activated T cells were higher for asymptomatic children. Strikingly, the pre-stimulation levels of two T cell activation phenotypes (CD8+CD69+ and CD8+CD25+CD69+) and the post-stimulation levels of two regulatory phenotypes (CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+) were significantly positively correlated with and explained 68% of the individual variation in parasitaemia. A machine-learning model based on levels of these four phenotypes accurately distinguished between

asymptomatic and symptomatic children (sensitivity = 86%, specificity = 94%), suggesting that these phenotypes govern the observed variation in disease status.

Compared to symptomatic *P. falciparum* infections, in children asymptomatic infections are characterized by lower levels of Tregs and activated T cells, which are associated with lower parasitaemia. The results indicate that T cell regulatory and activation phenotypes govern both parasitaemia and disease status in paediatric malaria in the studied sub-Saharan African population.

2.1 Background

Falciparum malaria occurs when sporozoites inoculated into the human host develop in the liver into merozoites that invade red cells and cause clinical disease. The acquisition of natural immunity to falciparum malaria is slow and requires frequent exposure to the parasite over a period of time [120]. Despite previous exposure to the parasite, people in endemic areas may remain susceptible to clinical disease or they may be asymptomatic carriers of parasites as clinical immunity is only partial and never sterile. Also, repeated parasite exposure has been associated with limited protectiveness to vaccine candidates [121, 122]. The lack of a proper understanding of the immune responses occurring during natural infections has, for example, resulted in an inability to develop effective interventions such as vaccines.

Understanding the regulatory and protective immune responses during asymptomatic and clinical infections remains necessary to comprehend mechanisms that enable the control of infections as well as the persistence and survival of the parasite. A number of studies in malaria have associated protection from clinical disease with having a broad antibody repertoire [123-125].

Nonetheless, the presence of asymptomatic infections in children who may not have a broad antibody repertoire suggests that there is some level of immunity to the parasite by the host and this is characterized by the absence of clinical manifestations of the disease. Moreover, during *Plasmodium falciparum* infections, it is believed that the effector function of immune cells will be compromised due to immune regulation [126]. This may be induced by the specific expansion of certain T or B cell sub-sets and modulation of certain antigen presenting cells, such as the dendritic cells [127]. T cells express receptors that enable co-stimulation, activation, memory formation, and immune regulation to ensure effective and timely immune response induction upon antigen recognition. The expansion of specific cell sub-sets, especially those that express regulatory markers, may either enhance or inhibit the development of immunity against an infection. However, the association between such cellular activation and regulatory markers and parasite control during asymptomatic infections is inadequately understood.

Regulatory T cells are unique cell phenotypes that function to maintain homeostasis when the immune response is activated. The establishment of immune homeostasis may result in blocking the activity of other immune cells. For instance, CTLA-4 (also known as CD152), once activated, functions to inhibit activation of both antigen presenting cells and other T cells. Even though the role of Tregs during *P. falciparum* infections remains controversial, it has been observed that in both human and rodent malaria an early induction of Tregs may result in an increased parasite density [94, 128-130]. Furthermore, the expansion of Tregs in malaria has been associated with decreased antigen-specific immune responses [128].

Also, a recent study by Kurup *et al.* [95] has shown that CTLA-4 Tregs expand

during symptomatic malaria in both human and murine models, which is associated with decreased parasite clearance and impedes the acquisition of immunity in murine models. Other studies have also reported the upregulation of TNFRII on Tregs with asymptomatic parasitaemia [131].There have also been reports on the upregulation of FOXP3 mRNA transcripts during acute malaria infections in children and naïve adults, which negatively correlated with Th1 memory responses [94, 132]. Nonetheless, other studies have also shown conflicting data whereby no association was found between the levels of Tregs and *Plasmodium* infection [133-136]. Collectively, these imply that the activity of Tregs associated with the development of protective immunity needs to be comprehended. The likely suggestions are that infections may cause the expansion of Tregs, which in turn may cause immune suppression and enhance parasite growth as observed in other studies [129, 137, 138].

This study aims to compare the expression levels of T cell activation and regulatory markers across symptomatic, asymptomatic and healthy control children living in hyperendemic areas with stable malaria transmission in Ghana. The Treg markers CD25+Foxp3+, the early activation marker CD69, and the late activation marker CD25 were measured. Tregs have an established role in suppressing effector immune responses to a variety of pathogens, including malarial parasites [139, 140]. CD69 expression in CD4+ T cells has been shown to correlate with the development of antigen-specific antibodies in experimental human falciparum malaria [141]. CD69 is a transmembrane

glycoprotein expressed during early activation and increases with inflammation with the potential to induce cytotoxic activity once crosslinked [142-146], whereas CD25 (IL-2α receptor) has been associated with T cell proliferation and differentiation through the IL-2 cytokine [145]. This suggests that their combined expression may lead to an enhanced cellular immune activity. Therefore, it was hypothesized that asymptomatic infections have reduced Treg levels, such that exposure to *P. falciparum* is associated with increased cellular response and lower parasitaemia, which in turn feeds back to reduce cellular activation.

2.2 Methods

2.2.1 Study sites

Participants for the study were recruited from Asutsuare and Paakro sub-districts, which are hyperendemic for malaria transmission. Asutsuare has two malaria transmission seasons; June to August (high transmission season) and November to December (low transmission season) with an entomological inoculation rate of 14.6 infective bites/man/year whereas Paakro has May to June as the high transmission season and September to October as the low transmission season [147, 148]. Samples from participants were obtained during the high transmission seasons.

2.2.2 Participants and sample collection

The study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana (Permit No. 096/15-16). A written informed consent was obtained from parents or guardians and assent appropriately received from the children before they were enrolled. Samples were obtained in a cross-sectional

study from 57 children under 13 years old who satisfied the inclusion criteria with no known conditions that could interfere with the experiments. The participants were grouped into *P. falciparum*-infected asymptomatic children (n=18), symptomatic malaria patients (n=22) and healthy controls (n=17). About 5 ml of venous blood was collected into heparin tubes before anti-malarial treatment. Both thick and thin smears were prepared and stained with Giemsa for parasite identification after screening for infection with rapid diagnostic tests. Haematological indices were determined by an automated haematological analyzer. PBMCs were isolated by ficoll gradient centrifugation and stored in liquid nitrogen until the time of the experiment. The PBMCs were cryopreserved in fetal bovine serum with 10% Dimethyl sulfoxide (DMSO).

2.2.3 Flow cytometry analysis

Stored PBMCs were thawed and washed. The viability was assessed by trypan blue dye exclusion method. Cells with viability greater than 95% were used in the assay. The cells were surface stained with the following antibodies for T cell sub-sets (anti-CD3-, anti-CD4, anti-CD8), co-stimulation markers (anti-CD28) and activation markers (anti-CD25, anti-CD69) (**Table S2.1**). The cells were washed, fixed and permeabilized using FOXP3 buffer set (BD) and intracellularly stained for regulatory markers Foxp3 (Biolegend) and CTLA-4 (BD). Fluorescence minus one controls and compensation were performed to set gates using single colour stained or unstained PBMCs. Data were compensated and analysed using Flowjo V10 software (Tree Star, San Carlos, CA, USA). The gating strategies are outlined in **Figures 1a and 2a**.

2.2.4 Stimulation of PBMCs with infected and uninfected red blood cells

Plasmodium falciparum parasites of the NF54 strain were cultured in O^+ red blood cells at 3% haematocrit in culture medium (RPMI 1640 medium, 25 µg/ml of gentamycin, 10%

heat-inactivated O^+ human serum). The culturing was done in the presence of 7.5% sodium bicarbonate at 37°C in a 5% O_2, 5% CO_2 and 90% N_2 atmosphere. PBMCs were later thawed and rested for 6 hours in 10% fetal bovine serum. About 400,000 cells were later stimulated with intact *P. falciparum* trophozoites/schizont (NF54 clone)-infected RBCs (iRBCs; 3 iRBCs: 1 PBMC) or uninfected RBCs (uRBCs; 3 uRBCs: 1 PBMC) and cultured in complete RPMI 1640 in 5% CO_2 at 37ºC. After 4 hours of stimulation, brefeldin A was added at a concentration of 10 µg/ml. Cells were washed and stained after 18 hours with the following monoclonal antibodies; anti-CD3 (APC-H7), CD4 (BUV 395), CD8 (PerCP-Cy5.5), CD25(PE-CF594), CD69(PE-Cy7), CD152/CTLA-4(APC), FOXP3 (PE) all from BD. The cells were washed by centrifugation before staining for both extracellular and intracellular markers.

2.2.5 Statistical analysis

Data analyses were performed with the GraphPad Prism version 6.01 (GraphPad Software, Inc.) and the R statistical software version 3.4.0 (R Foundation for Statistical Computing). The demographics and clinical characteristics of the study participants were compared among the 3 study groups using Chi-square test for categorical variables, Kruskal-Wallis or One- way ANOVA for continuous variables, Mann-Whitney U test and Wilcoxon-Signed Rank Test for paired comparisons for data that were not normally distributed. For comparing the markers of T cells among the 3 study populations, the Kruskal-Wallis test was used with a Dunn's *post hoc* test or a Bonferroni correction for multiple comparisons where necessary. Spearman's rank correlation was used to determine associations between markers. Support vector machine model, a supervised machine-learning algorithm, was used to predict disease status. Statistical significance was set at P-values less than 0.05.

2.3 Results

2.3.1 Characteristics of the study population

Venous blood samples were obtained from 57 children including 18 with asymptomatic *P. falciparum* infections, 22 with symptomatic malaria, and 17 with no *P. falciparum* parasites detected in blood by microscopy or rapid diagnostic test (**Table 2.1**). There was no statistically significant difference between the ages of children in the asymptomatic *versus* symptomatic groups. In contrast, the healthy controls were significantly older than the asymptomatic (P=0.0404) and symptomatic children (P=0.0123). Also, the mean haemoglobin levels in asymptomatic children were significantly higher compared to the symptomatic children (P=0.0348). However, levels were comparable between the children in the control group and asymptomatic or symptomatic groups. Total leukocyte counts were significantly higher in the asymptomatic children compared to symptomatic children (P=0.0478) but comparable to controls. Even though the median lymphocyte counts did not differ significantly amongst the groups, lower levels were found in the symptomatic group than in the asymptomatic and control groups. Also, platelet levels decreased with the severity of *P. falciparum* infections. The median platelet counts in the symptomatic children were significantly lower than in the asymptomatic (P=0.0029) and control (P<0.0001) groups. Children in the asymptomatic group had statistically similar platelet counts as the control group. Parasitaemia levels were significantly lower in asymptomatic children compared to children with symptomatic infection (P=0.0009).

Table 2.1 Demographics and clinical characteristics of the study participants

Characteristics	Control	Asymptomatic	Symptomatic	P values
Participants	n=17 9(8-11)	n=18 7(4.5-9)	n=22 6(4.8-7)	
Age (IQR), years				0.0087 [a]
Female/Male (%)	52.94/47.06	44.44/55.56	50/50	0.8765[b]
Mean hemoglobin (IQR), g/dl	11.5(10.8-12.1)	12.7(11.7-13.58)	10.7(8.8-13.1)	0.0402[c]
Parasitaemia (IQR), µl	NA	845(260.7-3812)	13973(7238-58764)	0.0009 [d]
Leukocytes (10^9/L)	7(5.7-8.0)	7.7(6.1-9.6)	5.1(1.2-8.3)	0.0436 [a]
Lymphocytes (10^6/L)	2.9(2.5-3.6)	2.1(1.2-3.45)	1.9(1.3-3.9)	0.0889 [a]
Platelets (10^9/L)	305(237-356)	223(193-280)	101(61-198)	
		>0.0001[a]		

IQR interquartile range,

[a] Kruskal Wallis test

[b] Chi-square test

[c] One-way ANOVA

[d] Mann-Whitney U test NA Not Applicable

2.3.2 Decreased levels of regulatory T cells in asymptomatic Plasmodium falciparum infections

To investigate if there are any differences between the *P. falciparum*-infected group and healthy controls with respect to T cell regulation, the levels of Treg populations in the 3 study groups were determined. Here, Treg populations were classified as CD3+, CD4+, CD25+ and FoxP3+ (**Figure 2.1a**).

For the Peripheral blood mononuclear cells (PBMCs) analysed directly without stimulation (*ex-vivo*), it was observed that Tregs had a lower frequency in the asymptomatic children compared to the symptomatic children (P=0.0065), but levels were comparable between asymptomatic and control groups (P>0.05). Also, the Treg frequency in the symptomatic children was higher than in the controls (P=0.0209, **Figure 2.1b**). This

trend remained the same after the cells were stimulated with iRBCs *in vitro*; lower levels of Tregs were found in the asymptomatic children than in the symptomatic children (P=0.0394), while levels were comparable to the controls. Similarly, levels in the symptomatic children were higher than in the controls (P=0.0391, **Figure 2.1b**).

Furthermore, the levels of activated Tregs based on the expression of CTLA4, an immunosuppressive marker that inhibits activation of immune cells by direct contact, were determined. The levels of CTLA4+ Tregs differed significantly across the study populations (P=0.0017). The levels of CTLA4+ Tregs in children with asymptomatic malaria were significantly lower than in those with clinical malaria (P=0.0174, **Figure. 2.1C**) but comparable with the control group. However, levels of CTLA4+ Tregs in the symptomatic group were significantly higher than in the control group (P=0.0034). In addition, after iRBC stimulation, CTLA-4+ Treg levels remained significantly lower in the asymptomatic group compared to the symptomatic group (P=0.0493) but were comparable to levels observed in healthy controls (P=0.5457, **Figure. 2.1C**).

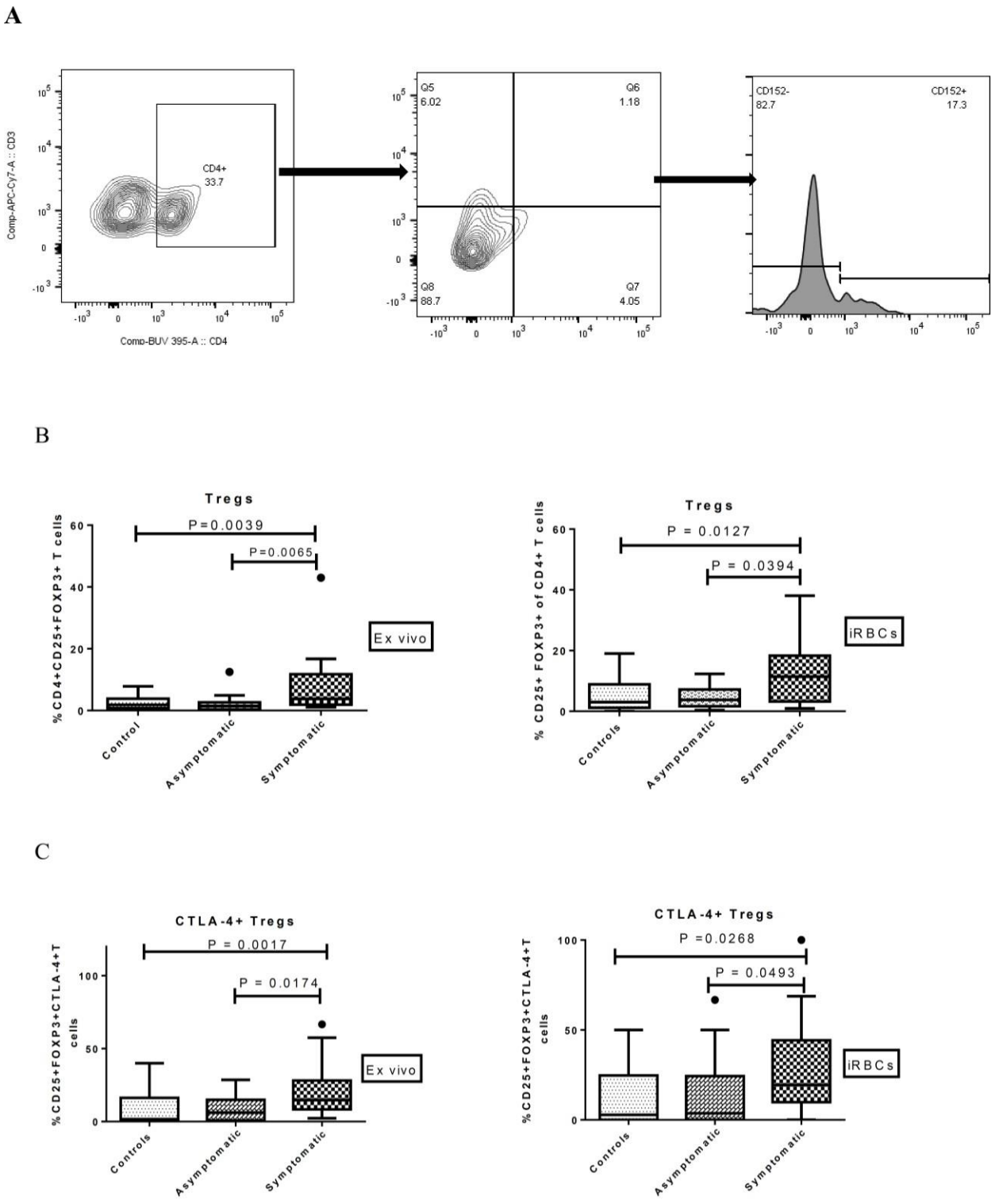


Figure 2.1. Percentage expression of CD4+CD25+FOXP3+ regulatory T cells among study groups, (A) Representative flow cytometry gating strategy for phenotyping CD4+CD25+FOXP3+ and CTLA-4+ Tregs in peripheral blood. The percentage expression

and activation of regulatory T cells were analysed in healthy controls (n=16), children with asymptomatic infections (n=18) and symptomatic falciparum malaria (n=22); for levels of (B) Tregs analysed as CD4+CD25+FOXP3+; and, (C) activated Tregs analysed as CD4+CD25+FOXP3+CTLA-4+ T cells both *ex vivo* and after iRBC stimulation. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are denoted by whiskers. Medians are indicated by the horizontal lines across the boxes. Kruskal-Wallis test was used for comparisons, followed by Dunn's test where necessary

2.3.3 Decreased expression of CD69 activation marker on T cells in asymptomatic Plasmodium falciparum infections before infected red blood cell stimulation

With the observed levels of Tregs being lower in the asymptomatic children compared to symptomatic children, the extent of cellular activation was measured to determine if they may differ across the study groups. The expression of the CD69 activation marker on both CD4+ and CD8+ T cell sub-sets before *in vitro* stimulation was investigated (**Figure 2.2a**). Children with asymptomatic malaria had significantly lower levels of CD69+ expression on CD4+ T cells compared to children with symptomatic disease (P=0.0016) but had comparable levels with the controls (P >0.05, **Figure 2.2b**). In addition, children with symptomatic malaria had a higher level of the CD4+CD69+ T cells than the controls (P=0.0068, **Figure 2.2b**). This trend was the same for the CD8+CD69+ T cells.

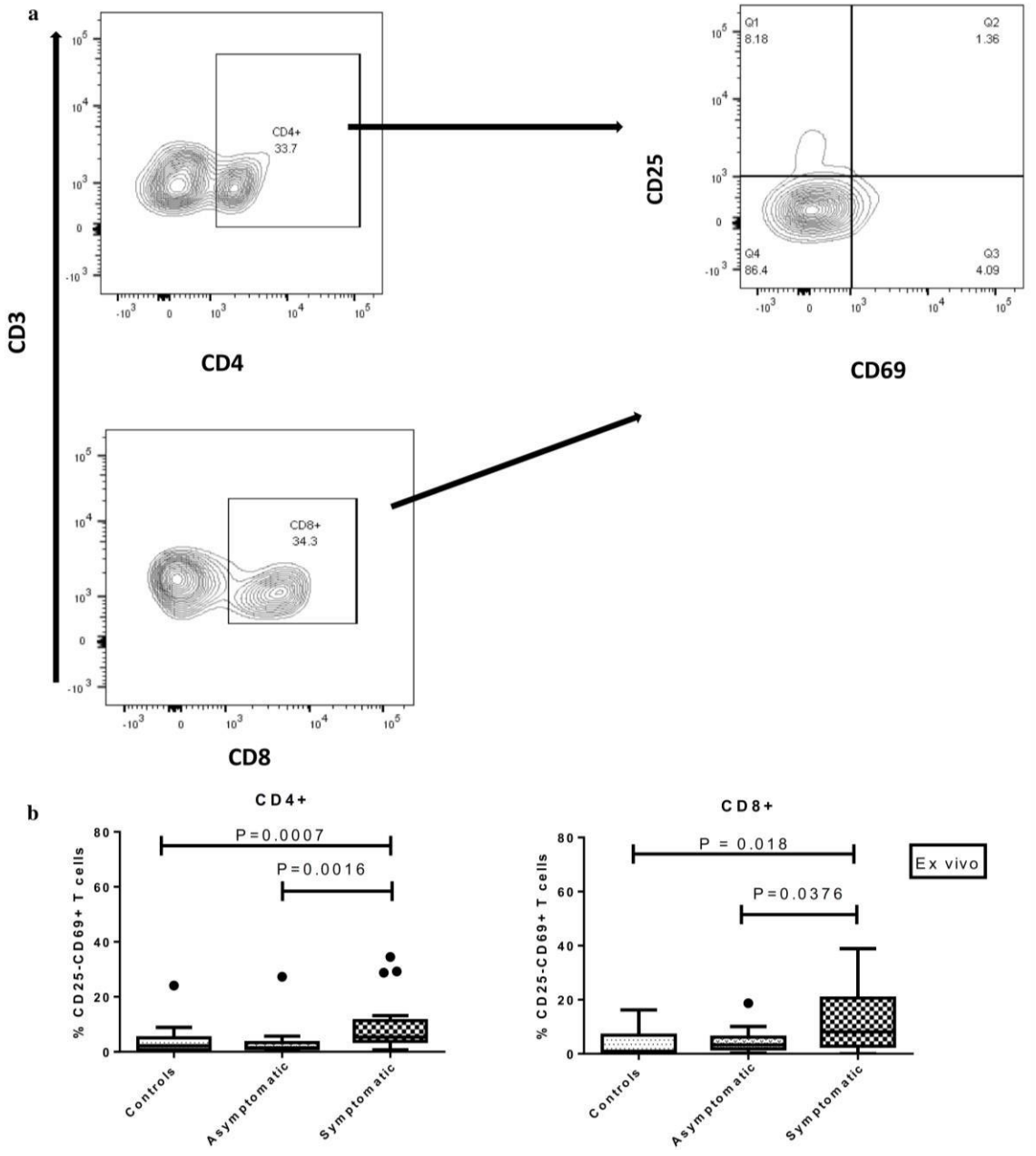


Figure 2.2. Expression of T-cell activation markers CD25/CD69 on T cells in PBMCs from the study cohort. (a) Representative flow cytometry gating strategy for phenotyping activation markers on CD4+ and CD8+ T cells ex vivo; the expression of the activation markers (**b)** CD25−CD69+ on T cells was analysed in healthy controls (n = 17), asymptomatic *P. falciparum*-infected children (n = 18), and symptomatic *P. falciparum*-infected children (n = 21). The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers. Medians are indicated by the horizontal lines across the boxes. Kruskal–Wallis test was used for comparisons, followed by Dunn's test where necessary

2.3.4 The pattern of expression of CD25 on T cells among symptomatic, asymptomatic Plasmodium falciparum infections and healthy controls before infected red blood cell stimulation

The expression levels of CD25, a late activation marker, on CD4+ and CD8+ T cells were determined (**Figure 2.2A**). Except for CD8+CD25+CD69- T cells, levels of CD25 were not significantly different between asymptomatic and symptomatic children. There was no significant difference in the expression of CD25 on CD4+ T cells across the study populations (P=0.4971, **Figure 2.3a**). However, for CD8+ T cells, the expression of CD25 was significantly lower in the asymptomatic group compared to the symptomatic children (P=0.0257), whereas levels between the asymptomatic and control groups were comparable (**Figure 2.3a**).

CD25+FOXP3- T cells have been classified as activated effector Th 1 cells capable of secreting effector cytokines, such as IFNγ (interferon gamma), TNF (tumour necrosis factor) and IL-10 (interleukin-10). Therefore, the *ex vivo* expression of these markers was compared across the study groups. No significant difference was observed in the expression of CD25+FOXP3- on either CD4+ or CD8+ T cell sub-sets between asymptomatic and symptomatic children. However, the levels of CD25+FOXP3- on CD4+ T cells were significantly lower in the healthy controls when compared to the asymptomatic (P=0.0063) or symptomatic children (P=0.0024). In addition, no significant difference was observed in the expression of CD8+CD25+FOXP3- T cells across the study groups. (P=0.1192, **Figure 2.3b**).

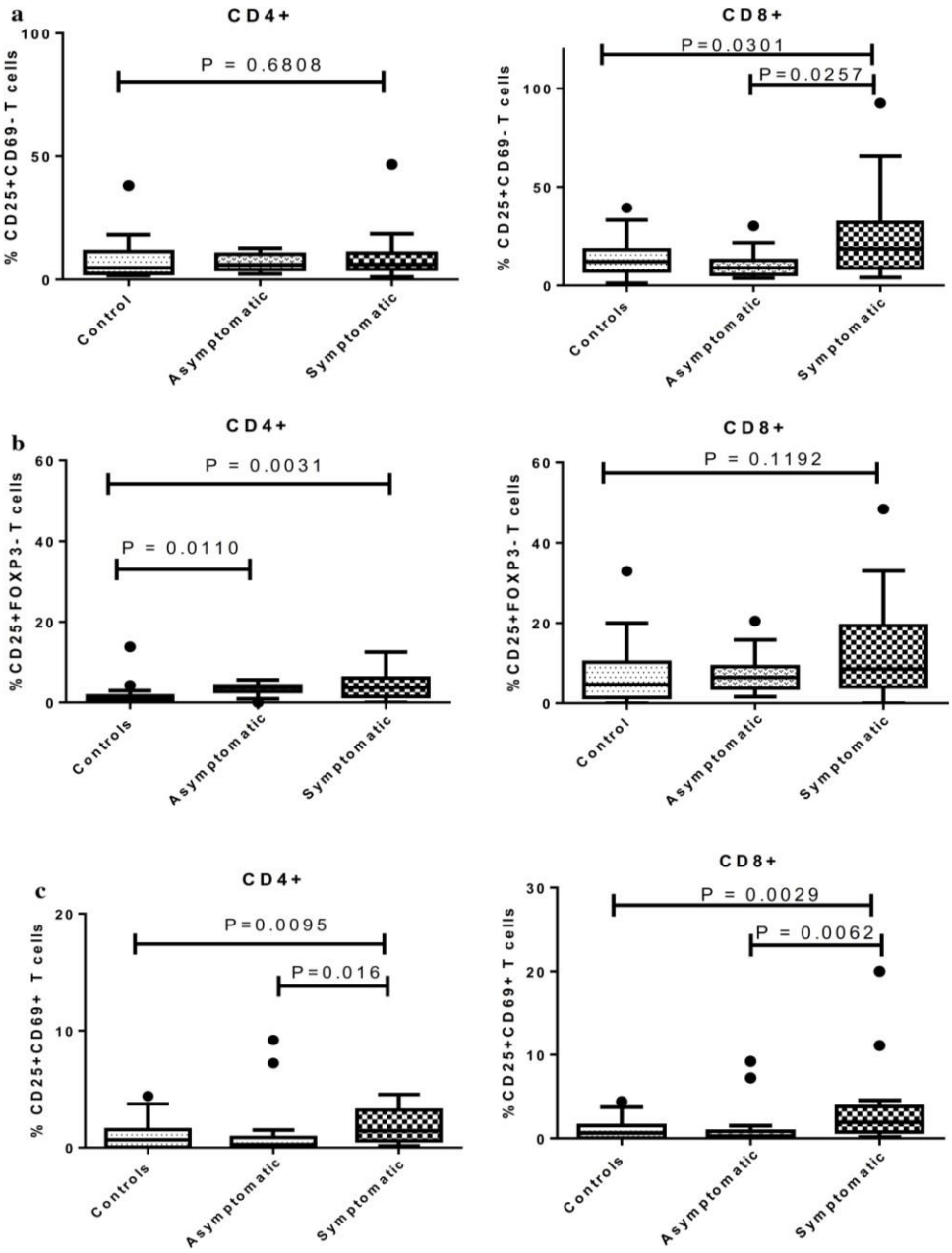


Figure 2.3. Expression of T-cell activation markers CD25/CD69 on T cells in PBMCs from the study cohort. a CD25+CD69−; **b** CD25+FOXP3− cells; and, **c** CD25+CD69+ on T cells was analyzed in healthy controls (n = 17), asymptomatic *P. falciparum*-infected children (n = 18), and symptomatic *P. falciparum*-infected children (n = 21). The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers. Medians are indicated by the horizontal lines across the boxes. Kruskal–Wallis test was used for comparisons, followed by Dunn's test where necessary

2.3.5 Decreased expression of CD25+CD69+ T cell activation markers during asymptomatic Plasmodium falciparum infections before infected red blood cell stimulation

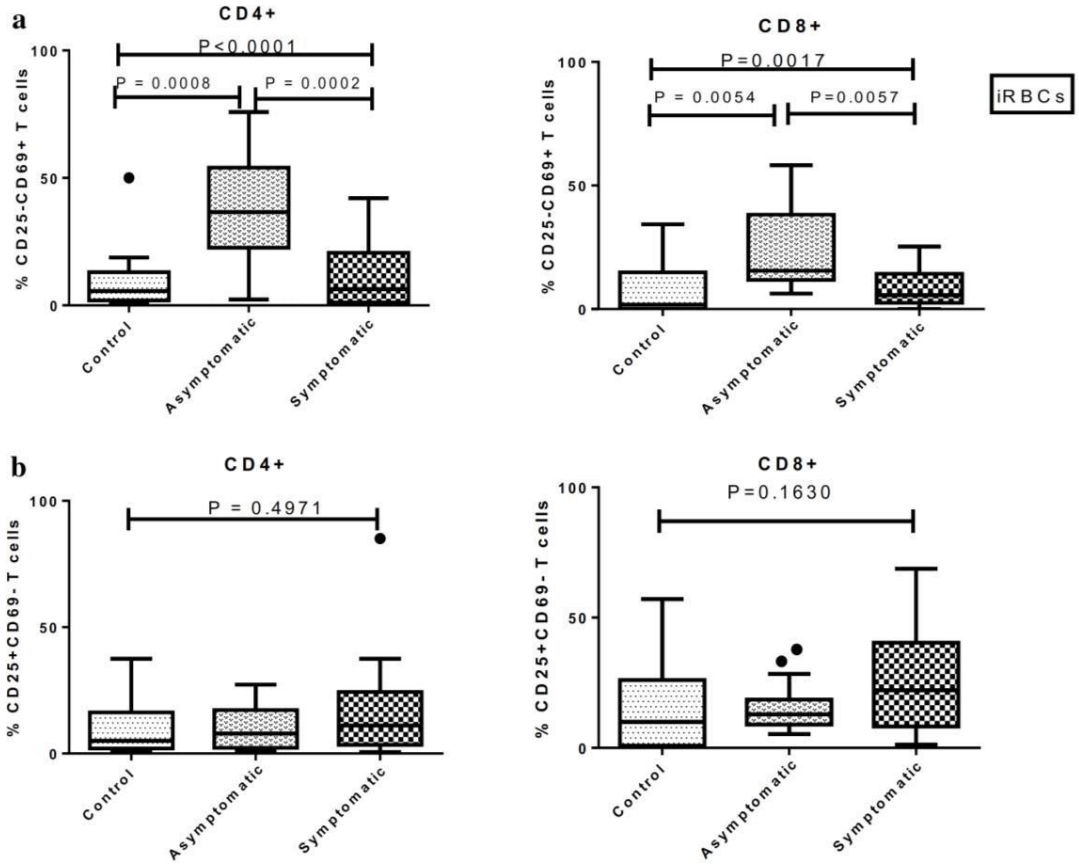
The expression of CD69 activation marker on both CD4+ and CD8+ T cell subsets before *in vitro* stimulation was investigated in the three study groups (**Figure 2.2a**). For the PBMCs analysed *ex vivo*, CD4+ and CD8+ T cells expressing both CD25 and CD69 were higher in the symptomatic when compared to the asymptomatic group (P=0.016 and P=0.0062) and healthy controls (P=0.047 and P=0.0232), respectively (**Figure 2.3c**). However, no significant difference was observed between the asymptomatic group and controls (P>0.05) for both T cell sub-sets (**Figure 2.3c**).

2.3.6 Increased expression of activation marker on T cells in asymptomatic infections after infected red blood cell stimulation

When PBMCs were stimulated *in vitro* with iRBCs, levels of activated CD4+ CD69+ T cells in the asymptomatic children increased significantly above those found in the symptomatic children (P=0.0002) and controls (P=0.0008**, Figure 2.4a**). Levels of CD4+CD69+ T cells did not differ significantly between symptomatic children and controls. Higher expression of CD8+ CD69+ cells was also observed in the asymptomatic cohort compared to both symptomatic children (P=0.0057) and controls (P=0.0054) (**Figure 2.4a**). As was observed in the CD4+ T cell compartment, levels of CD8+CD69+ T cells did not differ significantly between symptomatic children and controls (**Figure 2.4a**).

Also, no significant difference was found in the expression of CD25+CD69- T cells on any of the T cell sub-sets across the study groups (**Figure 2.4b**). Levels of

CD25+FOXP3- activated effector T cells were increased in the asymptomatic children compared to symptomatic and healthy controls (**Figure 2.4c**). Increased levels of the CD4+CD25+FOXP3- activation marker was found in the asymptomatic group compared to the symptomatic (P=0.035) and control (P=0.0007) groups. Likewise, CD8+CD25+FOXP3- activated T cells were significantly increased in the asymptomatic group compared to the symptomatic (P=0.0208) and control (P <0.0001) groups (**Figure 2.4c**). In addition, a significant increase in levels of double-positive CD4+CD25+CD69+ T cells were observed in the asymptomatic children compared to the symptomatic children (P=0.035) and controls (P=0.0025**, Figure 2.4d**). Likewise, higher expression of CD8+CD25+CD69+ cells was observed in the asymptomatic cohort when compared to the healthy controls (P=0.023) but not the symptomatic children (P >0.05, **Figure 2.4d**).



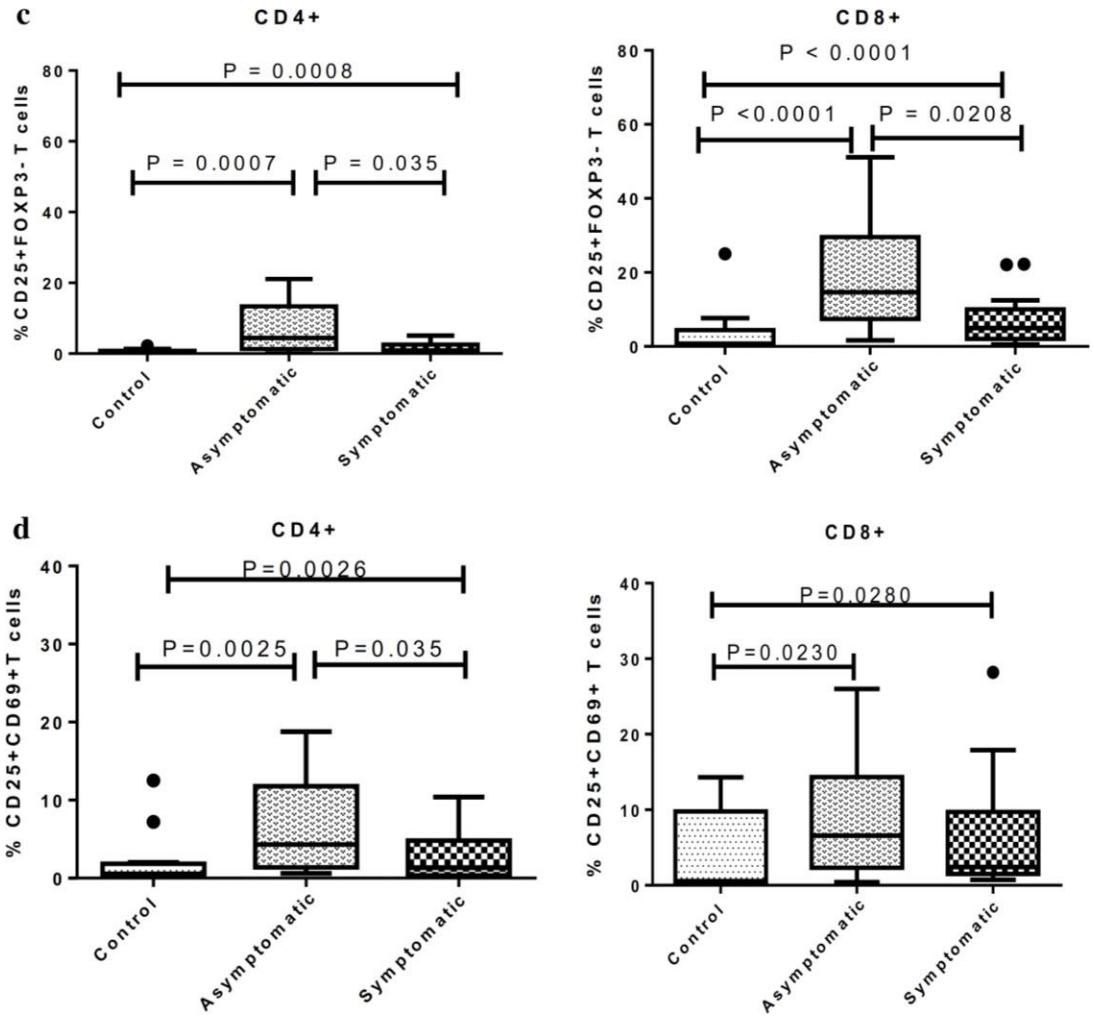


Figure 2.4 Expression of activation markers CD25/CD69 on T-cells from the study cohorts after iRBC stimulation. PBMCs were stimulated with iRBC lysates (iRBCs) to determine the levels of activation markers (CD25/CD69) on both CD4+ and CD8+ T cell sub-sets. The percentage expression of (**a)** CD25−CD69+; (**b)** CD25+CD69−; (**c)** CD25+FOXP3−; and, (**d)** CD25+CD69+ T cells was analysed in healthy controls (n = 17), asymptomatic *P. falciparum*-infected children (n = 18) and symptomatic *P. falciparum*-infected children (n = 21). The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles presented as whiskers. Medians are indicated by the horizontal lines across the boxes. The Kruskal–Wallis test was used for statistical comparisons between groups. P values < 0.05 were considered significant after Dunn's test to correct for multiple comparisons

2.3.7 Cellular activation and Treg frequency govern parasitaemia and disease status

Correlations between the 24 considered T cell phenotypes (including the pre- and post-iRBC stimulation levels of Tregs) and parasite control (as measured by parasitaemia levels) were investigated. After applying a Bonferroni correction for multiple comparisons, significant positive correlations were found between parasitaemia and the levels of both CD8+CD69+ (r=0.4128658, P=0.0016) and CD8+CD25+CD69+ (*r*=0.4070214, *P*=0.0018) T cells measured before iRBC stimulation, and the levels of both CD4+CD25+Foxp3+ (*r*=0.4772815, *P*=0.0002) and CD8+CD25+Foxp3+ (*r*=0.4772714, *P*=0.0003) T cells measured after stimulation. Strikingly, levels of these four T cell phenotypes together accounted for 68% of the variation in parasitaemia observed in asymptomatic and symptomatic children (Appendix 1; **Figure S2.1** and **S2.2**).

Machine learning was used to determine whether the levels of these four T cell phenotypes alone could be used to predict disease status in infected children. A model based on a support vector machine was fitted to the levels of the four T cell phenotypes measured in a sub-set of the infected children and then used to predict disease severity in the remaining children. Using a 5-fold cross-validation analysis to prevent overfitting, it was found that the model accurately distinguishes between asymptomatic and symptomatic children, with a sensitivity of 86%, a specificity of 94%, and an area under the receiver-operator-characteristic curve (AUC) of 90% (**Figure 2.5**). Together, the results suggest that expression levels of the considered regulatory and activation markers determine most of the individual variation in parasitaemia and predict disease status in asymptomatic and symptomatic *P. falciparum* infections.

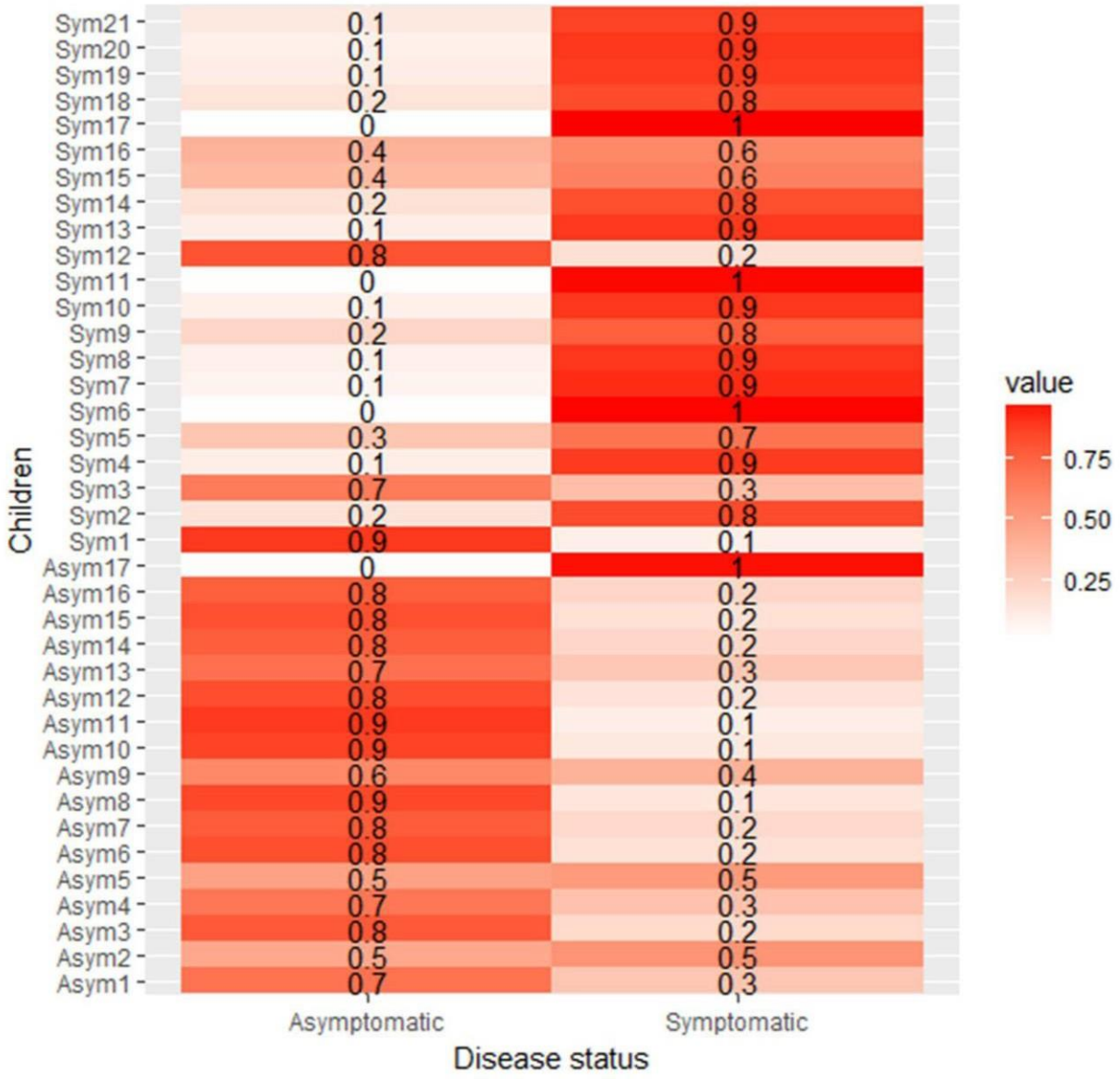


Figure 2.5 T cell regulatory and activation markers distinguish between asymptomatic and symptomatic *Plasmodium falciparum* infections. It was assessed whether a machine-learning model based on pre-iRBC stimulation levels of CD8+CD69+ and CD8+CD25+CD69+ T cells and post-stimulation levels of CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+ T cells could accurately predict disease status in asymptomatic and symptomatic children. Thirty-eight children had data for all the 4 T cell phenotypes considered. The children were randomly separated into 5 groups. Fixing one group as a test group, we trained a machine-learning model (precisely a support vector machine) on the other 4 groups and then predicted the disease status of children found in the test

group. The process was repeated until each of the groups was used exactly once as a test group. The plot shows a representative heatmap of the predicted probability that each child is either asymptomatic or symptomatic. Strikingly, as expected, most asymptomatic (respectively symptomatic) children have a higher predicted probability of being asymptomatic (respectively symptomatic)

2.4 Discussion

The aim of this study was to investigate the frequency of activated Tregs and T cell early and late activation markers during *P. falciparum* infections, and the correlations between these and levels of parasitaemia. It was observed that asymptomatic infections are associated with lower levels of Tregs with reduced Treg activation, and reduced expression of T cell activation markers compared to symptomatic infections. Also, T cells from asymptomatic *P. falciparum*-infected children were more responsive to iRBC stimulation compared to cells from symptomatic infections. Importantly, the measured variations in regulatory and activation marker levels explained most (68%) of the variation in parasitaemia observed in asymptomatic and symptomatic infections. These results indicate that in contrast to children with symptomatic malaria, there seems to be appropriate levels of immune regulation and activation in children with asymptomatic malaria, which favor the control of parasitaemia. Another, non-mutually exclusive possibility not ruled out by the analyses is that asymptomatic children might have higher levels of protective antibodies compared to symptomatic children, which might contribute to the observed differences in parasitaemia.

Previous data have shown that malaria-exposed individuals can harbour infection without clinical symptoms, implying that there is some level of immune restriction on parasite replication [124]. Increased levels of Tregs have been associated with higher

parasitaemia [128, 129] and delayed parasite clearance [137] as well as the development of clinical disease [132, 149, 150]. In this study, it was found that the level of Tregs is higher in children with clinical malaria compared to children with asymptomatic infections and healthy controls. This supports findings from other studies which have also associated increased Treg frequencies with symptomatic malaria infections [94, 140]. Also, the significant increase in the Treg frequency which was observed in the symptomatic children after iRBC stimulation may indicate that during clinical malaria Tregs from the pre-clinical state are expanded or being induced.

The low levels of Tregs observed in the asymptomatic children corroborates the findings of Boyle *et al*. [149] who identified lower levels of Tregs in children with asymptomatic infections. This was interesting since in another previous study by Jangpatarapongsa *et al*. [126] they also identified lower amounts of Treg cytokines in individuals with asymptomatic *Plasmodium vivax* infections, suggesting there is less Treg activation in individuals with asymptomatic *Plasmodium* infections. This supports the view that lower levels of Tregs may be associated with a decreased risk of developing clinical disease and possibly an increased likelihood of developing immunity to malaria.

It has recently been shown that Tregs expressing CTLA-4 in murine models of malaria interfere with the acquisition of long-term immunity to malaria infections [95]. The increase in CTLA-4 in Tregs observed in individuals with *P. falciparum* infections compared to uninfected controls could reflect their direct role in controlling immune responses during human malaria infections. Also, the increased expression of CTLA-4 on Tregs in the symptomatic children suggests that immune regulation associated with clinical

malaria may affect cellular activation, consequently, affecting the downstream development of anti-malaria immunity.

Importantly, persistent immune activation has been described as a major factor in predicting disease with increased levels of activation being associated with clinical disease progression [151-153]. Resting T cells are identified phenotypically by the absence of CD25/CD69 markers [154]. In this study, activated T cells were classified by the expression of CD25+/CD69+ markers. A significant increase in immune activation in the CD4 and CD8 T cells was observed in clinical malaria. This is in line with a previous study that observed increased immune activation during clinical malaria infections [155]. However, it should be noted that the increased activation in symptomatic children may not directly connote an effective T cell response since cytokine profiles were not measured.

In contrast, in the asymptomatic children, it was found that fewer cells expressed both activation markers on either CD4 or CD8 T cells indicating reduced cellular activation when compared to children with symptomatic malaria. A plausible interpretation of these results is that lower immune suppression by Tregs in asymptomatic children leads to more effector T cell activation and greater parasite control, which in turn feeds back to reduce T cell activation. Conversely, higher immune suppression in symptomatic children might limit parasite control leading to higher levels of parasitaemia and T cell activation. This is in line with the observation that *P. falciparum* activates the immune system in a dose-dependent manner [156]. In addition, the lack of symptoms, lower levels of immune activation, and lower parasitaemia in the asymptomatic children might also result from higher levels of parasite-specific antibodies that reduce parasitaemia levels below the

threshold required to induce a T cell response. Additional research is needed to elucidate these hypotheses.

It has been shown that asymptomatic children maintain levels of CD4+CD25+FOXP3- effector T cells that co-produce IFNγ, TNFα and IL-10, describing these cells as self-regulatory [93, 94]. Even though lower levels of Tregs and activated Tregs were observed in the asymptomatic children, CD4+CD25+FOXP3- T cells were not significantly different between the asymptomatic and symptomatic children. This may suggest that during asymptomatic malaria, restriction of parasite replication and inflammation may be mediated by these self-regulating effector T cells.

Unfortunately, this study had a number of limitations since a longitudinal study could not be conducted to determine if any of the asymptomatic cohorts may develop clinical disease since they were treated when diagnosed. Consequently, the possibility that the immune dynamics observed reflect changes that occur during the natural course of *P. falciparum* infections could not be ruled out. In addition, because parasitaemia were determined by microscopy, it was not possible to determine conclusively that none of the healthy cohort had sub-microscopic infection. It was also not possible to evaluate the humoral response in the study population to determine its contribution to the immune dynamics observed.

2.5 Conclusion

The study shows evidence that Tregs are lower and associated with reduced Treg activation in children with asymptomatic *P. falciparum* infections, which corresponds to reduce cellular activation and lower levels of parasitaemia. Also, the greater expansion of

activation markers after iRBC stimulation in asymptomatic children compared to symptomatic children suggests that the former children harbour a larger latent repertoire of parasite-responsive T cells. Alternatively, this observation could reflect less Treg-mediated suppression of T cell activation in cells from asymptomatic children. Together, these data support the view that the dynamics of T cell regulation and activation may contribute to the acquisition of anti-parasite and/or anti-disease immunity to malaria. Insights into these dynamics might inform the development of malaria vaccines that induce appropriate levels of cellular activation and regulation as well as optimal control of parasitaemia and disease.

Chapter Three

frontiers in Immunology

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Phenotypic Evidence of T Cell Exhaustion and Senescence During Symptomatic *Plasmodium falciparum* Malaria

Augustina Frimpong[1,2,3]*, *Kwadwo Asamoah Kusi*[1,2], *Dennis Adu-Gyasi*[4], *Jones Amponsah*[2], *Michael Fokuo Ofori*[1,2] and *Wilfred Ndifon*[3,5]*

[1] Department of Biochemistry, Cell, and Molecular Biology, West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, Ghana, [2] Immunology Department, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Accra, Ghana, [3] African Institute for Mathematical Sciences, Cape Coast, Ghana, [4] Kintampo Health Research Centre, Kintampo, Ghana, [5] African Institute for Mathematical Sciences, University of Stellenbosch, Cape Town, South Africa

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***Correspondence:**
Augustina Frimpong
tinafrimp@gmail.com
Wilfred Ndifon
wndifon@aims.ac.za

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T cells play significant roles during *Plasmodium falciparum* infections. Their regulation of the immune response in symptomatic children with malaria has been deemed necessary to prevent immune associated pathology. In this study, we phenotypically characterized the expression of T cell inhibitory(PD-1, CTLA-4) and senescent markers (CD28(-), CD57) from children with symptomatic malaria, asymptomatic malaria and healthy controls using flow cytometry. We observed increased expression of T cell exhaustion and senescence markers in the symptomatic children compared to the asymptomatic and healthy controls. T cell senescence markers were more highly expressed on CD8 T cells than on CD4 T cells. Asymptomatically infected children had comparable levels of these markers with healthy controls except for CD8+ PD-1+ T cells which were significantly elevated in the asymptomatic children. Also, using multivariate regression analysis, CTLA-4 was the only marker that could predict parasitaemia level. The results suggest that the upregulation of immune exhaustion and senescence markers during symptomatic malaria may affect the effector function of T cells leading to inefficient clearance of parasites, hence the inability to develop sterile immunity to malaria.

Keywords: malaria, *Plasmodium falciparum*, T-cell, exhaustion, immune senescence, PD-1, CTLA-4, CD57

3.0 Phenotypic evidence of T cell exhaustion and senescence during symptomatic *Plasmodium falciparum* malaria

Abstract

T cells play significant roles during *P. falciparum* infections. Their regulation of the immune response in symptomatic children with malaria has been deemed necessary to prevent immune-associated pathology. In this study, we phenotypically characterized the expression of T cell inhibitory (PD-1, CTLA-4) and senescent markers (CD28(-), CD57) from children with symptomatic malaria, asymptomatic malaria and healthy controls using flow cytometry.

We observed increased expression of T cell exhaustion and senescence markers in the symptomatic children compared to the asymptomatic and healthy controls. T cell senescence markers were more highly expressed on CD8 T cells than on CD4 T cells. Asymptomatically infected children had comparable levels of these markers with healthy controls except for CD8+ PD-1+ T cells whose levels were significantly elevated in the asymptomatic children. Also, using multivariate regression analysis, CTLA-4 was the only marker that could predict parasitaemia load. The results suggest that the upregulation of immune exhaustion and senescence markers during symptomatic malaria may affect the effector function of T cells leading to inefficient clearance of parasites, hence the inability to develop sterile immunity to malaria.

3.1 Background

Clinical malaria is a disease of public health importance due to its associated morbidity and mortality [157]. With the emergence of drug-resistant parasites and insecticide-resistant vectors, there is a need to develop effective interventions [158-160]. Despite promising results of candidate vaccines in naïve individuals, comparatively poorer responses are observed in people in endemic areas [121, 122], indicating that much effort needs to be focused on understanding host factors associated with the development of immunity, especially in malaria-endemic areas. Blood stage infection with malaria parasites may either be asymptomatic, result in uncomplicated malaria, or proceed to complications such as severe malaria anemia or cerebral malaria. Repeated exposure to parasites usually results in the acquisition of anti-disease immunity which is characterized by the absence of clinical symptoms, yet with susceptibility to the infection. This suggests that the naturally induced immune response generated against *P. falciparum* may not always be potent enough to eradicate the infection. Therefore, malaria vaccines that can protect against symptomatic disease and possibly also eliminate infections remain a global health priority.

Lymphocytes, including T cells, play a significant role in the generation of protective malaria-specific responses [92], and their mechanism of action may either be by controlling or decreasing parasitemia [161] or by exacerbating the infection promoting parasitemia [129]. However, looking at natural infections it can be presumed that the inability to eliminate *P. falciparum* malaria may be associated with immune dysfunction resulting from the expression of markers that negatively regulate T cell activity or result in their ineffective response. These may lead to the exhaustion of T cells, which has been well

described in viral infections including HIV and hepatitis B (HBV) [47, 48] as well as in protozoan infections like Toxoplasmosis and Leishmania [162, 163].

In malaria, work in both human and murine models has reported the upregulation of immune inhibitory markers such as T-cell immunoglobin and mucin domain-3 (TIM-3), lymphocyte-activation gene-3 (LAG-3) and programmed cell death-1 (PD-1) during acute infections [164, 165]. These have been shown to affect not only the effector functions of T cells including cell proliferation and cytokine production but also antibody generation by B cells [166]. Specifically, PD-1 has been associated with decreased cytokine production and proliferation in T cells as well as enhancing disease progression, whereas CTLA-4 has been associated with T cell anergy and establishment of immunological tolerance [167, 168]. Furthermore, it has been shown that the dysfunctional nature of exhausted T cells in murine models of malaria can be reversed by blockage of these receptors as this enhances effective parasite clearance and acquisition of immunity [95, 166].

In addition to immune exhaustion, infectious pathogens such as Cytomegalovirus (CMV) and Human Immune deficiency virus (HIV) have been associated with accelerated ageing of the body's immune defense system through the upregulation of CD57, a classical marker for immune senescence [169, 170]. CD57 is a terminally differentiated marker found on some cell subsets including T cells [171-173]. Naïve T cells express CD28, a co-stimulatory molecule that provides signaling for T cell activation)after antigen recognition and this may bind to B7 proteins to provide co-stimulatory signals[174, 175]. However, repeated T cell activation is associated with the progressive loss of CD28, a characteristic of memory or terminally differentiated cells, and the corresponding upregulation of CD57 [176-178]. These senescent cells are characterized by shortened telomeres, replicative

senescence, loss of CD27 resulting in a low proliferative capacity of the cells [179], eventually, leading to an inability to eradicate an infection. Importantly, the expression of CD57 is associated with repeated antigen stimulation[180] which was identified to accurately predict replicative senescence[171]. In addition, CD57 expression on CD28- T cells have been shown to differ from the normal ageing T cell phenotypes (CD28-CD57+, similarly observed in CMV)[180, 181] found in HIV infections [182].

Cellular aging has been described in wild birds chronically infected with malaria [183]. Interestingly, a recent study reported evidence of cellular aging in travelers with single acute *P. falciparum* infections, characterized by decreased telomerase activity and increased levels of CDKN2A, a molecular marker associated with cellular aging [184]. Nevertheless, it remains to be elucidated if frequent exposure to malaria is associated with increased expression of markers of T cell senescence in endemic areas. Here, we determined the expression profile of inhibitory or exhaustive, and immune-senescence markers on both CD4+ and CD8+ T cells. We characterized the expression of PD-1, CTLA-4, CD28 and CD57 markers in children with symptomatic malaria, asymptomatic malaria and healthy controls. In addition, we also determined the impact of these T cell phenotypes on parasitaemia and inflammation (using the platelet-to-lymphocyte ratio).

3.2 Materials and methods

3.2.1 Ethics statement

The study protocols were approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research at the University of Ghana. All participants were

children. Children were enrolled when they gave assent after parents or guardians approved and signed the informed consent forms. All methods were performed in accordance with the relevant guidelines and regulations

3.2.2 Study subjects

A total of 57 children within the age ranges of 1–12 years were recruited for the study, consisting of healthy children with no P. falciparum infections (n = 17), children with asymptomatic P. falciparum malaria (n = 18) and children with clinical malaria (n = 22) who were recruited from the Asutsuare and the Paakro sub-districts which are hyper-endemic areas for malaria transmission in Ghana. A volume of 5ml of venous blood was collected from all study participants after recruitment. Parasites were identified using Giemsa stained thick and thin blood films. Clinical cases of malaria recruited from the health centers were defined by a history of fever within 24 hours of health center attendance and presence of parasitaemia. For clinical cases, we collected venous blood samples from the children before anti-malarial treatment, based on the nationally recommended guidelines. Asymptomatic cases were recruited from the community and were defined by the presence of parasitaemia, absence of fever and no signs or symptoms of the disease. Healthy children, also recruited from the community were selected based on the absence of parasitaemia, fever and no signs or symptoms of the disease.

3.2.3 Peripheral Blood Mononuclear Cells (PBMC) and Plasma Isolation

Isolation of PBMCs was performed by density gradient centrifugation using ficoll paque. After isolation, PBMCs were enumerated and cryopreserved with fetal bovine serum containing 10% DMSO. PBMCs were kept at -80^{o}C overnight and subsequently transferred

to liquid nitrogen until required for the experiment.

3.2.4 Flow cytometry analysis

The cryopreserved cells were retrieved, thawed and washed. The viability of the PBMCs was measured using the trypan blue dye exclusion method. Cells having a viability ≥95% were used for the experiment. After cells were washed, they were stained for extracellular markers using: anti-CD3-(APC H7), anti-CD4 (BUV 395), anti-CD8 (PerCP Cy5.5), anti-CD28 (APCR700), anti-CD57 (FITC) and anti-PD-1 (BUV737) antibodies on ice for 30 minutes. The cells were later washed, and intracellularly stained for FOXP3 (PE) and CTLA-4 (APC) on ice for 40 minutes after fixation and permeabilization using FOXP3 buffer set (BD) according to manufacturer's instructions. All the antibodies were purchased from BD except anti-human FOXP3 fluorochrome-conjugated antibody (Biolegend). We gated for T cells by CD3, CD4 and CD8 lineage markers. Gates for inhibitory and senescence markers were defined using fluorescence minus one controls (**Figure S3.1**). Cells were acquired on a BD LSR Fortessa II-X20 cytometer. Data was compensated and analyzed using FlowJo V10 software (Tree Star, San Carlos, CA).

3.2.5 Statistical analysis

Data analyses were performed with R-studio for statistical analysis (version 2) and the GraphPad Prism version 6.01 (GraphPad Software, Inc.). For comparing the markers of T cells among the three study populations, the Kruskal-Wallis test with a Dunn's post hoc test for multiple comparisons was used. Spearman's rank correlation was used to determine associations between markers. Principal component analysis (PCA) was conducted to identify and visualize significant features of T cell phenotypes (degree of variation) that

can cluster our study populations by considering all phenotypes measured. PCA which is an unsupervised learning algorithm provides dimensions (linear combinations) along which the data are separable and reduces the noise associated with data whilst increasing its robustness. PCA was used since it reduces the data set to a small set of patterns and retain the significant features that are responsible for variation (separating the data into clusters). Multiple linear regression models with likelihood ratio test were also used to investigate the association between parasitaemia or inflammation and the measured cellular markers. Statistical significance was set at $P < 0.05$.

3.3 Results

3.3.1 Clinical characteristics of study participants

The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board. This was a cross-sectional study in which we recruited 57 children in the age range of 1–12 years. The participants included 22 symptomatic children, 18 asymptomatic children and 17 healthy controls (**Table 3.1**). The sexes of the children were comparable amongst the study groups ($p < 0.05$). Healthy children were older than the asymptomatic ($p < 0.05$) and symptomatic children ($p < 0.05$). Levels of parasitemia mirrored the intensity of infection, with symptomatic children having a higher parasite load compared to the asymptomatic children ($p < 0.001$). Hemoglobin levels were significantly decreased in the symptomatic children in comparison to the asymptomatic children ($p < 0.05$). Even though, the lymphocyte count was not significantly different amongst the study groups, they also mirrored the intensity of infection ($p > 0.05$). We found the granulocyte count to be comparable amongst the study groups ($p > 0.05$). Also, the platelet-to-lymphocyte ratio (PLR) was found to be comparable between the healthy controls and

asymptomatic groups ($p < 0.05$) but higher than the symptomatic group ($p < 0.05$).

Table 3.1 Clinical characteristics of participants enrolled in the study

Characteristics	Control (C)	Asymptomatic (A)	Symptomatic (S)	P values
Participants	n=17	n=18	n=22	
Age (IQR), years	9(8-11)	7(4.5-9)	6(4.8-7)	0.0087 [a]
Female (%)	52.94	44.44	50	0.8765[b]
Hemoglobin, g/dl[#]	11.5(0.994)	12.7(1.234)	10.7(3.025)	0.0402[c]
Parasitaemia (IQR), µl	NA	845(260.7-3812)	13973(7238-58764)	0.0009 [d]
Granulocytes (10^9/L)[#]	3.353(1.335)	3.069(2.115)	5.041(3.310)	0.0518 [c]
Lymphocytes (10^6/L)	2.9(2.5-3.6)	2.1(1.2-3.45)	1.9(1.3-3.9)	0.0889 [a]
Platelets (10^9/L)	305(237-356)	223(193-280)	101(61-198)	>0.0001[a]

IQR interquartile range, [a] Kruskal Wallis test, [b] Chi-square test, [c] One-way ANOVA, [d] Mann-Whitney U test, # Mean (Standard deviation)

3.3.2 Increased expression of PD-1 and CTLA-4 markers on T cells in children with symptomatic P. falciparum malaria

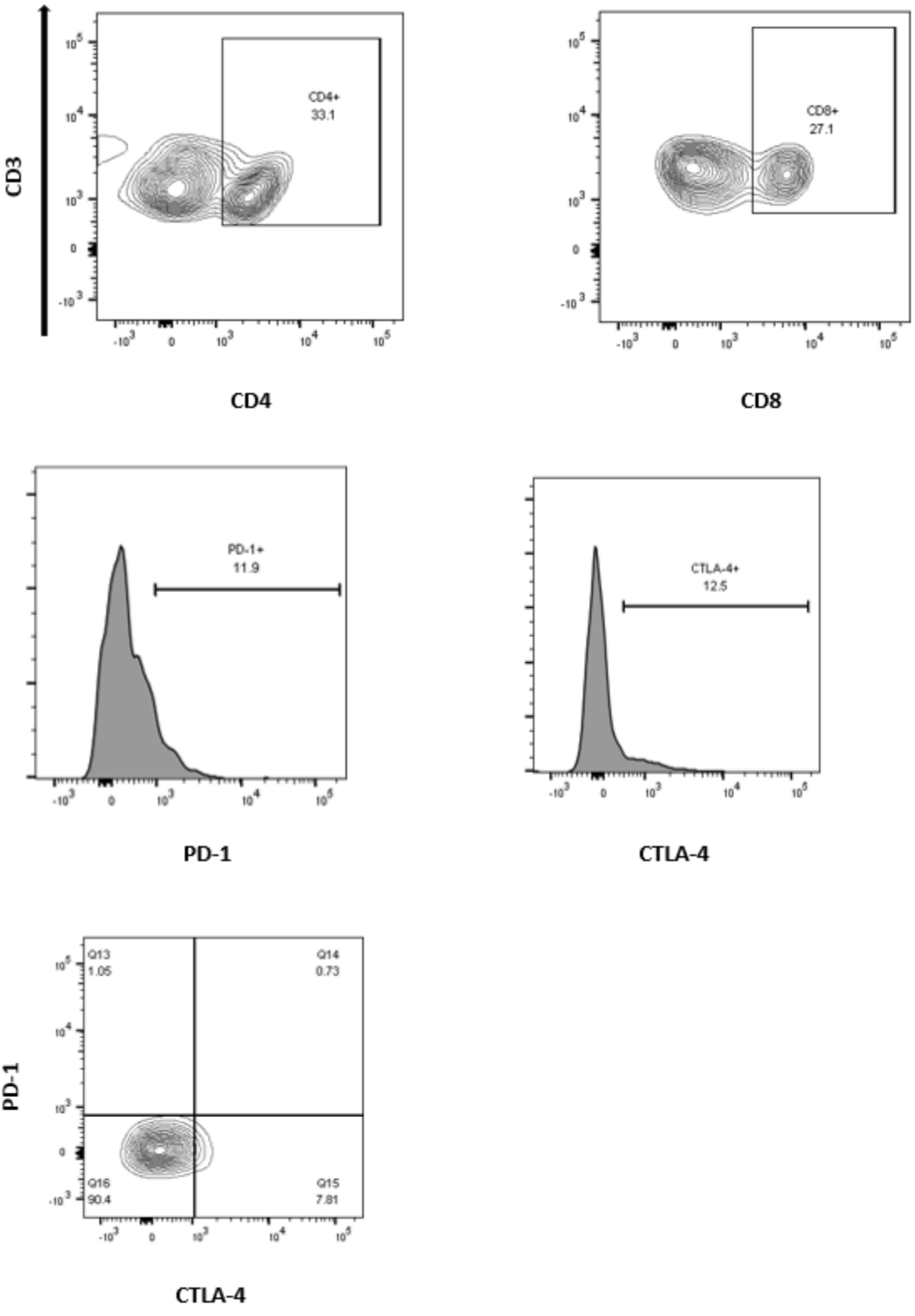
We first investigated the expression of the inhibitory markers PD-1 and CTLA-4 on T cells (**Figure 3.1A**). The expression levels of PD-1 were significantly upregulated in the symptomatic children compared to the asymptomatic ($p<0.0001$) and healthy groups ($p<0.0001$) for the CD4+ T cells (**Figure 3.1B**). Levels of PD-1 in the asymptomatic children and healthy children were comparable. Similarly, CD8+PD-1+ T cells were upregulated in children with symptomatic malaria compared to asymptomatic ($p=0.0312$) and uninfected controls ($p<0.0001$). Nevertheless, the expression of PD-1 on CD8+ T cells was increased significantly in the asymptomatic children compared to the healthy controls ($p=0.0359$). Of note, the levels of PD-1 were higher in CD8+ T cells compared to the CD4+ T cells in all study groups. Also, the expression levels of CTLA-4 on CD4+ T cells were

increased significantly in the symptomatic children compared to the asymptomatic ($p<0.001$) and healthy controls ($p<0.05$) whereas comparable levels of expression were found between asymptomatic children and healthy controls (**Figure 3.1C**). This trend was the same for the levels of CTLA-4 expression on CD8+ T cells among the study groups: symptomatic children had increased levels compared to asymptomatic ($p<0.001$) and healthy children ($p<0.05$).

Next, we assessed the expression of PD-1 and CTLA-4 double-positive markers on both T cell subsets. The symptomatic children had significantly higher levels of PD-1 and CTLA-4 double positive markers on CD4+ T cells in comparison to the asymptomatic children ($p<0.0001$) and healthy controls ($p=0.0091$). Similarly, levels on CD8+ T cells were higher in symptomatic children compared to the asymptomatic children ($p=0.0121$) and healthy controls ($p=0.0098$) (**Figure 3.1C**). In all, levels of PD-1 and CTLA-4 double positive markers between the asymptomatic children and healthy controls were comparable and not significantly different.

The significant levels of inhibitory markers observed in children with symptomatic malaria may be related to the inadequacy of effector functions in clearing parasitemia.

A


CD3
CD4+
33.1
CD4
CD8+
27.1
CD8
Count
PD-1+
11.9
PD-1
CTLA-4+
12.5
CTLA-4
PD-1
Q13
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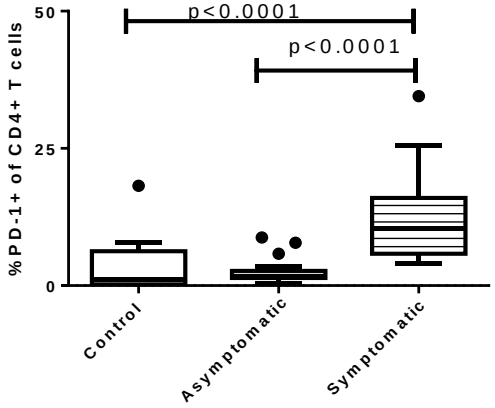
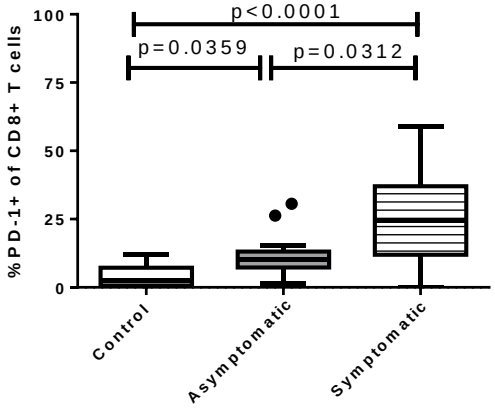
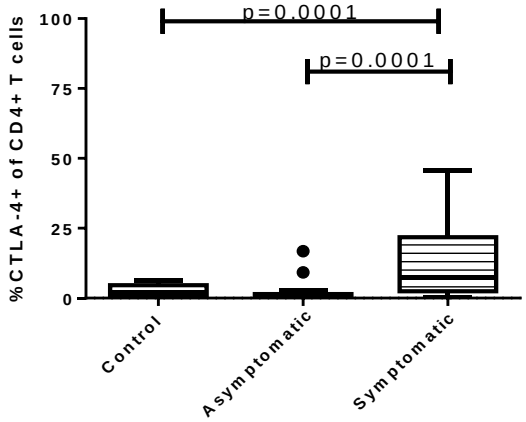
Q14
0.73
Q16
90.4
Q15
7.81
CTLA-4

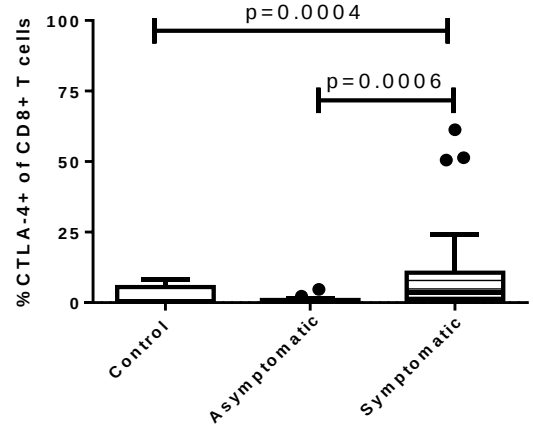
B


p<0.0001
p<0.0001
%PD-1+ of CD4+ T cells
50
25
0
Control
Asymptomatic
Symptomatic

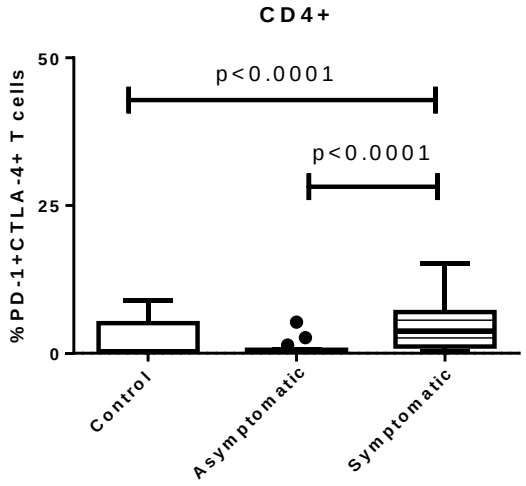

p<0.0001
p=0.0359
p=0.0312
%PD-1+ of CD8+ T cells
100
75
50
25
0
Control
Asymptomatic
Symptomatic

C


p=0.0001
p=0.0001
%CTLA-4+ of CD4+ T cells
100
75
50
25
0
Control
Asymptomatic
Symptomatic


p=0.0004
p=0.0006
%CTLA-4+ of CD8+ T cells
100
75
50
25
0
Control
Asymptomatic
Symptomatic

D


CD4+
p<0.0001
p<0.0001
%PD-1+CTLA-4+ T cells
50
25
0
Control
Asymptomatic
Symptomatic

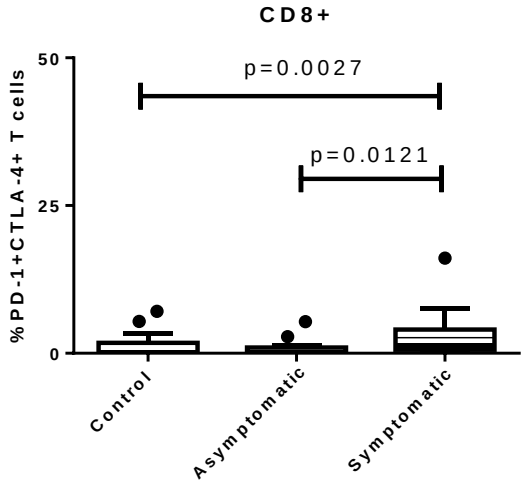

CD8+
p=0.0027
p=0.0121
%PD-1+CTLA-4+ T cells
50
25
0
Control
Asymptomatic
Symptomatic

Figure 3.1. Expression profile of exhaustion and regulatory markers on CD4+ and CD8+ T cells among symptomatic malaria patients (n=22), asymptomatic malaria(n=18) and healthy children (n=16). **(A)** The gating strategy to identify expression levels of PD-1 and CTLA-4 markers. The expression profile comparing levels of **(B).** PD-1, **(C)**. CTLA-4, **(D).** PD-1 and CTLA-4 double-positive markers, on both CD4+ and CD8+ T cells across the study subjects. Levels of expression were compared using the Kruskal-Wallis with Dunn's test to correct for multiple comparisons. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers with medians indicated by the bold horizontal lines across the boxes. P values less than 0.05 were considered statistically significant.

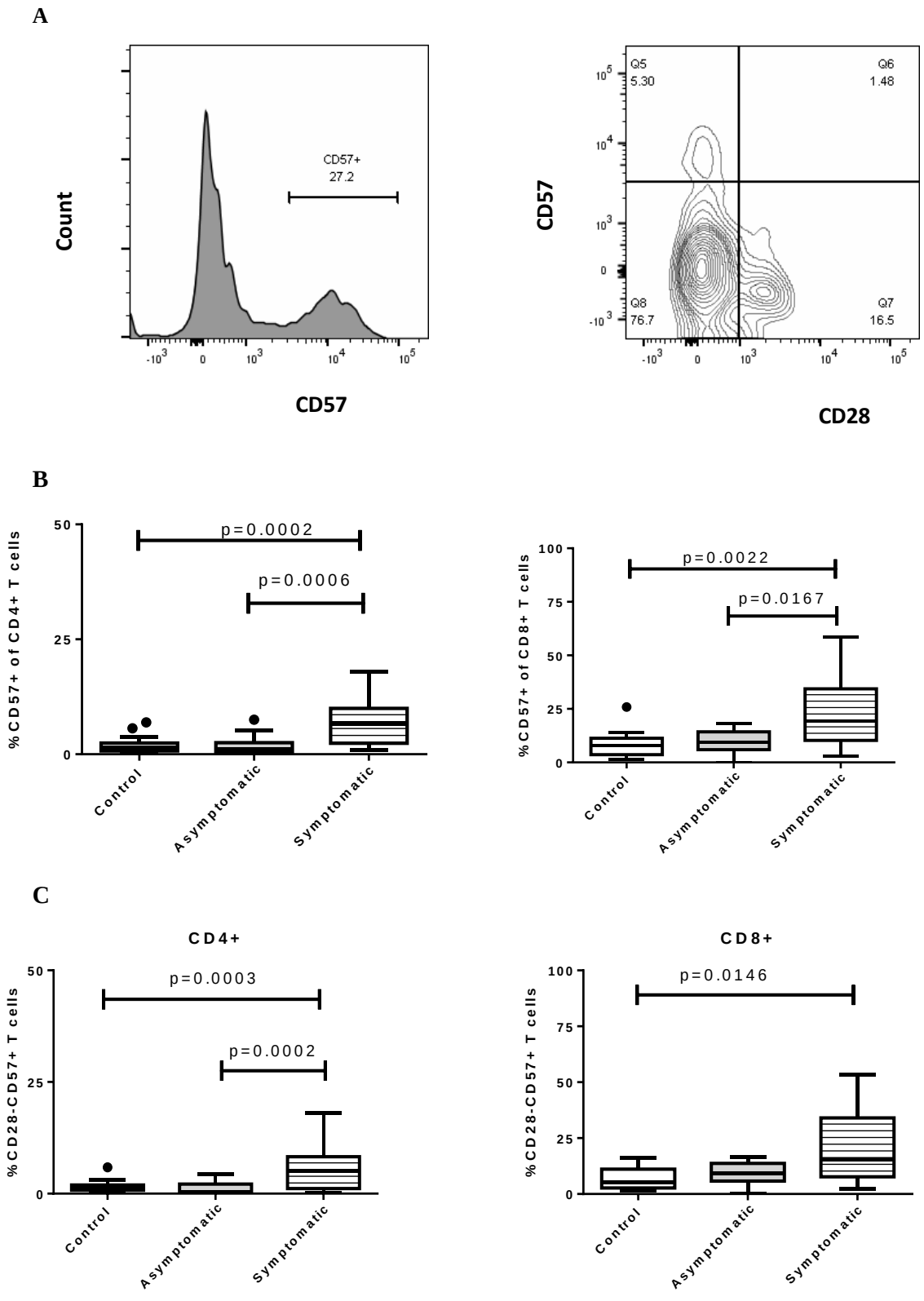
3.3.3 Symptomatic P. falciparum infection is associated with the upregulation of T- cell senescence markers

We also determined if symptomatic malaria may be associated with the biological aging of T cells, by measuring senescent markers using CD28 and CD57 and comparing the proportions with the asymptomatic and healthy groups. We first determined the proportions of T cells expressing CD57 which were found to higher on CD8+ T cells compared to the CD4+ T cells. This trend was similar in all 3 study groups (**Figure 3.2**). Levels of CD4+CD57+ T cells were significantly higher in children with symptomatic malaria compared to asymptomatic ($p = 0.0006$) and healthy controls ($p = 0.0041$). A similar trend was observed for the CD8+ T cell subsets where a significant difference was observed between symptomatic and asymptomatic children ($p = 0.0167$) and healthy controls ($p = 0.0050$) (**Figure 3.2A**). Secondly, we checked for the percentage expression of CD28-CD57+ T cells, a marker frequently associated with T cell aging in the elderly. Levels of CD28-CD57+CD4+ T cells were also increased in children with symptomatic malaria compared to children with asymptomatic infections ($p = 0.0002$) and healthy controls ($p = 0.0064$). In contrast, levels of the CD28-CD57+ marker on CD8+ T cells did not differ

between the symptomatic children and asymptomatic children ($p = 0.1115$), but was increased in the symptomatic group compared to healthy controls ($p = 0.0178$) (**Figure 3.2B**).

Later, we gated on CD28- T cells and measured the expression of CD57 on CD28- T cells (CD57 of CD28- T cells) to determine if the expression levels may be similar or differ from what is observed in normal aging or HIV infections. We found that the percentage expression of CD57+ on CD28-CD4+ T cells remained increased in children with symptomatic malaria compared to children with asymptomatic infections ($p < 0.0001$) and healthy controls ($p = 0.0261$). Also, the percentage expression of CD57 on CD28- CD8+ T cells was significantly increased in children with symptomatic malaria compared to those with asymptomatic malaria ($p = 0.0175$) or healthy controls ($p = 0.0147$) (**Figures 3.2C and D**).

We further compared the expression of CD57 and PD-1 double-positive markers (commonly associated with increased apoptosis) on T cells in the study participants. We observed that CD4+ T cells expressing both CD57 and PD-1 were increased in children with symptomatic malaria compared to asymptomatic ($p = 0.0127$) and healthy controls ($p = 0.0071$; **Figure 3.2E**). This trend was similarly observed in the CD8+ T cells: levels of PD-1+CD57+CD8+ T cells were increased in children with symptomatic malaria in comparison to asymptomatic children ($p < 0.0001$) and healthy controls ($p = 0.0001$). Overall, T cells from symptomatic P. falciparum infected children showed phenotypic evidence of T cell senescence.


A
Count
CD57+
27.2
CD57
CD57
Q5
5.30
Q6
1.48
Q8
76.7
Q7
16.5
CD28
B
% CD57+ of CD4+ T cells
p=0.0002
p=0.0006
Control
Asymptomatic
Symptomatic
% CD57+ of CD8+ T cells
p=0.0022
p=0.0167
Control
Asymptomatic
Symptomatic
C
CD4+
% CD28-CD57+ T cells
p=0.0003
p=0.0002
Control
Asymptomatic
Symptomatic
CD8+
% CD28-CD57+ T cells
p=0.0146
Control
Asymptomatic
Symptomatic

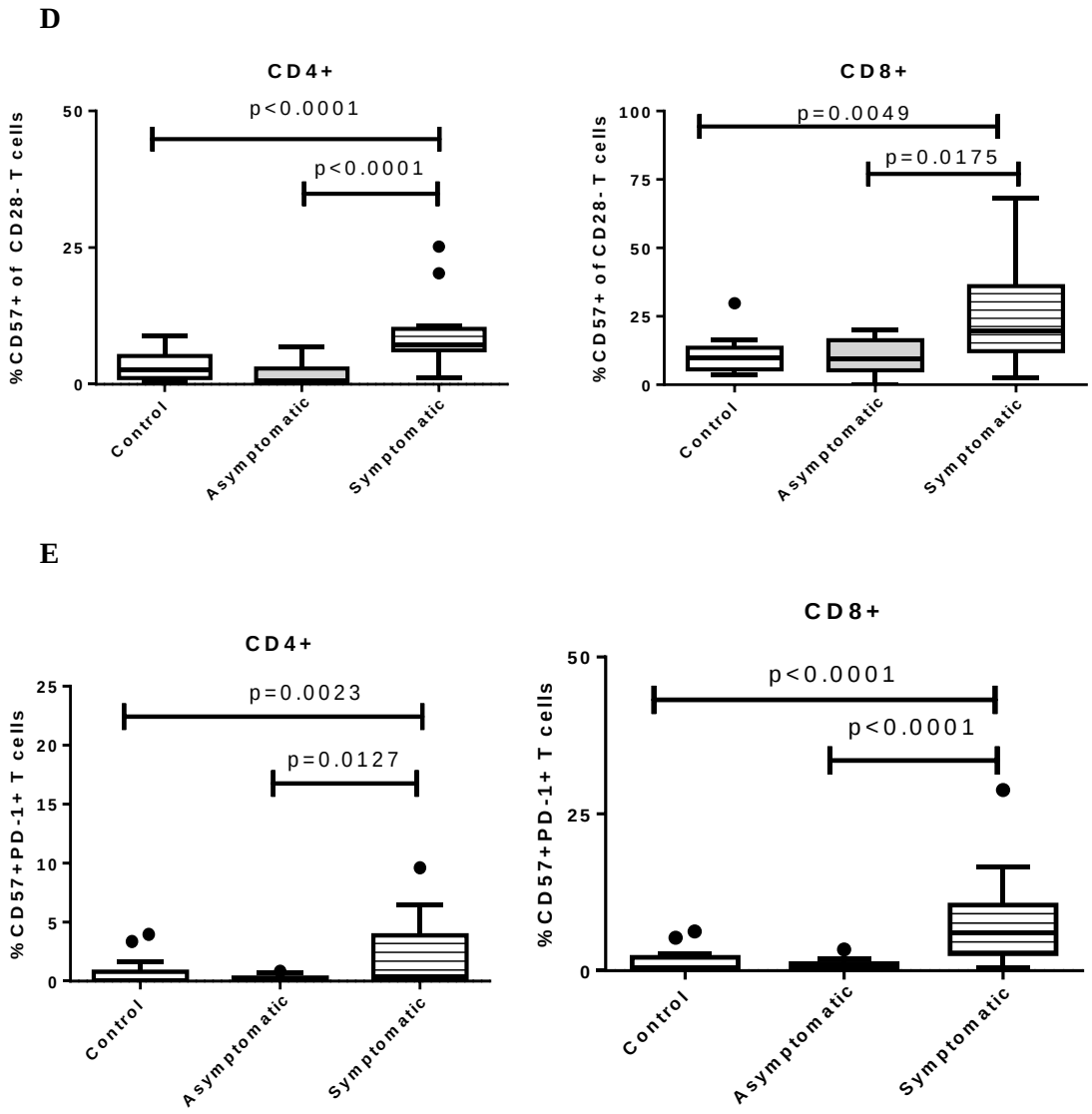


Figure 3.2. Symptomatic *P. falciparum* infection is associated with the upregulation of T-cell senescence markers. The expression profile of immune senescence markers on CD4+ and CD8+ T cells among symptomatic malaria patients, asymptomatic malaria and healthy children. **(A)** The gating strategy for identifying CD57 and CD28 surface markers. The expression profile comparing levels of **(B)**. CD57, **(C).** CD28-CD57+, **(D)**. CD57+ of CD28- and **(E)**. PD-1+CD57+ markers on both CD4+ and CD8+ T cells across the study subjects. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers with medians indicated by the bold horizontal lines across the boxes. Levels of expression were compared among the study population using

the Kruskal-Wallis with Dunn's test to correct for multiple comparisons. P values less than 0.05 were considered statistically significant.

3.3.4 CTLA-4 is a major predictor of parasite load during Plasmodium falciparum infection

The effect of cellular markers on parasitemia and inflammation was investigated using multivariate regression analysis. We analysed 7 T cell phenotypic markers; inhibitory (PD-1+, CTLA-4+, PD-1CTLA-4+) and senescence (CD57+, CD28-CD57+, CD57+ of CD28-, PD-1+CD57+) each on both CD4+ and CD8+ T cells to determine if any of these markers could predict parasitaemia or inflammation (PLR). We defined inflammation by the ratio of platelet to lymphocyte count (PLR)[185-187]. Before the multivariate analysis, we initially performed a correlation analysis to determine if any of the phenotypes may be significantly associated with PLR or parasitaemia. The proportions of CD4PD-1 ($r=-0.65$, $p<0.01$) and CD8CTLA-4 ($r=-0.506$, $p<0.05$) were inversely correlated with PLR (**Figure S3.2**) but positively correlated with parasitaemia (for CD4PD-1, $r = 0.4631$, $p<0.05$; CD8CTLA-4, $r = 0.4831$, $p<0.05$). However, using the multiple linear regression model and performing a likelihood ratio test, expression levels of CTLA-4 on both CD4+ and CD8+ T cells were found to significantly predict the level of parasitemia in the symptomatic children (**Figure 3.3, Table 3.2 and 3.3**). Likewise, the levels of CD8+CD28-CD57+ and CD57 on CD8+CD28- T cells could significantly explain some of the variation observed in parasitemia (**Table 3.2 and 3.3**). Even though all the coefficients from the regression analysis for the T cell phenotypes were inversely associated with inflammation, none could be a predictor of inflammation (**Table S3.1**).

On the other hand, among the asymptomatic malaria group, levels of CD4+PD-1+ and CD4+PD-1+CD57+ could predict and explain some of the variation observed in

parasitemia ($p<0.05$; $p<0.0001$) whereas for CD8+ T cells, the expression of CTLA-4 ($p<0.001$) and PD-1+CTLA-4+ ($p<0.0001$) were good predictors of parasitemia (**FigureS3.3; Table S3.2**).

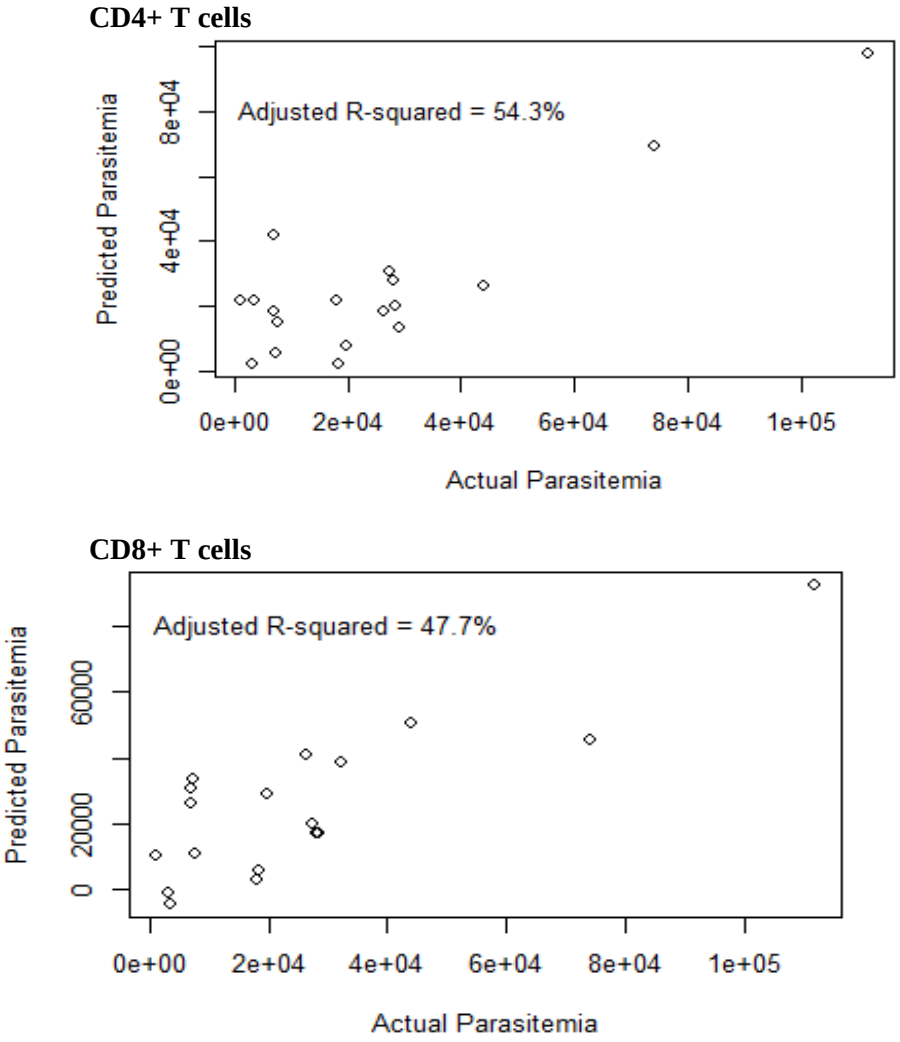


Figure 3.3. The relationship between the surface markers and parasitemia. A multiple linear regression plot. Actual parasitaemia levels were indicated on the x-axis with the predicted values of parasitaemia on the y-axis. The model was designed using 7 T cell phenotypes measured in **(A)** CD4 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) and **(B)** CD8 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) T cells from PBMCs obtained from the symptomatic children.

3.3.5 Multivariate analysis of T cell inhibitory and senescence markers

In order to identify significant immunological signatures (T cell phenotypes) that can explain the variation in our study population and separate our study population into clusters, we performed a principal component analysis (PCA). Using the loadings and scores generated from the analysis, From the eigen values we obtained, we selected principal components that best explained the variations in the datasets. Components 1 and 2 for the CD4+ T cells accounted for 73.1% (62 and 11.7% respectively) of the variation in data whereas, for CD8+ T cells, PC1 and PC2 accounted for 81.1% (56.3 and 24.8 respectively). From the plots, it can be observed that mostly the symptomatic group had higher PC values compared to the asymptomatic group. Using the entire datasets, the principal components clustered our population into three groups based on the frequencies of the phenotypes (**Figure 3.4A and B**). Also, from the CD8+ T cells, it can be observed that PC1 is associated with inhibitory markers located in the upper right quadrant whereas PC2 is associated with senescent markers, located in the lower right quadrant. In addition, the loadings of PD-1 and CD57 were significant for PC1 and PC2 respectively. Further analysis indicated the T cell phenotypes contributing to most of the variation for CD4 T cells were CTLA-4 and PD-1 whereas, for CD8 T cells, the important markers were PD-1, CD57 and CTLA4 **(Figure S3.4**).

A. PCA biplot for CD4 T cells **B. PCA biplot for CD8 T cells**

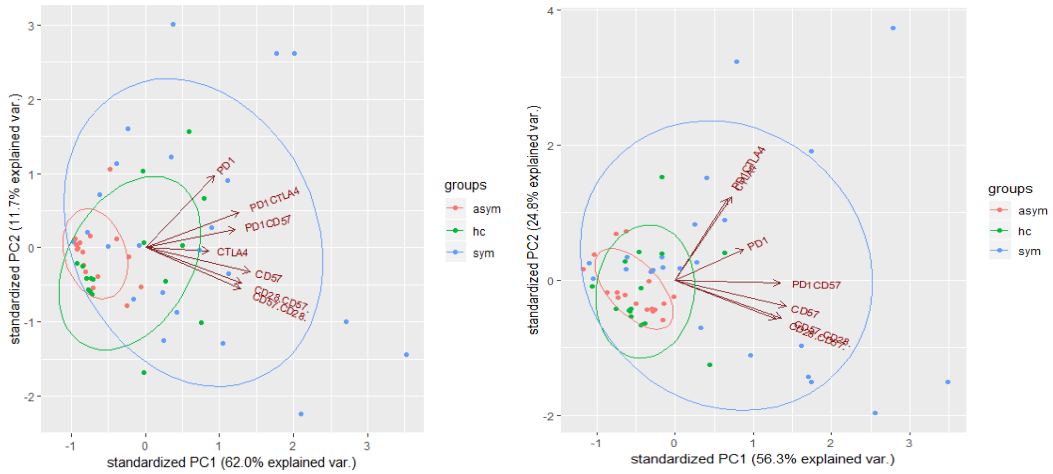


Figure 3.4. Principal component analysis of cellular markers determined in the healthy groups (n=17), asymptomatic malaria (n=18) and symptomatic malaria (n=22), for (**A**) CD4 and (**B**) CD8 T cells. The green, red and blue symbols as well as ellipses denote the healthy, asymptomatic and symptomatic groups respectively. The red arrows indicate the cellular markers.

3.3.6 The interrelationship between the cellular inhibitory markers

Next, we determined the interrelationship between the surface markers (including FOXP3) using partial correlation, a multiparametric correlation analysis that controls for confounding factors (**Table 3.2 and 3.3**). For instance, significant positive correlations for CD4 T cells were observed between FOXP3 and PD1, FOXP3 and CD57, PD-1CTLA-4 and CD57 ($p<0.05$). Generally, significant correlations were all positively related.

Table 3.2 A partial correlation matrix with covariates between the cellular markers measured on CD4+ T cells for the symptomatic malaria population

CD4	CTLA4	PD1	PD1CTLA4	CD57	CD57+CD28-	PD1CD57	CD28-CD57+	FOXP3
CTLA4	1							
PD1	-0.28392916	1						
PD1CTLA4	0.48244305	**0.7425784****	1					
CD57	-0.09261014	-0.4476809	**0.6287185****	1				
CD57+CD28-	0.28077369	0.1731718	-0.0862313	-0.06116931	1			
PD1CD57	0.36438345	0.1282666	-0.179054	-0.08438154	-0.2904794	1		
CD28-CD57+	0.03186114	0.3141221	-0.456166	**0.91009944******	0.363051	0.1875853	1	
FOXP3	0.2481085	**0.6309751***	-0.4845044	**0.55752428***	-0.432212	-0.0890901	-0.3706132	1

Significant correlations were determined using a permutation test. P < 0. 05 were considered statistically significant; *p<0.05, **p<0.01, ****p<0.0001

Table 3.3 A partial correlation matrix with covariates between the cellular markers measured on CD8+ T cells for the symptomatic malaria population

CD8	CTLA4	PD1	PD1CTLA4	CD57	CD57+CD28-	PD1CD57	CD28-CD57+	FOXP3
CTLA4	1							
PD1	0.4365899	1						
PD1CTLA4	**0.8497852******	−0.267313	1					
CD57	−0.230747	0.0385105	0.136794	1				
CD57+CD28-	0.2025701	0.0277434	−0.1928562	**0.6714082****	1			
PD1CD57	−0.2022586	**0.720932****	0.1076203	0.0563088	0.14287562	1		
CD28-CD57+	0.1714043	−0.4517704	−0.0132945	0.2164709	0.46299502	0.2318686	1	
FOXP3	0.2319811	−0.3906262	0.1694245	0.0678562	−0.00587128	0.3006289	−0.29398143	1

Significant correlations were determined using a permutation test. P < 0. 05 were considered statistically significant; *p<0.05, **p<0.01, ****p<0.0001

3.4 Discussion

The upregulation of inhibitory and senescent markers on T cells has been associated with the impairment of effector T cell responses. In this study, we sought to identify T-cell immune signatures that may be associated with the development of symptomatic malaria. We analyzed the pattern of expression of co-inhibitory and senescent markers in children with symptomatic *P. falciparum* malaria, asymptomatic malaria and healthy controls. We found that the expression of these exhaustive and senescent markers was increased in children with symptomatic malaria compared to those with asymptomatic infections and healthy controls. Using multivariate regression analysis with likelihood ratio test, we found CTLA-4 to be a strong predictor of parasitemia levels. Also, none of the T cell phenotypes measured was a good predictor of inflammation, even though PD-1 and CTLA-4 were inversely correlated with inflammation. Using a principal component analysis our study population was clustered into three groups based on the level of expression of the cellular markers. Further analysis revealed that, for CD4+ T cells, the levels of CD4+CTLA4+ and CD4+PD-1+ markers could explain the clustering pattern of the study groups, whereas for CD8+ T cells the important markers were CD8+PD-1+ , CD8+CD57+ and CD8+CTLA-4+. In addition, we observed a lower platelet to lymphocyte ratio in the symptomatic malaria group probably resulting from the decreased platelets and lymphocytes counts that are associated with clinical malaria.[188, 189].

The activation of T cells by pathogens leads to the induction of inhibitory receptors such as CTLA-4 and PD-1 [190]. PD-1 and CTLA-4 are some of the well-characterized inhibitory receptors associated with the exhaustion of T cells [166, 191, 192]. Levels of expression of the inhibitory markers PD-1 and CTLA-4 were upregulated in children with

symptomatic malaria, confirming recent studies that have observed increased levels of PD-1 and CTLA-4 during acute infections[164, 193, 194], resulting in decreased production of cytokines[195]. This, therefore, suggests that the increased frequency of inhibitory markers during clinical disease may alter the effector function of T cells.

It has been shown by Butler et al.,[166] that levels of CD4+PD-1+ phenotypes correlate with parasitemia in clinical malaria. In this study, we found that CD4+PD-1+ and CD8+CTLA-4+ could also predict parasitemia levels in the asymptomatic malaria group. Importantly, in the symptomatic group, the expression of CTLA-4 was a major predictor in determining parasitemia load. This suggests that T cell exhaustion may induce tolerance which may promote parasitemia. Since both PD-1 and CTLA-4 are negative regulators of the immune response, their observed increase in symptomatic children may indicate that PD-1 and CTLA-4 contribute in regulating T cell activity or inflammation[164, 195]. Interestingly, we found an inverse association between these inhibitory markers and inflammation, which is in line with previous observations in murine models that blockage of T cell inhibitory markers exacerbated the immune response, increased susceptibility to severe disease and decreased survival[196, 197]Furthermore, the strong association we observed between T cell exhaustion and clinical parameters such as parasitemia and inflammation suggests that T cell exhaustion plays a vital role in malaria pathogenesis.

We have previously shown that asymptomatic *P. falciparum* infections are characterized by a reduced frequency of regulatory T cells [198]. Here, we hypothesized that asymptomatic infections may have reduced expression of inhibitory markers compared to symptomatic children. We found that the expression levels of inhibitory markers in asymptomatic and healthy controls were mostly comparable except for PD-1 which was

increased in the *P. falciparum* infected groups compared to the healthy controls [192]. It may indicate PD-1 expression may be driven by *P. falciparum* infections. This could also imply that continuous exposure to *P. falciparum* infections may render T cells to be defective in function. Unfortunately, we could not determine the functionality of these T cells to confirm this.

Immunosenescence is the aging of immune cells characterized by shortened telomeres and inability to replicate,[199] sensitivity to apoptosis and, phenotypically, the expression of CD57 [171]. T cell senescent markers were more associated with CD8+ T cells compared to CD4+ T cells consistent with earlier reports that they accumulate at lower frequencies for CD4+ T cells in human periphery[200]. Here, we found an increased expression of CD57+T cell subsets in children with symptomatic malaria[201]. This may suggest that malaria accelerates the aging of the T cell pool. In addition, the increased expression of CD28-CD57+ marker observed in symptomatic children indicates a greater proportion of effector T cells in symptomatic malaria have a memory phenotype since these cell subsets have been described to be antigen experienced [171, 180, 200]. It has previously been shown that PD-1+CD57+CD8+ T cells have increased sensitivity to apoptosis mediated by PD-1 [202]. The observed increase in expression of CD57 and PD-1 double-positive markers on CD8+ T cells, therefore, indicates a greater risk of apoptosis of these cells in clinical malaria. Additionally, T cell ageing has been well characterized in the elderly population, CMV and HIV infections[170, 179, 182]. In contrast to CMV infections which leads to expansion of T cell senescent markers, HIV leads to a decrease in the expression of CD57 on CD28-CD8+ gated T cells, whereas levels of CD8+CD28-CD57+ remains unchanged. In our study, we observed that both the proportion of CD28-

T cells that express CD57 were expanded in the symptomatic malaria group, suggesting that T cell aging in falciparum infections is more similar to that observed during CMV infections than in HIV infections. Together, these observed phenotypic changes might reduce the responsiveness of the T cell repertoire to *P. falciparum* antigens resulting in an impaired ability to eliminate parasitemia.

FOXP3 is an immune regulatory marker associated with preventing immunopathology during inflammation. Both FOXP3 and PD-1 have been shown to suppress host immune response. Importantly, *P. falciparum* infections has been reported to cause the induction of PD-1+CTLA4+ T cells that control T cell activity[164]. In this study, the positive correlation observed between FOXP3 and PD-1 T cells as well as between PD-1 and PD-1CTLA4 T cells could indicate that these markers play complementary roles in mediating the increasing immune activation that is associated with symptomatic malaria. There are conflicting reports about the role of CD57+ T cells in clinical disease, with some reports describing them as immunosuppressive and others suggesting they exacerbate immune activity through IFNγ production[180, 203] . Here, we observed a significant positive correlation between CD4+CD57+ T cells and CD4+FOXP3+ T cells as well as between CD4+CD57+ and CD4+PD-1+CTLA-4+ T cell subsets. However, since we could not determine the functionality of the CD57 T cell subsets, we can only suggest that the positive relationship observed between CD4+CD57 and CD4+FOXP3+ as well as CD4+ PD-1+CTLA-4+ T cell subsets may indicate that CD4+CD57+ T cells play suppressive roles during clinical malaria. This further supports the view that *Plasmodium* infections induce immunosuppressive immune responses that enhance the development of tolerance to the parasite, a mechanism affecting the

development of sterile immunity.

Also, the results from the principal component analysis may indicate that a selection panel of the considered markers may serve as a biomarker for identifying individuals with symptomatic disease. It may probably be used to predict the outcome or immune response to vaccination. These results provide a basis to perform functional assays to determine the impact of the considered markers on the acquisition of anti-disease immunity during *P. falciparum* infections, preferably in a longitudinal cohort

Studies have reported a low ratio of platelet to lymphocyte count (PLR) as a marker for inflammation in various infectious diseases such as HBV[185] and HCV[186]. In this study, even though none of our markers was a good predictor of inflammation as previously stated using the PLR, we show that symptomatic malaria is characterized by low ratio of platelet -to-lymphocyte counts, which is indicative of on-going inflammatory response. Nonetheless, additional studies are needed to determine the significance of other hematological markers of inflammation (such as the neutrophil to lymphocyte ratio) to ascertain their clinical relevance during symptomatic malaria.

This study had a number of limitations due to the cross-sectional nature. We could not determine the effect of anti-malarial treatment on the expression of these inhibitory and senescent markers since samples were taking before the initiation of treatment. Furthermore, we defined *P. falciparum* infections by microscopy which is not able to distinguish between microscopic and sub-microscopic infections. In addition, we could not determine the effect of these markers on T cell cytokine production since cytokine profile analysis was not performed.

Despite these shortcomings, this study shows evidence that the phenotypic defect of T cells during *P. falciparum* infections are more pronounced in clinical malaria and associated with higher expression of exhaustive and senescent markers compared to asymptomatic infections. CTLA-4 was a good predictor of parasitemia in both symptomatic and asymptomatic malaria groups. Also, using the platelet to lymphocyte ratio, none of the markers measured could predict inflammation. In addition, we observed that the aging phenotype of T cells in malaria infection is similar to that observed with normal aging and CMV infections. These may imply that the increased expression of these markers may be associated with the absence of sterile immunity to *P. falciparum* malaria.

phenotypes could predict inflammation using a simple linear regression model. A direct association between the expression levels of PD-1 and CTLA-4 on the one hand and disease severity as well as the extent of parasitaemia on the other hand provides evidence for prolonged parasite exposure being largely responsible for upregulation of these markers. These may imply that the increased expression of these markers may, in part, be associated with the absence of sterile immunity to *P. falciparum* malaria.

Chapter Four

4.0 Dynamic changes in the T cell receptor β repertoire during paediatric malaria infection

Abstract

Plasmodium falciparum malaria infection induces a complex effector immune response involving a myriad of immune cells. The immune activity via T cells has been projected to influence the disease condition and outcome.

In order to understand the dynamics of the T cell receptor repertoire during *P. falciparum* infection, we profiled TCRβ chain sequences via high through-put sequencing from children with *Plasmodium* infections (asymptomatic, uncomplicated and severe malaria cases) and compared with similar sequences from aparasitemic (healthy) children as controls.

We observed significant differences in the V and J gene distribution and usage, with biased selection of specific gene segments in the *Plasmodium* infected group. T cells from the asymptomatic group exhibited increased diversity which was inversely associated with parasitaemia. Also, there was increased TCR sharing in the asymptomatic group compared to the uncomplicated and severe malaria groups. Importantly we further identified 9 unique TCR specificity clusters and predicted binding motifs within these clusters found in multiple individuals in the malaria infected group.

In summary, this study has characterized the TCR repertoire in peripheral blood during malaria infection and provides important information that is crucial in developing effective therapeutics and vaccines against malaria.

4.1 Background

Malaria is an infectious disease caused by protozoans from the genus *Plasmodium.* According to WHO, there was no significant reduction in the incidence of the disease in 2018 [1]. In addition, there is no licensed effective malaria vaccine meeting the efficacy targets set in the Malaria Vaccine Technology Roadmap [204]. Natural infections in malaria endemic areas may result in anti-disease or anti-parasite protection but not sterile immunity [6, 198, 205]. The magnitude of host immunity is in large part determined by the activity of T cells, which are central to the immune activity of other cells [141, 206].

The antigenic receptor of T cells (TCR) confers specificity during their development and maturation in the thymus. The TCR is generated by somatic recombination of Variable (V), Joining (J) and Diversity (D) gene segments, which form the TCR β chain. The diversity of the TCR is further enhanced by the addition and deletion of nucleotides in the junctional regions between the gene segments, ensuring recognition and effective T cell responses against a diverse range of pathogen-derived antigens. Such responses further modify TCR diversity, as the total frequency of the TCRs that recognize the pathogen may increase by up to 10^5 fold before declining to above the pre-response levels [207, 208]. If we adopt the common definition of TCR diversity based on the Simpson's D [209], then it follows that a preferential increase in frequency of a subset of TCRs will lead to a decline in TCR diversity. TCR diversity is largely restricted to the hypervariable loops or complementarity determining region 3 (CDR3) of TCR chains [13] . Therefore, profiling the CDR3 sequences can provide insight into a person's infection history as well as the progression or outcome of new infections.

Indeed, previous work has documented changes in TCR repertoires occurring in various clinical conditions such as autoimmune diseases [51, 210], infectious diseases [211-213], and ageing [214]. For instance, in tuberculosis, limited diversity has been correlated with severe disease progression [70]. However, there have been few studies that have investigated the TCR repertoire and diversity in malaria infections. These studies have revealed an upregulation or usage of specific Vβ gene segments. However, only one of the studies focused on the repertoire in human falciparum malaria. Using a medium-throughput approach based on flow cytometry, the Vβ repertoire was characterized in children with severe malaria, uncomplicated malaria, asymptomatic malaria and during convalescence. They observed an upregulation of the Vβ21.3 segment during severe disease and of the Vβ20 segment during convalescence [215]. Also, rodent malaria models have ascribed a pathogenic role for Vβ8.1, an immune receptor gene that reacts with *P. berghei* specific antigen [216, 217] segment believed to be crucial for the pathogenesis of cerebral malaria. Nevertheless, the sequence diversity in the CDR3 region of the TCR during clinical disease remains to be investigated. Therefore, in this study, we profiled, for the first time, the TCR repertoire in children with *P. falciparum* infections using high throughput sequencing. We hypothesized that clonotypic expansions that occur during *P. falciparum* infections, will contribute to the generation of a TCR repertoire that is unique to each disease state. Thousands of PBMCs obtained from 33 children with or without *P. falciparum* malaria were sequenced. We found significant differences in Vβ and Jβ gene usage between the study groups. Remarkably, we found the level of TCR sharing and diversity during malaria to be markedly higher in the asymptomatic cohort, consistent with a protective role of TCR diversity in disease. Severe malaria was characterized by short

CDR3 sequences. Importantly, we identified sequence specificity groups in our *P. falciparum* malaria-infected cohort, which varied in composition with disease status. Taken together, this information will be critical in targeting protective T cell responses in the design of interventions against malaria.

4.2 Materials and methods

4.2.1 Study design and cohort

Protocol for the study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana and the Regional Health Directorate of Greater Accra region. Exclusion criteria for the study were children with evidence of concomitant infection including bacterial infections, history of an underlying disease, those who had taken anti-malaria drugs 2 weeks preceding the study. The inclusion criteria for enrollment was presence of parasitaemia in blood, no axillary temperature of < 37.5^{o}C or any other ailment (for asymptomatic children); axillary temperature ≥37.5 ^{o}C, presence of parasitaemia, fever (uncomplicated malaria), or presence of comma with a Blantyre score ≤ 2, with greater 2 or more episodes of seizure with no other sources of underlying ailment for the severe malaria (cerebral malaria) group. The control group were screened by both microscopy and PCR to determine the absence of parasitaemia and submicroscopic infections. Participants were recruited after informed consent and assent were received from guardians and the children respectively. For this experiment, a total of 33 children were included; healthy controls (n=9), asymptomatic (n=7), uncomplicated malaria (n=9), severe malaria (n=8). Children with clinical malaria were recruited from the health facilities whereas asymptomatic and aparasitemic children were recruited from households within the community. All participants were children under 12 years of age

(**Table 4.1**). Study participants were recruited from a hyper-endemic malaria transmission zone in Ghana. About 5ml of peripheral blood was obtained from the participants into heparin tubes before anti-malarial treatment. PBMCs were isolated by Ficoll gradient centrifugation. About 1 x 10^5 cells were stored in trizol for RNA isolation.

4.2.2 Library preparation and sequencing

Library preparation and sequencing were performed according to the miodx Clonomap™ immune repertoire sequencing assay using the Illumina MiSeq platform (miodx, San Hose, CA). Briefly, total RNA was extracted from PBMC obtained from malaria positive and negative controls. For an input RNA of 50-2000ng per sample, CDR3 regions of the TCRβ gene were amplified using various primers from the β chain consisting of the V and J gene segments and sequenced. PCR amplification bias were corrected using the 5' Rapid Amplification of cDNA ends (5'RACE) with unique molecular identifiers (UMI) to obtain the full length of the TCRβ transcripts. The 5' barcodes were used to multiplex the libraries to reduce sequencing costs. The libraries were purified, quantified using real-time PCR, and paired-end sequenced using Illumina MiSeq.

4.2.3 Bioinformatic analysis

The sequencing data were de-multiplexed, quality-filtered, and clustered based on the UMIs to accurately quantify the frequencies of unique TCR clones. V, D and J gene segments of each uniquely identified clonal sequences were determined by alignment to germline sequences found in the ImMunoGeneTics database [218]. The nucleotide sequences were subsequently translated into amino acid sequences. For VDJ annotation, CDR3 regions were identified as the subsequence occurring between the last conserved cysteine found at the 3' end of the Vβ and the conserved phenylalanine found at the 5' end

of the Jβ gene segment. CDR3 sequences with stop codons were classified as out-of-frame or non-productive sequences.

4.2.4 Estimating TCR repertoire diversity

TCR repertoire diversity was estimated using the Renyi's entropy [219]. Renyi's entropy unifies various diversity indices into a diversity profile characterized by an order parameter, α. Mathematically, the Renyi entropy of a given TCR repertoire X is defined as:

$$H_\alpha(X) = \frac{1}{1-\alpha} \log \left(\sum_x (f_x)^\alpha\right)$$

where $\alpha \neq 1$, $f_x(x)$ is the frequency of TCR x, and the sum is over all distinct TCRs x in X.

When α=0, the Renyi entropy equals the number of distinct TCRs found in the TCR repertoire under consideration. In ecology, this is called the richness of the repertoire.

In the limit as α tends to 1, the Renyi entropy equals the exponent of the famous Shannon entropy used in information theory. Imagine picking a TCR x from the repertoire X. The Shannon entropy of X measures your uncertainty about the identity of x. If all TCRs in X were identical, then you would be certain about the identity of any x you pick from X. Accordingly, the Shannon entropy of X would be zero and its exponent would equal 1, which is the diversity of X.

When α=2, the Renyi entropy equals the reciprocal of the famous Simpson's index used in ecology. Once again, imagine picking a TCR x from the repertoire X, replacing it, and then picking another TCR y from X. Simpson's index measures the probability that both x and y are identical. If all TCRs in X were identical, then the reciprocal of Simpson's

index would equal 1, which is the diversity of X.

4.2.5 Estimating TCR repertoire divergence

The Kullback-Leibler divergence (KLD) was used to estimate the difference between the distributions of V, J and VJ combinations found between the study groups. For example, in the case of V gene segments, the KLD between the two groups with V distributions given by g and h, respectively, is defined as:

$$\mathrm{KLD}(g||h) = \sum_{v} g(v)\log\left(\frac{g(v)}{h(v)}\right)$$

$g(v)$ and $h(v)$ denote the frequency of segment v in groups 1 and 2, respectively.

Bootstrap samples of the V, J, and VJ segments were generated under the null hypothesis that there were no differences between the considered groups. KLD values were also calculated based on the boostrap samples. The proportion of these bootstrap KLD values that were lower than or equal to the corresponding real KLD value was determined and used to determine statistical significance of the latter value at a false discovery rate of 5% [220].

4.2.6 Identification of public and private TCRs

CDR3 amino acid sequences were designated as public if they satisfied one of two criteria:

1) they occurred in all individuals in a group, or 2) they occurred in at least 75% of the individuals in a group.

4.2.7 Identification of TCRs with specificity

We identified groups of TCRs with similar antigen specificity using the GLIPH algorithm developed by Glanville et al, [221], which clusters TCR sequences based on their global and local similarities. Briefly, global similarity identifies TCR sequences that differ in at most one amino acid. In contrast, local similarity identifies TCRs that share CDR3 motifs (2-mers, 3-mers, 4-mers) which are statistically enriched in pathogen-exposed versus naïve TCR repertoires.

4.2.8 Statistical analyses

The students' t-test and ANOVA (for normally distributed data) and Mann-Whitney or Kruskal-Wallis test (for non-normally distributed data) were used to assess the significant differences between the groups. Benjamini-Hochberg's multiple comparison test was used to correct for multiple testing at a false discovery rate of 5%. Correlation was performed with the Spearman's rank correlation coefficient. Proportions were assessed for significance using the Chi-square test. Graphs were developed with R statistical software (Rstudio version 3.5.2, R Development Core Team) and GraphPad Prism (version 6.01 GraphPad Software, Inc.). P values were significant at $p< 0.05$.

4.3 Results

4.3.1 Summary characteristics on study population

To determine the TCR repertoire in malaria infections, 33 children were sampled: 9 aparasitemic children, 7 asymptomatic, 9 uncomplicated and 8 severe malaria cases. The characteristics of the study population are summarized in (**Table 4.1**). The median age of children with severe malaria was lower when compared to the other groups. Levels of parasitaemia were higher in the severe malaria group compared to the other groups.

Asymptomatic children had the lowest parasitaemia load.

The total number of sequences differed among the groups and was higher in the asymptomatic group compared to the aparasitemic ($p<0.0001$), uncomplicated ($p<0.05$), and severe malaria groups ($p<0.01$; **Table 4.1**). There was a significant difference in the number of productive and non-productive sequences among the groups. The *P. falciparum*-infected group contained a higher number of productive sequences compared to the controls, with the asymptomatic group having the highest number of sequences compared to control ($p<0.0001$) and severe ($p<0.05$) groups. The number of non-productive sequences was significantly higher in the asymptomatic group compared to the control group ($p<0.01$). The CDR3 nucleotide length distribution differed between the healthy controls and *P. falciparum*-infected group ($p<0.0001$) whereas no significant difference was observed within the infected group (**Figure 4.1**). However, the CDR3 nucleotide lengths were observed to be lower in the severe malaria group.

Table 4.1: Characteristics of the study population

Characteristics	Aparasitemic Controls	Asymptomatic	Uncomplicated	Severe malaria
Sample size (n)	9	7	9	8
Age (IQR), years	9(6-10.5)	7(2-9)	8.5(7.25-10.5)	4.5 (2.25-7)
Female (n)	5	3	3	4
Parasitemia (IQR), µl	NA	1,762(621.4–4,193)	26,357 (7,143–28,341)	47,713(7,777–67563)
Productive sequences	7838	355177	192424	40725
Non-productive sequences	915	38789	31895	16905

IQR interquartile range, *NA* not applicable, # Mean ± standard error

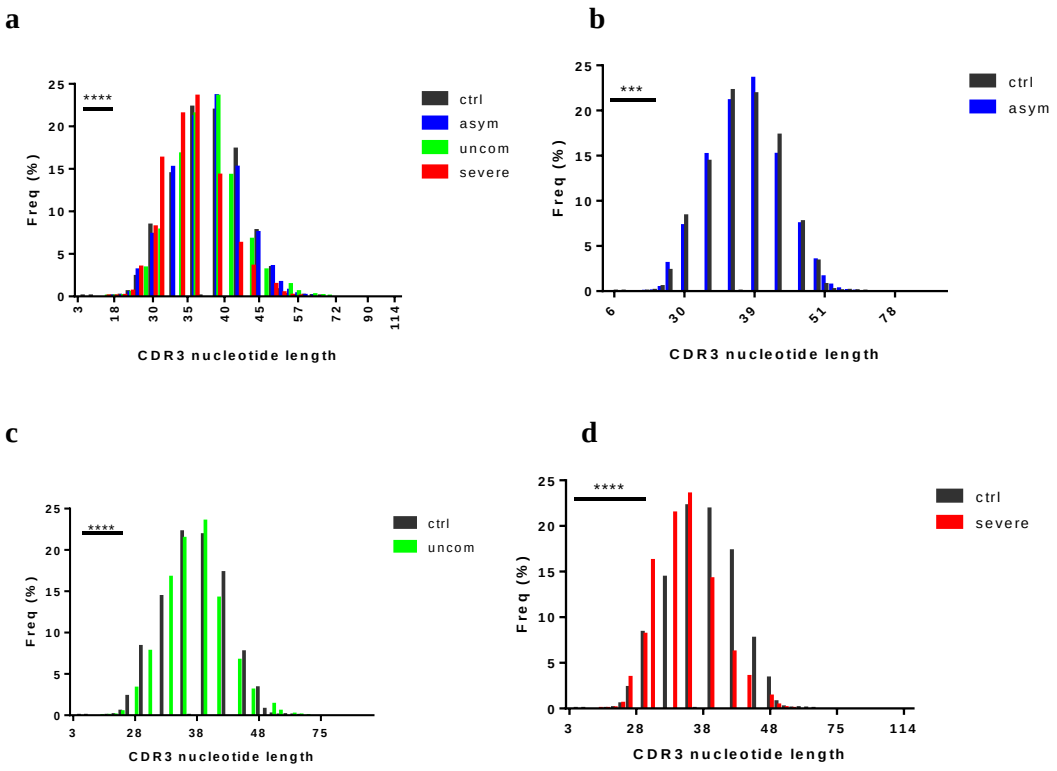


Figure 4.1. Decreased CDR3 length in *P. falciparum* infected group. A histogram plot showing the comparison of the CDR3 nucleotide length distribution for the **(a)** aparasitemic, asymptomatic, uncomplicated and severe groups, aparasitemic and **(b)** asymptomatic (**c**) uncomplicated (**d**) severe malaria groups. The asterisk (*) shows significant difference; **** ($p<0.0001$), ***($p<0.001$), **($p<0.01$), *($p<0.05$).

4.3.2 Pattern of Vβ and Jβ gene segment usage within the study groups

We identified 56 Vβ gene segments. We observed 2 Vβ gene segments (Vβ5-1 and Vβ20-1) to be used at higher frequencies in the aparasitemic group whereas 10 Vβ gene segments (Vβ1, Vβ13-2, Vβ16, Vβ17, Vβ3-2, Vβ5-7, Vβ6-8, Vβ6-9, Vβ7-1, Vβ7-4) were used at much lower frequencies (10^{-6}) followed by 4 gene segments (Vβ12-5, Vβ23-1, Vβ5-3, Vβ6-7) used at frequencies of 10^{-5} (**Figure 4.2a**). The Jβ gene segment consists of 2 clusters; Jβ 1 and Jβ 2. Jβ 1 consists of 6 subunits which are functional whilst 7 functional

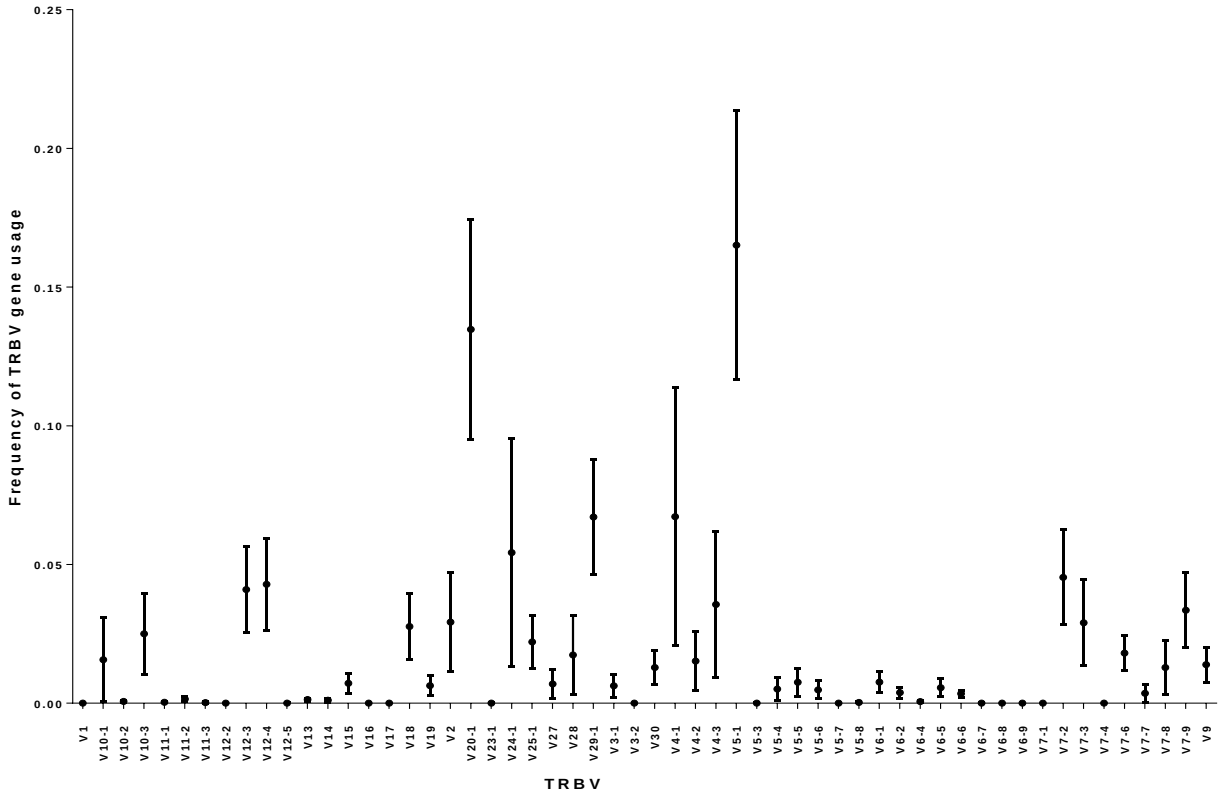
subunits are found in the Jβ2 luster. We observed that almost all Jβ2 subunits were used frequently in the aparasitemic except for Jβ2-6 (**Figure 4.2c**).

Among the asymptomatic group, Vβ5-1 was preferentially used followed by Vβ20-1 whereas Vβ segments 12-2 and 7-1 were used at low frequencies (10^{-6}) with segments Vβ17, Vβ 3-2, Vβ 5-3, Vβ 5-7 and Vβ 6-8 used at frequencies of 10^{-5} (**Figure S4.1a**). For the Jβ gene segments, Jβ2 cluster was preferentially used except for Jβ2-4 and Jβ2-6. Even though Jβ1 subunits were used at very low frequencies, Jβ1-1 was much preferred in the Jβ1 cluster (**Figure S4.1b**).

Within the uncomplicated malaria group, there was the selective usage of Vβ 20-1. Also, 3 Vβ gene segments (Vβ3-2, Vβ6-8, Vβ7-1) were used at frequencies of 10^{-6}, and 5 Vβ gene segments (Vβ12-2, Vβ16, Vβ17, Vβ5-3, Vβ5-7) at frequencies of 10^{-5} (**Figure S4.1b**). However, Jβ2 cluster was preferentially used at high frequencies compared to Jβ1 clusters except for Jβ2-4 and Jβ2-6. In the Jβ1 cluster, there was preferential usage of Jβ1-2 subunit followed by Jβ1-1 (**Figure S4.1d, Figure S4.4**).

Similarly, as observed in the uncomplicated malaria group, the severe malaria group had a preferentially selection of Vβ20-1 gene segment. With Vβ12-2, Vβ3-2 and Vβ7-1 segments being expressed at very low frequencies (10^{-6}) followed by Vβ1, Vβ17, Vβ5-3, Vβ5-7 and Vβ6-8 being expressed at frequencies of 10^{-5} (**Figure 4.2b**). Even though, Jβ2 gene segments were preferentially used compared to Jβ1, the usage of Jβ1-2, Jβ2-4 and Jβ2-6) followed by Jβ1-1 (**Figure 4.2d**).

a


Frequency of TRBV gene usage
0.25
0.20
0.15
0.10
0.05
0.00
V1
V10-1
V10-2
V10-3
V11-1
V11-2
V11-3
V12-2
V12-3
V12-4
V12-5
V13
V14
V15
V16
V17
V18
V19
V2
V20-1
V23-1
V24-1
V25-1
V27
V28
V29-1
V3-1
V3-2
V30
V4-1
V4-2
V4-3
V5-1
V5-3
V5-4
V5-5
V5-6
V5-7
V5-8
V6-1
V6-2
V6-4
V6-5
V6-6
V6-7
V6-8
V6-9
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V7-2
V7-3
V7-4
V7-6
V7-7
V7-8
V7-9
V9
TRBV

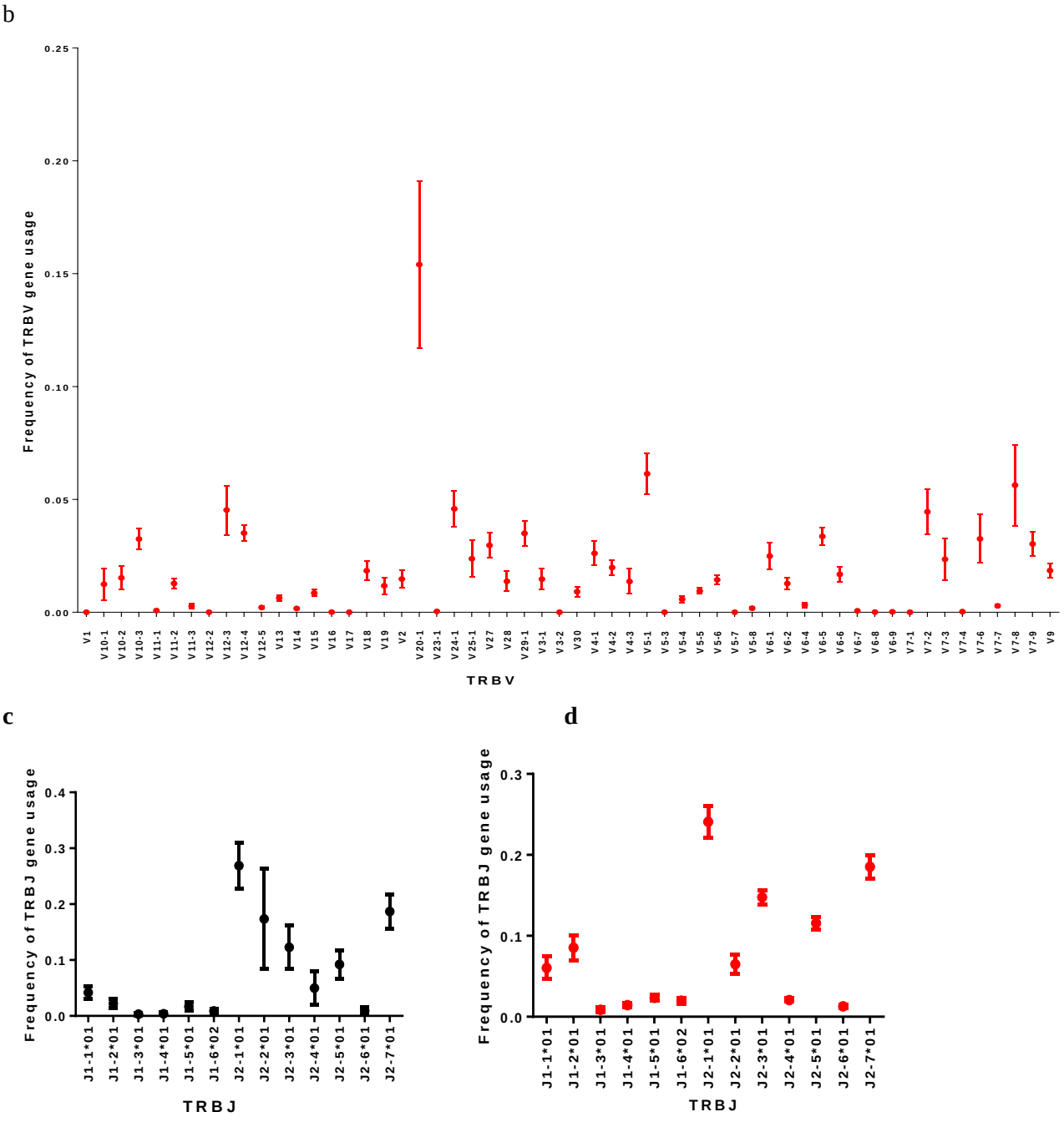


Figure 4.2. Pattern of Vβ and Jβ gene usage. The frequency of Vβ usage within **(a)** aparasitemic **(b)** severe malaria groups. The frequency of Jβ gene usage between **(c)** aparasitemic and **(d)** severe malaria groups. Data are presented as means with standard error.

4.3.3 Divergence in the TCRβ repertoire between study populations.

We further assessed the global differences in the distribution of V, J and VJ gene usage in the study groups using the Kullback-Leibler (KL) divergence for the aparasitemic and parasitemic groups (asymptomatic, uncomplicated and severe malaria group), the asymptomatic and the symptomatic group (uncomplicated and severe malaria) as well as between the uncomplicated and severe malaria group (**Figure 4.3**). We found V, J, and VJ usage in the control group to be more similar to that found in the asymptomatic group compared to the symptomatic groups. In addition, V, J and VJ usage in the asymptomatic group was more similar to that found in the uncomplicated malaria group compared to the severe malaria group.

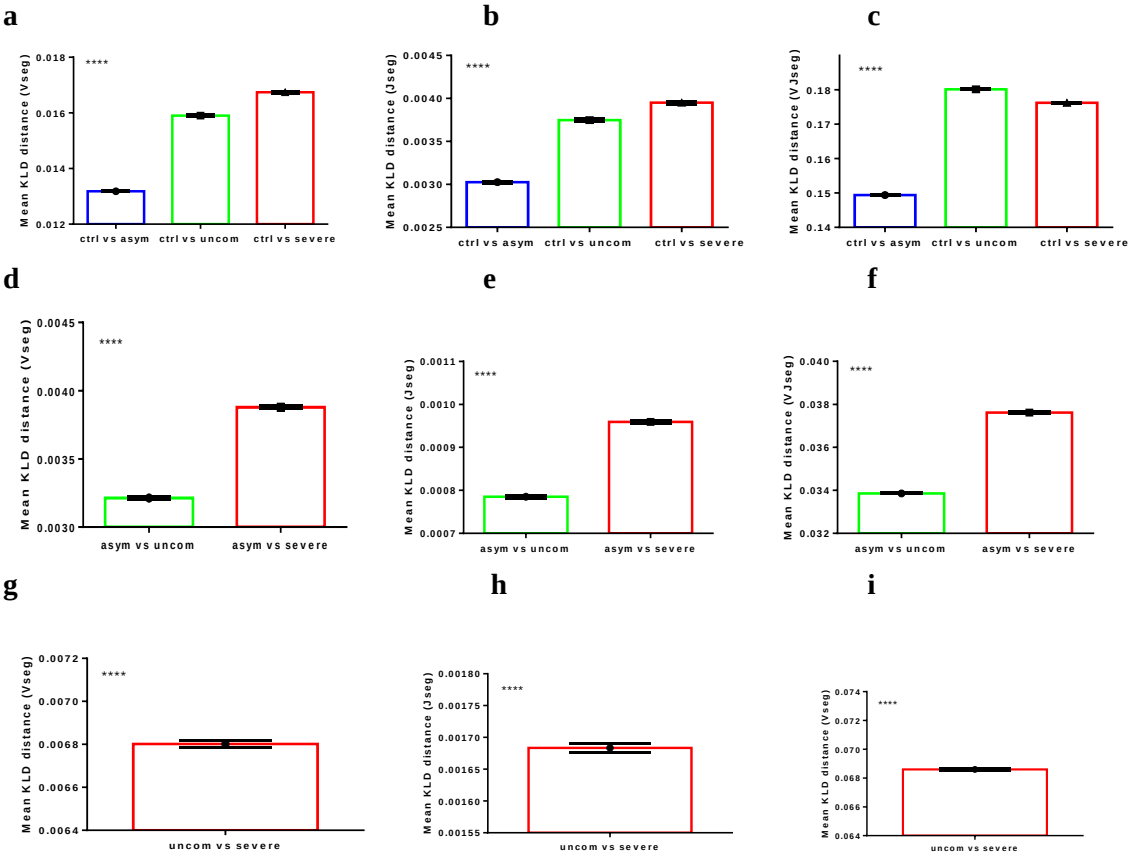


Figure 4.3. Mean Kullback-Leibler divergence for Vβ and Jβ gene segment usage compared between groups. Group differences in gene segment usage measured by

Kullback-Leibler divergence between (**a-c**) aparasitemic and malaria group, (**d-f**) asymptomatic and symptomatic group, (g-i) uncomplicated and severe group for V gene, J gene and VJ gene combinations. The asterisk (*) indicate significant differences, ****($p<0.0001$)

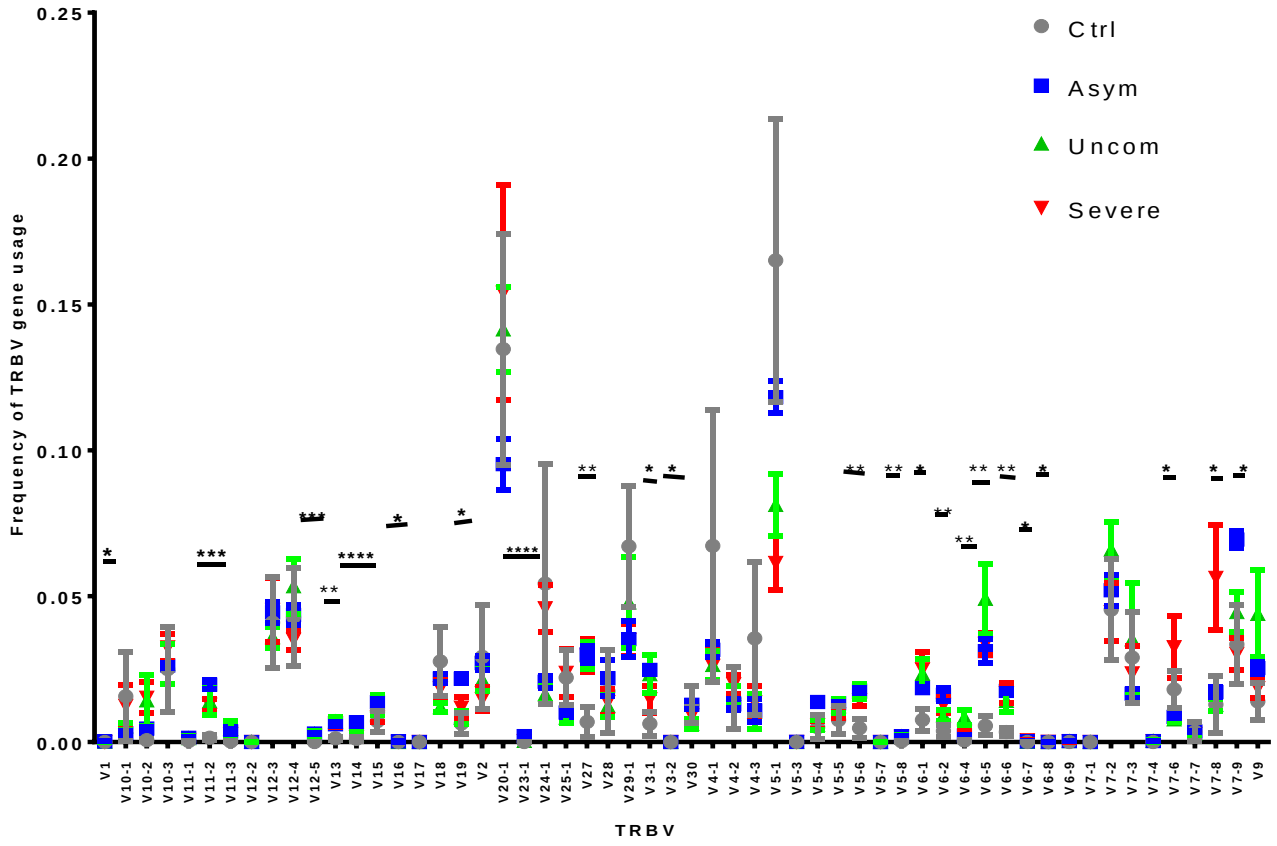
4.3.4 Biased V gene and J gene segment usage during falciparum malaria

The pattern of expression of the individual V gene segments differed significantly between the groups (**Figure 4.4a**). The usage of 23 V genes was significantly different in the *P. falciparum* infected group compared to the aparasitemic group by parametric testing. Within the *P. falciparum*-infected group, the usage of TRBV genes 7-9, 11-2, 14 and 23-1 was higher in the asymptomatic group compared to the uncomplicated and severe malaria groups. TRBV 6-5 usage was higher in the uncomplicated group versus the severe malaria group, whereas the usage of TRBV 7-6 and 7-9 was higher in the latter group versus the former group. These results demonstrate preferential usage of particular V gene segments in each group.

For the Jβ gene segment, significant difference in expression levels for Jβ1-2 ($p<0.0001$), Jβ1-3 and Jβ1-6 ($P<0.05$) (**Figure 4.4b**) were found between the aparasitemic and *P. falciparum*-infected group (asymptomatic, uncomplicated and severe malaria). However, when we compared within the infected group, only the usage of Jβ1-2 was found to be significantly increased in the asymptomatic group compared to the severe malaria group ($p=0.0173$).

We also observed skewed global VJ combinations within the study groups (**Figure 4.5**). For instance, the severe malaria group had dominant combinations of Vβ12-3/Jβ2-7, Vβ7-8/Jβ2-1, and Vβ7-6/Jβ2-1 gene segments (**Figure 4.5d**).

a


Ctrl
Asym
Uncom
Severe
Frequency of TRBV gene usage
0.25
0.20
0.15
0.10
0.05
0.00
TRBV
V1
V10-1
V10-2
V10-3
V11-1
V11-2
V11-3
V12-2
V12-3
V12-4
V12-5
V13
V14
V15
V16
V17
V18
V19
V2
V20-1
V23-1
V24-1
V25-1
V27
V28
V29-1
V3-1
V3-2
V30
V4-1
V4-2
V4-3
V5-1
V5-3
V5-4
V5-5
V5-6
V5-7
V5-8
V6-1
V6-2
V6-4
V6-5
V6-6
V6-7
V6-8
V6-9
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V7-3
V7-4
V7-6
V7-7
V7-8
V7-9
V9

b

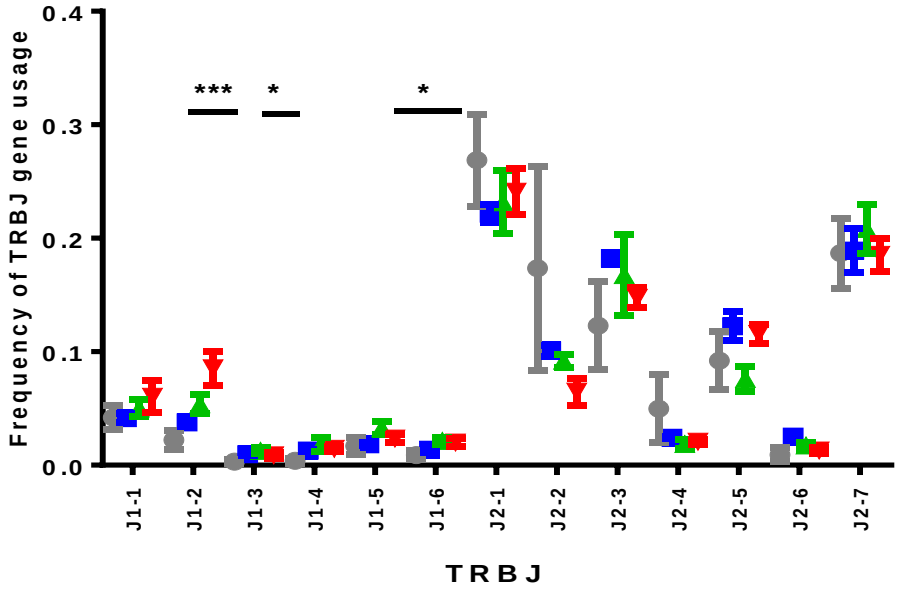
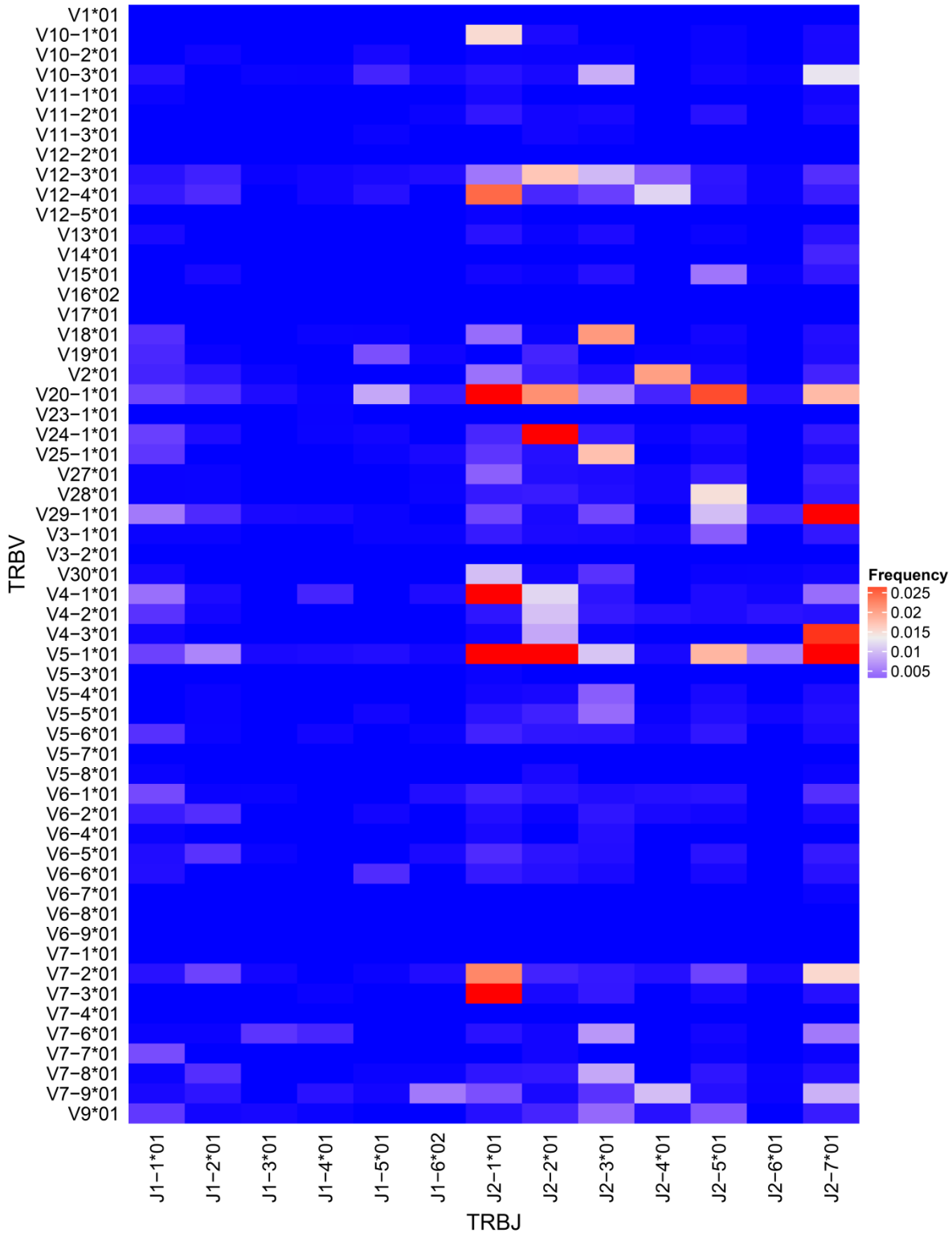
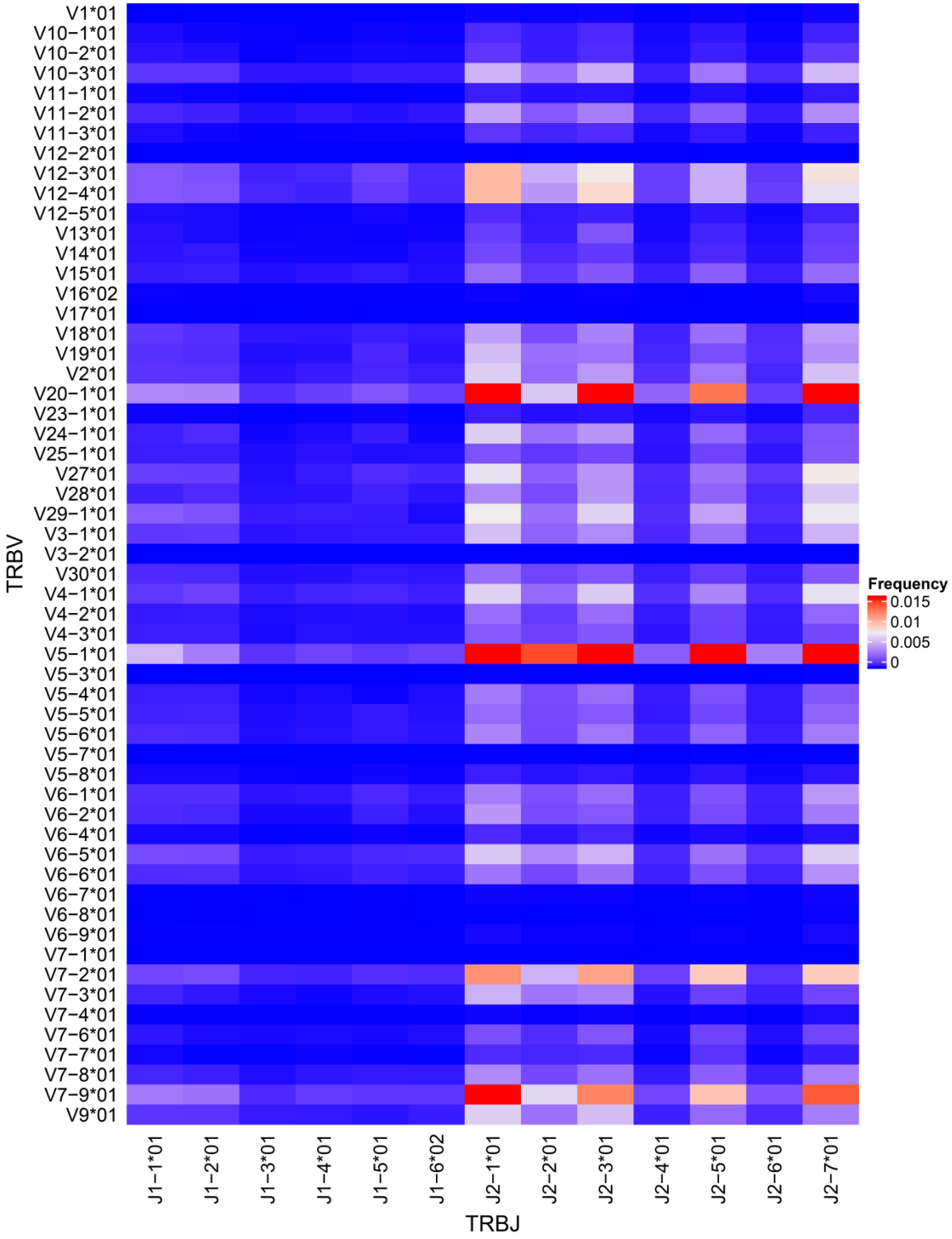


Figure 4.4. **Frequency of V and J gene usage**. The data show the frequency of (**a**) V genes (**b**) J gene usage among the study population. The plots show the mean proportions with the standard error. The asterisk (*) shows the significant difference in the gene usage; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. One-way ANOVA was used for comparisons followed by Holm Sidak's correction for multiple testing.

a


TRBV
V1*01
V10−1*01
V10−2*01
V10−3*01
V11−1*01
V11−2*01
V11−3*01
V12−2*01
V12−3*01
V12−4*01
V12−5*01
V13*01
V14*01
V15*01
V16*02
V17*01
V18*01
V19*01
V2*01
V20−1*01
V23−1*01
V24−1*01
V25−1*01
V27*01
V28*01
V29−1*01
V3−1*01
V3−2*01
V30*01
V4−1*01
V4−2*01
V4−3*01
V5−1*01
V5−3*01
V5−4*01
V5−5*01
V5−6*01
V5−7*01
V5−8*01
V6−1*01
V6−2*01
V6−4*01
V6−5*01
V6−6*01
V6−7*01
V6−8*01
V6−9*01
V7−1*01
V7−2*01
V7−3*01
V7−4*01
V7−6*01
V7−7*01
V7−8*01
V7−9*01
V9*01
J1−1*01
J1−2*01
J1−3*01
J1−4*01
J1−5*01
J1−6*02
J2−1*01
J2−2*01
J2−3*01
J2−4*01
J2−5*01
J2−6*01
J2−7*01
TRBJ
Frequency
0.025
0.02
0.015
0.01
0.005

b


TRBV
V1*01
V10−1*01
V10−2*01
V10−3*01
V11−1*01
V11−2*01
V11−3*01
V12−2*01
V12−3*01
V12−4*01
V12−5*01
V13*01
V14*01
V15*01
V16*02
V17*01
V18*01
V19*01
V2*01
V20−1*01
V23−1*01
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V25−1*01
V27*01
V28*01
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V3−1*01
V3−2*01
V30*01
V4−1*01
V4−2*01
V4−3*01
V5−1*01
V5−3*01
V5−4*01
V5−5*01
V5−6*01
V5−7*01
V5−8*01
V6−1*01
V6−2*01
V6−4*01
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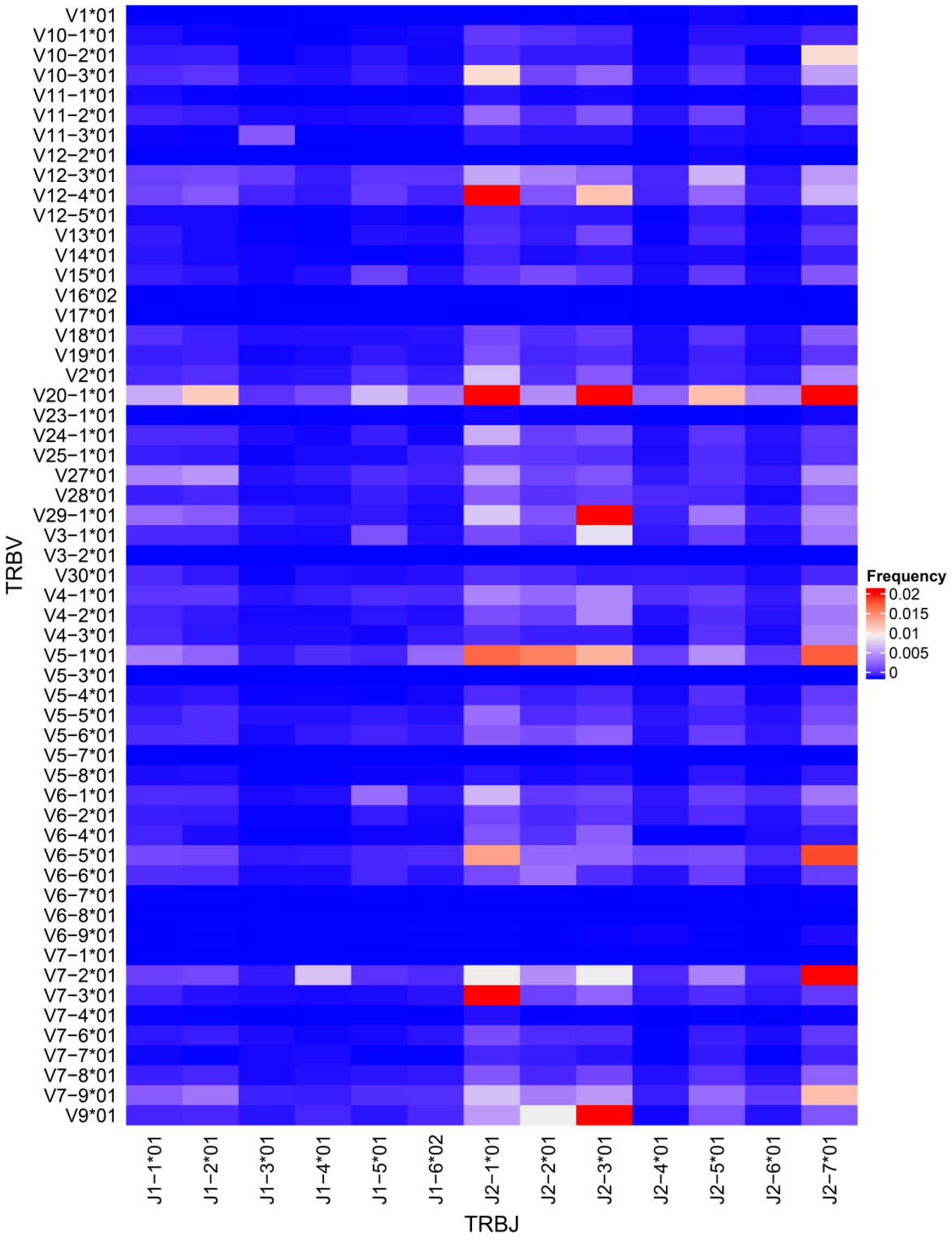
V7−2*01
V7−3*01
V7−4*01
V7−6*01
V7−7*01
V7−8*01
V7−9*01
V9*01
J1−1*01
J1−2*01
J1−3*01
J1−4*01
J1−5*01
J1−6*02
J2−1*01
J2−2*01
J2−3*01
J2−4*01
J2−5*01
J2−6*01
J2−7*01
TRBJ
Frequency
0.015
0.01
0.005
0

c


V1*01
V10−1*01
V10−2*01
V10−3*01
V11−1*01
V11−2*01
V11−3*01
V12−2*01
V12−3*01
V12−4*01
V12−5*01
V13*01
V14*01
V15*01
V16*02
V17*01
V18*01
V19*01
V2*01
V20−1*01
V23−1*01
V24−1*01
V25−1*01
V27*01
V28*01
V29−1*01
V3−1*01
V3−2*01
V30*01
V4−1*01
V4−2*01
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V5−4*01
V5−5*01
V5−6*01
V5−7*01
V5−8*01
V6−1*01
V6−2*01
V6−4*01
V6−5*01
V6−6*01
V6−7*01
V6−8*01
V6−9*01
V7−1*01
V7−2*01
V7−3*01
V7−4*01
V7−6*01
V7−7*01
V7−8*01
V7−9*01
V9*01
TRBV
J1−1*01
J1−2*01
J1−3*01
J1−4*01
J1−5*01
J1−6*02
J2−1*01
J2−2*01
J2−3*01
J2−4*01
J2−5*01
J2−6*01
J2−7*01
TRBJ
Frequency
0.02
0.015
0.01
0.005
0

d

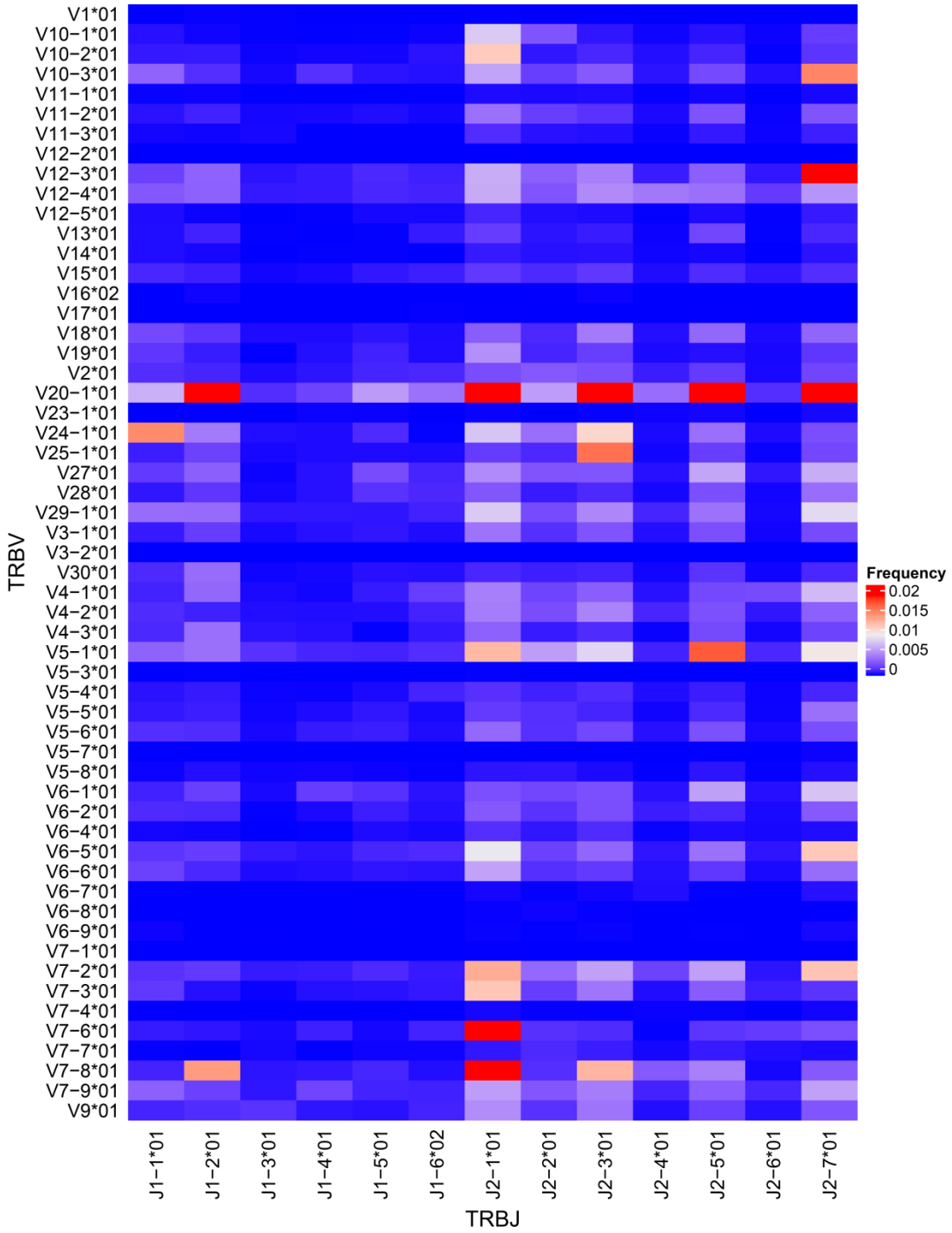

Figure 4.5. Frequency of V and J gene pairing. The VJ repertoire in each group (**a**) control; (**b**) asymptomatic; (**c**) uncomplicated; (**d**) severe malaria group were plotted. The X and Y axes list all possible V and J gene combinations displaying the frequency of usage; with red being the highest frequency, blue representing the lowest frequency.

4.3.5 Characteristics of Vβ and Jβ gene usage after bootstrap resampling

In order to avoid sampling bias which may occur from directly measuring the counts of observed sequences, we further performed bootstrap resampling. This is a non-parametric resampling with 10^5 iterations and we re-analyzed the usage of the various genes (Vβ and Jβ) gene segments between the groups to determine their probability of occurrence. We observed that, at least from resampling, the usage of 24 variable genes (**Figures S4.3a-f**) were significantly different between the aparasitemic and malaria groups (asymptomatic = 34, uncomplicated = 31, severe = 24). In the malaria group, the usage of Vβ gene segments differed between the asymptomatic and uncomplicated (33 Vβ genes), asymptomatic and severe group (32 Vβ genes), and uncomplicated and severe groups respectively (24 Vβ genes).

We also observed significant differences in the expression of specific Jβ gene segments between the control and *P. falciparum*-infected groups; asymptomatic and symptomatic groups; and the uncomplicated and severe malaria groups (**Figure S4.3g-i**).

In addition, we compared the frequencies of the different combinations of TRBV and TRBJ gene usage to their expected values in the various study populations. We observed the selection of specific Vβ and Jβ gene pairs within individuals in each group (**Figure 4.4** and **Figure S4.3**). For instance, the usage of some Vβ and Jβ combinations was common in the various groups (TRBV 20-1; J2-1), whereas, other Vβ and Jβ pairings were specific for the *P. falciparum*-infected group (such as TRBV20-1; J2-3; J2-7, J2-5). Interestingly, significant Jβ1 pairings were observed in only the severe malaria group (**Figure S4.3d**). However, each group had unique V/J gene pairings even after re-sampling as shown in **Figure 4.6**, with high expressions or hotspots indicating high mean frequencies

or usage.

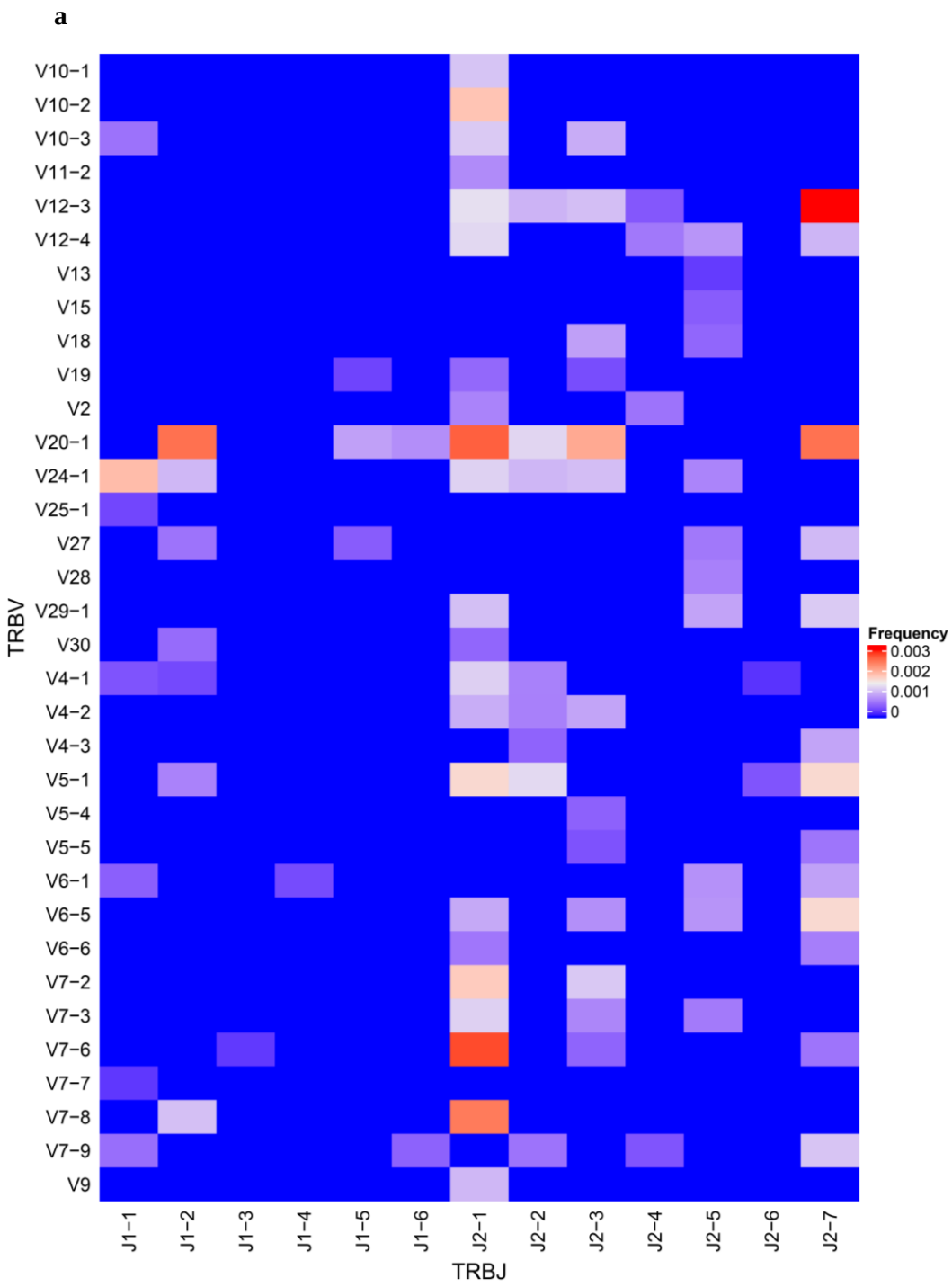

Figure 4.6. V and J gene pairing after bootstrap re-sampling. The distribution of V gene and J gene repertoire for (**a**) control and severe malaria groups. The X and Y axes list all possible V and J gene combinations displaying the frequency of usage; with red being the highest frequency, blue representing the lowest frequency.

4.3.6 Increased richness and diversity during asymptomatic P. falciparum infection

TCR repertoire diversity depends on the species richness and relative abundance of the clonotypes, which in turn shape the T cell response [38, 194]. Diversity is likely to be affected by clonal expansion after antigenic stimulation. To determine the diversity, we used Renyi's entropy which is based on Hill's diversity profiles, in order to obtain a continuum of diversity indices based on a range of alpha values. At smaller alpha values, the diversity estimate accords a greater weight to low-abundance TCRs than it does at larger alpha values. In fact, at larger alpha values the diversity estimate is determined almost exclusively by the clonally expanded TCRs. Comparison of the infected groups yields some striking observations. Firstly, all the infected repertoires have a relatively high TCR richness, ranging from 5870.88 TCRs in the severe malaria group to 59870.3 TCRs in the asymptomatic malaria group. As alpha increases from 0 to 20, the diversity estimate declines in all groups (**Figure 4.7a**), as it accounts for increasingly smaller subsets of TCRs with high frequencies. In all these TCR subsets, diversity is higher in the asymptomatic group compared to the other groups (**Figure 4.7a**). In contrast, while richness is higher in the uncomplicated malaria group compared to the severe malaria group, the diversity of clonally expanded TCRs is higher in the severe group (**Figure 4.7a**). Strikingly, for the most inclusive estimates of diversity (corresponding to alpha values of 0-1.6), diversity is inversely correlated with parasitaemia (Spearman's correlation; -0.4794—0.5223; $p<0.05$, **Figure 4.7b**).

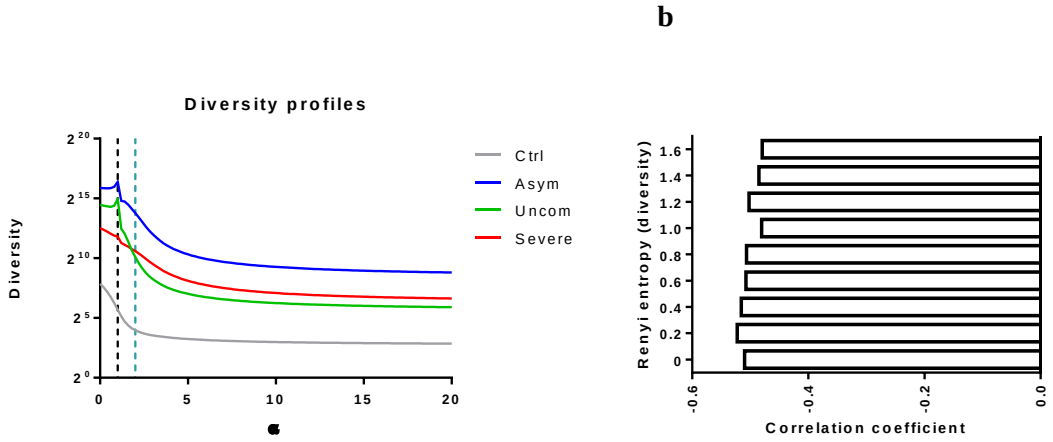

Figure 4.7. Increased diversity during asymptomatic *P. falciparum* infection. (**a**) The diversity of the CDR3 measured using Renyi's entropy. The black dotted lines indicate the Shannon's entropy and the blue dotted lines indicate the Simpson's index of diversity. (**b**) The bar plot displays the significant correlation ($p<0.05$) between the diversity and parasitaemia in the malaria-infected group.

4.3.7 Convergent recombination events in the T cell repertoire

Next, we determined the degree of convergent recombination in creating public or private clones. We observed that the frequency of recombination was higher in the aparasitemic group compared to the asymptomatic and uncomplicated malaria groups ($p<0.05$; **Figure. 4.8b**). In order to understand the genetic manipulations that may be associated with the development of public TCR clones, we determined the degree of convergent recombination in both public or private clones. However, since public clones were only predominant in

the asymptomatic group (**Figure S4.5b**), we could only compare the global recombination events between these clones (public and private) for this group. Even though the frequency of convergent recombination in the TCRs was elevated within the public clones, no significant difference was observed ($p=0.0511$; **Figure S4.5d**).

We further determined whether there is a significant difference between groups in the number of recombination events that exceed different thresholds ($r = 3, 5, 10, 15$) (**Figure S4.5c**). We observed, a trend of increased recombination events within the asymptomatic group compared to the other groups. However, there was a significant difference between the groups only for large values of r ($r =5$ and 15, $p<0.05$) indicating that the asymptomatic groups have a greater occurrence of sequences with a large number of convergent recombination events.

Focusing on the public and private clones found in the asymptomatic group, we determined the number of convergent recombination events greater than or equal to different r thresholds, $2\leq r \leq 15$. Interesting, as r increased from 2 to 15, it was observed that the number of convergent recombination events decline rapidly in private clones, reaching 0 at r=9 (**Figure 4.8c**). In contrast, the number of recombination events decline much more slowly in the public clones. These observations are consistent with the existence of a greater likelihood of producing public clones during V(D)J recombination as well as a greater degree of antigen-driven selection of the same clones in the studied asymptomatic individuals.

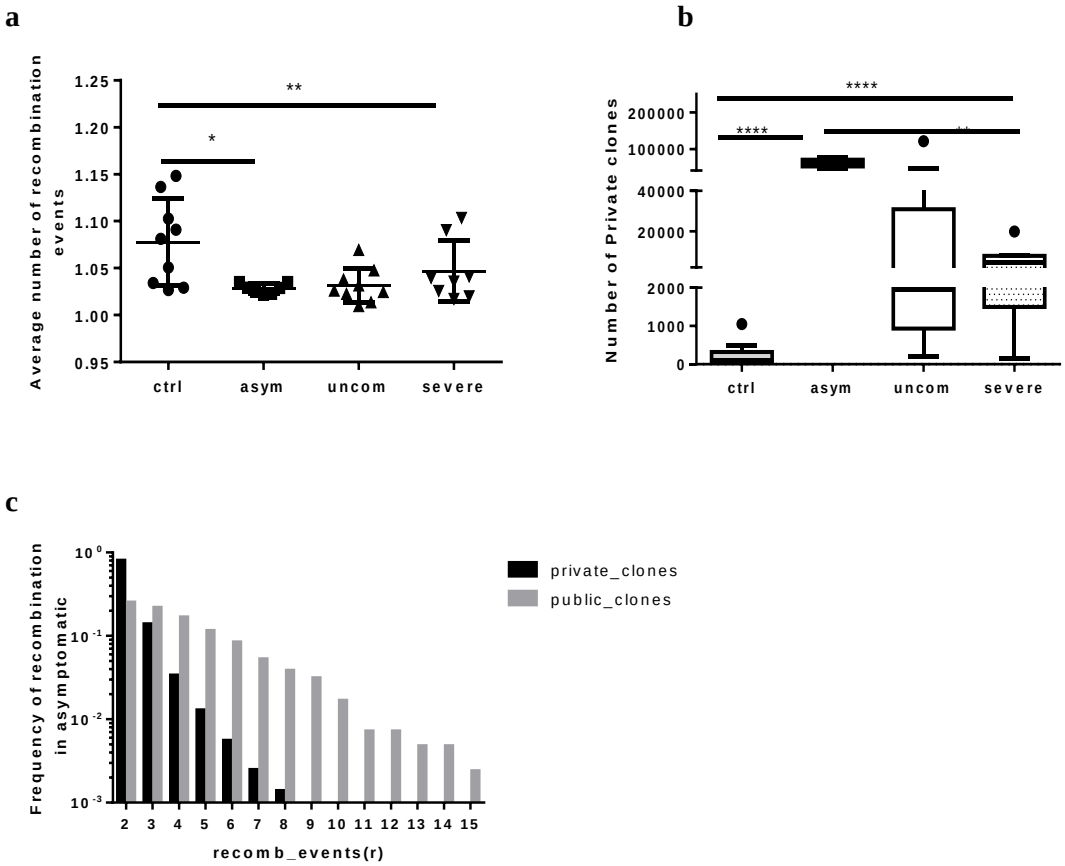

Figure 4.8. Frequency of recombination events in public and private clones. (**a**) The scatter plot denotes the frequency of recombination events found in each data set. The plot shows the mean and standard deviation. The number of (**b**) private clones found in individuals in each group. (**c**) Degree of convergent recombination events in the public and private clones occurring in the asymptomatic group at threshold of 2<=r<=15, recombination events. The plot shows the mean and standard deviation. Data presented in boxplot shows inter-quartile ranges with horizontal lines indicating the median. The asterisks (*) indicate statistical significance; ****($p<0.0001$), ***($p<0.001$), **($p<0.01$), *($p<0.05$).

4.3.8 Asymptomatic TCR repertoire shares an increased number of CDR3 sequences

Recombination biases result in the generation of public or private clones. TCR clones are termed “public” when they are found in many individuals and termed “private” when they are found in few individuals. We analyzed the number of TCR sequences that occur in at

least 75% of individuals in each group which we classified as public T cell clones. We found 318 public T cell clones in the asymptomatic group (**Figure S4.5b**), compared to only one in the severe group and none in the control and uncomplicated groups.

4.3.9 Grouping CDR3 sequences based on their antigenic specificity

Using GLIPH (grouping of lymphocytes based on paratope hotspots), we interrogated our datasets to determine which set of TCR sequences have similar antigen specificity. We compiled a list of the top 100 most abundant clones from each infected individual and then clustered them using GLIPH. We obtained a total of 1846 specificity clusters. We looked for clusters with four unique clones found in at least 3 individuals. There were 9 such clusters containing a total of 46 unique clones (**Table 4.2**). We found a specificity cluster, with the representative clone CASCEADRDTDTQYF, that appears in a much higher fraction of the asymptomatic group (57%) compared to the uncomplicated (22%) and severe (0%) groups. This specificity cluster contains a 4-mer amino acid sequence motif with a DRD nucleus. In contrast, a specificity cluster with the representative clone CASSLEPSRTYNEQFF appears in a much higher fraction of the severe group (75%) compared to the uncomplicated (67%) and asymptomatic (43%) groups.

We aligned these specificity clusters to TCR sequences found in the McPAS-TCR catalogue [195] to determine if any of these specificity groups may have an already existing pathological conditions or specificities in either human or mice. None of the CDR3β sequences were found to be associated with any known pathologies. We found that the PDW motif, which is enriched in our TCRs from P. falciparum-infected individuals, is also used by human CD8 T cells that recognize the Protein Nef in HIV.

Table 4.2: TCR specificity clusters observed in the *P. falciparum*-infected cohort.

Specificity cluster	CDR3 clones	Motifs	TRBV	TRBJ	Patient proportion asym(n =7)	uncom (n =9)	seve (n=8)	Freq (mean)	Freq (SD)
CASSLVAVTDTQYF	casslvatqetqyf		V7-8	J2-5				1.63E-05	9.65E-05
	cassliaaqetqyf		V7-8	J2-5				5.45E-05	0.000201
	casslvaaqetqyf		V7-8	J2-5				1.16E-05	5.25E-05
	cass**LVAV**hneqff	LVAV	V7-8	J2-1	4	3	3	4.65E-05	0.000155
	cass**LVAV**nyneqff	LVAV	V7-9	J2-1				8.40E-06	4.97E-05
	cass**LVAV**qetqyf	LVAV	V7-8	J2-5				1.01E-05	4.58E-05
	casslvasqetqyf		V7-8	J2-5				1.05E-05	5.40E-05
	cass**LVAV**tdtqyf	LVAV	V7-8	J2-3				1.98E-05	0.000106
CASCEADRDTDTQYF*	casslg**DRDA**dtqyf	DRDA	V7-9	J2-3				0.00011871	0.0007
	csve**ADRD**stdtqyf	ADRD	V29-1	J2-3				1.23E-05	7.25E-05
	castf**DRDA**rneqff	DRDA	V28	J2-1				6.49E-06	3.84E-05
	cast**ADRD**ypkniqyf	ADRD	V12-3	J2-4	4	2	0	1.78E-05	0.000106
	cats**DRDA**niqyf	DRDA	V15	J2-4				7.97E-06	4.72E-05
	casce**ADRD**tdtqyf	ADRD	V12-4	J2-3				5.06E-06	2.99E-05
	csar***ADRDA***yneqff	ADRD, DRDA	V20-1	J2-1				0.00010187	0.000557
CASSLEPSRTYNEQFF	cassl***G PSRT***nneqff	GPSR, PSRT	V7-3	J2-1				4.64E-05	0.0002
	casnl***G PSRTY***neqff	GPRS, PSRT, SRTY	V7-3	J2-1				0.00011161	0.00066
	cassle***PSRTY***neqff	PSRT, SRTY	V7-3	J2-1	3	6	6	8.28E-06	4.90E-05
	cassm***G PSRTY***neqff	GPRS, PSRT, SRTY	V7-3	J2-1				0.00017257	0.000972
	ctssl***G PSRTY***neqff	GPRS, PSRT, SRTY	V7-3	J2-1				8.28E-06	4.90E-05
	cassl***G PSRTY***neqff	GPRS, PSRT, SRTY	V7-3	J2-1				0.01195658	0.032472
CASSLKRNAYEQYF	cass**LKR**vtnsplhf	LKR	V7-3	J1-6				4.49E-05	0.000266
	csa**LKR**eradtqyf	LKR	V20-1	J2-7	2	1	3	2.49E-06	1.48E-05

	cass**LKR**drvasyeqyf	LKR	V4-1	J2-7				0.0001616	0.000708
	cqq**LKR**ggrgdpvf	LKR	V7-2	J2-5				3.80E-07	2.25E-06
	cass**LKR**nayeqyf	LKR	V27	J2-7				5.64E-05	0.000334
CASSQEYSSGGRYEQYF	cas**SQEY**rvfyeqyf	SQEY	V4-1	J2-7				2.07E-05	0.000123
	cas**SQEY**asqpagplhf	SQEY	V4-3	J1-6				5.78E-05	0.000342
	cas**SQEY**rifyeqyf	SQEY	V4-1	J2-7	2	3	0	1.39E-05	8.23E-05
	cas**SQEY**ssggryeqyf	SQEY	V4-2	J2-7				0.00016951	0.000711
CASSEDRADQPQHF	cass**EDRA**dqpqhf	EDRA	V12-4	J1-5				8.46E-05	0.000501
	cassqp**EDRA**ntgelff	EDRA	V4-1	J2-2				0.00011343	0.000602
	csv**EDRA**ryeqyf	EDRA	V29-1	J2-7	2	3	0	3.37E-06	2.00E-05
	cassq**EDRA**yyeqyf	EDRA	V4-1	J2-7				1.42E-05	8.41E-05
CASSKMTGASTDTQYF	casslal**MTGA**qetqyf	MTGA	V5-1	J2-5				3.37E-06	2.00E-05
	cassk**MTGA**stdtqyf	MTGA	V3-1	J2-3				5.41E-05	0.000306
	casssy**MTGA**dteaff	MTGA	V5-1	J1-1	1	2	1	1.75E-05	0.000104
	cassq**MTGA**stdtqyf	MTGA	V4-1	J2-3				7.45E-05	0.000401
CSASLIGWNGGEQFF	cassypgt**NGGE**lff	NGGE	V6-5	J2-2				1.62E-05	9.59E-05
	csaslfg**W*NGGE***qff	WNGG, NGGE	V20-1	J2-1				0.00130294	0.004429
	csaslig**W*NGGE***qff	WNGG, NGGE	V20-1	J2-1	1	4	2	3.09E-05	0.000183
	csasifg**W*NGGE***qff	WNGG, NGGE	V20-1	J2-1				2.61E-05	0.000155
CASVPDWGTGELFF	csa**PDW**sneqff	PDW	V20-1	J2-1				0.00019368	0.000773
	casv**PDW**gtgelff	PDW	V6-1	J2-2				2.49E-05	0.000147
	csa**PDW**sygvgeqyf	PDW	V20-1	J2-7	1	3	2	1.23E-05	7.30E-05
	csa**PDW**nneqff	PDW	V20-1	J2-1				0.0001142	0.000449

Red coloured letters indicate predicted binding motifs with 10 fold enrichment of probability < 0.001. The asterisk (*) indicate specificity cluster that significantly occur($p<0.05$) at unequal distributions in the groups.

4.4 Discussion and Conclusion

The role of T cells is crucial in malaria infection, with dynamic changes occurring in both CD4 and CD8 T cell activity in response to the pathogen both in human and animal models [92]**.** Usually, the activity of malaria-specific T cells has been measured by medium throughput approaches such as ELISPOT and flow cytometry. Studying human malaria-specific T cells through next- generation sequencing approach to obtain information such as the diversity of the TCR repertoire, gene usage, convergent recombination events and grouping them into antigen specificities has never been performed. However, this information is necessary to help identify and target antigen- specific responses in malaria infections to improve the performance of malaria vaccine candidates. Using 5'RACE amplification and bulk TCRβ sequencing, we have characterized the dynamic changes that may occur during *P. falciparum* malaria infection in children with asymptomatic, uncomplicated and severe disease. From our data summary, due to the low number of sequencing depth (under sampling) observed in the aparasitemic group, which may affect the measurement of TCR diversity [222, 223], our conclusions are mainly drawn within the *P. falciparum*-infected group. We show that TCR repertoire within the malaria-infected group differs in diversity, clonality, TCR sharing and gene segment usage. In addition, the diversity of the repertoire is inversely correlated with parasitaemia. Strikingly, the magnitude of TCR sharing was very high between the asymptomatic cohort.

Additionally, the increased number of unique sequences observed in the asymptomatic group suggested increased diversity which may further suggest a polyclonal response upon antigen stimulation. Thymic selection affects the T cell repertoire as well as the CDR3 distribution and length and the specificity of the TCRs in recognizing antigens

[224]. The length of the CDR3 sequence may affect the folding property of the TCR loop [225], which may result in confirmation change in the TCR as well as the receptor interaction with MHC molecules [226]. Shorter CDR3 lengths have been associated with pathologic outcomes in autoimmune diseases such as ulcerative colitis [227] and diabetes [51, 228]. Apparently, previous studies in mice have identified a role for pathogenic T cells in the development of severe malaria (cerebral malaria) under the inflammation hypothesis [112, 229]. These pathogenic T cells have been restricted to those expressing the Vβ 8.1 TCR gene segment [216, 217, 230]. In this study, we found shorter CDR3 lengths in the severe malaria group compared to the other groups. Whether this intriguing observation is relevant to the pathogenic role of T cells during human cerebral malaria infection deserves further study.

The usage of Vβ and Jβ gene segments have been described to be non-uniform in healthy controls. The extent of repertoire skewing within the malaria-infected group varied significantly with clonal dominance observed in specific T cell clones. The preferential use of selected TCR Vβ and Jβ gene segments could be as a result of *P falciparum* antigen stimulation leading to the expansion of specific T cell clones. Likewise, the selective usage of gene segments may be influenced by the severity of the infection or disease. Also, the segments which were present in all infected groups could reflect gene segments selection by a common antigen present in all the infected groups. These observations are consistent with the view that malaria infection influences the TCR repertoire resulting in the activation and expansion of specific T cell clones. Delineating the immune response from these specific clones may help in identifying the antigens stimulating the activation and expansion of such specific TCRs. Also, the consistently lower KL divergence observed for

the J gene segment usage amongst all groups could be as a result of the low number of genes in the Jβ gene cluster compared to the 56 Vβ gene segments. Among the infected group, we observed increased diversity in the asymptomatic group compared to uncomplicated and severe groups. Because a more diverse repertoire is better able to protect against a large diversity of pathogen-derived antigens, this observation is consistent with the absence of symptoms in the asymptomatic group [231]. The decreased diversity in the uncomplicated and severe malaria group could be as a result of oligoclonal T cell response during infection. It could also be that the low parasitaemia levels may have minimal impact in affecting the TCR diversity. Likewise, higher parasitaemia levels may create T cell perturbations due to increased activation, exhaustion and apoptosis (Chapters 2 and 3), which are more likely to decrease TCR diversity as found in the diseased groups.

The frequency and development of public clones are mostly predicted during convergent recombination and germline somatic recombination [232]. Public T cell clones have been proposed to be crucial for preservation of tolerance to self-antigens [56], shared to respond to same pathogen [233]. Importantly, public TCRs have been associated with favorable outcomes from disease [58] as well as immune escape [234]. Public TCR clones may be expanded during antigen stimulation either via an infection or vaccination [235, 236]. Apart from the asymptomatic group, the degree of TCR sharing among the infected group was rare in the uncomplicated and severe groups. This may be due to exposure to the large antigenic repertoire of *P. falciparum* antigens, coupled with diversity and polymorphisms of the parasite. In addition, public T cell responses have been denoted to be cross-reactive [235], therefore, providing for an improved protection against disease. Apparently, understanding the mechanism of public TCRs will inform in the development of interventions.

TCRβ sequences with same specificity tend to harbor short sequence motifs which

are conserved [237] with predicted interaction with antigenic peptides [221]. We found multiple groups of TCR sequences predicted to have similar antigen specificities in our infected group. Interestingly, some specificity groups were more prevalent in the symptomatic group. However, we could not determine if these clusters were HLA specific. Nevertheless, it will be fascinating if these CDR3β similarity clusters can be validated since this has the potential to aid in the identification of antigenic peptides.

Intriguingly, the complexity of the TCR repertoire observed during *P. falciparum* infections may explain some of the complexities observed in immune responses to the pathogen and help tailor specific interventions.

Chapter Five

5.0 General discussion and conclusions

Despite continuous advances in medicine, children remain vulnerable to infectious diseases. According to World Health Organization (WHO), morbidity in children under 5 caused by infections was 4.4 million in 2018 [238]. Malaria, an infectious disease of public health importance, is endemic in sub-Saharan Africa, where it causes serious morbidities in children and non-immune adults. It has been observed that the immune system of children is less robust, making them prone to infectious diseases such as malaria. However, only a handful of studies has focused on understanding the immune repertoire in children with malaria using high-throughput approaches. Indeed, to our knowledge there has been no previous study that focused on understanding the quantitative response of T cells to falciparum infections in children using immune repertoire sequencing. To address this knowledge gap, the studies described in this thesis were designed to determine the T cell perturbations that occur during *Plasmodium falciparum* infections in children under the age of 12 years living in a malaria endemic area. We used both flow cytometry and next generation sequencing approaches to profile the changes occurring in T cells during infection.

The human immune system is constantly challenged with a wide array of pathogens which it identifies and combats with the help of myriad immune cell receptors that are somatically generated. The activity of T cells during infections is crucial since T cells fight infections directly and also indirectly by controlling the activity of other immune cells. T cell activation is essential for T cells to proliferate to attain frequencies that are necessary for effective responses to infections. T cell activation and differentiation are intertwined

as, for example, a subset of activated T cells eventually differentiate into memory T cells. Nevertheless, prolonged T cell activation has been associated with poor disease outcome.

A number of outcomes were generated from the data analyzed from this thesis. We have shown that symptomatic malaria is associated with increased T cell activation that correlates with immune regulation. In Chapter 2, we showed that clinical malaria is associated with both activation and immune regulation, resulting in T cells which are unresponsive when further stimulated with iRBCs. This contrasted with what we observed in asymptomatic children, whose PBMCs significantly increased in number upon stimulation with iRBCs. Using a machine learning model, we showed that levels of T cell activation and regulation govern parasitaemia and disease status. Also, the low levels of activation and regulation in the asymptomatic group was quite interesting, since initially it could be presumed that the asymptomatic group are more likely to have an increased suppressive response. However, neither the expression of markers associated with activation, regulation, exhaustion or senescence were significantly upregulated in the asymptomatic group compared to the symptomatic. Importantly almost all levels in the asymptomatic were relatively comparable to the healthy controls, supporting the view that development of clinical symptoms is associated with the degree of T cell activation and regulation.

Sustained antigen exposure over a sufficiently long time eventually causes T cells to lose their replicative ability and become exhausted, leading to decreased immune function or effectiveness. In fact, with the application of a mathematical model, T cell exhaustion has been shown to be closely related to T cell senescence [213]. In Chapter 3,

we showed that clinical malaria is associated with increased expression of exhaustive markers as well as senescence markers in T cells which were more pronounced on CD8 T cells. We further found that exhaustive markers play compensatory regulatory roles and are negatively associated with inflammation. This may indicate that malaria infection per se plays a key role in the premature ageing of the immune system which occurs profoundly on CD8+ T cells. This has implications in the acquisition of immunity to not just malaria but other infectious diseases, creating the need for early diagnosis and treatment of the infection. In addition, it is likely to have profound impact on the efficacy of malaria interventions.

Furthermore, in Chapter 4 using immune repertoire sequencing data we found a direct association between T cell diversity and parasite load. This indicates that increasing parasitaemia may select for an oligoclonal T cell response Also, low TCR diversity has been directly linked to immune dysregulation, mechanisms that are associated with disturbing the immune homeostasis [239]. Dysregulation which occurs as a result of immune imbalance is triggered by factors such as increasing T cell activation and regulation [94, 130], including the expression of exhaustive [166, 194] and senescent markers on T cells [178, 240]. All these factors could have contributed to the low diversity observed in the uncomplicated and severe malaria groups when compared to the asymptomatic group. These patterns we can say may be associated to the increased clonotype diversity as well as the prevalence of public/shared clones found in this group. This signifies the importance of T cell selection during an immune response. In addition, prevalence of public clones has been related to increased convergent recombination [241] and TCR production [236] which contribute to a positive outcome from disease. Finally using the GLIPH (grouping of

lymphocytes interactions by Paratope hotspots) algorithm [221], we identified TCRs that share antigen specificities. These TCRs have predicted binding motifs which are more likely to interact with antigenic peptides.

Also, another interesting outcome was the increased number of TCR public clones observed in the asymptomatic group. This is very intriguing since it went on to confirm that the development of anti-disease immunity to malaria is associated with prevalence of public clones that are mostly cross reactive to various antigens, with the potential to suppress parasite replication. We further believe our data explains some of the complexities that is associated with malaria infection and its impact on host T cell responses. With immune repertoire sequencing approach, we observed biases in V and J gene usage, as well as significant differences in the CDR3 nucleotide length among study groups. Noticeably, the CDR3 nucleotide length in the severe malaria groups were of shorter length, which was quite fascinating due to the reports of pathogenic T cells being involved in the development of severe disease in murine models [188, 189, 204], implicating genetic association linking susceptibility to disease status.

In conclusion, we show that interrogating host immune responses using repertoire sequencing provides a variety of information that helps explain the complexities of host pathogen interactions. In addition, coupled with applied mathematics, predictions as well as new hypothesis can be made, generating more insight into mechanisms of disease.

5.1 Future studies

The application of TCR sequencing to understand diseases provides important information that is relevant for designing therapeutics and interventions. There remains much follow-

up research to be done based on the information provided in this thesis. Firstly, there is a need to validate the TCR specificity groups we identified in patients with *P. falciparum* infections. Validating such TCR sequences would provide information about their cognate antigenic ligands and HLA restrictions, decreasing the amount of time it normally takes to find antigenic peptides for vaccine design. Secondly, additional information about the TCR response to falciparum infections will come from studying longitudinal cohorts with multiple sampling time points – e.g. before, during and after the malaria season. Such a longitudinal cohort study would provide more samples for in- depth sequencing coverage. It would make it possible to track the generation and maintenance of T cell repertoire, identifying TCR clones contributing to protective immunity and the series of infections required to generate such protective TCRs. Finally, it would be important to study both the α and β chain to provide a more complete picture of the structural determinants of specificity to *P. falciparum*. This kind of follow-up study is important since understanding the mechanism of cellular immunity to malaria through high throughput and predictive approaches will help improve malaria vaccine design strategies and enhance the discovery of vaccine candidates with an increased breadth of protection.

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APPENDICES

Appendix 1 Supplementary data for Chapter 2

Table S2.1 List of antibody panels

Antibody	Clone
Anti-CD3	SK7
Anti-CD4	SK3
Anti-CD8	SK1
Anti-CD25	M-A 251
Anti-CD28	CD28.2
Anti-CD57	NK-1
Anti-CD69	FN50
Anti-CD279	EH12.1
Anti-CD152	BN13
Anti-FOXP3	206D

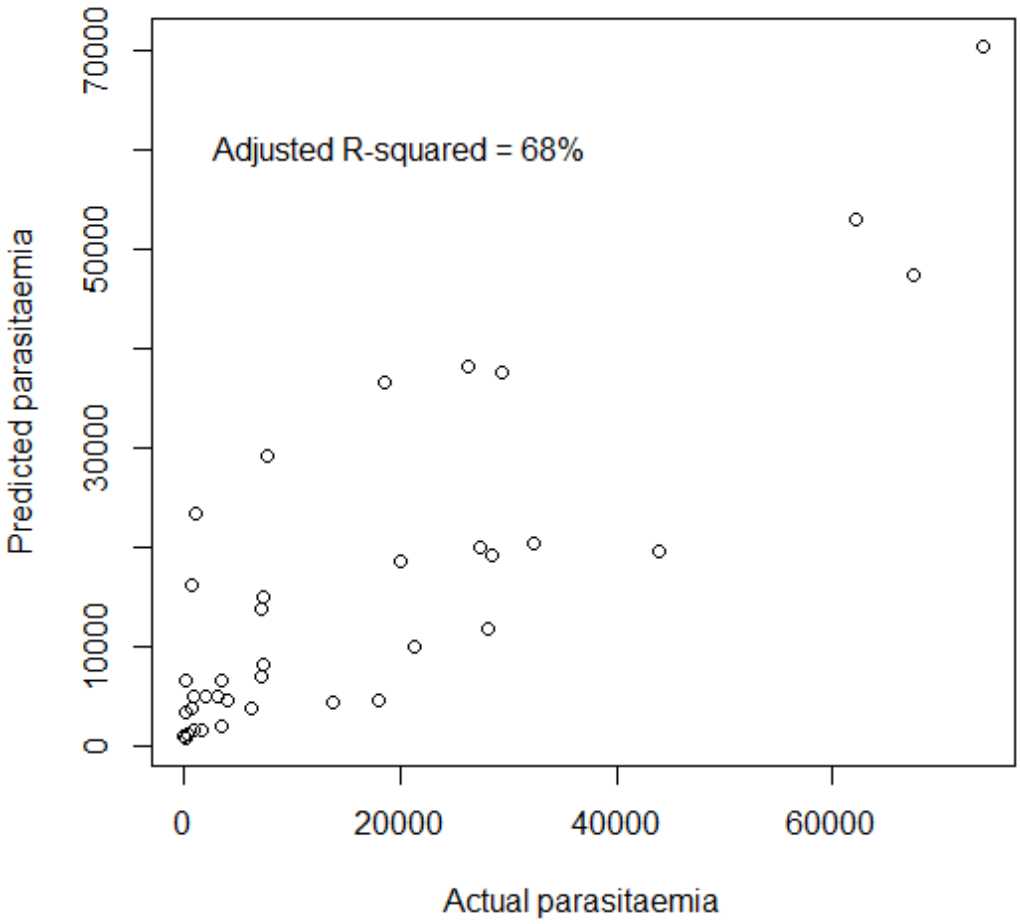


Figure S2.1 T cell activation and regulatory markers govern parasitaemia. We analyzed a dataset consisting of levels of parasitaemia and of 24 T cell phenotypic markers measured in PBMCs from 57 children, before and after stimulating the PBMCs with *P. falciparum*-infected red blood cells (iRBCs). Two asymptomatic children had no parasitaemia data and were excluded from the analysis. An initial correlation analysis identified significant Spearman's rank correlations between parasitaemia vs. levels of CD8+CD69+ (r=0.4128658, P=0.0016) and CD8+CD25+CD69+ (*r*=0.4070214, *P*=0.0018) T cells measured before iRBC stimulation, and levels of CD4+CD25+Foxp3+ (*r*=0.4772815, *P*=0.0002) and CD8+CD25+Foxp3+ (*r*=0.4772714, *P*=0.0003) T cells measured after iRBC stimulation. Significance of the correlations was determined using a permutation test. We next performed a linear regression analysis to determine the amount of variation in parasitaemia explained by levels of these four T cell phenotypes in the 41 asymptomatic and symptomatic children. A diagnostic test identified one out of these 41

children to be an outlier (see **Figure S2.2**). After removing this outlying child, levels of the four considered phenotypes were able to explain 68% of the variation in parasitaemia. In the plot, parasitaemia levels measured in the children are shown on the x-axis while the levels predicted by the regression model are shown on the y-axis.

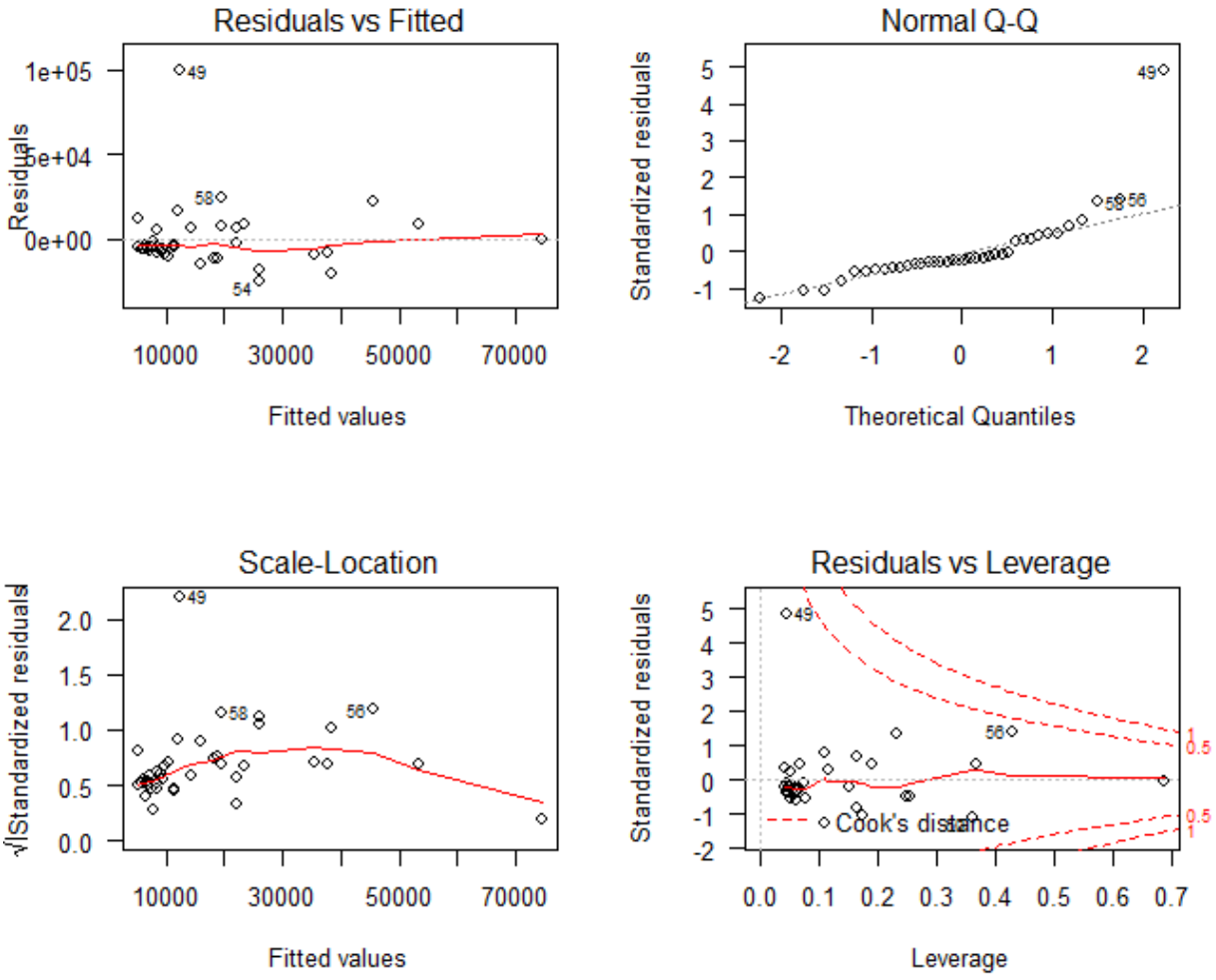
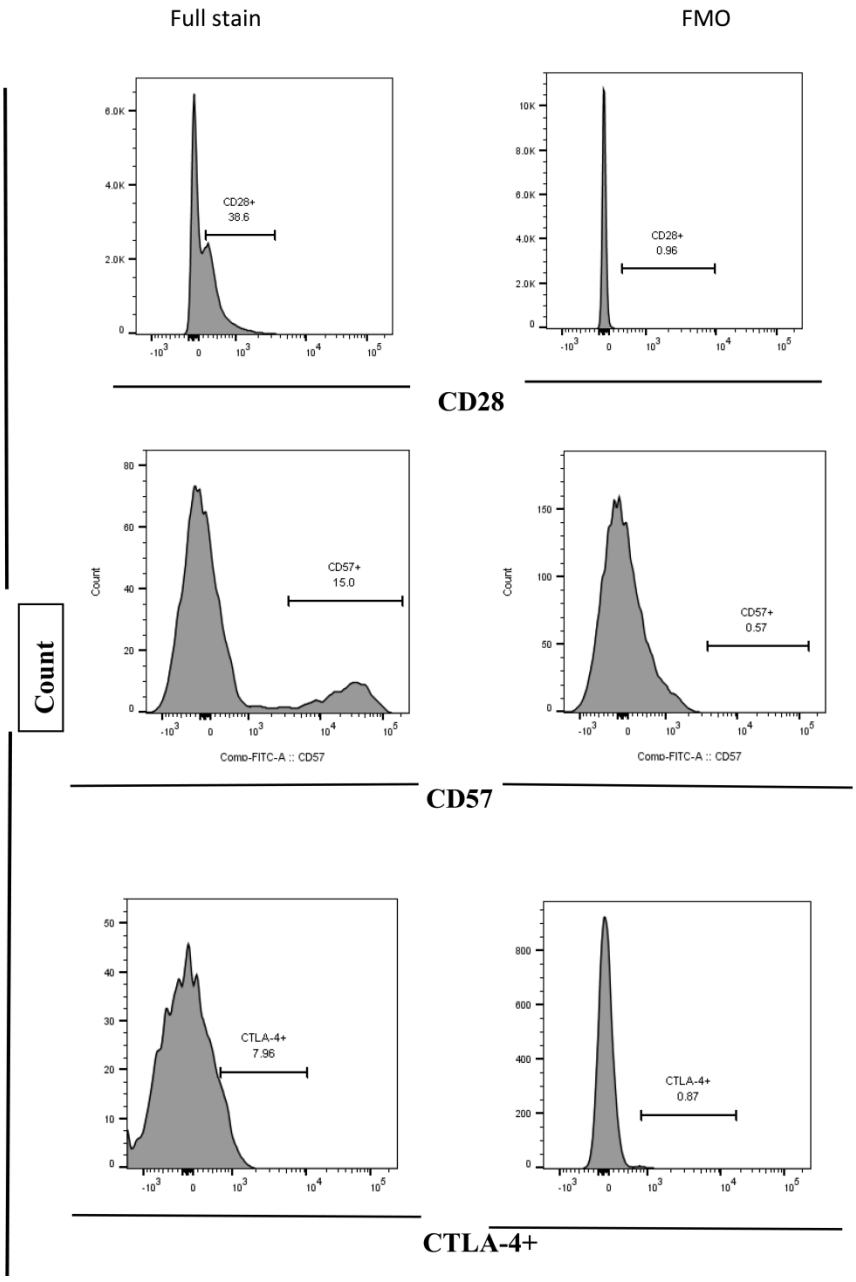


Figure S2.2 Diagnostic plots for the regression analysis described in the legend of Figure S2.1 The plots reveal data points from one of the subjects (subject #49) to be outliers. This subject was excluded from the regression analysis.

Appendix 2 Supplementary data for Chapter 3



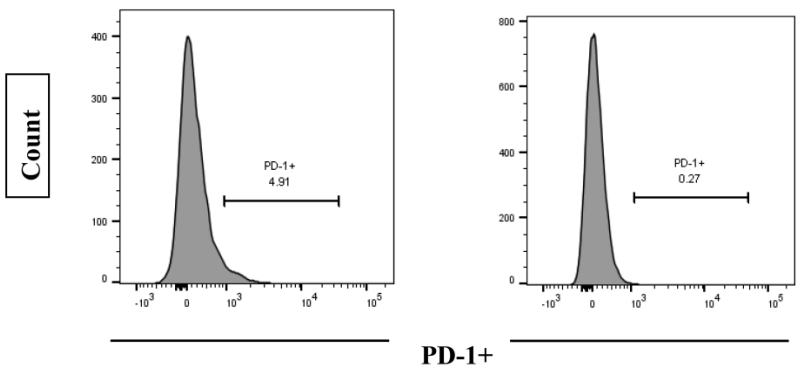


Figure S3.1 Fluorescence-minus-one (FMO) controls. Shown here are examples of the FMOs performed for the markers CD28, CD57, CTLA-4 and PD-1. The graphs are paired with full stain on the left and FMO on the right.

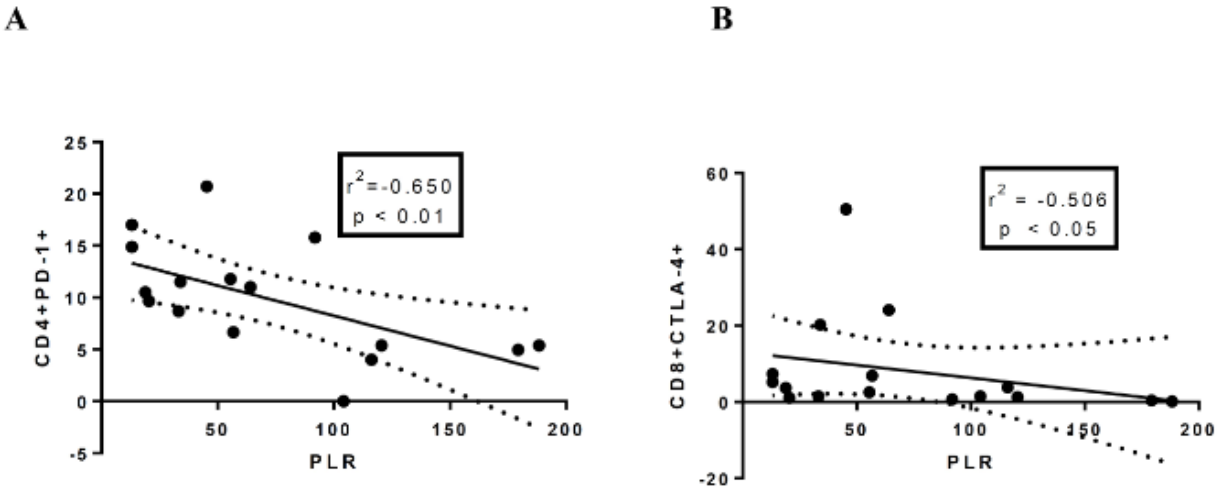
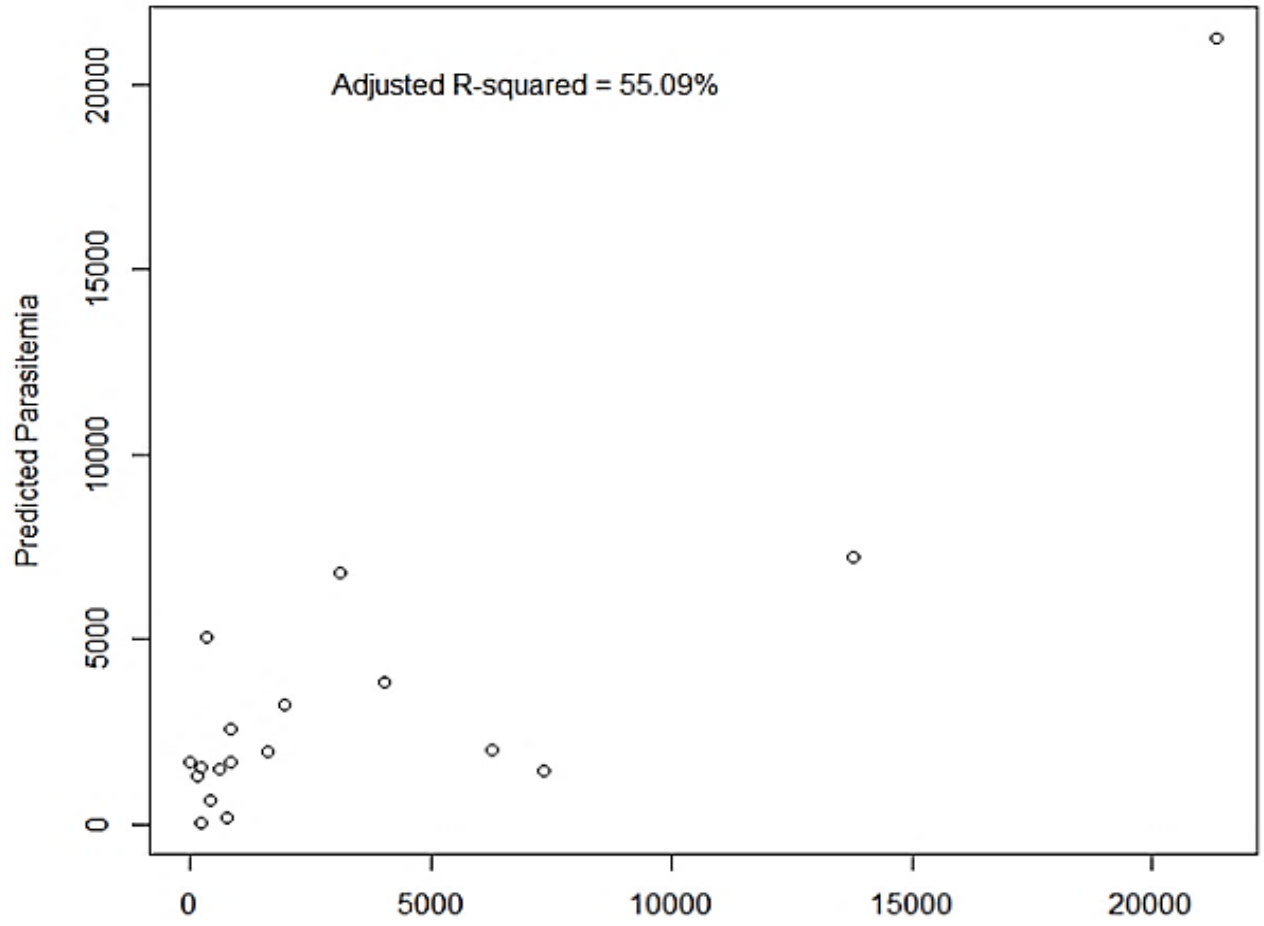


Figure S3.2 Correlation between CD4+PD-1+ and CD8+ CTLA-4+ T cells and inflammation in children with symptomatic malaria.

A. CD4+ T cells


Adjusted R-squared = 55.09%
Predicted Parasitemia
0
5000
10000
15000
20000
0
5000
10000
15000
20000

B. CD8+ T cells

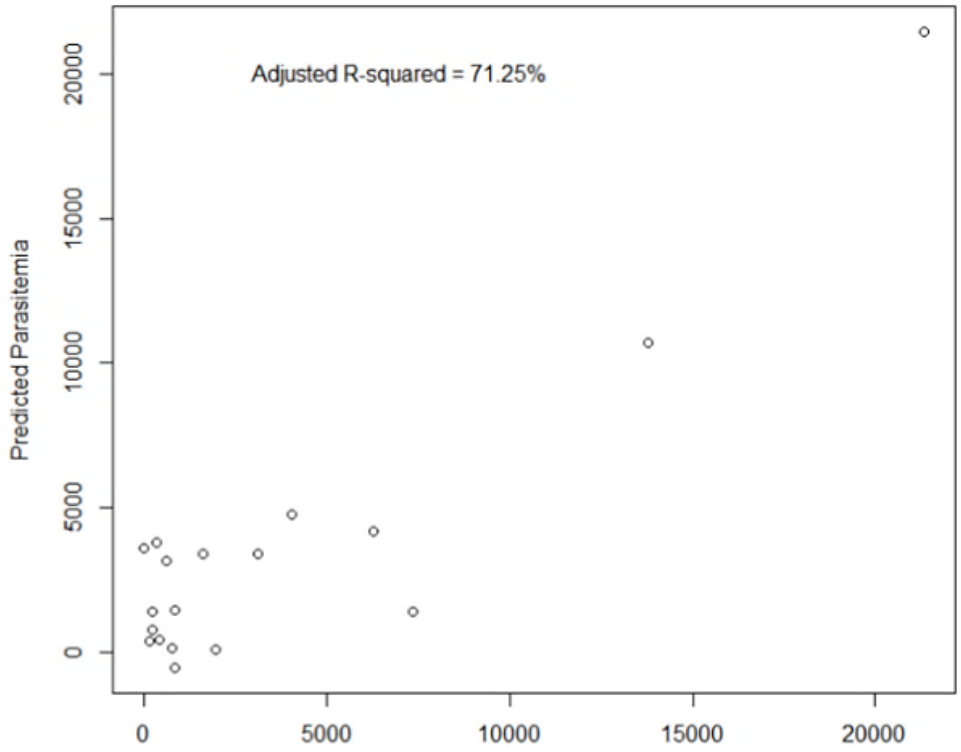


Figure 3.3. The relationship between the surface markers and parasitemia for the asymptomatic malaria group. Using a multiple linear regression plot, actual parasitaemia levels were indicated on the x-axis with the predicted values of parasitaemia on the y-axis. The model was designed using 7 T cell phenotypes measured in **(A)** CD4 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) and **(B)** CD8 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) T cells by immunophenotyping PBMCs obtained from the asymptomatic children.

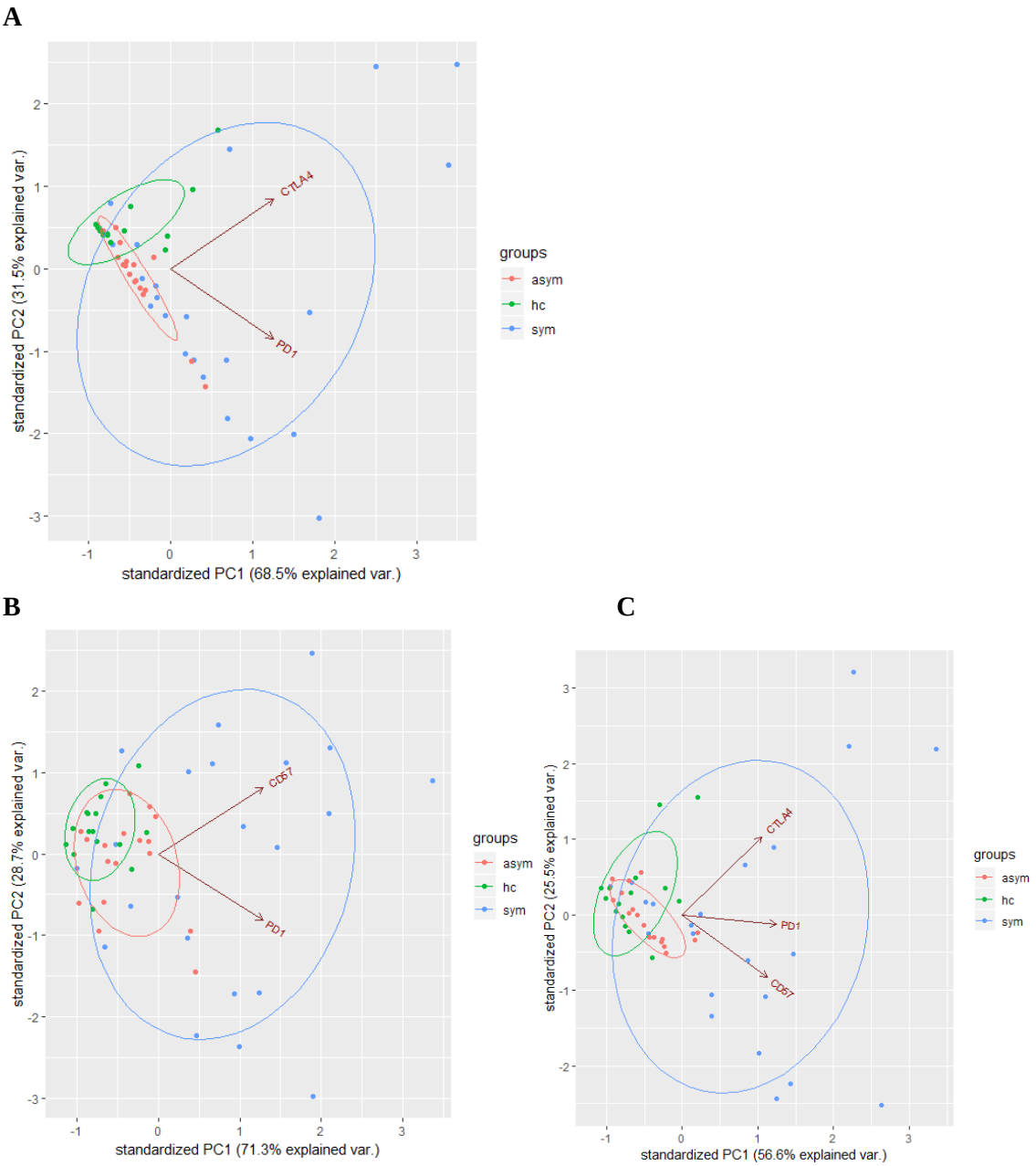


Figure S3.1. Principal component analysis (PCA) biplot for the selected dataset from measured cellular markers for (**a**) CD4; (**b-c**) CD8 T cells in the 17 healthy (hc), 18 asymptomatic malaria (asym) and 22 symptomatic malaria(sym) children. The datasets have been plotted in two dimensions using the first (PC1) and second (PC2) principal components. The groups are indicated by symbols with ellipses representing the group clusters. Blue, red and green colours denote for symptomatic, asymptomatic and healthy children.

Table S3.1. The association between inflammation and inhibitory markers on CD4+ and CD8+ T cells for the symptomatic malaria population

CD4				**CD8**			
Covariate	**Coefficient**	**p value**	**LR test(p value)**	**Covariate**	**Coefficient**	**p value**	**LR test(p value)**
CTLA4	-0.26	0.54	0.32	CTLA4	-0.572	0.97	0.95
PD1	-0.439	0.29	0.093	PD1	-0.572	0.96	0.95
PD1CTLA4	-0.224	0.69	0.45	PD1CTLA4	-0.598	0.74	0.62
CD57	-0.283	0.49	0.27	CD57	-0.58	0.85	0.77
CD57CD28	-0.239	0.59	0.39	CD57CD28	-0.578	0.87	0.8
PD1CD57	-0.313	0.43	0.21	PD1CD57	-0.572	0.96	0.94
CD28CD57	-0.317	0.43	0.21	CD28CD57	-0.571	1	1

We adjusted the effect of each phenotype in predicting the degree of inflammation using the other markers measured. The likelihood ratio test was used to test for the significance of each variable on the model by comparing the adjusted model with another model for which the covariate is absent. P<0.05 was considered statistically significant. Both models were not significant.

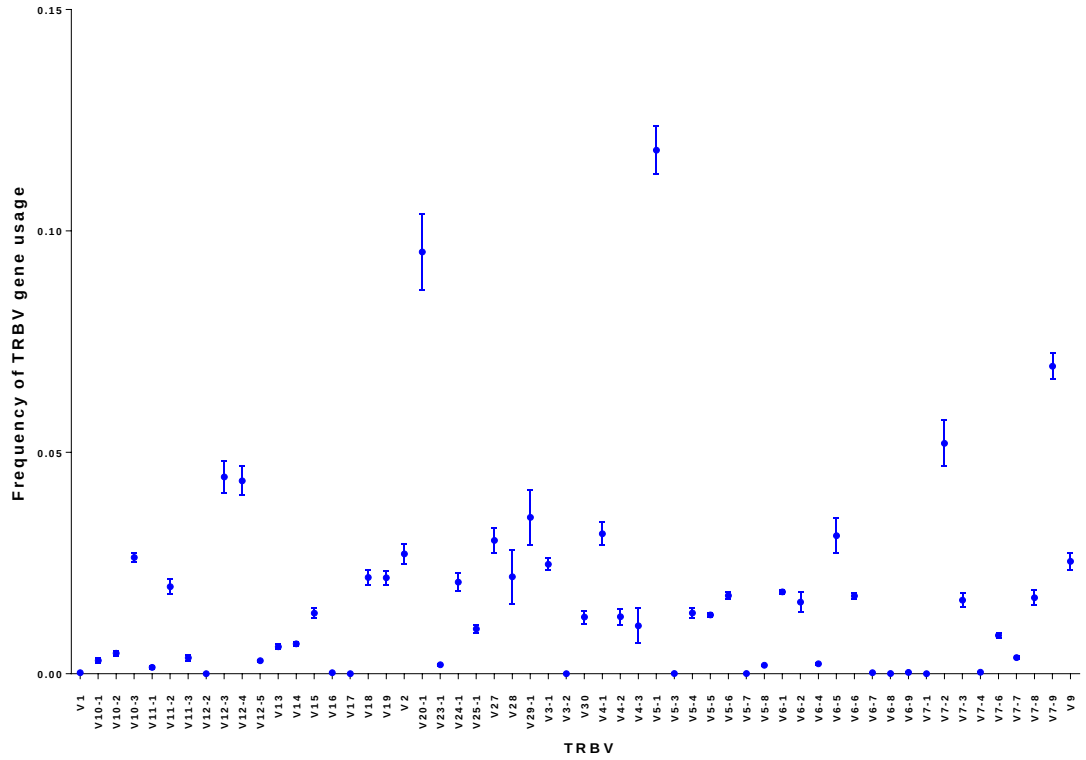
Table S3.2. The association between parasitemia and inhibitory markers on CD4+ and CD8+ T cells for the asymptomatic malaria population

CD4				**CD8**			
Covariate	**Coefficient**	**p value**	**LR test(p value)**	**Covariate**	**Coefficient**	**p value**	**LR test(p value)**
CTLA4	0.5443	0.306	0.1596	CTLA4	-0.08919	**0.0002**	**<0.0001**
PD1	0.4268	0.0722	**0.0135**	PD1	0.7092	0.313	0.1654
PD1CTLA4	0.5626	0.418	0.2656	PD1CTLA4	0.3793	**0.0041**	**<0.0001**
CD57	0.5678	0.462	0.3116	CD57	0.6717	0.14	0.0427
CD57CD28	0.5702	0.484	0.3359	CD57CD28	0.7229	0.455	0.3042
PD1CD57	0.3701	**0.0007**	**<0.0001**	PD1CD57	0.6939	0.22	0.0917
CD28CD57	0.565	0.437	0.2849	CD28CD57	0.6932	0.217	0.0894

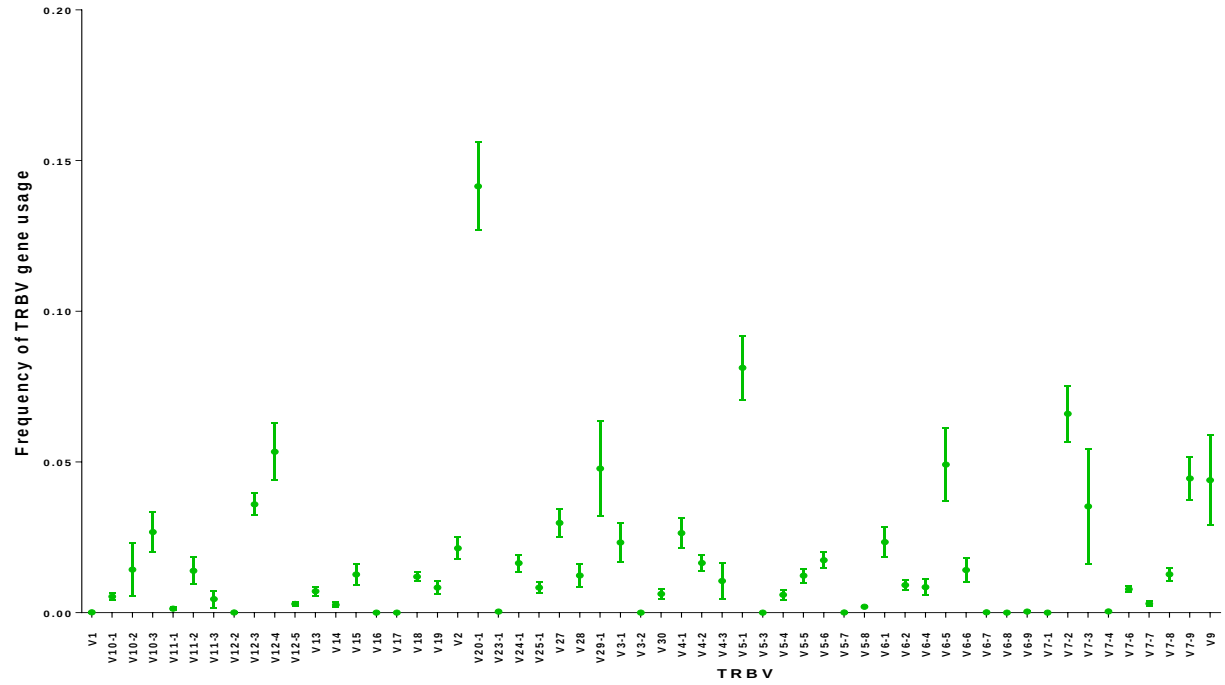
We adjusted the effect of each phenotype in predicting the degree of inflammation using the other markers measured. The likelihood ratio test was used to test for the significance of each variable on the model by comparing the adjusted model with another model for which the covariate is absent. P<0.05 was considered statistically significant.

Appendix 3 Supplementary data for Chapter 4

a



b



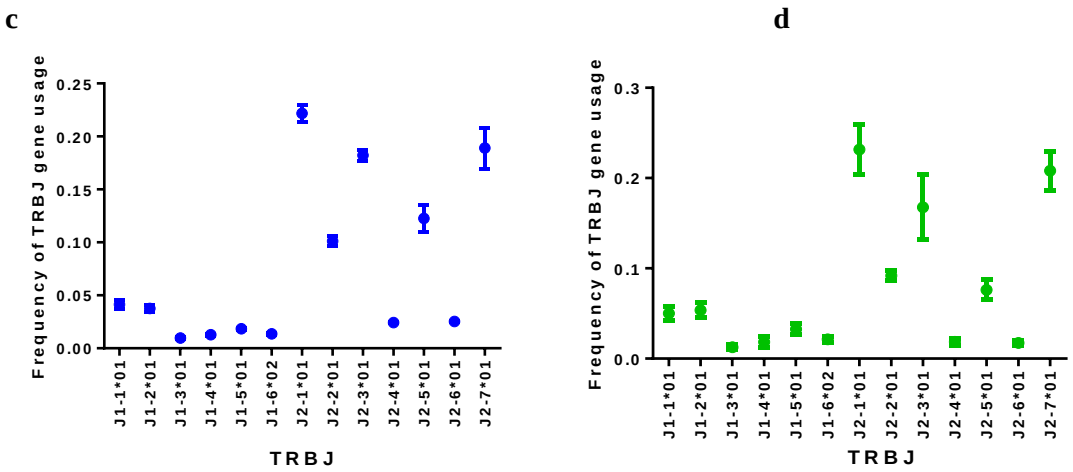
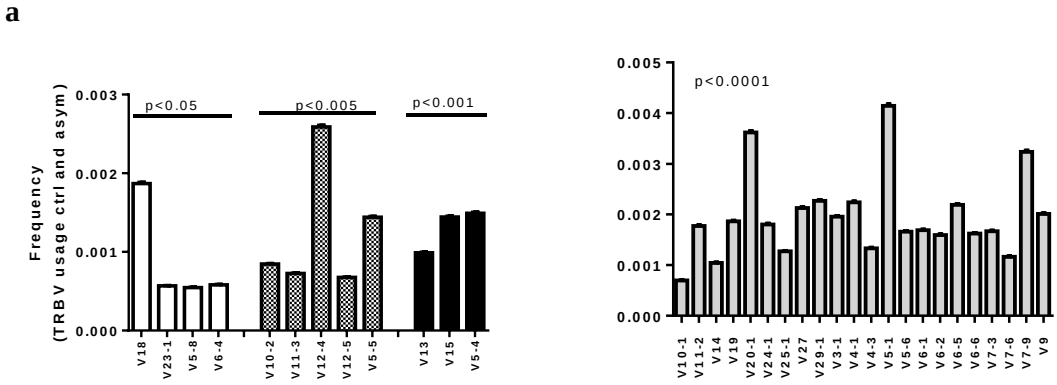
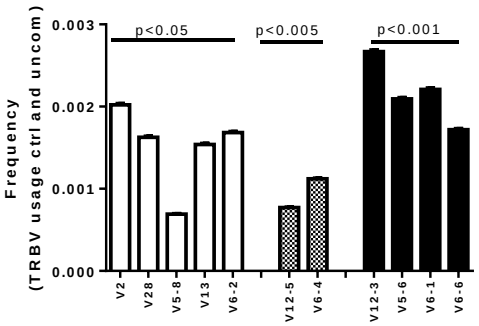
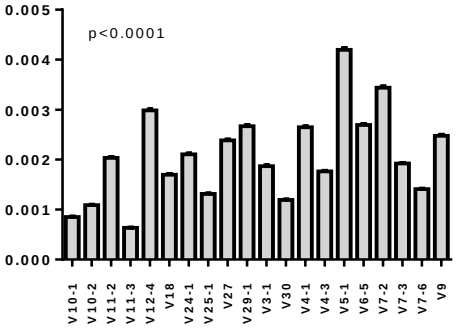


Figure S4.1. Pattern of Vβ and Jβ gene usage within the study groups. The frequency of Vβ usage within **(a)** asymptomatic **(b)** uncomplicated malaria groups. The frequency of Jβ gene usage between **(c)** asymptomatic and **(d)** uncomplicated malaria groups. Data are presented as means with standard error.

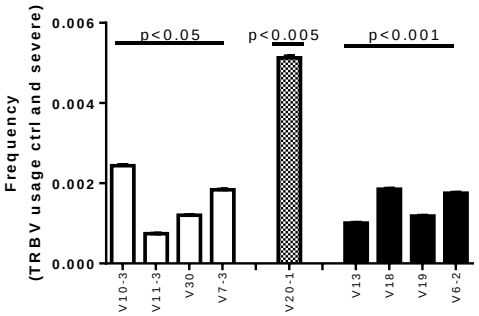


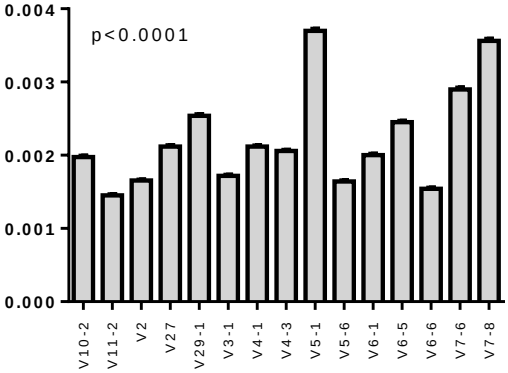
b


Frequency
(TRBV usage ctrl and uncom)
0.003
0.002
0.001
0.000
p<0.05
p<0.005
p<0.001
V2
V28
V5-8
V13
V6-2
V12-5
V6-4
V12-3
V5-6
V6-1
V6-6

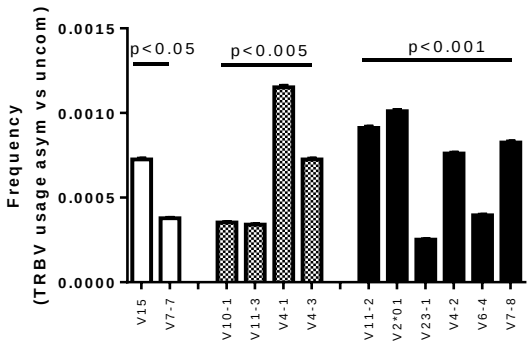

0.005
0.004
0.003
0.002
0.001
0.000
p<0.0001
V10-1
V10-2
V11-2
V11-3
V12-4
V18
V24-1
V25-1
V27
V29-1
V3-1
V30
V4-1
V4-3
V5-1
V6-5
V7-2
V7-3
V7-6
V9

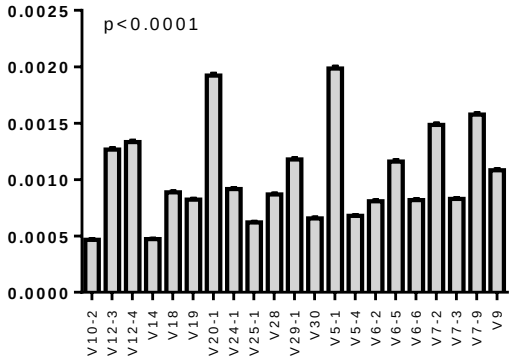
c


Frequency
(TRBV usage ctrl and severe)
0.006
0.004
0.002
0.000
p<0.05
p<0.005
p<0.001
V10-3
V11-3
V30
V7-3
V20-1
V13
V18
V19
V6-2

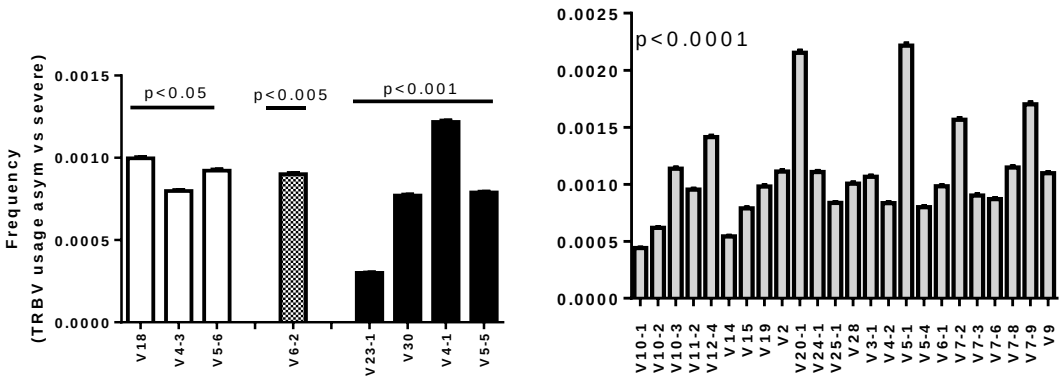

0.004
0.003
0.002
0.001
0.000
p<0.0001
V10-2
V11-2
V2
V27
V29-1
V3-1
V4-1
V4-3
V5-1
V5-6
V6-1
V6-5
V6-6
V7-6
V7-8

d

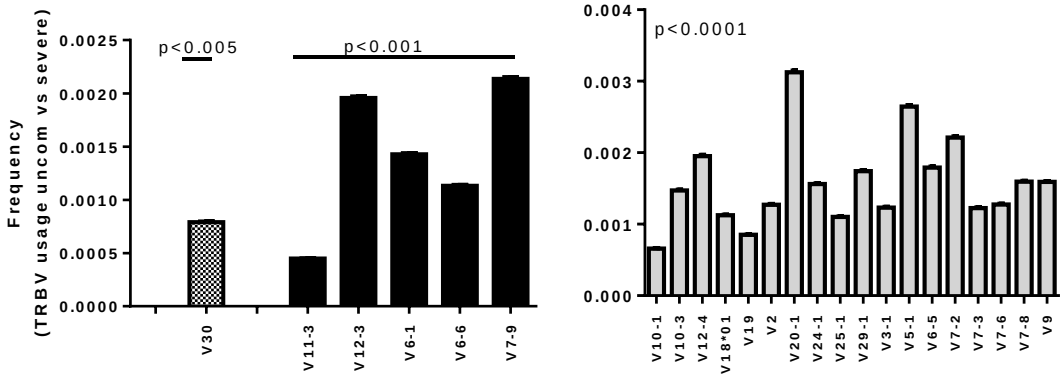

Frequency
(TRBV usage asym vs uncom)
0.0015
0.0010
0.0005
0.0000
p<0.05
p<0.005
p<0.001
V15
V7-7
V10-1
V11-3
V4-1
V4-3
V11-2
V2*01
V23-1
V4-2
V6-4
V7-8


0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
p<0.0001
V10-2
V12-3
V12-4
V14
V18
V19
V20-1
V24-1
V25-1
V28
V29-1
V30
V5-1
V5-4
V6-2
V6-5
V6-6
V7-2
V7-3
V7-9
V9

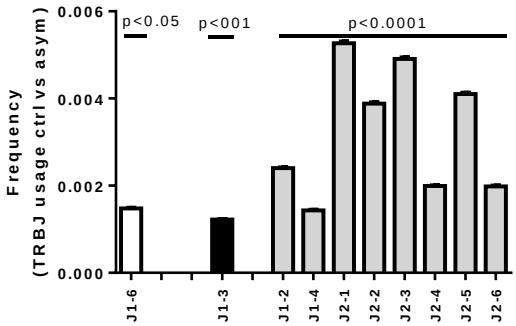
e


Frequency
(TRBV usage asym vs severe)
0.0015
0.0010
0.0005
0.0000
p<0.05
p<0.005
p<0.001
V18
V4-3
V5-6
V6-2
V23-1
V30
V4-1
V5-5
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
p<0.0001
V10-1
V10-2
V10-3
V11-2
V12-4
V14
V15
V19
V2
V20-1
V24-1
V25-1
V28
V3-1
V4-2
V5-1
V5-4
V6-1
V7-2
V7-3
V7-6
V7-8
V7-9
V9

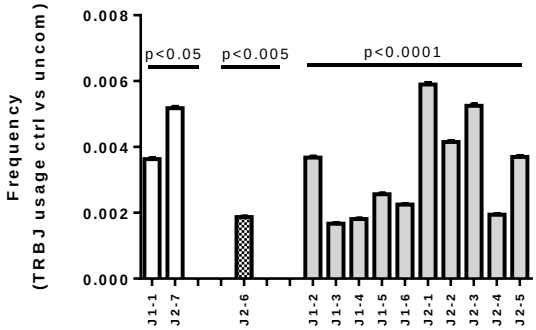
f


Frequency
(TRBV usage uncom vs severe)
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
p<0.005
p<0.001
V30
V11-3
V12-3
V6-1
V6-6
V7-9
0.004
0.003
0.002
0.001
0.000
p<0.0001
V10-1
V10-3
V12-4
V18*01
V19
V2
V20-1
V24-1
V25-1
V29-1
V3-1
V5-1
V6-5
V7-2
V7-3
V7-6
V7-8
V9

g


Frequency
(TRBJ usage ctrl vs asym)
0.006
0.004
0.002
0.000
p<0.05
p<001
p<0.0001
J1-6
J1-3
J1-2
J1-4
J2-1
J2-2
J2-3
J2-4
J2-5
J2-6

h


Frequency
(TRBJ usage ctrl vs uncom)
0.008
0.006
0.004
0.002
0.000
p<0.05
p<0.005
p<0.0001
J1-1
J2-7
J2-6
J1-2
J1-3
J1-4
J1-5
J1-6
J2-1
J2-2
J2-3
J2-4
J2-5

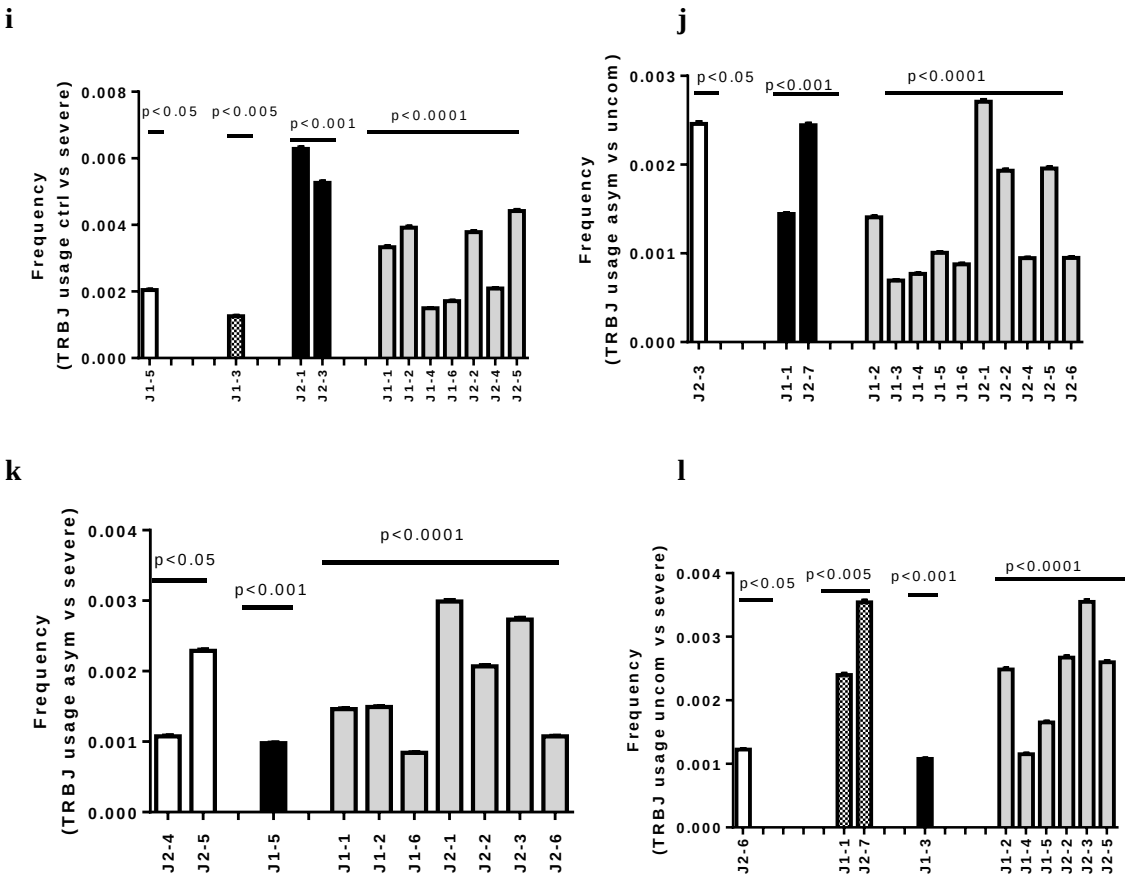
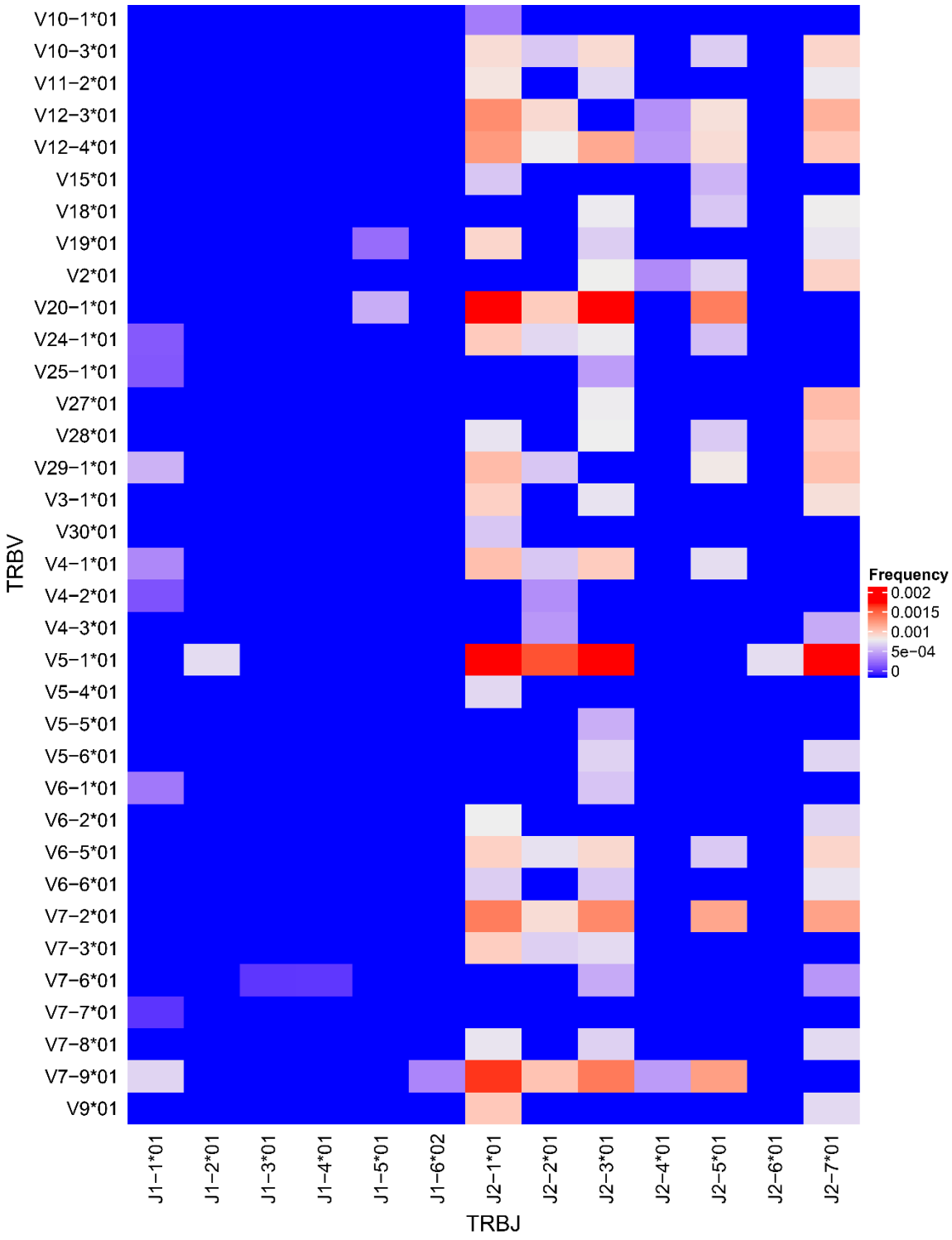
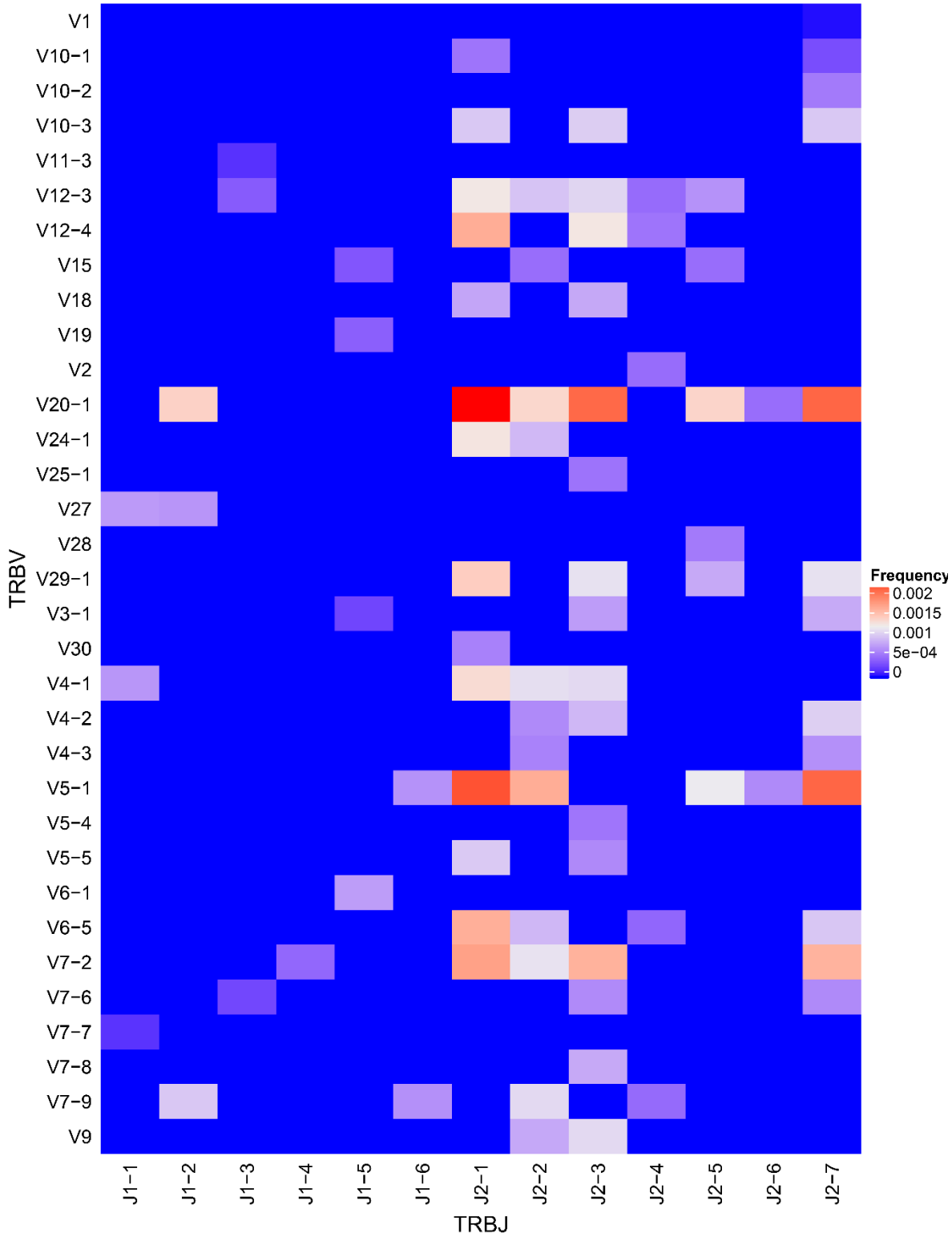


Figure S4.2. **Frequency of V and J gene usage after bootstrap re-sampling**. The data show significant (**a-f**) V gene between (**a**) control and asymptomatic (**b**) control and uncomplicated (**c**) control and severe, (**d**) asymptomatic and uncomplicated, (**e**) asymptomatic and severe, (**f**) uncomplicated and severe malaria groups; .**g-l**) J gene usage between (**g**) control and asymptomatic (**h**) control and uncomplicated (**i**) control and severe, (**j**) asymptomatic and uncomplicated, (**k**) asymptomatic and severe, (**l**) uncomplicated and severe malaria groups after bootstrapping (10^5) with non-parametric test for significance. The bars show the mean proportions with the standard error. Benjamin-Hochberg test was used to correct for multiple comparisons.

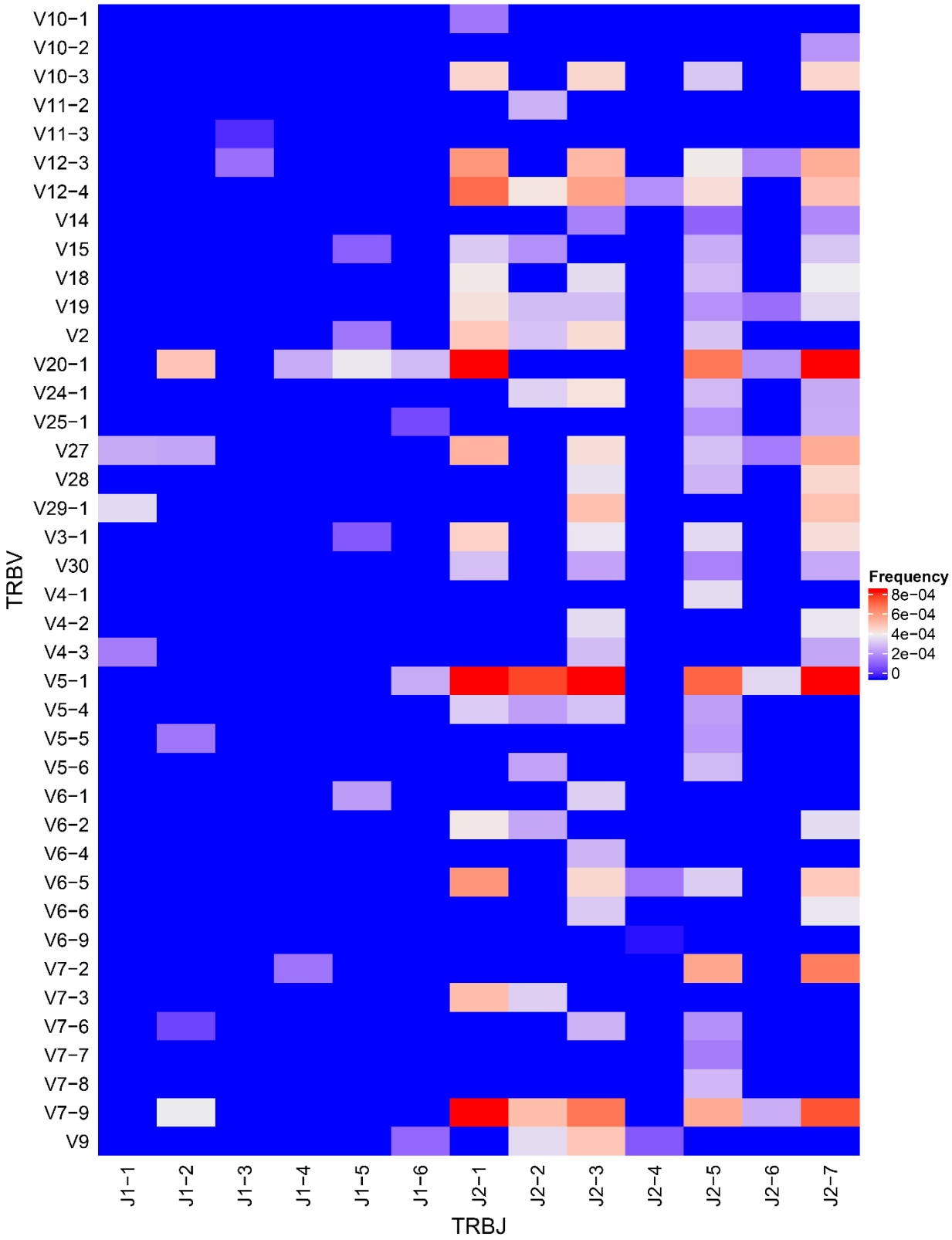
a


TRBV
V10−1*01
V10−3*01
V11−2*01
V12−3*01
V12−4*01
V15*01
V18*01
V19*01
V2*01
V20−1*01
V24−1*01
V25−1*01
V27*01
V28*01
V29−1*01
V3−1*01
V30*01
V4−1*01
V4−2*01
V4−3*01
V5−1*01
V5−4*01
V5−5*01
V5−6*01
V6−1*01
V6−2*01
V6−5*01
V6−6*01
V7−2*01
V7−3*01
V7−6*01
V7−7*01
V7−8*01
V7−9*01
V9*01
J1−1*01
J1−2*01
J1−3*01
J1−4*01
J1−5*01
J1−6*02
J2−1*01
J2−2*01
J2−3*01
J2−4*01
J2−5*01
J2−6*01
J2−7*01
TRBJ
Frequency
0.002
0.0015
0.001
5e−04
0

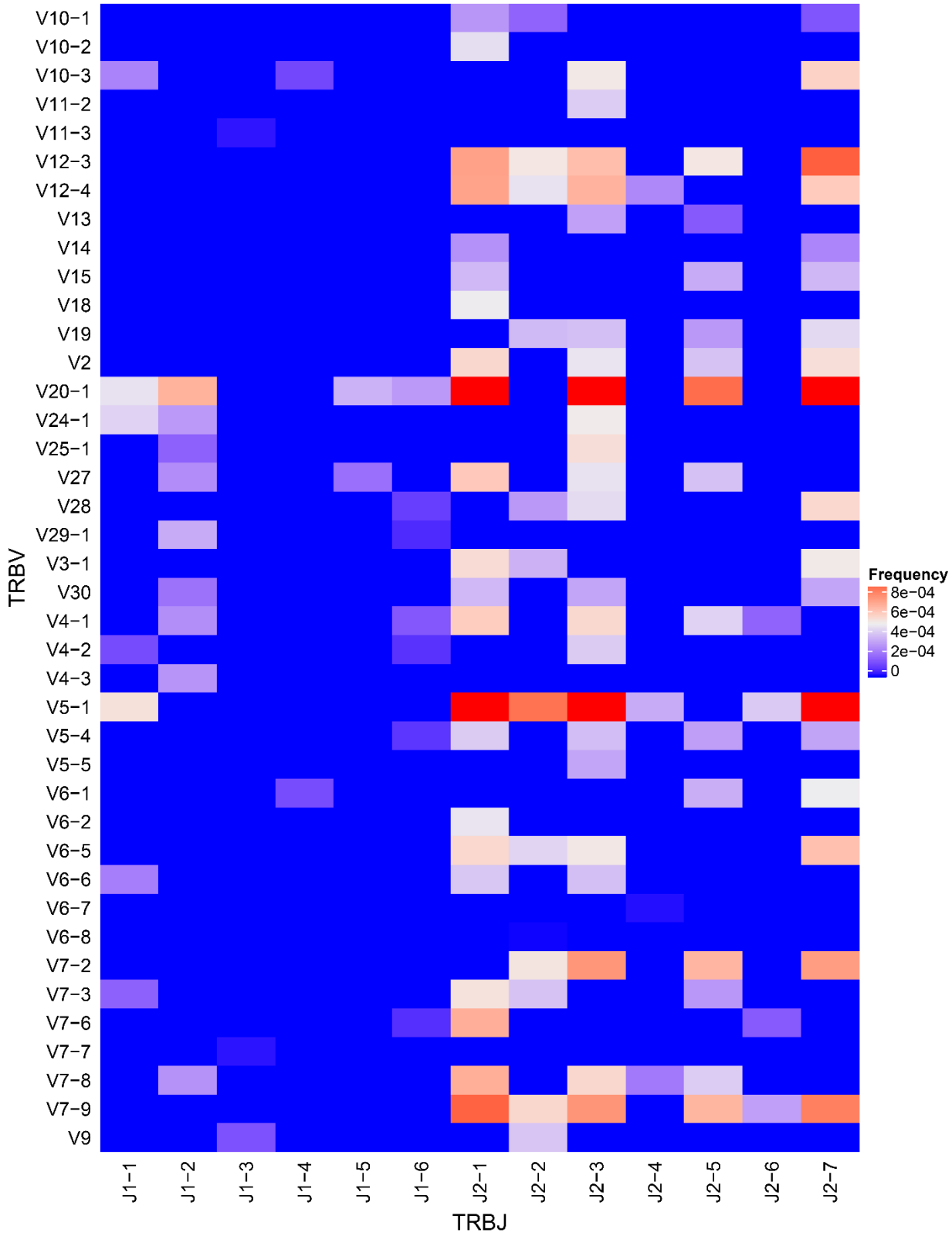
b


V1
V10-1
V10-2
V10-3
V11-3
V12-3
V12-4
V15
V18
V19
V2
V20-1
V24-1
V25-1
V27
V28
V29-1
V3-1
V30
V4-1
V4-2
V4-3
V5-1
V5-4
V5-5
V6-1
V6-5
V7-2
V7-6
V7-7
V7-8
V7-9
V9
TRBV
J1-1
J1-2
J1-3
J1-4
J1-5
J1-6
J2-1
J2-2
J2-3
J2-4
J2-5
J2-6
J2-7
TRBJ
Frequency
0.002
0.0015
0.001
5e-04
0

c


V10−1
V10−2
V10−3
V11−2
V11−3
V12−3
V12−4
V14
V15
V18
V19
V2
V20−1
V24−1
V25−1
V27
V28
V29−1
V3−1
V30
V4−1
V4−2
V4−3
V5−1
V5−4
V5−5
V5−6
V6−1
V6−2
V6−4
V6−5
V6−6
V6−9
V7−2
V7−3
V7−6
V7−7
V7−8
V7−9
V9
TRBV
J1−1
J1−2
J1−3
J1−4
J1−5
J1−6
J2−1
J2−2
J2−3
J2−4
J2−5
J2−6
J2−7
TRBJ
Frequency
8e−04
6e−04
4e−04
2e−04
0

d


V10−1
V10−2
V10−3
V11−2
V11−3
V12−3
V12−4
V13
V14
V15
V18
V19
V2
V20−1
V24−1
V25−1
V27
V28
V29−1
V3−1
V30
V4−1
V4−2
V4−3
V5−1
V5−4
V5−5
V6−1
V6−2
V6−5
V6−6
V6−7
V6−8
V7−2
V7−3
V7−6
V7−7
V7−8
V7−9
V9
TRBV
J1−1
J1−2
J1−3
J1−4
J1−5
J1−6
J2−1
J2−2
J2−3
J2−4
J2−5
J2−6
J2−7
TRBJ
Frequency
8e−04
6e−04
4e−04
2e−04
0

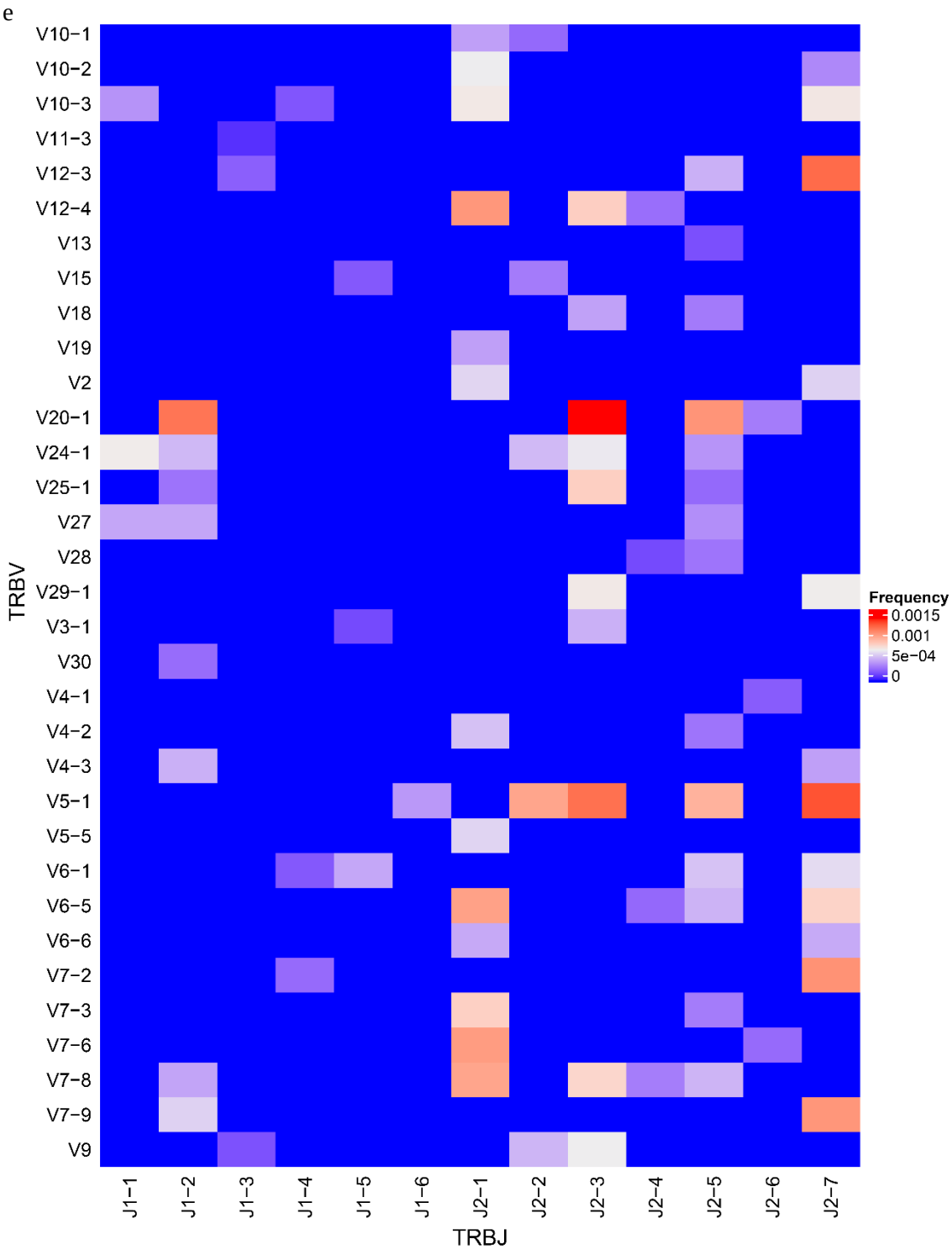


Figure S4.3. V and J gene pairing after bootstrap re-sampling. The distribution of V gene and J gene repertoire for (**a**) control and asymptomatic; (**b**) control and uncomplicated; (**c**) asymptomatic and uncomplicated; (**d**) asymptomatic and severe; (**e**) uncomplicated and severe malaria groups. The X and Y axes list all possible V and J gene combinations displaying the frequency of usage; with red being the highest frequency, blue representing the lowest frequency.

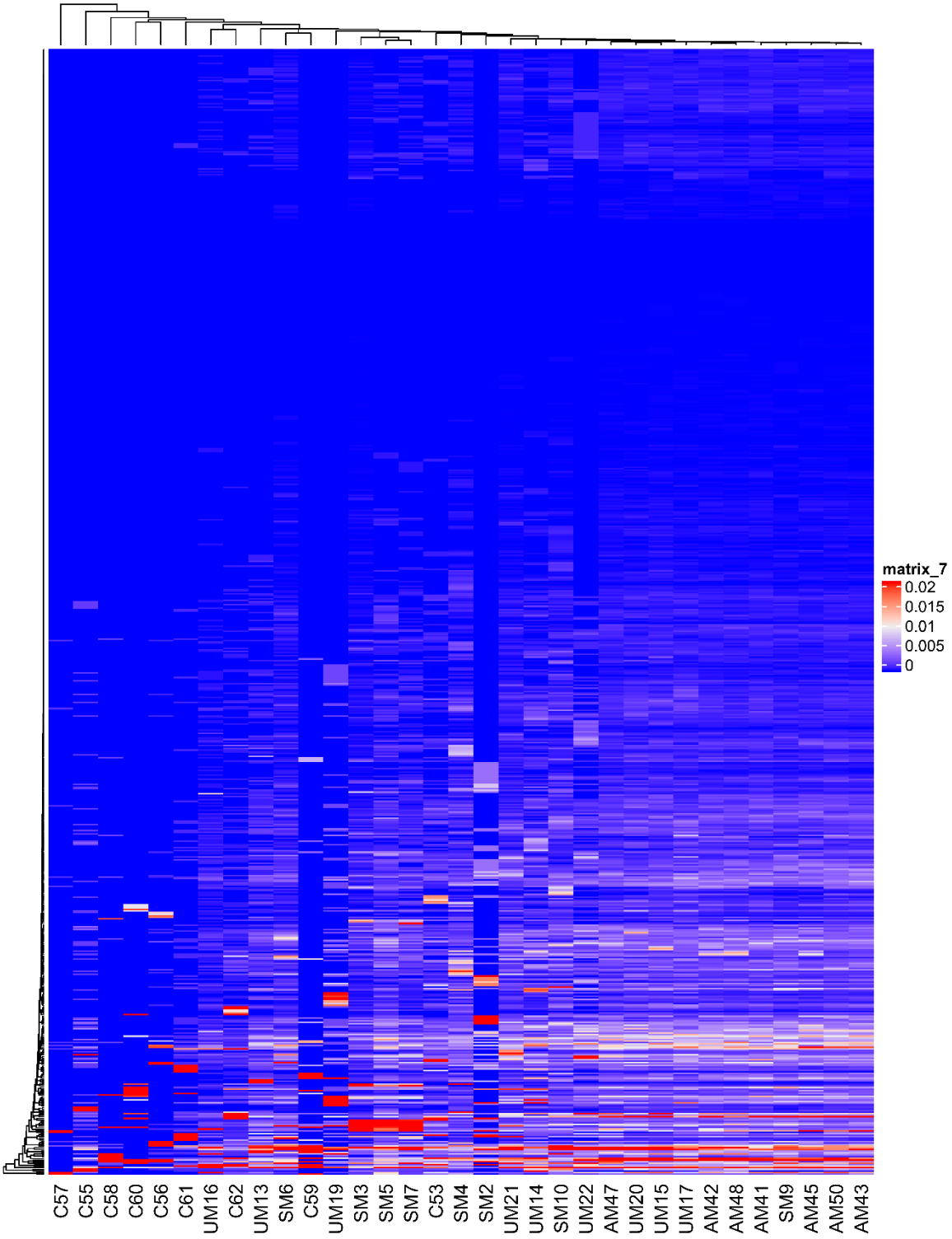


Figure S4.4. Hierachical clustering of study cohort based on Vβ and Jβ gene pairing.
C =control; AM = asymptomatic; UM = uncomplicated; SM = severe

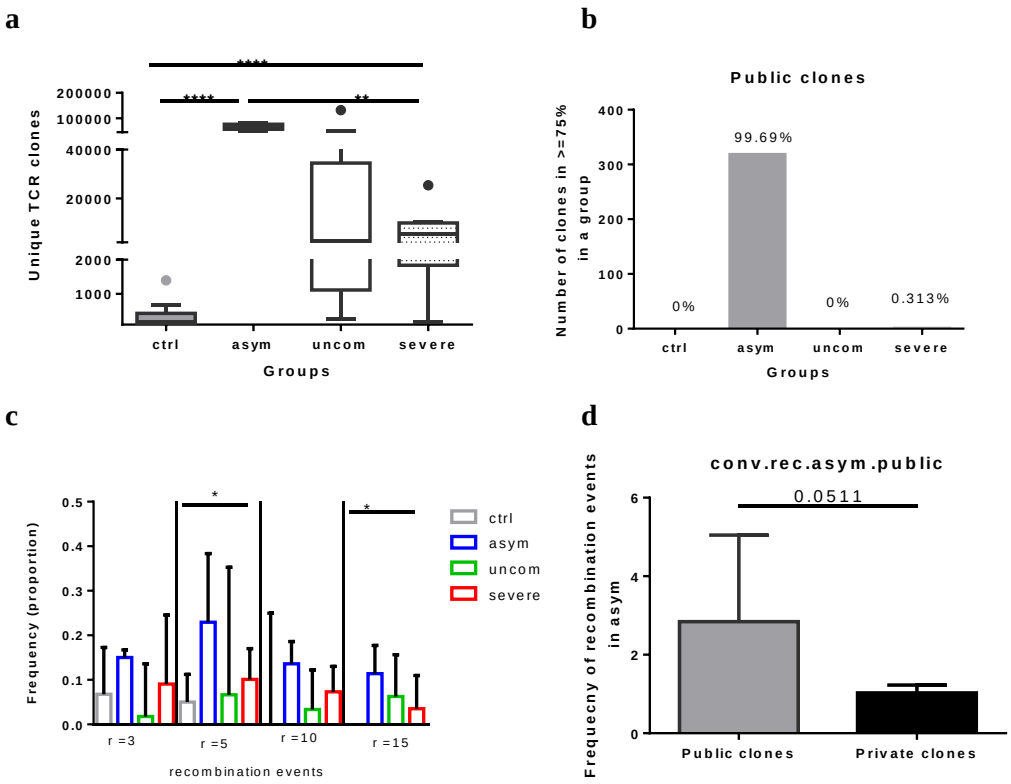


Figure S4.5: **Increased number of clones in the asymptomatic *P. falciparum* infected group** (**a**)The total unique CDR3 sequences. (**b**) The number of recombination events (**c**) at different r values across the compared between the study population, (**d**) between public and private clones for the asymptomatic population. P<0.05 were considered statistically significant, ns indicates not significant. Ctrl = aparasitemic control; asym = asymptomatic; uncom = uncomplicated.

Appendix 4 **Manuscripts generated from this dissertation**

Frimpong *et al. Malar J (2018) 17:263*
https://doi.org/10.1186/s12936-018-2410-6

Malaria Journal

RESEARCH

Open Access

Characterization of T cell activation and regulation in children with asymptomatic *Plasmodium falciparum* infection

Augustina Frimpong[1,2,3*], Kwadwo Asamoah Kusi[1,2], Bernard Tornyigah[2], Michael Fokuo Ofori[1,2] and Wilfred Ndifon[3,4*]

Abstract

Background: Asymptomatic *Plasmodium* infections are characterized by the absence of clinical disease and the ability to restrict parasite replication. Increasing levels of regulatory T cells (Tregs) in *Plasmodium falciparum* infections have been associated with the risk of developing clinical disease, suggesting that individuals with asymptomatic infections may have reduced Treg frequency. However, the relationship between Tregs, cellular activation and parasite control in asymptomatic malaria remains unclear.

Methods: In a cross-sectional study, the levels of Tregs and other T cell activation phenotypes were compared using flow cytometry in symptomatic, asymptomatic and uninfected children before and after stimulation with infected red blood cell lysates (iRBCs). In addition, the association between these T cell phenotypes and parasitaemia were investigated.

Results: In children with asymptomatic infections, levels of Tregs and activated T cells were comparable to those in healthy controls but significantly lower than those in symptomatic children. After iRBC stimulation, levels of Tregs remained lower for asymptomatic versus symptomatic children. In contrast, levels of activated T cells were higher for asymptomatic children. Strikingly, the pre-stimulation levels of two T cell activation phenotypes (CD8+CD69+ and CD8+CD25+CD69+) and the post-stimulation levels of two regulatory phenotypes (CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+) were significantly positively correlated with and explained 68% of the individual variation in parasitaemia. A machine-learning model based on levels of these four phenotypes accurately distinguished between asymptomatic and symptomatic children (sensitivity = 86%, specificity = 94%), suggesting that these phenotypes govern the observed variation in disease status.

Conclusion: Compared to symptomatic *P. falciparum* infections, in children asymptomatic infections are characterized by lower levels of Tregs and activated T cells, which are associated with lower parasitaemia. The results indicate that T cell regulatory and activation phenotypes govern both parasitaemia and disease status in paediatric malaria in the studied sub-Saharan African population.

Keywords: Malaria, Regulatory T-cells, T-cell activation, Asymptomatic, Symptomatic, Children, *falciparum*, Immunity

*Correspondence: tinafrimp@gmail.com; wndifon@aims.ac.za
[1] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, P. O. Box LG 54, Accra, Ghana
[4] African Institute for Mathematical Sciences, University of Stellenbosch, 7 Melrose Rd, Muizenberg, Cape Town 7945, South Africa
Full list of author information is available at the end of the article

Background

Falciparum malaria occurs when sporozoites inoculated into the human host develop in the liver into merozoites that infect red blood cells and cause clinical disease. The acquisition of natural immunity to falciparum malaria is slow and requires frequent exposure to the parasite over a period of time [1]. Despite previous exposure to the parasite, people in endemic areas may remain susceptible to clinical disease or they may be asymptomatic carriers of parasites as clinical immunity is only partial and never sterile. Also, repeated parasite exposure has been associated with limited protectiveness to vaccine candidates [2, 3]. The lack of a proper understanding of the immune responses occurring during natural infections has, for example, resulted in an inability to develop effective interventions such as vaccines.

Understanding the regulatory and protective immune responses during asymptomatic and clinical infections remain necessary to comprehend mechanisms that enable the control of infections as well as the persistence and survival of the parasite. A number of studies in malaria have associated protection from clinical disease with having a broad antibody repertoire [4–6]. Nonetheless, the presence of asymptomatic infections in children who may not have a broad antibody repertoire suggests that there is some level of immunity to the parasite by the host and this is characterized by the absence of clinical manifestations of the disease. Moreover, during *Plasmodium falciparum* infections, it is believed that the effector function of immune cells will be compromised due to immune regulation [7]. This may be induced by the specific expansion of certain T or B cell sub-sets and modulation of certain antigen presenting cells, such as the dendritic cells [8]. T cells express receptors that enable co-stimulation, activation, memory formation, and immune regulation to ensure effective and timely immune response induction upon antigen recognition. The expansion of specific cell sub-sets, especially those that express regulatory markers, may either enhance or inhibit the development of immunity against an infection. However, the association between such cellular activation and regulatory markers and parasite control during asymptomatic infections is inadequately understood.

Regulatory T cells are unique cell phenotypes that function to maintain homeostasis when the immune response is activated. The establishment of immune homeostasis may result in blocking the activity of other immune cells. For instance, CTLA-4 (also known as CD152), once activated, functions to inhibit activation of both antigen presenting cells and other T cells. Even though the role of Tregs during *P. falciparum* infections remains controversial, it has been observed that in both human and rodent malaria an early induction of Tregs may result in an increased parasite density [9–12]. Furthermore, the expansion of Tregs in malaria has been associated with decreased antigen-specific immune responses [11].

Also, a recent study by Kurup et al. [13] has shown that CTLA-4 Tregs expand during symptomatic malaria in both human and murine models, which is associated with decreased parasite clearance and impedes the acquisition of immunity in murine models. Other studies have also reported the upregulation of TNFRII on Tregs with asymptomatic parasitaemia [14]. There have also been reports on the upregulation of FOXP3 mRNA transcripts during acute malaria infections in children and naïve adults, which negatively correlated with Th1 memory responses [9, 15]. Nonetheless, other studies have also shown conflicting data whereby no association was found between the levels of Tregs and *Plasmodium* infection [16–19]. Collectively, these imply that the activity of Tregs associated with the development of protective immunity needs to be comprehended. The likely suggestions are that infections may cause the expansion of Tregs, which in turn may cause immune suppression and enhance parasite growth as observed in other studies [11, 20, 21].

This study aims to compare the expression levels of T cell activation and regulatory markers across symptomatic, asymptomatic and healthy control children living in hyperendemic areas with stable malaria transmission in Ghana. The Treg markers CD25+Foxp3+, the early activation marker CD69, and the late activation marker CD25 were measured. Tregs have an established role in suppressing effector immune responses to a variety of pathogens, including malarial parasites [22, 23]. CD69 expression in CD4+ T cells has been shown to correlate with the development of antigen-specific antibodies in experimental human falciparum malaria [24]. CD69 is a transmembrane glycoprotein expressed during early activation and increases with inflammation with the potential to induce cytotoxic activity once crosslinked [25–29], whereas CD25 (IL-2α receptor) has been associated with T cell proliferation and differentiation through the IL-2 cytokine [28]. This suggests that their combined expression may lead to an enhanced cellular immune activity. Therefore, it was hypothesized that asymptomatic infections have reduced Treg levels, such that exposure to *P. falciparum* is associated with increased cellular response and lower parasitaemia, which in turn feeds back to reduce cellular activation.

Methods

Study sites

Participants for the study were recruited from Asutsuare and Paakro sub-districts, which are hyperendemic for malaria transmission. Asutsuare has two malaria

transmission seasons; June to August (high transmission season) and November to December (low transmission season) with an entomological inoculation rate of 14.6 infective bites/man/year whereas Paakro has May to June as the high transmission season and September to October as the low transmission season [30, 31]. Samples from participants were obtained during the high transmission seasons.

Participants and sample collection

The study was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research, University of Ghana (Permit No. 096/15-16). A written informed consent was obtained from parents or guardians and assent appropriately received from the children before they were enrolled. Samples were obtained in a cross-sectional study from 57 children under 13 years old who satisfied the inclusion criteria with no known conditions that could interfere with the experiments. The participants were grouped into *P. falciparum*-infected asymptomatic children (n = 18), symptomatic malaria patients (n = 22) and healthy controls (n = 17). About 5 ml of venous blood was collected into heparin tubes before anti-malarial treatment. Both thick and thin smears were prepared and stained with Giemsa for parasite identification after screening for infection with rapid diagnostic tests. Haematological indices were determined by an automated haematological analyzer. PBMCs were isolated by ficoll gradient centrifugation and stored in liquid nitrogen until the time of the experiment. The PBMCs were cryopreserved in fetal bovine serum with 10% dimethyl sulfoxide (DMSO).

Flow cytometry analysis

Stored PBMCs were thawed and washed. The viability was assessed by trypan blue dye exclusion method. Cells with viability greater than 95% were used in the assay. The cells were surface stained with the following antibodies for T cell sub-sets (anti-CD3, anti-CD4, anti-CD8), co-stimulation markers (anti-CD28, anti-CD57) and activation markers (anti-CD25, anti-CD69) (Additional file 1). The cells were washed, fixed and permeabilized using FOXP3 buffer set (BD) and intracellularly stained for regulatory markers Foxp3 (Biolegend) and CTLA-4 (BD). Fluorescence minus one controls and compensation were performed to set gates using single colour stained or unstained PBMCs. Data were compensated and analysed using Flowjo V10 software (Tree Star, San Carlos, CA, USA). The gating strategies are outlined in Figs. 1a and 2a.

Stimulation of PBMCs with infected and uninfected red blood cells

Plasmodium falciparum parasites of the NF54 strain were cultured in O^+ red blood cells at 3% haematocrit in culture medium (RPMI 1640 medium, 25 µg/ml of gentamycin, 10% heat-inactivated O^+ human serum). The culturing was done in the presence of 7.5% sodium bicarbonate at 37 °C in a 5% O_2, 5% CO_2 and 90% N_2 atmosphere. PBMCs were later thawed and rested for 6 h in 10% fetal bovine serum. About 400,000 cells were later stimulated with intact *P. falciparum* trophozoites/schizont (NF54 clone)-infected RBCs (iRBCs; 3 iRBCs: 1 PBMC) or uninfected RBCs (uRBCs; 3 uRBCs: 1 PBMC) and cultured in complete RPMI 1640 in 5% CO_2 at 37 °C. After 4 h of stimulation, brefeldin A was added at a concentration of 10 µg/ml. Cells were washed and stained after 18 h with the following monoclonal antibodies; anti-CD3 (APC-H7), CD4 (BUV 395), CD8 (PerCP-Cy5.5), CD25 (PE-CF594), CD69 (PE-Cy7), CD152/CTLA-4 (APC), FOXP3 (PE) all from BD. The cells were washed by centrifugation before staining for both extracellular and intracellular markers.

Statistical analysis

Data analyses were performed with the GraphPad Prism version 6.01 (GraphPad Software, Inc.) and the R statistical software version 3.4.0 (R Foundation for Statistical Computing). The demographics and clinical characteristics of the study participants were compared among the 3 study groups using Chi square test for categorical variables, Kruskal–Wallis or One-way ANOVA for continuous variables, Mann–Whitney U test and Wilcoxon-Signed Rank Test for paired comparisons for data that were not normally distributed. For comparing the markers of T cells among the 3 study populations, the Kruskal–Wallis test was used with a Dunn's post hoc test or a Bonferroni correction for multiple comparisons where necessary. Spearman's rank correlation was used to determine associations between markers. Support vector machine model, a supervised machine-learning algorithm was used to predict disease status. Statistical significance was set at P-values < 0.05.

Results

Characteristics of the study population

Venous blood samples were obtained from 57 children including 18 with asymptomatic *P. falciparum* infections, 22 with symptomatic malaria, and 17 with no *P. falciparum* parasites detected in blood by microscopy or rapid diagnostic test (Table 1). There was no statistically significant difference between the ages of children in the asymptomatic versus symptomatic groups. In

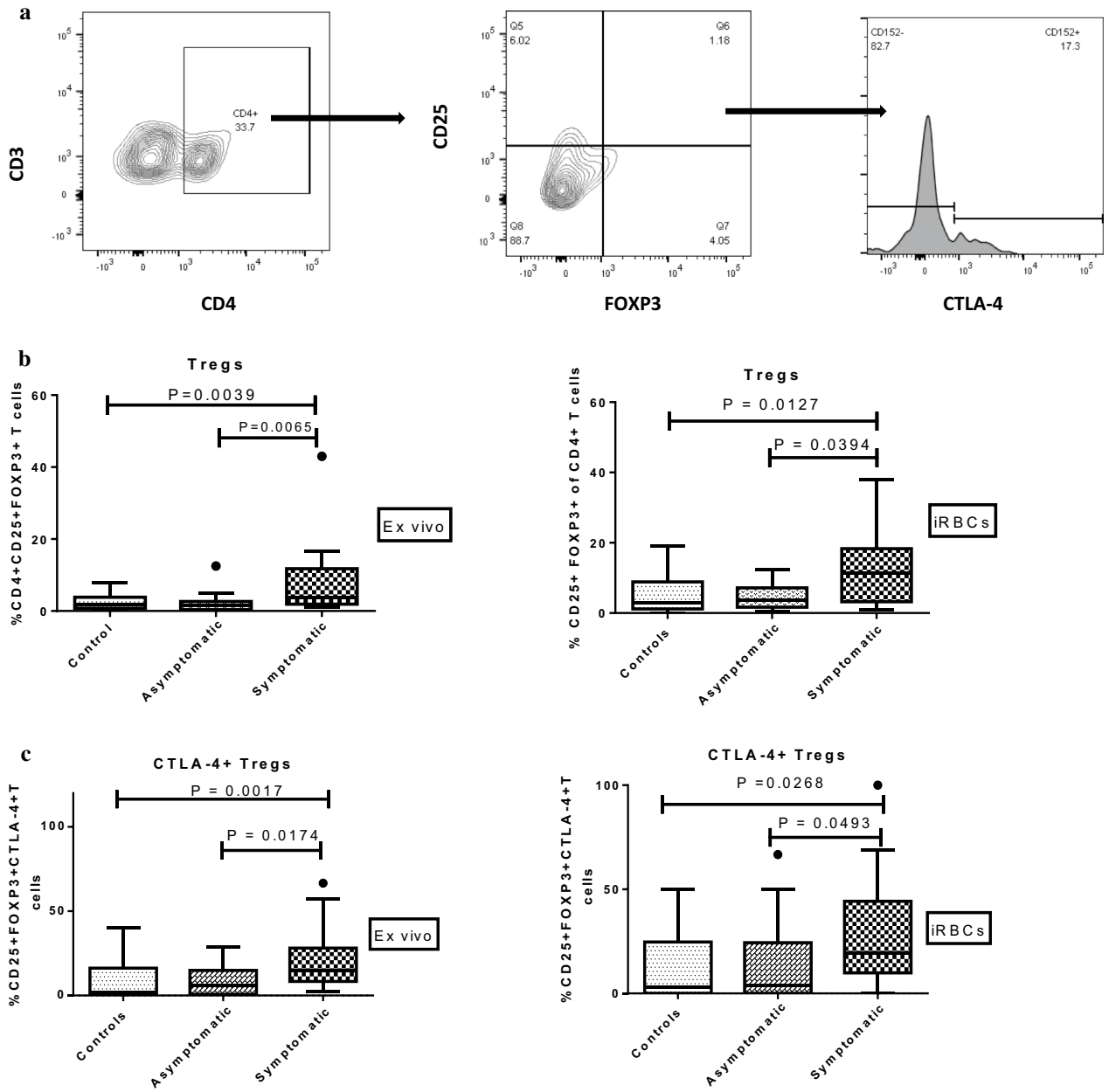


Fig. 1 Percentage expression of CD4+CD25+FOXP3+ regulatory T cells among study groups. **a** Representative flow cytometry gating strategy for phenotyping CD4+CD25+FOXP3+ and CTLA-4+ Tregs in peripheral blood. The percentage expression and activation of regulatory T cells were analysed in healthy controls (n = 16), children with asymptomatic infections (n = 18) and symptomatic falciparum malaria (n = 22); for levels of **b** Tregs analysed as CD4+CD25+FOXP3+; and, **c** activated Tregs analysed as CD4+CD25+FOXP3+CTLA-4+ T cells both ex vivo and after iRBC stimulation. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are denoted by whiskers. Medians are indicated by the horizontal lines across the boxes. Kruskal–Wallis test was used for comparisons, followed by Dunn's test where necessary

contrast, the healthy controls were significantly older than the asymptomatic ($P = 0.0404$) and symptomatic children ($P = 0.0123$). Also, the mean haemoglobin levels in asymptomatic children were significantly higher compared to the symptomatic children ($P = 0.0348$). However, levels were comparable between the children in the control group and asymptomatic or symptomatic groups. Total leukocyte counts were significantly higher in the asymptomatic children compared to symptomatic children ($P = 0.0478$) but comparable to controls. Even though the median lymphocyte counts did not differ significantly amongst the groups, lower levels were found in the symptomatic group than in the asymptomatic and control groups. Also, platelet levels decreased with the

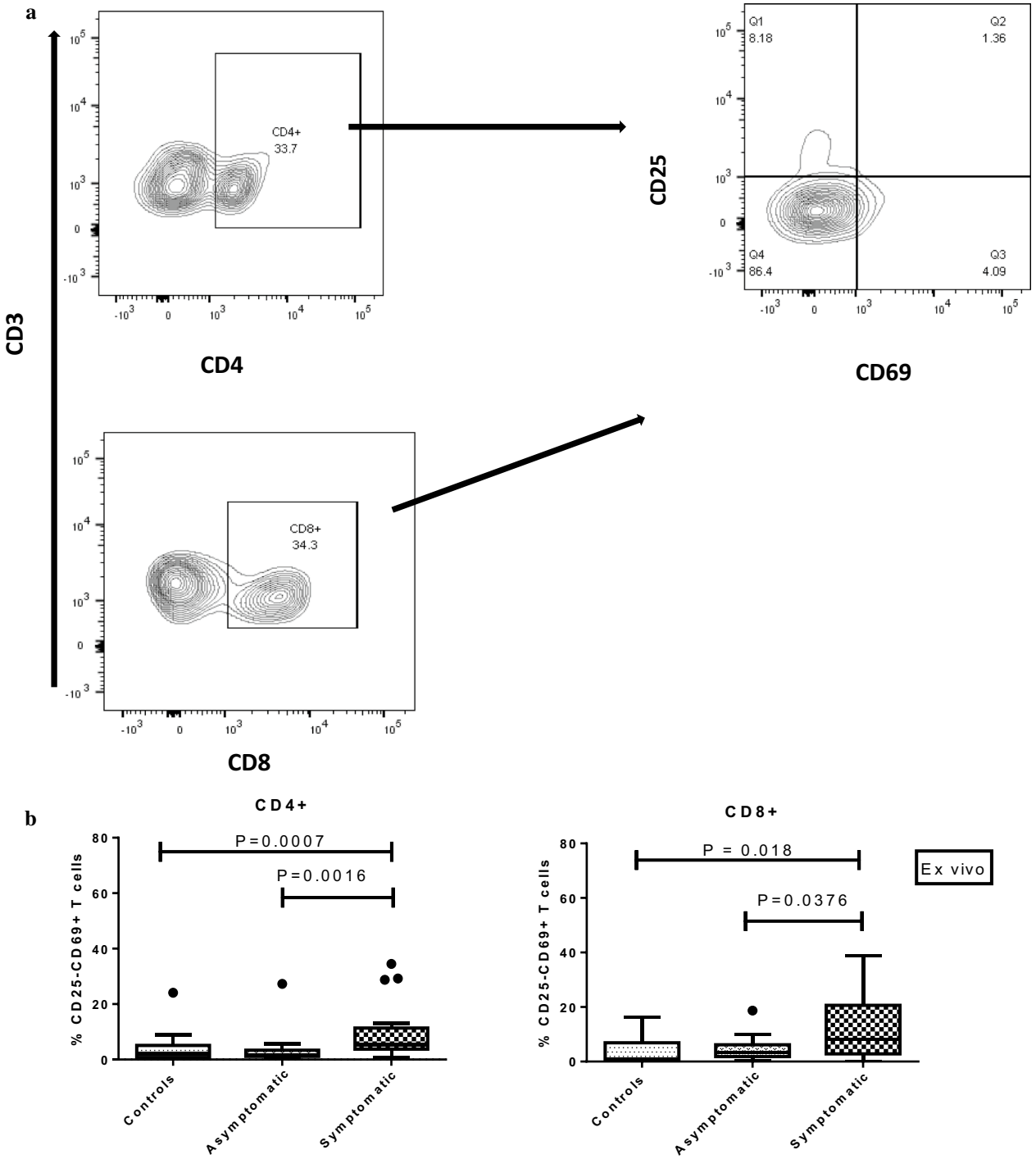


Fig. 2 Expression of T-cell activation markers CD25/CD69 on T cells in PBMCs from the study cohort. **a** Representative flow cytometry gating strategy for phenotyping activation markers on CD4+ and CD8+ T cells ex vivo; the expression of the activation markers **b** CD25−CD69+ on T cells was analysed in healthy controls (n = 17), asymptomatic *P. falciparum*-infected children (n = 18), and symptomatic *P. falciparum*-infected children (n = 21). The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers. Medians are indicated by the horizontal lines across the boxes. Kruskal–Wallis test was used for comparisons, followed by Dunn's test where necessary

Table 1 Demographics and clinical characteristics of the study participants

Characteristics	Control	Asymptomatic	Symptomatic	P values
Sample size	n = 17	n = 18	n = 22	
Age (IQR), years	9 (8–11)	7 (4.5–9)	6 (4.8–7)	0.0087[a]
Female (%)	52.94	44.44	50	0.8765[b]
Mean haemoglobin (IQR), g/dl	11.5 (10.8–12.1)	12.7 (11.7–13.58)	10.7 (8.8–13.1)	0.0402[c]
Parasitaemia (IQR), µl	NA	845 (260.7–3812)	13,973 (7238–58,764)	0.0009[d]
Leukocytes (10^9/l)	7 (5.7–8.0)	7.7 (6.1–9.6)	5.1 (1.2–8.3)	0.0436[a]
Lymphocytes (10^6/l)	2.9 (2.5–3.6)	2.1 (1.2–3.45)	1.9 (1.3–3.9)	0.0889[a]
Platelets (10^9/l)	305 (237–356)	223 (193–280)	101 (61–198)	> 0.0001[a]

IQR interquartile range, *NA* not applicable

[a] Kruskal–Wallis test

[b] Chi square test

[c] One-way ANOVA

[d] Mann–Whitney U test

severity of *P. falciparum* infections. The median platelet counts in the symptomatic children were significantly lower than in the asymptomatic ($P = 0.0029$) and control ($P < 0.0001$) groups. Children in the asymptomatic group had statistically similar platelet counts as the control group. Parasitaemia levels were significantly lower in asymptomatic children compared to children with symptomatic infection ($P = 0.0009$).

Decreased levels of regulatory T cells in asymptomatic *Plasmodium falciparum* infections

To investigate if there are any differences between the *P. falciparum*-infected group and healthy controls with respect to T cell regulation, the levels of Treg populations in the 3 study groups were determined. Here, Treg populations were classified as CD3+, CD4+, CD25+ and FoxP3+ (Fig. 1a).

For the peripheral blood mononuclear cells (PBMCs) analysed directly without stimulation (ex vivo), it was observed that Tregs had a lower frequency in the asymptomatic children compared to the symptomatic children ($P = 0.0065$), but levels were comparable between asymptomatic and control groups ($P > 0.05$). Also, the Treg frequency in the symptomatic children was higher than in the controls ($P = 0.0209$, Fig. 1b). This trend remained the same after the cells were stimulated with iRBCs in vitro; lower levels of Tregs were found in the asymptomatic children than in the symptomatic children ($P = 0.0394$), while levels were comparable to the controls. Similarly, levels in the symptomatic children were higher than in the controls ($P = 0.0391$, Fig. 1b).

Furthermore, the levels of activated Tregs based on the expression of CTLA4, an immunosuppressive marker that inhibits activation of immune cells by direct contact, were determined. The levels of CTLA4+ Tregs differed significantly across the study populations ($P = 0.0017$). The levels of CTLA4+ Tregs in children with asymptomatic malaria were significantly lower than in those with clinical malaria ($P = 0.0174$, Fig. 1c) but comparable with the control group. However, levels of CTLA4+ Tregs in the symptomatic group were significantly higher than in the control group ($P = 0.0034$). In addition, after iRBC stimulation, CTLA-4+ Treg levels remained significantly lower in the asymptomatic group compared to the symptomatic group ($P = 0.0493$) but were comparable to levels observed in healthy controls ($P = 0.5457$, Fig. 1c).

Decreased expression of CD69 activation marker on T cells in asymptomatic *Plasmodium falciparum* infections before infected red blood cell stimulation

With the observed levels of Tregs being lower in the asymptomatic children compared to symptomatic children, the extent of cellular activation was measured to determine if they may differ across the study groups. The expression of the CD69 activation marker on both CD4+ and CD8+ T cell sub-sets before in vitro stimulation was investigated (Fig. 2a). Children with asymptomatic malaria had significantly lower levels of CD69+ expression on CD4+ T cells compared to children with symptomatic disease ($P = 0.0016$) but had comparable levels with the controls ($P > 0.05$, Fig. 2b). In addition, children with symptomatic malaria had a higher level of the CD4+CD69+ T cells than the controls ($P = 0.0068$, Fig. 2b). This trend was the same for the CD8+CD69+ T cells.

The pattern of expression of CD25 on T cells among symptomatic, asymptomatic *Plasmodium falciparum* infections and healthy controls before infected red blood cell stimulation

The expression levels of CD25, a late activation marker, on CD4+ and CD8+ T cells were determined (Fig. 2a). Except for CD8+CD25+CD69− T cells, levels of CD25 were not

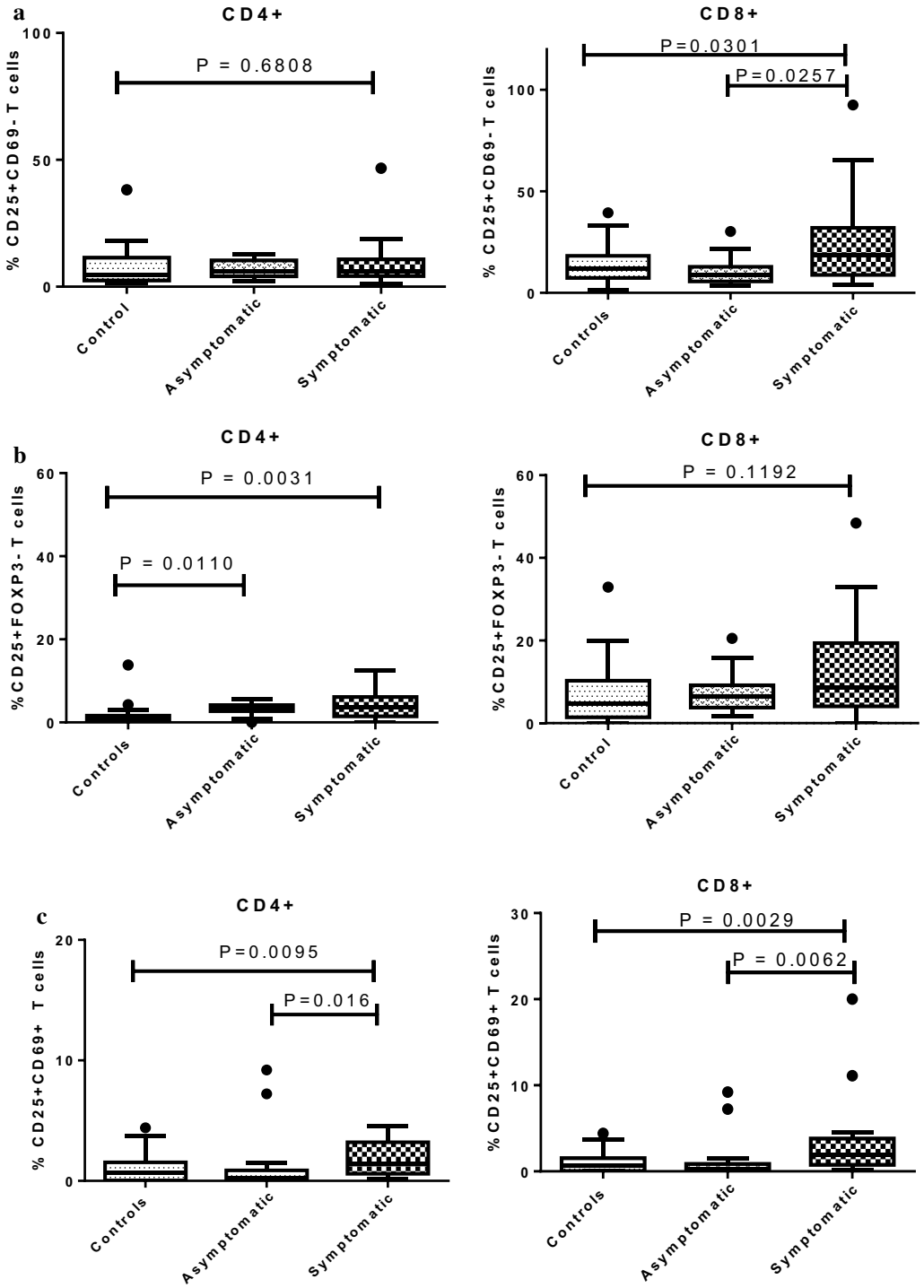


Fig. 3 Expression of T-cell activation markers CD25/CD69 on T cells in PBMCs from the study cohort. **a** CD25+CD69−; **b** CD25+FOXP3− cells; and, **c** CD25+CD69+ on T cells was analysed in healthy controls (n = 17), asymptomatic *P. falciparum*-infected children (n = 18), and symptomatic *P. falciparum*-infected children (n = 21). The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers. Medians are indicated by the horizontal lines across the boxes. Kruskal–Wallis test was used for comparisons, followed by Dunn's test where necessary

significantly different between asymptomatic and symptomatic children. There was no significant difference in the expression of CD25 on CD4+ T cells across the study populations ($P=0.4971$, Fig. 3a). However, for CD8+ T cells, the expression of CD25 was significantly lower in the asymptomatic group compared to the symptomatic children ($P=0.0257$), whereas levels between the asymptomatic and control groups were comparable (Fig. 3a).

CD25+FOXP3− T cells have been classified as activated effector Th 1 cells capable of secreting effector cytokines, such as IFNγ (interferon gamma), TNF (tumour necrosis factor) and IL-10 (interleukin-10). Therefore, the ex vivo expression of these markers was compared across the study groups. No significant difference was observed in the expression of CD25+FOXP3− on either CD4+ or CD8+ T cell sub-sets between asymptomatic and symptomatic children. However, the levels of CD25+FOXP3− on CD4+ T cells were significantly lower in the healthy controls when compared to the asymptomatic ($P=0.0063$) or symptomatic children ($P=0.0024$). In addition, no significant difference was observed in the expression of CD8+CD25+FOXP3− T cells across the study groups ($P=0.1192$, Fig. 3b).

Decreased expression of CD25+CD69+ T cell activation markers during asymptomatic *Plasmodium falciparum* infections before infected red blood cell stimulation

The expression of CD69 activation marker on both CD4+ and CD8+ T cell subsets before in vitro stimulation was investigated in the three study groups (Fig. 2a). For the PBMCs analysed ex vivo, CD4+ and CD8+ T cells expressing both CD25 and CD69 were higher in the symptomatic when compared to the asymptomatic group ($P=0.016$ and $P=0.0062$) and healthy controls ($P=0.047$ and $P=0.0232$), respectively (Fig. 3c). However, no significant difference was observed between the asymptomatic group and controls ($P>0.05$) for both T cell sub-sets (Fig. 3c).

Increased expression of activation marker on T cells in asymptomatic infections after infected red blood cell stimulation

When PBMCs were stimulated in vitro with iRBCs, levels of activated CD4+CD69+ T cells in the asymptomatic children increased significantly above those found in the symptomatic children ($P=0.0002$) and controls ($P=0.0008$, Fig. 4a). Levels of CD4+CD69+ T cells did not differ significantly between symptomatic children and controls. Higher expression of CD8+CD69+ cells was also observed in the asymptomatic cohort compared to both symptomatic children ($P=0.0057$) and controls ($P=0.0054$) (Fig. 4a). As was observed in the CD4+ T cell compartment, levels of CD8+CD69+ T cells did not differ significantly between symptomatic children and controls (Fig. 4a).

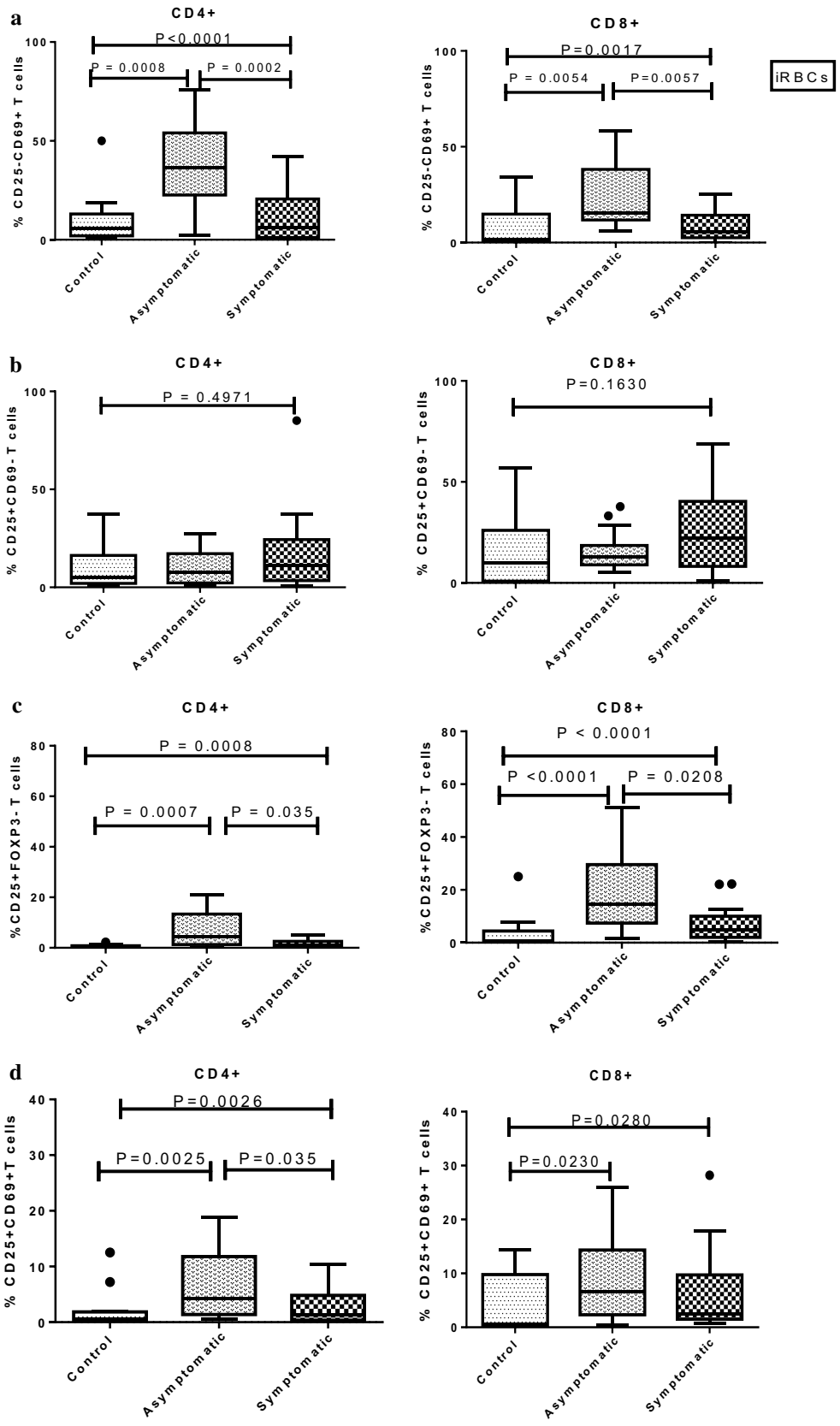
Also, no significant difference was found in the expression of CD25+CD69− T cells on any of the T cell sub-sets across the study groups (Fig. 4b). Levels of CD25+FOXP3− activated effector T cells were increased in the asymptomatic children compared to symptomatic and healthy controls (Fig. 4c). Increased levels of the CD4+CD25+FOXP3− activation marker were found in the asymptomatic group compared to the symptomatic ($P=0.035$) and control ($P=0.0007$) groups. Likewise, CD8+CD25+FOXP3− activated T cells were significantly increased in the asymptomatic group compared to the symptomatic ($P=0.0208$) and control ($P<0.0001$) groups (Fig. 4c). In addition, a significant increase in levels of double-positive CD4+CD25+CD69+ T cells were observed in the asymptomatic children compared to the symptomatic children ($P=0.035$) and controls ($P=0.0025$, Fig. 4d). Likewise, higher expression of CD8+CD25+CD69+ cells was observed in the asymptomatic cohort when compared to the healthy controls ($P=0.023$) but not the symptomatic children ($P>0.05$, Fig. 4d).

Cellular activation and Treg frequency govern parasitaemia and disease status

Correlations between the 24 considered T cell phenotypes (including the pre- and post-iRBC stimulation levels of Tregs) and parasite control (as measured by parasitaemia levels) were investigated. After applying a Bonferroni correction for multiple comparisons, significant positive correlations were found between parasitaemia and the levels of both CD8+CD69+ ($r=0.4128658$, $P=0.0016$) and CD8+CD25+CD69+ ($r=0.4070214$, $P=0.0018$) T cells measured before iRBC

(See figure on next page.)

Fig. 4 Expression of activation markers CD25/CD69 on T-cells from the study cohorts after iRBC stimulation. PBMCs were stimulated with iRBC lysates (iRBCs) to determine the levels of activation markers (CD25/CD69) on both CD4+ and CD8+ T cell sub-sets. The percentage expression of **a** CD25−CD69+; **b** CD25+CD69−; **c** CD25+FOXP3−; and, **d** CD25+CD69+ T cells was analysed in healthy controls (n = 17), asymptomatic *P. falciparum*-infected children (n = 18) and symptomatic *P. falciparum*-infected children (n = 21). The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles presented as whiskers. Medians are indicated by the horizontal lines across the boxes. The Kruskal–Wallis test was used for statistical comparisons between groups. P values < 0.05 were considered to be significant after Dunn's test to correct for multiple comparisons


a
CD4+
P<0.0001
P = 0.0008
P = 0.0002
% CD25-CD69+ T cells
Control
Asymptomatic
Symptomatic
CD8+
P=0.0017
P = 0.0054
P=0.0057
iRBCs
b
CD4+
P = 0.4971
% CD25+CD69- T cells
CD8+
P=0.1630
c
CD4+
P = 0.0008
P = 0.0007
P = 0.035
% CD25+FOXP3- T cells
CD8+
P < 0.0001
P <0.0001
P = 0.0208
d
CD4+
P=0.0026
P=0.0025
P=0.035
% CD25+CD69+T cells
CD8+
P=0.0280
P=0.0230
% CD25+CD69+ T cells

stimulation, and the levels of both CD4+CD25+Foxp3+ ($r=0.4772815$, $P=0.0002$) and CD8+CD25+Foxp3+ ($r=0.4772714$, $P=0.0003$) T cells measured after stimulation. Strikingly, levels of these four T cell phenotypes together accounted for 68% of the variation in parasitaemia observed in asymptomatic and symptomatic children (Additional files 2, 3).

Machine learning was used to determine whether the levels of these four T cell phenotypes alone could be used to predict disease status in infected children. A model based on a support vector machine was fitted to the levels of the four T cell phenotypes measured in a sub-set of the infected children and then used to predict disease severity in the remaining children. Using a 5-fold cross-validation analysis to prevent overfitting, it was found that the model accurately distinguishes between asymptomatic and symptomatic children, with a sensitivity of 86%, a specificity of 94%, and an area under the receiver-operator-characteristic curve (AUC) of 90% (Fig. 5). Together, the results suggest that expression levels of the considered regulatory and activation markers determine most of the individual variation in parasitaemia and predict disease status in asymptomatic and symptomatic *P. falciparum* infections.

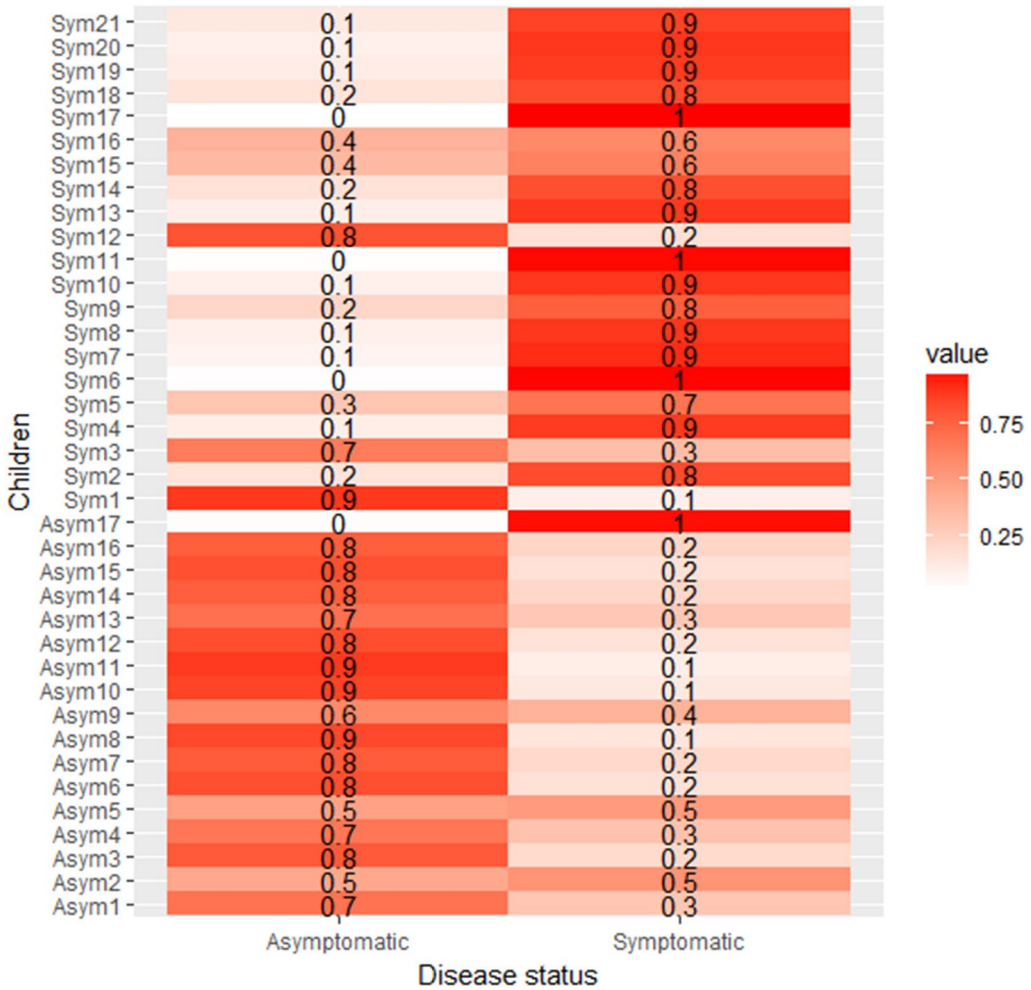


Fig. 5 T cell regulatory and activation markers distinguish between asymptomatic and symptomatic *Plasmodium falciparum* infections. It was assessed whether a machine-learning model based on pre-iRBC stimulation levels of CD8+CD69+ and CD8+CD25+CD69+ T cells and post-stimulation levels of CD4+CD25+Foxp3+ and CD8+CD25+Foxp3+ T cells could accurately predict disease status in asymptomatic and symptomatic children. Thirty-eight children had data for all the 4 T cell phenotypes considered. The children were randomly separated into 5 groups. Fixing one group as a test group, we trained a machine-learning model (precisely a support vector machine) on the other 4 groups and then predicted the disease status of children found in the test group. The process was repeated until each of the groups was used exactly once as a test group. The plot shows a representative heatmap of the predicted probability that each child is either asymptomatic or symptomatic. Strikingly, as expected, most asymptomatic (respectively symptomatic) children have a higher predicted probability of being asymptomatic (respectively symptomatic)

Discussion

The aim of this study was to investigate the frequency of activated Tregs and T cell early and late activation markers during *P. falciparum* infections, and the correlations between these and levels of parasitaemia. It was observed that asymptomatic infections are associated with lower levels of Tregs with reduced Treg activation, and reduced expression of T cell activation markers compared to symptomatic infections. Also, T cells from asymptomatic *P. falciparum*-infected children were more responsive to iRBC stimulation compared to cells from symptomatic children. Importantly, the measured variations in regulatory and activation marker levels explained most (68%) of the variation in parasitaemia observed in asymptomatic and symptomatic infections. These results indicate that in contrast to children with symptomatic malaria, there seems to be appropriate levels of immune regulation and activation in children with asymptomatic malaria, which favor the control of parasitaemia. Another, non-mutually exclusive possibility not ruled out by the analyses is that asymptomatic children might have higher levels of protective antibodies compared to symptomatic children, which might contribute to the observed differences in parasitaemia.

Previous data have shown that malaria-exposed individuals can harbour infection without clinical symptoms, implying that there is some level of immune restriction on parasite replication [5]. Increased levels of Tregs have been associated with higher parasitaemia [10, 11] and delayed parasite clearance [20] as well as the development of clinical disease [15, 32, 33]. In this study, it was found that the level of Tregs is higher in children with clinical malaria compared to children with asymptomatic infections and healthy controls. This supports findings from other studies which have also associated increased Treg frequencies with symptomatic malaria infections [9, 23]. Also, the significant increase in the Treg frequency which was observed in the symptomatic children after iRBC stimulation may indicate that during clinical malaria Tregs from the pre-clinical state are expanded or being induced.

The low levels of Tregs observed in the asymptomatic children corroborates the findings of Boyle et al. [32] who identified lower levels of Tregs in children with asymptomatic infections. This was interesting since in another previous study by Jangpatarapongsa et al. [7] they also identified lower amounts of Treg cytokines in individuals with asymptomatic *Plasmodium vivax* infections, suggesting there is less Treg activation in individuals with asymptomatic *Plasmodium* infections. This supports the view that lower levels of Tregs may be associated with a decreased risk of developing clinical disease and possibly an increased likelihood of developing immunity to malaria.

It has recently been shown that Tregs expressing CTLA-4 in murine models of malaria interfere with the acquisition of long-term immunity to malaria infections [13]. The increase in CTLA-4 in Tregs observed in individuals with *P. falciparum* infections compared to uninfected controls could reflect their direct role in controlling immune responses during human malaria infections. Also, the increased expression of CTLA-4 on Tregs in the symptomatic children suggests that immune regulation associated with clinical malaria may affect cellular activation, consequently, affecting the downstream development of anti-malaria immunity.

Importantly, persistent immune activation has been described as a major factor in predicting disease with increased levels of activation being associated with clinical disease progression [34–36]. Resting T cells are identified phenotypically by the absence of CD25/CD69 markers [37]. In this study, activated T cells were classified by the expression of CD25+/CD69+ markers. A significant increase in immune activation in the CD4 and CD8 T cells was observed in clinical malaria. This is in line with a previous study that observed increased immune activation during clinical malaria infections [38]. However, it should be noted that the increased activation in symptomatic children may not directly connote an effective T cell response since cytokine profiles were not measured.

In contrast, in the asymptomatic children, it was found that fewer cells expressed both activation markers on either CD4 or CD8 T cells indicating reduced cellular activation when compared to children with symptomatic malaria. A plausible interpretation of these results is that lower immune suppression by Tregs in asymptomatic children leads to more effector T cell activation and greater parasite control, which in turn feeds back to reduce T cell activation. Conversely, higher immune suppression in symptomatic children might limit parasite control leading to higher levels of parasitaemia and T cell activation. This is in line with the observation that *P. falciparum* activates the immune system in a dose-dependent manner [39]. In addition, the lack of symptoms, lower levels of immune activation, and lower parasitaemia in the asymptomatic children might also result from higher levels of parasite-specific antibodies that reduce parasitaemia levels below the threshold required to induce a T cell response. Additional research is needed to elucidate these hypotheses.

It has been shown that asymptomatic children maintain levels of CD4+CD25+FOXP3- effector T cells that co-produce IFNγ, TNF and IL-10, describing these cells as self-regulatory [9, 40]. Even though lower levels of Tregs and activated Tregs were observed in the

asymptomatic children, CD4+CD25+FOXP3− T cells were not significantly different between the asymptomatic and symptomatic children. This may suggest that during asymptomatic malaria, restriction of parasite replication and inflammation may be mediated by these self-regulating effector T cells.

Unfortunately, this study had a number of limitations since a longitudinal study could not be conducted to determine if any of the asymptomatic cohorts may develop clinical disease because they were treated when diagnosed. Consequently, the possibility that the immune dynamics observed reflect changes that occur during the natural course of *P. falciparum* infections could not be ruled out. In addition, because parasitaemia was determined by microscopy, it was not possible to determine conclusively that none of the healthy cohort had submicroscopic infection. It was also, not possible to evaluate the humoral response in the study population to determine its contribution to the immune dynamics observed.

Conclusion

The study shows evidence that Tregs are lower and associated with reduced Treg activation in children with asymptomatic *P. falciparum* infections, which corresponds to reduce cellular activation and lower levels of parasitaemia. Also, the greater expansion of activation markers after iRBC stimulation in asymptomatic children compared to symptomatic children suggests that the former children harbour a larger latent repertoire of parasite-responsive T cells. Alternatively, this observation could reflect less Treg-mediated suppression of T cell activation in cells from asymptomatic children. Together, these data support the view that the dynamics of T cell regulation and activation may contribute to the acquisition of anti-parasite and/or anti-disease immunity to malaria. Insights into these dynamics might inform the development of malaria vaccines that induce appropriate levels of cellular activation and regulation as well as optimal control of parasitaemia and disease.

Additional files

Additional file 1. Antibody panel and clones used.

Additional file 2. Linear regression analysis to determine the level of variation in parasitaemia using 4 T cell phenotypes.

Additional file 3. Diagnostic plots for the regression analysis described in the legend of Additional file 2.

Abbreviations

Tregs: regulatory T cells; CTLA-4: cytotoxic T-cell lymphocyte antigen 4; PBMC: peripheral blood mononuclear cells; iRBCs: infected red blood cells; uRBCs: uninfected red blood cells; IFNγ: interferon gamma; TNF: tumour necrosis factor; IL-10: interleukin 10; DMSO: dimethyl sulfoxide.

Authors' contributions

AF and WN conceived the idea, designed the experiments. WN, MFO and KAK supervised the work. AF performed the experiments in the study. BT assisted in the experiment. AF, KAK, MFO and WN wrote the paper. All authors read and approved the final manuscript.

Author details

[1] West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), Department of Biochemistry, Cell and Molecular Biology, University of Ghana, Legon, P. O. Box LG 54, Accra, Ghana. [2] Immunology Department, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, P.O. Box LG 581, Accra, Ghana. [3] African Institute for Mathematical Sciences, P.O. Box DL 676, Cape-Coast, Ghana. [4] African Institute for Mathematical Sciences, University of Stellenbosch, 7 Melrose Rd, Muizenberg, Cape Town 7945, South Africa.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author(s) on reasonable request.

Consent for publication

The authors have read and agreed to the content of this manuscript and its publication upon acceptance.

Ethics approval and consent to participate

Ethical approval was obtained from the ethics committee of the Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana. Participation was voluntary and written informed consent and assent were obtained from parents/guardians and the children respectively.

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Phenotypic Evidence of T Cell Exhaustion and Senescence During Symptomatic *Plasmodium falciparum* Malaria

Augustina Frimpong[1,2,3]*, *Kwadwo Asamoah Kusi*[1,2], *Dennis Adu-Gyasi*[4], *Jones Amponsah*[2], *Michael Fokuo Ofori*[1,2] and *Wilfred Ndifon*[3,5]*

[1] Department of Biochemistry, Cell, and Molecular Biology, West African Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana, Accra, Ghana, [2] Immunology Department, Noguchi Memorial Institute for Medical Research, College of Health Sciences, University of Ghana, Accra, Ghana, [3] African Institute for Mathematical Sciences, Cape Coast, Ghana, [4] Kintampo Health Research Centre, Kintampo, Ghana, [5] African Institute for Mathematical Sciences, University of Stellenbosch, Cape Town, South Africa

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Faith H. A. Osier,
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Johanna Daily,
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***Correspondence:**
Augustina Frimpong
tinafrimp@gmail.com
Wilfred Ndifon
wndifon@aims.ac.za

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T cells play significant roles during *Plasmodium falciparum* infections. Their regulation of the immune response in symptomatic children with malaria has been deemed necessary to prevent immune associated pathology. In this study, we phenotypically characterized the expression of T cell inhibitory(PD-1, CTLA-4) and senescent markers (CD28(-), CD57) from children with symptomatic malaria, asymptomatic malaria and healthy controls using flow cytometry. We observed increased expression of T cell exhaustion and senescence markers in the symptomatic children compared to the asymptomatic and healthy controls. T cell senescence markers were more highly expressed on CD8 T cells than on CD4 T cells. Asymptomatically infected children had comparable levels of these markers with healthy controls except for CD8+ PD-1+ T cells which were significantly elevated in the asymptomatic children. Also, using multivariate regression analysis, CTLA-4 was the only marker that could predict parasitaemia level. The results suggest that the upregulation of immune exhaustion and senescence markers during symptomatic malaria may affect the effector function of T cells leading to inefficient clearance of parasites, hence the inability to develop sterile immunity to malaria.

Keywords: malaria, *Plasmodium falciparum*, T-cell, exhaustion, immune senescence, PD-1, CTLA-4, CD57

BACKGROUND

Clinical malaria is a disease of public health importance due to its associated morbidity and mortality (1). With the emergence of drug-resistant parasites and insecticide resistant vectors, there is a need to develop effective interventions (2–4). Despite promising results of candidate vaccines in naïve individuals, comparatively poorer responses are observed in people in endemic areas (5, 6), indicating that much effort needs to be focused on understanding host factors associated with the development of immunity, especially in malaria-endemic areas. Blood stage infection with malaria parasites may either result in asymptomatic malaria, uncomplicated malaria, or proceed to complications such as severe malaria anemia or cerebral malaria. Repeated exposure to parasites usually results in the acquisition of anti-disease immunity which is characterized by the absence of

clinical symptoms, yet with susceptibility to the infection. This suggests that the naturally induced immune response generated against *P. falciparum* may not always be potent enough to eradicate the infection. Therefore, malaria vaccines that can protect against symptomatic disease and possibly also eliminate infections remain a global health priority.

Lymphocytes, including T cells, play a significant role in the generation of protective malaria-specific responses (7), and their mechanism of action may either be by controlling or decreasing parasitemia (8) or by exacerbating the infection promoting parasitemia (9). However, looking at natural infections it can be presumed that the inability to eliminate *P. falciparum* malaria may be associated with immune dysfunction resulting from the expression of markers that negatively regulate T cell activity or result in their ineffective response. These may lead to the exhaustion of T cells, which has been well-described in viral infections including HIV and hepatitis B (HBV) (10, 11) as well as in protozoan infections like Toxoplasmosis and Leishmania (12, 13).

In malaria, work in both human and murine models has reported the upregulation of immune inhibitory markers such as T-cell immunoglobin and mucin domain-3 (TIM-3), lymphocyte-activation gene-3 (LAG-3) and programmed cell death-1 (PD-1) during acute infections (14, 15). These have been shown to affect not only the effector functions of T cells including cell proliferation and cytokine production but also antibody generation by B cells (16). Specifically, PD-1 has been associated with decreased cytokine production and proliferation in T cells as well as enhancing disease progression, whereas CTLA-4 has been associated with T cell anergy and establishment of immunological tolerance (17, 18). Furthermore, it has been shown that the dysfunctional nature of exhausted T cells in murine models of malaria can be reversed by blockage of these receptors as this enhances effective parasite clearance and acquisition of immunity (16, 19).

In addition to immune exhaustion, infectious pathogens such as Cytomegalovirus (CMV) and Human Immune deficiency virus (HIV) have been associated with accelerated aging of the body's immune defense system through the upregulation of CD57, a classical marker for immune senescence (20, 21). CD57 is a terminally differentiated marker found on some cell subsets including T cells (22–24). Naïve T cells express CD28, a co-stimulatory molecule that provides signaling for T cell activation) after antigen recognition and this may bind to B7 proteins to provide co-stimulatory signals (25, 26). However, repeated T cell activation is associated with the progressive loss of CD28, a characteristic of memory or terminally differentiated cells, and the corresponding upregulation of CD57 (27–29). These senescent cells are characterized by shortened telomeres, replicative senescence, loss of CD27 resulting in a low proliferative capacity of the cells (30), eventually, leading to an inability to eradicate an infection. Importantly, the expression of CD57 is associated with repeated antigen stimulation (31) which was identified to accurately predict replicative senescence (22). In addition, CD57 expression on CD28- T cells has been shown to differ from the normal aging T cell phenotypes (CD28-CD57+, similarly observed in CMV) (31, 32) found in HIV infections (33).

Cellular aging has been described in wild birds chronically infected with malaria (34). Interestingly, a recent study reported evidence of cellular aging in travelers with single acute *P. falciparum* infections, characterized by decreased telomerase activity and increased levels of CDKN2A, a molecular marker associated with cellular aging (35). Nevertheless, it remains to be elucidated if frequent exposure to malaria is associated with increased expression of markers of T cell senescence in endemic areas. Here, we determined the expression profile of inhibitory or exhaustive, and immune-senescence markers on both CD4+ and CD8+ T cells. We characterized the expression of PD-1, CTLA-4, CD28 and CD57 markers in children with symptomatic malaria, asymptomatic malaria and healthy controls. In addition, we also determined the impact of these T cell phenotypes on parasitaemia and inflammation (using the platelet-to-lymphocyte ratio).

MATERIALS AND METHODS

Ethics Statement

The study protocols were approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research at the University of Ghana. All participants were children and informed consent was obtained from parents or guardians and assent properly received from the children before they were enrolled in the study. All methods were performed in accordance with the relevant guidelines and regulations.

Study Subjects

A total of 57 children within the age ranges of 1–12 years were recruited for the study, consisting of healthy children with no *P. falciparum* infections ($n = 17$), children with asymptomatic *P. falciparum* malaria ($n = 18$) and children with clinical malaria ($n = 22$) who were recruited from the Asutsuare and the Paakro sub-districts which are hyper-endemic areas for malaria transmission in Ghana. A volume of 5 ml of venous blood was collected from all study participants after recruitment. Parasites were identified using Giemsa stained thick and thin blood films. Clinical cases of malaria recruited from the health centers were defined by a history of fever within 24 h of health center attendance and presence of parasitaemia. For clinical cases, we collected venous blood samples from the children before anti-malarial treatment, based on the nationally recommended guidelines. Asymptomatic cases were recruited from the community and were defined by the presence of parasitaemia, absence of fever and no signs or symptoms of the disease. Healthy children, also recruited from the community were selected based on the absence of parasitaemia, fever and no signs or symptoms of the disease.

Peripheral Blood Mononuclear Cells (PBMC) and Plasma Isolation

Isolation of PBMCs was performed by density gradient centrifugation using ficoll paque. After isolation, PBMCs were enumerated and cryopreserved in fetal bovine serum with 10%

dimethyl sulfoxide. PBMCs were kept at −80°C overnight and subsequently transferred to liquid nitrogen until required for the experiment.

Flow Cytometry Analysis

PBMCs were retrieved, thawed and washed. The viability was measured by the trypan blue dye exclusion method and cells with viability > 95% were used in the assay. All the antibodies were purchased from BD except anti-human FOXP3 fluorochrome-conjugated antibody (Biolegend). After washing, the cells were extracellularly stained with the following antibodies: anti-CD3-(APC H7), anti-CD4 (BUV 395), anti-CD8 (PerCP Cy5.5), anti-CD28 (APCR700), anti-CD57 (FITC) and anti-PD-1 (BUV737) on ice for 30 min. The cells were washed, fixed and permeabilized using FOXP3 buffer set (BD) according to manufacturer's instructions and intracellularly stained for FOXP3 (PE) and CTLA-4 (APC) on ice for 40 min. We gated for T cells by CD3, CD4, and CD8 lineage markers. Gates for inhibitory and senescence markers were defined using fluorescence minus one controls (**Figure S1**). Cells were acquired on a BD LSR Fortessa II-X20 cytometer. Data were compensated and analyzed using Flowjo V10 software (Tree Star, San Carlos, CA).

Statistical Analysis

Data analyses were performed with R-studio for statistical analysis (version 2) and the GraphPad Prism version 6.01 (GraphPad Software, Inc.). For comparing the markers of T cells among the three study populations, the Kruskal-Wallis test with a Dunn's *post hoc* test for multiple comparisons was used. Spearman's rank correlation was used to determine associations between markers. Principal component analysis (PCA) was conducted to identify and visualize significant features of T cell phenotypes (degree of variation) that can cluster our study populations by considering all phenotypes measured. PCA which is an unsupervised learning algorithm provides dimensions (linear combinations) along which the data are separable and reduces the noise associated with data whilst increasing its robustness. PCA was used since it reduces the data set to a small set of patterns and retain the significant features that are responsible for variation (separating the data into clusters). Multiple linear regression models with likelihood ratio test were also used to investigate the association between parasitaemia or inflammation and the measured cellular markers. Statistical significance was set at $P < 0.05$.

RESULTS

Clinical Characteristics of the Study Participants

The study was approved by the Noguchi Memorial Institute for Medical Research Institutional Review Board. This was a cross-sectional study in which we recruited 57 children in the age range of 1–12 years. The participants included 22 symptomatic children, 18 asymptomatic children and 17 healthy controls (**Table 1**). The sexes of the children were comparable amongst the study groups ($p < 0.05$). Healthy children were older than the asymptomatic($p < 0.05$) and symptomatic children ($p < 0.05$). Levels of parasitemia mirrored the intensity of infection, with symptomatic children having a higher parasite load compared to the asymptomatic children ($p < 0.001$). Hemoglobin levels were significantly decreased in the symptomatic children in comparison to the asymptomatic children ($p < 0.05$). Even though, the lymphocyte count was not significantly different amongst the study groups, they also mirrored the intensity of infection (p>0.05). We found the granulocyte count to be comparable amongst the study groups ($p > 0.05$). Also, the platelet-to-lymphocyte ratio (PLR) was found to be comparable between the healthy controls and asymptomatic groups ($p < 0.05$) but higher than the symptomatic group ($p < 0.05$).

Increased Expression of PD-1 and CTLA-4 Markers on T Cells in Children With Symptomatic *P. falciparum* Malaria

We first investigated the expression of the inhibitory markers PD-1 and CTLA-4 on T cells **(Figure 1A)**. The expression levels of PD-1 were significantly upregulated in the symptomatic children compared to the asymptomatic ($p < 0.0001$) and healthy groups ($p < 0.0001$) for the CD4+ T cells (**Figure 1B**). Levels of PD-1 in the asymptomatic children and healthy children were comparable. Similarly, CD8+PD-1+ T cells were upregulated in children with symptomatic malaria compared to asymptomatic ($p = 0.0312$) and uninfected controls ($p < 0.0001$). Nevertheless, the expression of PD-1 on CD8+ T cells was increased significantly in the asymptomatic children compared to the healthy controls ($p = 0.0359$). Of note, the levels of PD-1 were higher in CD8+ T cells compared to the CD4+ T cells in all study groups. Also, the expression levels of CTLA-4 on CD4+ T cells were increased significantly in the symptomatic children compared to the asymptomatic ($p < 0.001$) and healthy controls ($p < 0.05$) whereas comparable levels of expression were found between asymptomatic children and healthy controls (**Figure 1C**). This trend was the same for the levels of CTLA-4 expression on CD8+ T cells among the study groups: symptomatic children had increased levels compared to asymptomatic ($p < 0.001$) and healthy children ($p < 0.05$).

Next, we assessed the expression of PD-1 and CTLA-4 double-positive markers on both T cell subsets. The symptomatic children had significantly higher levels of PD-1 and CTLA-4 double positive markers on CD4+ T cells in comparison to the asymptomatic children ($p < 0.0001$) and healthy controls ($p = 0.0091$). Similarly, levels on CD8+ T cells were higher in symptomatic children compared to the asymptomatic children ($p = 0.0121$) and healthy controls ($p = 0.0098$) (**Figure 1D**). In all, levels of PD-1 and CTLA-4 double positive markers between the asymptomatic children and healthy controls were comparable and not significantly different.

The significant levels of inhibitory markers observed in children with symptomatic malaria may be related to the inadequacy of effector functions in clearing parasitemia.

TABLE 1 | Clinical characteristics of the study participants.

Characteristics	Control (C)	Asymptomatic (A)	Symptomatic (S)	*P*-values
Participants	$n = 17$	$n = 18$	$n = 22$	
Age (IQR), years	9(8–11)	7(4.5–9)	6(4.8–7)	0.0087[a]
Female (%)	52.94	44.44	50	0.8765[b]
Hemoglobin, g/dl[#]	11.5(0.994)	12.7(1.234)	10.7(3.025)	0.0402[c]
Parasitemia (IQR), μl	NA	845(260.7–3812)	13973(7238–58764)	0.0009[d]
Granulocytes (10^9/L)[#]	3.353(1.335)	3.069(2.115)	5.041(3.310)	0.0518[c]
Lymphocytes (10^6/L)	2.9(2.5–3.6)	2.1(1.2–3.45)	1.9(1.3–3.9)	0.0889[a]
Platelets (10^9/L)	305(237–356)	223(193–280)	101(61–198)	>0.0001[a]
PLR(IQR)[&]	95.31(82.79–133.9)	70.97(27.52–205.3)	53.54(31.19–95.31)	0.0345[a]

IQR, interquartile range. [a]Kruskal Wallis test. [b]Chi-square test. [c]One-way ANOVA. [d]Mann-Whitney U-test. [#]Mean(Standard deviation). [&](C = 17, A = 16, S = 19).

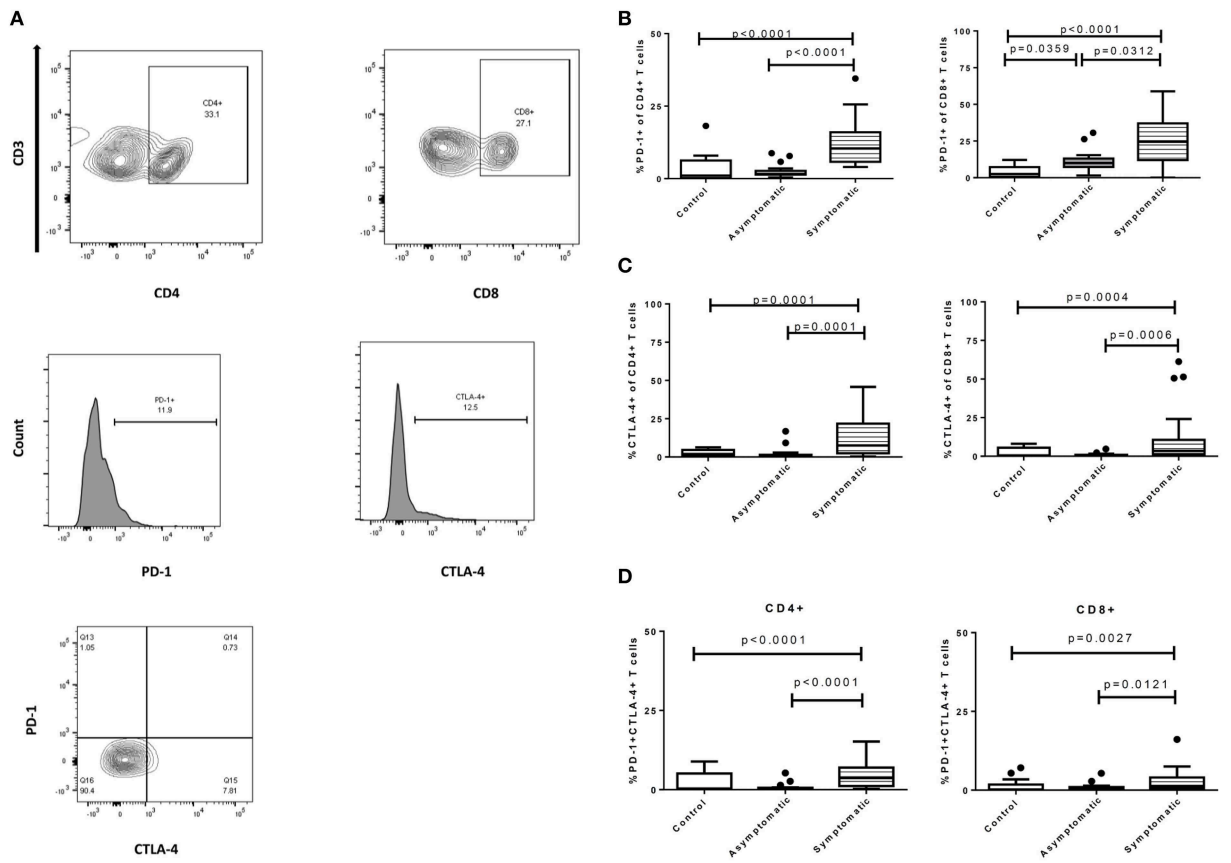

FIGURE 1 | Expression profile of exhaustion and regulatory markers on CD4+ and CD8+ T cells among symptomatic malaria patients ($n = 22$), asymptomatic malaria($n = 18$) and healthy children ($n = 16$). **(A)** The gating strategy to identify expression levels of PD-1 and CTLA-4 markers. The expression profile comparing levels of **(B)**. PD-1, **(C)**. CTLA-4, **(D)**. PD-1 and CTLA-4 double-positive markers, on both CD4+ and CD8+ T cells across the study subjects. Levels of expression were compared using the Kruskal-Wallis with Dunn's test to correct for multiple comparisons. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers with medians indicated by the bold horizontal lines across the boxes. $P < 0.05$ were considered statistically significant.

Symptomatic *P. falciparum* Infection Is Associated With the Upregulation of T-Cell Senescence Markers

We also determined if symptomatic malaria may be associated with the biological aging of T cells, by measuring senescent markers using CD28 and CD57 and comparing the proportions with the asymptomatic and healthy groups. We first determined the proportions of T cells expressing CD57 which were found to higher on CD8+ T cells compared to the CD4+ T cells. This trend was similar in all 3 study groups (**Figure 2**).

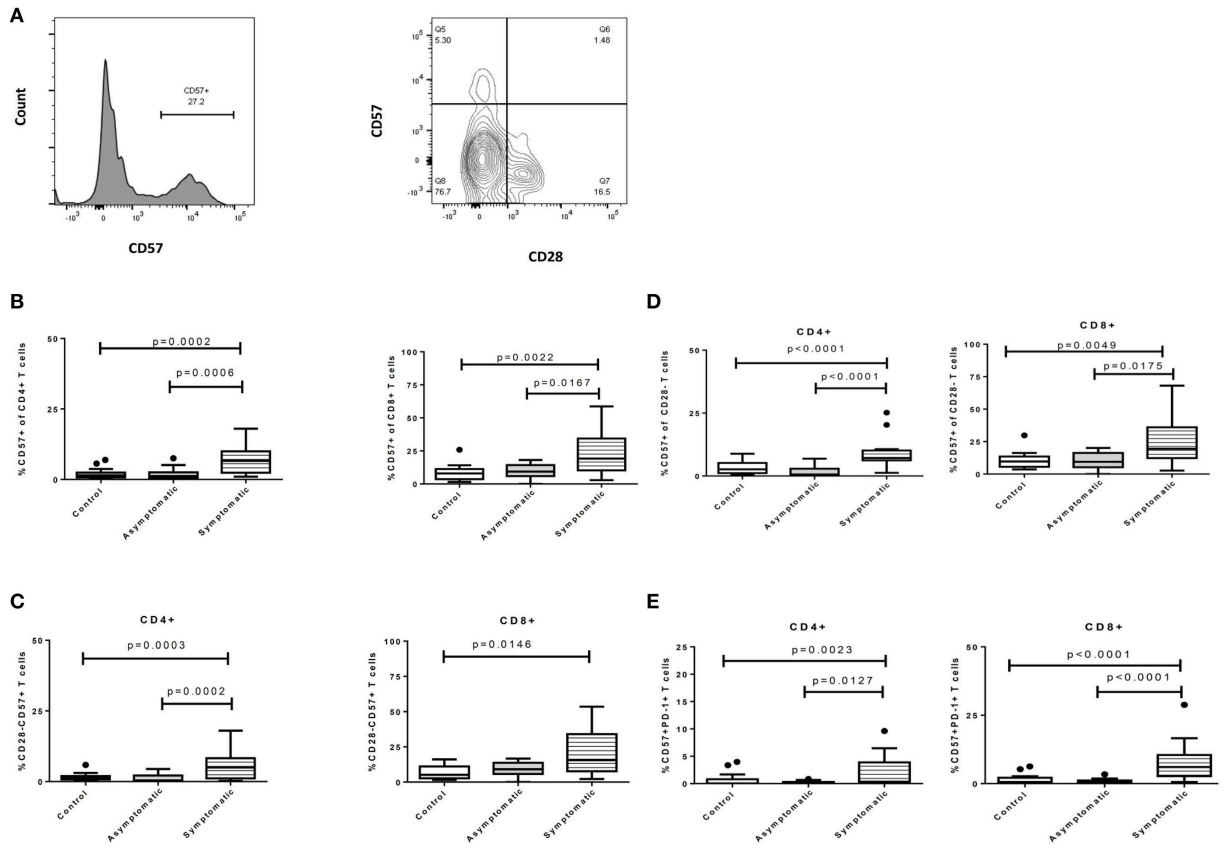

FIGURE 2 | Symptomatic *P. falciparum* infection is associated with the upregulation of T-cell senescence markers. The expression profile of immune senescence markers on CD4+ and CD8+ T cells among symptomatic malaria patients, asymptomatic malaria and healthy children. **(A)** The gating strategy for identifying CD57 and CD28 surface markers. The expression profile comparing levels of **(B)**. CD57, **(C)**. CD28-CD57+, **(D)**. CD57+ of CD28- and **(E)**. PD-1+CD57+ markers on both CD4+ and CD8+ T cells across the study subjects. The data are presented as box plots with inter-quartile ranges. The 10th and 90th percentiles are presented as whiskers with medians indicated by the bold horizontal lines across the boxes. Levels of expression were compared among the study population using the Kruskal-Wallis with Dunn's test to correct for multiple comparisons. $P < 0.05$ were considered statistically significant.

Levels of CD4+CD57+ T cells were significantly higher in children with symptomatic malaria compared to asymptomatic ($p = 0.0006$) and healthy controls ($p = 0.0041$). A similar trend was observed for the CD8+ T cell subsets where a significant difference was observed between symptomatic and asymptomatic children ($p = 0.0167$) and healthy controls ($p = 0.0050$) (**Figure 2A**). Secondly, we checked for the percentage expression of CD28-CD57+ T cells, a marker frequently associated with T cell aging in the elderly. Levels of CD28-CD57+CD4+ T cells were also increased in children with symptomatic malaria compared to children with asymptomatic infections ($p = 0.0002$) and healthy controls ($p = 0.0064$). In contrast, levels of the CD28-CD57+ marker on CD8+ T cells did not differ between the symptomatic children and asymptomatic children ($p = 0.1115$), but was increased in the symptomatic group compared to healthy controls($p = 0.0178$) (**Figure 2B**).

Later, we gated on CD28- T cells and measured the expression of CD57 on CD28- T cells (CD57 of CD28- T cells) to determine if the expression levels may be similar or differ from what is observed in normal aging or HIV infections. We found that the percentage expression of CD57+ on CD28-CD4+ T cells remained increased in children with symptomatic malaria compared to children with asymptomatic infections ($p < 0.0001$) and healthy controls ($p = 0.0261$). Also, the percentage expression of CD57 on CD28- CD8+ T cells was significantly increased in children with symptomatic malaria compared to those with asymptomatic malaria ($p = 0.0175$) or healthy controls ($p = 0.0147$) (**Figures 2B,C**).

We further compared the expression of CD57 and PD-1 double-positive markers (commonly associated with increased apoptosis) on T cells in the study participants. We observed that CD4+ T cells expressing both CD57 and PD-1 were increased in children with symptomatic malaria compared to asymptomatic ($p = 0.0127$) and healthy controls ($p = 0.0071$; **Figure 2D**). This trend was similarly observed in the CD8+ T cells: levels of PD-1+CD57+CD8+ T cells were increased in children with symptomatic malaria in comparison to asymptomatic children ($p < 0.0001$) and healthy

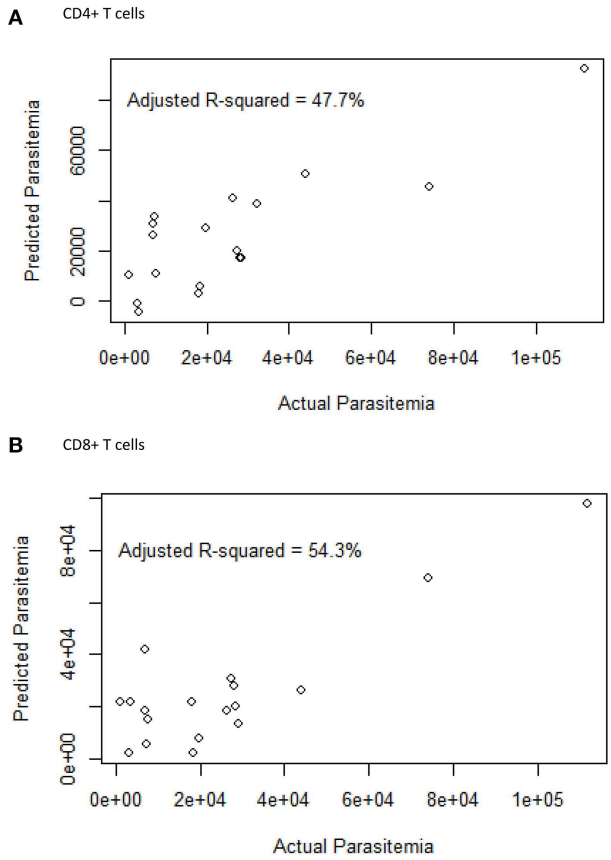

FIGURE 3 | The relationship between the surface markers and parasitemia. A multiple linear regression plot. Actual parasitaemia levels were indicated on the x-axis with the predicted values of parasitaemia on the y-axis. The model was designed using 7 T cell phenotypes measured in **(A)** CD4 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) and **(B)** CD8 (PD-1, CTLA-4, CD57, PD-1CTLA-4, PD-1CD57, CD28-CD57+, CD57+ of CD28-) T cells from PBMCs obtained from the symptomatic children.

controls ($p = 0.0001$). Overall, T cells from symptomatic *P. falciparum* infected children showed phenotypic evidence of T cell senescence.

CTLA-4 Is a Major Predictor of Parasite Load During *Plasmodium falciparum* Infection4

The effect of cellular markers on parasitemia and inflammation was investigated using multivariate regression analysis. We analyzed 7 T cell phenotypic markers; inhibitory (PD-1+, CTLA-4+, PD-1CTLA-4+) and senescence (CD57+, CD28-CD57+, CD57+ of CD28-, PD-1+CD57+)each on both CD4+ and CD8+ T cells to determine if any of these markers could predict parasitaemia or inflammation (PLR). We defined inflammation by the ratio of platelet-to-lymphocyte count (PLR) (36–38). Before the multivariate analysis, we initially performed a correlation analysis to determine if any of the phenotypes may be significantly associated with PLR or parasitaemia. The proportions of PD-1 ($r = -0.65$, $p < 0.01$) and CTLA-4 ($r = -0.506$, $p<0.05$) were inversely correlated with PLR (**Figure S4**) but positively correlated with parasitaemia (for PD-1, $r = 0.4631$, $p < 0.05$; CTLA-4, $r = 0.4831$, $p < 0.05$). However, using the multiple linear regression model and performing a likelihood ratio test, expression levels of CTLA-4 on both CD4+ and CD8+ T cells were found to significantly predict the level of parasitemia in the symptomatic children (**Figure 3**; **Tables 2**, **3**). Likewise, the levels of CD8+CD28-CD57+ and CD57 on CD8+CD28- T cells could significantly explain some of the variation observed in parasitemia (**Tables 2**, **3**). Even though all the coefficients from the regression analysis for the T cell phenotypes were inversely associated with inflammation, none could be a predictor of inflammation (**Table S1**).

On the other hand, among the asymptomatic malaria group, levels of CD4+PD-1+ and CD4+PD-1+CD57+ could predict and explain some of the variation observed in parasitemia ($p < 0.05$; $p < 0.0001$) whereas for CD8+ T cells, the expression of CTLA-4 ($p < 0.001$) and PD-1+CTLA-4+ ($p < 0.0001$) were good predictors of parasitemia (**Figure S3**; **Table S2**).

Multivariate Analysis of T Cell Inhibitory and Senescent Markers

In order to identify significant immunological signatures (T cell phenotypes) that can explain the variation in our study population and separate our study population into clusters, we performed a principal component analysis (PCA). From the eigen values we obtained, we selected principal components that best explained the variations in the datasets. Components 1 and 2 for the CD4+ T cells accounted for 73.1% (62 and 11.7%, respectively) of the variation in data whereas, for CD8+ T cells, PC1 and PC2 accounted for 81.1% (56.3 and 24.8, respectively). From the plots, it can be observed that mostly the symptomatic group had higher PC values compared to the asymptomatic group. Using the entire datasets, the principal components clustered our population into three groups based on the frequencies of the phenotypes (**Figures 4A,B**). Also, from the CD8+ T cells, it can be observed that PC1 is associated with inhibitory markers located in the upper right quadrant whereas PC2 is associated with senescent markers, located in the lower right quadrant. In addition, the loadings of PD-1 and CD57 were significant for PC1 and PC2, respectively. Further analysis indicated the T cell phenotypes contributing to most of the variation for CD4 T cells were CTLA-4 and PD-1 whereas, for CD8 T cells, the important markers were PD-1, CD57 and CTLA4 (**Figure S2**).

The Interrelationship Between the Cellular Inhibitory Markers

Next, we determined the interrelationship between the surface markers (including FOXP3) using partial correlation, a multiparametric correlation analysis that controls for confounding factors (**Tables 4**, **5**). For instance, significant positive correlations for CD4 T cells were observed between

FOXP3 and PD1, FOXP3 and CD57, PD-1CTLA-4 and CD57 ($p < 0.05$). Generally, significant correlations were all positively related.

DISCUSSION

The upregulation of inhibitory and senescent markers on T cells has been associated with the impairment of effector T cell responses. In this study, we sought to identify T-cell immune signatures that may be associated with the development of symptomatic malaria. We analyzed the pattern of expression of co-inhibitory and senescent markers in children with symptomatic *P. falciparum* malaria, asymptomatic malaria and healthy controls. We found that the expression of these exhaustive and senescent markers was increased in children with symptomatic malaria compared to those with asymptomatic infections and healthy controls. Using multivariate regression analysis with likelihood ratio test, we found CTLA-4 to be a strong predictor of parasitemia levels. Also, none of the T cell phenotypes measured was a good predictor of inflammation, even though PD-1 and CTLA-4 were inversely correlated with inflammation. Using a principal component analysis, our study population was clustered into three groups based on the level of expression of the cellular markers. Further analysis revealed that, for CD4+ T cells, the levels of CD4+CTLA4+ and CD4+PD-1+ markers could explain the clustering pattern of the study groups, whereas for CD8+ T cells the important markers were CD8+PD-1+, CD8+CD57+ and CD8+CTLA-4+. In addition, we observed a lower platelet-to-lymphocyte ratio in the symptomatic malaria group probably resulting from the decreased platelets and lymphocytes counts that are associated with clinical malaria (39, 40).

The activation of T cells by pathogens leads to the induction of inhibitory receptors such as CTLA-4 and PD-1 (41). PD-1 and CTLA-4 are some of the well-characterized inhibitory receptors associated with the exhaustion of T cells (16, 42, 43). Levels of expression of the inhibitory markers PD-1 and CTLA-4 were upregulated in children with symptomatic malaria, confirming recent studies that have observed increased levels of PD-1 and CTLA-4 during acute infections (14, 44, 45), resulting in decreased production of cytokines (46). This, therefore, suggests that the increased frequency of inhibitory markers during clinical disease may alter the effector function of T cells.

It has been shown by Butler et al. (16) that levels of CD4+PD-1+ phenotypes correlate with parasitemia in clinical malaria. In this study, we found that CD4+PD-1+ and CD8+CTLA-4+ could also predict parasitemia levels in the asymptomatic malaria group. Importantly, in the symptomatic group, the expression of CTLA-4 was a major predictor in determining parasitemia load. This suggests that T cell exhaustion may induce tolerance which may promote parasitemia. Since both PD-1 and CTLA-4 are negative regulators of the immune response, their observed increase in symptomatic children may indicate that PD-1 and CTLA-4 contribute in regulating T cell activity or inflammation (14, 46). Interestingly, we found an inverse association between these inhibitory markers and inflammation, which is in line with previous observations in murine models that blockage of T cell inhibitory markers exacerbated the immune response, increased susceptibility to severe disease and decreased survival (47, 48). Furthermore, the strong association we observed between T cell exhaustion and clinical parameters such as parasitemia and inflammation suggests that T cell exhaustion plays a vital role in malaria pathogenesis.

We have previously shown that asymptomatic *P. falciparum* infections are characterized by a reduced frequency of regulatory T cells (49). Here, we hypothesized that asymptomatic infections may have reduced expression of inhibitory markers compared to symptomatic children. We found that the expression levels of inhibitory markers in asymptomatic and healthy controls were mostly comparable except for PD-1 which was increased in the *P. falciparum* infected groups compared to the healthy controls (43). It may indicate PD-1 expression may be driven by *P. falciparum* infections. This could also imply that continuous exposure to *P. falciparum* infections may render T cells to be defective in function. Unfortunately,

TABLE 2 | The association of inhibitory markers on CD4+ T cells and parasitaemia for the symptomatic malaria population.

Covariate	Coefficient	*p*-value	LR test (*p*-value)
CTLA4	0.386	0.12	**0.032***
PD1	0.453	0.25	0.11
PD1CTLA4	0.509	0.57	0.44
CD57	0.499	0.48	0.33
CD57–CD28+	0.434	0.2	0.075
PD1CD57	0.52	0.75	0.66
CD28–CD57+	0.503	0.51	0.37

*$p < 0.05$.
Statistically significant values are highlighted in bold.

TABLE 3 | The association of inhibitory markers on CD8+ T cells and parasitaemia for the symptomatic malaria population.

Covariate	Coefficient	*p*–value	LR test (*p*-value)
CTLA4	0.253	**0.018***	**0.0012****
PD1	0.525	0.26	0.12
PD1CTLA4	0.545	0.351	0.2
CD57	0.563	0.491	0.34
CD57+CD28-	0.447	0.098	**0.023***
PD1CD57	0.582	0.792	0.72
CD28-CD57+	0.438	0.09	**0.02***

*Regression coefficients and p-values were determined using a multiple linear regression model. The effect of each phenotype in predicting the degree of inflammation was adjusted using the other considered phenotypes. The likelihood ratio (LR) test was used to assess the significance of each variable's effect by comparing the adjusted model with another model in which the covariate is absent. $P < 0.05$ was considered statistically significant. Statistically significant values are highlighted in bold. * $p < 0.05$, ** $p < 0.01$.*

TABLE 4 | A partial correlation matrix with covariates between the cellular markers measured on CD4+ T cells for the symptomatic malaria population.

CD4	CTLA4	PD1	PD1CTLA4	CD57	CD57+CD28-	PD1CD57	CD28-CD57+	FOXP3
CTLA4	1							
PD1	−0.28392916	1						
PD1CTLA4	0.48244305	**0.7425784****	1					
CD57	−0.09261014	−0.4476809	**0.6287185****	1				
CD57+CD28-	0.28077369	0.1731718	−0.0862313	−0.06116931	1			
PD1CD57	0.36438345	0.1282666	−0.179054	−0.08438154	−0.2904794	1		
CD28-CD57+	0.03186114	0.3141221	−0.456166	**0.91009944****	0.363051	0.1875853	1	
FOXP3	0.2481085	**0.6309751***	−0.4845044	**0.55752428***	−0.432212	−0.0890901	−0.3706132	1

*Significant correlations were determined using a permutation test. P < 0. 05 were considered statistically significant; *p < 0.05, **p < 0.01, ****p < 0.0001. Statistically significant values are highlighted in bold.*

TABLE 5 | A partial correlation matrix with covariates between the cellular markers measured on CD8+ T cells for the symptomatic malaria population.

CD8	CTLA4	PD1	PD1CTLA4	CD57	CD57+CD28-	PD1CD57	CD28-CD57+	FOXP3
CTLA4	1							
PD1	0.4365899	1						
PD1CTLA4	**0.8497852****	−0.267313	1					
CD57	−0.230747	0.0385105	0.136794	1				
CD57+CD28-	0.2025701	0.0277434	−0.1928562	**0.6714082****	1			
PD1CD57	−0.2022586	**0.720932****	0.1076203	0.0563088	0.14287562	1		
CD28-CD57+	0.1714043	−0.4517704	−0.0132945	0.2164709	0.46299502	0.2318686	1	
FOXP3	0.2319811	−0.3906262	0.1694245	0.0678562	−0.00587128	0.3006289	−0.29398143	1

*Significant correlations were determined using a permutation test. P < 0. 05 were considered statistically significant; **p < 0.01, ****p < 0.0001. Statistically significant values are highlighted in bold.*

we could not determine the functionality of these T cells to confirm this.

Immunosenescence is the aging of immune cells characterized by shortened telomeres and inability to replicate, (50) sensitivity to apoptosis and, phenotypically, the expression of CD57 (22). T cell senescent markers were more associated with CD8+ T cells compared to CD4+ T cells consistent with earlier reports that they accumulate at lower frequencies for CD4+ T cells in human periphery (51). Here, we found an increased expression of CD57+T cell subsets in children with symptomatic malaria (52). This may suggest that malaria accelerates the aging of the T cell pool. In addition, the increased expression of CD28-CD57+ marker observed in symptomatic children indicates a greater proportion of effector T cells in symptomatic malaria have a memory phenotype since these cell subsets have been described to be antigen experienced (22, 31, 51). It has previously been shown that PD-1+CD57+CD8+ T cells have increased sensitivity to apoptosis mediated by PD-1 (53). The observed increase in expression of CD57 and PD-1 double-positive markers on CD8+ T cells, therefore, indicates a greater risk of apoptosis of these cells in clinical malaria. Additionally, T cell aging has been well characterized in the elderly population, CMV and HIV infections (21, 30, 33). In contrast to CMV infections which leads to expansion of T cell senescent markers, HIV leads to a decrease in the expression of CD57 on CD28-CD8+ gated T cells, whereas levels of CD8+CD28-CD57+ remains unchanged. In our study, we observed that both the proportion of CD28- T cells that express CD57 were expanded in the symptomatic malaria group, suggesting that T cell aging in falciparum infections is more similar to that observed during CMV infections than in HIV infections. Together, these observed phenotypic changes might reduce the responsiveness of the T cell repertoire to *P. falciparum* antigens resulting in an impaired ability to eliminate parasitemia.

FOXP3 is an immune regulatory marker associated with preventing immunopathology during inflammation. Both FOXP3 and PD-1 have been shown to suppress host immune response. Importantly, *P. falciparum* infections has been reported to cause the induction of PD-1+CTLA4+ T cells that control T cell activity (14). In this study, the positive correlation observed between FOXP3 and PD-1 T cells as well as between PD-1 and PD-1CTLA4 T cells could indicate that these markers play complementary roles in mediating the increasing immune activation that is associated with symptomatic malaria. There are conflicting reports about the role of CD57+ T cells in clinical disease, with some reports describing them as immunosuppressive and others suggesting

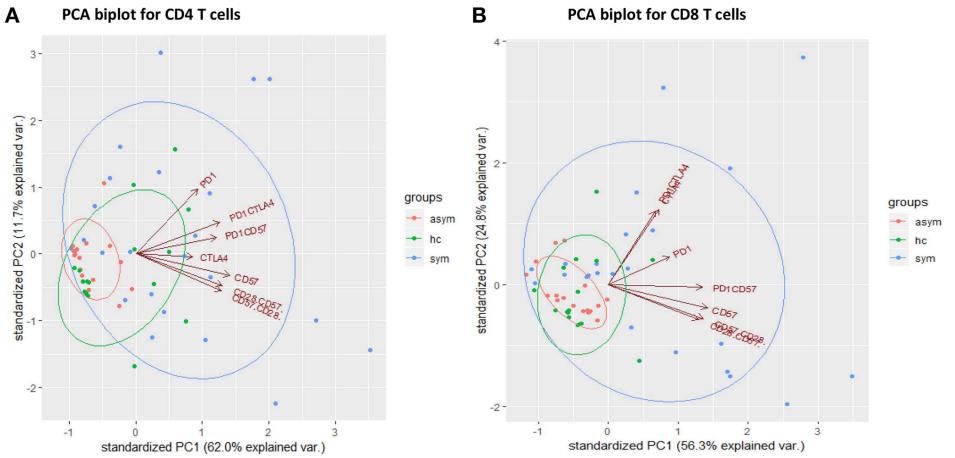

FIGURE 4 | Principal component analysis of cellular markers determined in the healthy groups ($n = 17$), asymptomatic malaria ($n = 18$) and symptomatic malaria ($n = 22$), for **(A)** CD4 and **(B)** CD8 T cells. The green, red and blue symbols as well as ellipses denote the healthy, asymptomatic and symptomatic groups respectively. The red arrows indicate the cellular markers.

they exacerbate immune activity through IFNγproduction (31, 54). Here, we observed a significant positive correlation between CD4+CD57+ T cells and CD4+FOXP3+ T cells as well as between CD4+CD57+ and CD4+PD-1+CTLA-4+ T cell subsets. However, since we could not determine the functionality of the CD57 T cell subsets, we can only suggest that the positive relationship observed between CD4+CD57 and CD4+FOXP3+ as well as CD4+ PD-1+CTLA-4+ T cell subsets may indicate that CD4+CD57+ T cells play suppressive roles during clinical malaria. This further supports the view that *Plasmodium* infections induce immunosuppressive immune responses that enhance the development of tolerance to the parasite, a mechanism affecting the development of sterile immunity.

Also, the results from the principal component analysis may indicate that a selection panel of the considered markers may serve as a biomarker for identifying individuals with symptomatic disease. It may probably be used to predict the outcome or immune response to vaccination. These results provide a basis to perform functional assays to determine the impact of the considered markers on the acquisition of anti-disease immunity during *P. falciparum* infections, preferably in a longitudinal cohort.

Studies have reported a low ratio of platelet-to-lymphocyte count (PLR) as a marker for inflammation in various infectious diseases such as HBV (36) and HCV (37). In this study, even though none of our markers was a good predictor of inflammation as previously stated using the PLR, we show that symptomatic malaria is characterized by low ratio of platelet-to-lymphocyte counts, which is indicative of on-going inflammatory response. Nonetheless, additional studies are needed to determine the significance of other hematological markers of inflammation (such as the neutrophil to lymphocyte ratio) to ascertain their clinical relevance during symptomatic malaria.

This study had a number of limitations due to the cross-sectional nature. We could not determine the effect of anti-malarial treatment on the expression of these inhibitory and senescent markers since samples were taking before the initiation of treatment. Furthermore, we defined *P. falciparum* infections by microscopy which is not able to distinguish between microscopic and sub-microscopic infections. In addition, we could not determine the effect of these markers on T cell cytokine production since cytokine profile analysis was not performed.

Despite these shortcomings, this study shows evidence that the phenotypic defect of T cells during *P. falciparum* infections are more pronounced in clinical malaria and associated with higher expression of exhaustive and senescent markers compared to asymptomatic infections. CTLA-4 was a good predictor of parasitemia in both symptomatic and asymptomatic malaria groups. Also, using the platelet to lymphocyte ratio, none of the markers measured could predict inflammation. In addition, we observed that the aging phenotype of T cells in malaria infection is similar to that observed with normal aging and CMV infections. These may imply that the increased expression of these markers may be associated with the absence of sterile immunity to *P. falciparum* malaria.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Noguchi Memorial Institute for Medical Research, ethical review committee with written informed consent from all subjects. All subjects gave written informed consent and assent in accordance with the Declaration of Helsinki. The protocol was approved by the Institutional Review Board of the Noguchi Memorial Institute for Medical Research at the University of Ghana (NMIMR-IRB CPN 096/15-16).

CONSENT FOR PUBLICATION

All authors have read and agreed to the content of this manuscript and its publication upon acceptance.

AUTHOR CONTRIBUTIONS

AF and MO conceived the idea and designed the experiments. WN, MO, and KK supervised the work. AF performed the experiments in the study and was assisted by DA-G and JA. AF, KK, MO and, WN wrote the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01345/full#supplementary-material

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Novel Strategies for Malaria Vaccine Design

Augustina Frimpong[1,2,3]*, *Kwadwo Asamoah Kusi*[1,2], *Michael Fokuo Ofori*[1,2] and *Wilfred Ndifon*[3,4]*

[1] Department of Biochemistry, Cell and Molecular Biology, West African Centre for Cell Biology of Infectious Pathogens, College of Basic and Applied Sciences, University of Ghana, Accra, Ghana, [2] Immunology Department, College of Health Sciences, Noguchi Memorial Institute for Medical Research, University of Ghana, Accra, Ghana, [3] African Institute for Mathematical Sciences, Cape Coast, Ghana, [4] African Institute for Mathematical Sciences, University of Stellenbosch, Cape Town, South Africa

The quest for a licensed effective vaccine against malaria remains a global priority. Even though classical vaccine design strategies have been successful for some viral and bacterial pathogens, little success has been achieved for *Plasmodium falciparum,* which causes the deadliest form of malaria due to its diversity and ability to evade host immune responses. Nevertheless, recent advances in vaccinology through high throughput discovery of immune correlates of protection, lymphocyte repertoire sequencing and structural design of immunogens, provide a comprehensive approach to identifying and designing a highly efficacious vaccine for malaria. In this review, we discuss novel vaccine approaches that can be employed in malaria vaccine design.

Keywords: *Plasmodium falciparum,* malaria, vaccine, immunoinformatics, structure-based, lymphocyte repertoire sequencing

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***Correspondence:**
Augustina Frimpong
tinafrimp@gmail.com
Wilfred Ndifon
wndifon@aims.ac.za

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THE GLOBAL MALARIA SITUATION

Malaria caused by *Plasmodium* parasites remains a major infectious disease of public health importance. The disease is caused by five protozoan species, namely *Plasmodium falciparum*, *P. vivax, P. malariae, P. ovale,* and *P. knowlesi.* The deadliest of these is *P. falciparum* which is predominant in sub-Saharan Africa (SSA). In 2016, approximately $2.7 billion was invested globally in control and elimination programs (1). Meanwhile, it was estimated in 2016 that nearly half of the world's population was at risk of infection, with 91% of the estimated deaths being in Africa and 70% of the mortality occurring in children under 5 years (1). Notwithstanding, preventive control and intervention measures have helped decrease the burden between 2000 and 2015. For instance, the incidence of new malaria cases was down by 37% world wide and 42% for the WHO African region. In addition, the incidence of mortality over the same period decreased by about 60% globally and 66% for the African region (2). Yet, malaria imposes huge economic losses for people in the African Region and there is a need to upscale the available interventions and introduce new ones such as a licensed cost-effective vaccine (3).

CHALLENGES TO THE ERADICATION OF MALARIA

Malaria eradication faces many challenges including insecticide resistance, emerging anti-malarial drug resistance and the presence of asymptomatic and submicroscopic infections.

Indoor residual spraying (IRS) and long-lasting insecticidal nets (LLINs), have been among the most effective tools for malaria control and elimination (4). So far, pyrethroids are the only

recommended class of insecticides for LLINs. However, more than 30 countries have reported resistance to pyrethroids, which has the potential to spread to new areas (5–9).

The rapid development of pyrethroid resistance suggests that alternative classes of insecticides need to be identified. As a result, WHO has cautioned against the use of pyrethroids (8), raising the need for alternative measures of control. The development of resistance to malaria drugs by *P. falciparum* remains a major threat to malaria elimination. The WHO-recommended first line treatment for uncomplicated malaria caused by *Plasmodium falciparum* is the artemisinin-based combination therapies (ACTs). Historically, *P. falciparum* has been able to develop resistance to almost all previous first-line antimalarial drugs (10, 11). The development of resistance to these drugs almost always begins from South-East Asia, where mutant parasites resistant to antimalarial drugs are more likely to survive due to lower levels of acquired immunity, poor adherence to administered drugs and higher parasite burdens (11–14). *P. falciparum* resistance to artemisinin-based drugs seems to have emerged sporadically (15), with mutations for resistance found within the kelch 13 propeller gene (15, 16). An inevitable fact is that artemisinin resistance may be imminent and other intervention avenues such as the development of highly effective vaccines need to be rapidly explored.

Also, the presence of asymptomatic and submicroscopic infections poses a major threat to malaria eradication and control. Continuous exposure to infectious mosquito bites leads to the development of anti-disease and anti-parasite immunity. The level of this immunity is determined by the transmission intensity and epidemiology of the disease (17, 18). It has been shown that the microscopic prevalence of malaria is almost half of that detected by nucleic acid amplification techniques and lower in low transmission areas (19, 20). The prevalence of submicroscopic infections has been found to be high in low transmission areas and common in children, probably as a result of a less robust immune response, leading to insufficient time for the development of protective immunity. In addition, asymptomatic infections may persist for several months and serve as a major threat to malaria eradication (21) as they sustain disease transmission (22–25).

CURRENT APPROACHES TO DEVELOPING A MALARIA VACCINE

Malaria Vaccines

The acquisition of partial immunity and the successful treatment of clinical symptoms of malaria in children with purified immunoglobulins from semi-immune adults (26) are positive indications of the feasibility of a vaccine against malaria. This is also supported by the induction of sterile immunity in both animal models and controlled human malaria infection (CHMI) through immunization with either live or attenuated sporozoites and merozoite-infected red cells (27–29). Attenuated sporozoites, even though they still maintain their natural hepatocyte invasion ability, do not fully mature in the liver and hence do not form merozoites that are responsible for the clinical symptoms of malaria (30).

Vaccine Targets

There are three stages to target for a potential malaria vaccine candidate. The first target of vaccine development is the pre-erythrocytic stage. This is the period where sporozoites travel through blood and infect hepatocytes to undergo schizogony, the vigorous multiplication stage that precedes the invasion of red blood cells (RBCs). The main purpose of developing a vaccine against this stage is to inhibit hepatocyte infections and hepatic parasite development, thus limiting RBC invasion (27, 30). The mechanisms of protection for this stage may involve antibody responses that prevent sporozoites from invading hepatocytes or cytotoxic T cells that destroy infected liver cells. So far, the licensed RTS,S, subunit vaccine remains the most advanced malaria vaccine to be developed. Other candidate vaccines include the whole-parasite vaccine candidates such as *Pf* sporozoite (PfSPZ), PfSPZ vaccination with chemoprophylaxis (PfSPZ-CVac) and the genetically attenuated parasite (PfSPZ-GAP).

The second target for malaria vaccine candidate design is the blood-stage of the parasite. The motivation for developing such vaccine candidates comes from evidence that people with repeated malaria infections in endemic areas develop some level of protective immunity, a state in which there is immune-controlled RBC invasion, resulting in fewer disease symptoms or asymptomatic infections (26, 31). Accordingly, vaccine candidates have been designed to elicit immune responses that will block/limit merozoite invasion of RBCs and stop the rapid replication of merozoites by targeting parasite surface proteins such merozoite surface proteins, apical membrane antigen 1 (AMA1), and the reticulocyte homolog (Rh) proteins (32–35). Other blood-stage vaccines target parasite antigens embedded in infected RBC membranes, such as *P. falciparum* Erythrocyte Membrane Protein-1 (PfEMP1) (36).

Despite being highly immunogenic and showing good promise as vaccine candidates, most of these antigens are also highly polymorphic and hence elicit antigen and parasite strain-specific responses (32, 33). Conversely, antigens such as the Rh proteins that show a high level of conservation (34, 35) tend to be less immunogenic (37).

The third malaria vaccine candidate target is the sexual parasite forms or gametocytes. Malaria transmission-blocking vaccines (TBVs) are designed to interrupt parasite transmission between humans and the mosquito vector through host immunological response to parasite targeted proteins such as Pfs230, Pfs45, Pfs48 (pre-fertilization antigens) and Pfs25, Pfs28 (post-fertilization antigens). Successful malaria transmission depends on the availability of infectious gametocytes in human

Abbreviations: ACTs, artemisinin-based combination therapies; PfEMP1, *P. falciparum* Erythrocyte Membrane Protein-1; MSP1, Merozoite Surface Protein; AMA1, Apical Membrane Antigen 1; Rh, Reticulocyte homolog (Rh); MHC, Major histocompatibility complex; HLA, Human leukocyte antigen; PfSPZ, *Pf* sporozoite; PfCVac, *Pf* chemoprophylaxis vaccination; PfGAP, *Pf* genetically attenuated parasite; TBV, transmission-blocking vaccines; BCR, B cell receptor; bnMAb, broadly neutralizing monoclonal antibody; TCR, T cell receptor; VIMT, Vaccine that interrupt malaria transmission; SSM-VIMT, Sexual, sporogenic or mosquito stage VIMT; PE-VIMT, pre-erythrocytic VIMT.

peripheral blood that can be taken up by mosquitoes during a blood meal. Studies have reported that the degree of infectivity of gametocytes to mosquitoes is based on the gametocyte density, drug stress, clonality of infection and immune defenses of the mosquito (38–42). However, according to Churcher et al. (38), even at very low densities, gametocytes remain infectious to mosquitoes. Also, it has been reported that in the human host, transmission can be stable at very low densities and is not directly proportional to the gametocyte density in peripheral blood (43, 44). Basically, a TBV exploits the fact that there is a functional immunological activity against the sexual stage parasite proteins which is able to reduce the infectivity of the parasite, thereby decreasing malaria transmission (45, 46). Vaccine candidates that seek to interrupt malaria transmission (VIMT) are of two main types: (1) sexual, sporogenic or mosquito stage VIMT (SSM-VIMT) candidates which are expected to interrupt human-to-mosquito transmission; and (2) the pre-erythrocytic VIMT (PE-VIMT) candidates, which are expected to interrupt mosquito-to-human transmission (47). Among the TBV candidates, only Pfs25 and Pfs230 have undergone clinical trials in human (48–51). Unfortunately, a major challenge with these candidate vaccines is the inability to elicit higher antibody titers. In regards, there are considerations to conjugate these candidate vaccines (50, 52).

Current Status of Malaria Vaccine Development

After decades of extensive research, the pre-erythrocytic stage vaccine, RTS,S has been licensed and is expected to undergo further testing in malaria endemic areas before possible approval for immunization. Currently, together with RTS,S, only 20 candidate vaccines are undergoing clinical trials (**Table 1**). For RTS,S, a recent evaluation on the safety and immunogenicity of the vaccine co-administered with the recommended expanded programme on immunization showed the vaccine to be safe and immunogenic with no related adverse events (58). The RTS,S/AS01 consists of a recombinant protein of the *P. falciparum* circumsporozoite protein (CSP) conjugated to a hepatitis B virus surface antigen. During clinical trials, the efficacy of the vaccine after 4 doses was observed to be 43.9% in children aged 5–17 months and 27.8% in children 6–12 weeks old (59). However, vaccine efficacy wanes with time and fails to meet the target set by the Malaria Vaccine Technology Roadmap (60). Consequently, other vaccination regimens such as the number of doses, time of immunization, and alternative approaches for vaccination are being evaluated (61).

Also, the R21, a virus-like particle vaccine which is a biosimilar of RTS,S consists of the CSP conjugated to a single hepatitis B surface antigen. The RTS,S-like vaccine has been shown to provide sterile protection in mice at very low doses. In addition, it was observed that most of the immune responses elicited against the candidate vaccine targeted the CSP in contrast to the hepatitis B surface antigen which is often targeted in the RTS,S vaccinated individuals (53). Importantly, this candidate vaccine is designed such that more epitopes of the CSP may be exposed to host immune system to enhance the efficacy of R21.

Furthermore, the whole sporozoite vaccine has been reported to provide significant protection against falciparum malaria. The whole organism candidate vaccine design approaches include the radiation-attenuated sporozoites (PfSPZ), whole PfSPZ with chemoprophylaxis (PfCVac) and the genetically modified sporozoites (PfGAP). Even though PfCVac showed complete protection to homologous *P. falciparum strain,* moderate protection has been observed with heterologous strains in non-exposed vaccines (62). Clinical trials with PfSPZ in endemic areas have been shown to be safe and well tolerated, however, inducing low level of immune responses compared to naïve individuals (63, 64). These may suggest that the breadth of immune responses to PfSPZ vaccines need to be increased by considering other vaccination regimens.

PROMISING APPROACHES TO MALARIA VACCINE DEVELOPMENT

Recent technological advances have greatly improved the prospects for designing an effective malaria vaccine through advances in high-throughput biology and computation. These alternative approaches may be focused on the parasite- or host immune system.

The Parasite-Focused Approach

The technologies involved in this approach center on the identification of immunogenic antigens from the pathogen by interrogating the parasite's genome, transcriptome or proteome. It may modify the structure of the antigenic component(s) identified with the aim of targeting various strains of the pathogen. The parasite-focused approach further tests the immunogenicity and safety of the candidate antigens to design novel and improved vaccines. This approach may involve the application of reverse vaccinology, structural vaccinology, and immunoinformatics.

Reverse Vaccinology

Reverse vaccinology, developed by Rappouli et al. is a technology first used in *Meningococcus* serogroup B bacteria to identify novel vaccine antigens (**Figure 1**). Here, the pathogen's genome is sequenced and analyzed to have access to the entire repertoire of proteins and enable comparison of conserved sequences shared among pathogens of the same species (65). Genomic data is analyzed using bioinformatics tools, taking into consideration all open reading frames. Also, with the use of computational tools, genomic sequences that are homologous to those of humans are eliminated from the vaccine candidates identified. The remaining genes are isolated and inserted into a suitable vector to obtain proteins for testing in animal models. Responses to the vaccine antigens are analyzed in immunized mice to validate their immunogenicity and efficacy levels. Importantly, molecular epidemiology studies are undertaken using various strains of the pathogen to ascertain whether the selected antigens are conserved or highly variable in a given population (66). This approach has been used to develop vaccines against serogroup B *Neisseria meningitidis* (67); and identify vaccine candidates for, *S. agalactia* and *S. pyogenes* (68, 69). This vaccine design approach

TABLE 1 | Current malaria vaccines in clinical trials.

Vaccine candidate	Clinical trial registration number	Clinical trial stage
PRE-ERYTHROCYTIC		
RTS,S/AS01	NCT01345240	Phase 3
R21/AS01B	NCT02600975	Phase 1
R21/ME-TRAP	NCT02905019 (53)	Phase 2
ChAd63/MVA ME-TRAP	NCT01635647 (54–56)	Phase 2
R21/Matrix-M1	NCT02572388/NCT02925403	Phase 1/2
PfSPZ Vaccine	NCT03510481	Phase 1
PfSPZ-CVac (PfSPZ Challenge + chloroquine or + chloroquine/pyrimethamine	NCT03083847	Phase 1
GAP 3KO (52-/36-/sap1-)	NCT02313376	Phase 1
BLOOD-STAGE		
pfAMA1-DiCo	NCT02014727 (57)	Phase 1
P27A	NCT01949909	Phase 2
PAMVAC	NCT02647489	Phase 1
PRIMVAC	NCT02658253	Phase 1
SEXUAL-STAGE		
ChAd63 Pfs25-IMX313/MVA Pfs25-IMX313	NCT02532049	Phase 1
Pfs25-EPA/Alhydrogel	NCT01867463, 51	Phase 1
Pfs230D1M-EPA/Alhydrogel and/or Pfs25-EPA/Alhydrogel	NCT02334462	Phase 1
Pfs25M-EPA/AS01 and/or Pfs230D1M-EPA/ASOI	NCT02942277	Phase 1
Pfs25 VLP-FhCMB	NCT02013687	Phase 1
Pfs25-Pfs25	NCT00977899	Phase 1
Pfs25 & Pvs/Monatide ISA 51	NCT00295581	Phase 1

Adapted from WHO. 9/28/2018. Malaria Vaccine Rainbow Tables. http://www.who.int/vaccine_research/links/Rainbow/en/index.html.

has greatly enhanced the discovery and characterization of several pathogen antigens.

Reverse vaccinology has been applied in malaria to identify parasite proteins either secreted or involved in signaling for consideration as possible vaccine candidates. The genomic sequence of *P. falciparum* has been available since 2002 (70). In addition, the sequence of other diverse *Plasmodium spp* including primate (71, 72) and rodent (73) parasites have been published. Comparative analysis of these sequences has shown similar homologs between species with possibly similar functions. For instance, the conserved Pf48/45 and PfHAP2 genes, both of which are transmission blocking vaccine candidates, functions were determined based on the role of their homologs in other *Plasmodium* spp. The functional analysis of the P48/45 and PHAP2 genes in *P. berghei* established their significant role in reducing the fertility of male gametes during fertilization, promoting these genes as vaccine candidates (74, 75), which are currently in the preclinical stage (76–78).

Despite the success of reverse vaccinology, it cannot be used to identify non-peptide antigens but can identify operons that code for synthesis of such molecules (79). For pathogens with complex genomes such as malaria parasites, no successful vaccine has as yet been developed via this approach. Further progress requires, among other things, improved predictive algorithms to identify the T and B cell epitopes as well as accurate quantitative assessments before inclusion in vaccines.

Structural Vaccinology

An improved understanding of the native structures of biological macromolecules such as proteins and how changes in their structure affect their functions can assist the identification of suitable epitopes (80, 81). Such epitopes can be designed into accessible forms for easy uptake by immune cells. These structural considerations make it possible to improve vaccine immunogenicity and safety and mitigate the effects of sequence variability within different strains of a pathogen (82). For instance, the bacterium *Meningococcus* is able to evade the host's immune system with the aid of a factor H binding protein (Hbp), which inactivates the host complement pathway by blocking factor H. Structural considerations allowed immunodominant epitopes of Hbp from various meningococcal strains to be identified and grafted into a single variant molecule to form a single antigen. This antigen was used in the MenB vaccine, responses against which are able to neutralize all the targeted strains (83).

Also, in an earlier study, short conserved α-helical coiled coil structural domains were identified from the asexual blood stage of the *P. falciparum* by examining the *Plasmodium* genome (84). Upon further screening, an unstructured peptide (P27A) that unfolds in native confirmation was selected. The peptide was the target of human antibodies which were able to restrict parasite replication (85). The vaccine candidate P27A has been considered immunogenic and safe with mild adverse events after Phase1 clinical trials (86).

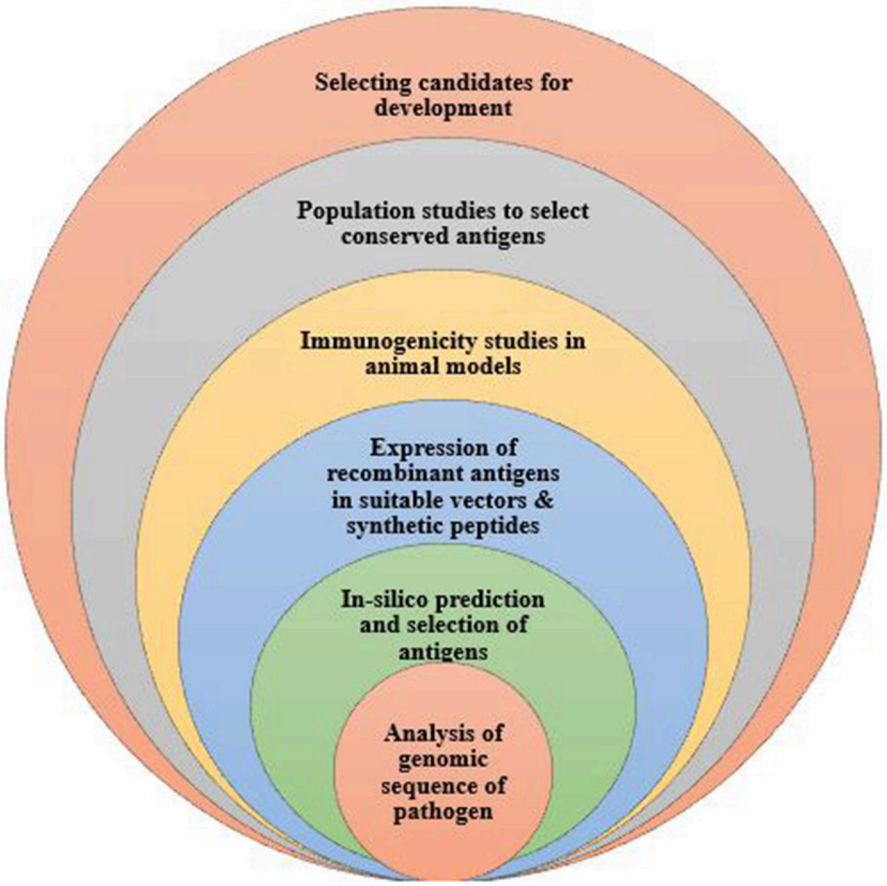

FIGURE 1 | The process of developing a vaccine using reverse vaccinology. Reverse vaccinology starts with obtaining the genomic sequence of the pathogen and using bioinformatics tools to identify all open reading frames to predict protein antigens. The predicted antigens are subsequently expressed in suitable vectors to produce the recombinant proteins which are tested to evaluate the immunogenicity in animal models. Expressed antigens that yield high immunogenicity are selected as vaccine candidates, further tested in population studies to determine and identify conserved antigens for further vaccine development.

For pathogens like *P. falciparum*, structural vaccinology may also help overcome antigenic variation. For instance, the application of structural vaccinology enabled the characterization of the less polymorphic DBL4ε domain of VAR2CSA to identify novel properties in the motif that affects the functional features of the antigen (87); identification and confirmation of the three-dimensional structure of the invasion ligand Cysteine-Rich Protective Antigen (CyRPA) (88). For example, the CyRPA was identified as a protective epitope providing an additive effect with the Reticulocyte binding-like Homologous protein 5 (*Pf*RH5) such that antibodies against *Pf*RH5 and CyRPA can inhibit parasite replication in host RBCs (88). Hopefully malaria vaccines incorporating these epitopes may elicit strong protective immune responses. Combining these protective antigens to create hybrid protein vaccines with enhanced efficacy may be a viable option for malaria.

A key challenge with this approach is the identification of suitable B and T cell epitopes for incorporation into vaccine candidates.

Immunoinformatics Based Approach to Vaccine Design

Immunoinformatics integrates both computational approaches and experimental immunology to develop machine learning algorithms that attempt to predict the immunogenicity of antigens. These approaches can be either pattern- or theory-based and may operate at either the amino acid sequence or the protein structure level. The pattern-based approaches conceive the prediction problem as one of finding sequence/structural patterns associated with immunogenicity. In contrast, the theory-based approaches attempt to model the basis for immunogenicity, for example, by using physical principles. Examples of algorithmic tools employed by pattern-based approaches include quantitative structure-activity relationship analysis, support vector machines, and artificial neural networks (89, 90). Theory-based approaches often employ Markovian and/or Bayesian models as well as models based on statistical mechanics (91).

Immunoinformatic approaches have already been applied to *P. falciparum* to predict possible cytotoxic T cell epitopes coupled

with HLA A/B molecules for malaria peptide vaccine design (92). For example, the PfEMP1 gene, a member of the var gene family has been associated with parasite evasion from host immune mechanisms due to its multiple variation and ability to bind to different host receptors (36). In a recent study, both *in-silico* and experimental approaches were used to identify antigenic epitopes from CIDR-1 and DBL-3γ conserved domains of PfEMP1. These epitopes were predicted to have good binding affinity to HLA molecules as well as the capability to induce IFN-γ, IL-4 secretion and T cell proliferation in exposed individuals (93).

Classically, HLA class I molecules optimally require peptides that are 8-10 amino acids long for presentation to CD8 T cells while HLA Class II molecules optimally require 12-25 amino acids long peptides for presentation to CD4 T cells. Of note high predictive accuracies have been achieved for bioinformatics methods for predicting peptide binding to HLA I molecules; whereas those for predicting binding to HLA II molecules require further improvement. An even greater challenge is prediction of peptide binding to B cell receptors for effective antibody responses. On-going work by us and other groups is aimed at addressing some of these challenges (91, 94). However, not all HLA binders are good epitopes for T cells and this poses a major challenge for approaches that predict HLA binders without considering the global picture of HLA-peptide-TCR interactions. Nonetheless, these computational approaches, which are quite cost-effective and are important down-selection tools in instances where there are too many peptides to evaluate experimentally, have the potential to aid in the development of effective vaccines against malaria.

Immune-Focused Approach

Due to the sophisticated immune-evasion mechanisms of *P. falciparum* that allow it to coexist with the host, vaccinologists require new paradigms in vaccine development. One such new paradigm that has been developed to target these pathogens is the immune-focused approach (**Figure 2**). In contrast to the parasite-focused approach, which centers on the pathogen of interest, this new approach seeks to harness the host immune system to more rapidly design effective vaccines. It focuses on studying the host immune system to discover protective immune signatures. It is expected that these protective signatures can be induced *de novo* in susceptible hosts to protect them against infection and/or disease. Compared to the pathogen-focused approach, the immune-focused approach has, in principle, a greater potential for success against pathogens like malaria parasites, which have highly variable genomes. In particular, it may be able to identify and design immune cells with broadly neutralizing antibodies (95) and enhanced cellular immune responses, which has proved difficult to accomplish by using conventional approaches. To provide context for the discussion of opportunities for vaccine development, we begin with a brief overview of human immunity to malaria.

Immunity to Malaria

In contrast to many pathogens against which highly potent, long-lived immunity is achieved, human immunity to malarial parasites is less potent and relatively short-lived (17). In malaria, acquired immunity to infection is rare; rather, what develops naturally, generally over a long period, is acquired immunity to disease (96). Such clinical immunity generally targets the disease-causing asexual blood stage of malarial parasites. It tends to be acquired faster in moderate-to-high transmission settings compared to low transmission areas, and with a higher number of clinical episodes (97–99). As with other infectious diseases, the development of clinical immunity to malaria is dependent on the adaptive arm of the immune system, and the principal mediators consist of specific subsets of B and T cells. Some progress has been made to elucidate the underlying mechanisms, although the key immune determinants remain unclear.

In addition, the ability to predict the beginning and end of transmission seasons have made it possible to study host responses to infection and some immune dynamics that occur before, during and after infection as well as, drug interventions (100) and how they may affect the immunity acquired (101, 102).

In natural infections, acquisition of immunity to sporozoite stage infections is limited, probably due to the low number of sporozoites that are inoculated as well as the limited time that sporozoites have extracellular, prior to hepatocyte invasion. In addition, it has been reported that *Plasmodium* sporozoites are able to modulate the cytokine environment by downregulating Th1 responses and antigen presentation to T cells (103). Recently, it was reported that continuous exposure to *P. falciparum* leads to the induction and expression of immunoregulatory cytokines such as IL-10 and affects the function of dendritic cells (104). These, coupled with frequent infection and immune activation, may profoundly impact on the tolerogenic environment leading to the escape of sporozoites from immune cells. Nevertheless, functional properties of antibodies to sporozoite-stage infections have been associated with natural protection from clinical disease. It has been reported that these antibodies kill sporozoites through complement fixation and inhibit hepatocyte invasion. However, the response to sporozoite antigens was age-dependent and acquired slowly compared to blood-stage antigens (105).

The blood-stage parasite is associated with the clinical symptoms of the disease as it causes an upregulation of pro-inflammatory cytokines, regulatory T cells and parasite sequestration in small blood vessels in host organs. Antibodies have been reported to play functional roles in preventing parasite invasion of red blood cells (106, 107). Antibodies to parasite antigens are associated with clinical immunity in endemic areas (108–110). The mechanisms of antibody activity may include blocking invasion of erythrocytes (111); opsonizing parasites to facilitate their clearance (110, 112) enhancing the killing of infected cells by monocytes (113); complement-mediated lysis of infected cells (114); and inhibiting adherence of infected erythrocytes to vascular endothelium (115). However, the generation of atypical memory B cells which have reduced effector functions has been observed under chronic conditions (116, 117).

T cells have also been shown to play protective roles during blood-stage infection. For instance, protection from the disease has been associated with $FOXP3^-$ Th1 cells which are self-regulatory and produce IFNγ, TNF and IL-10 (101, 118, 119). These cells are believed to prevent the production of pyrogenic

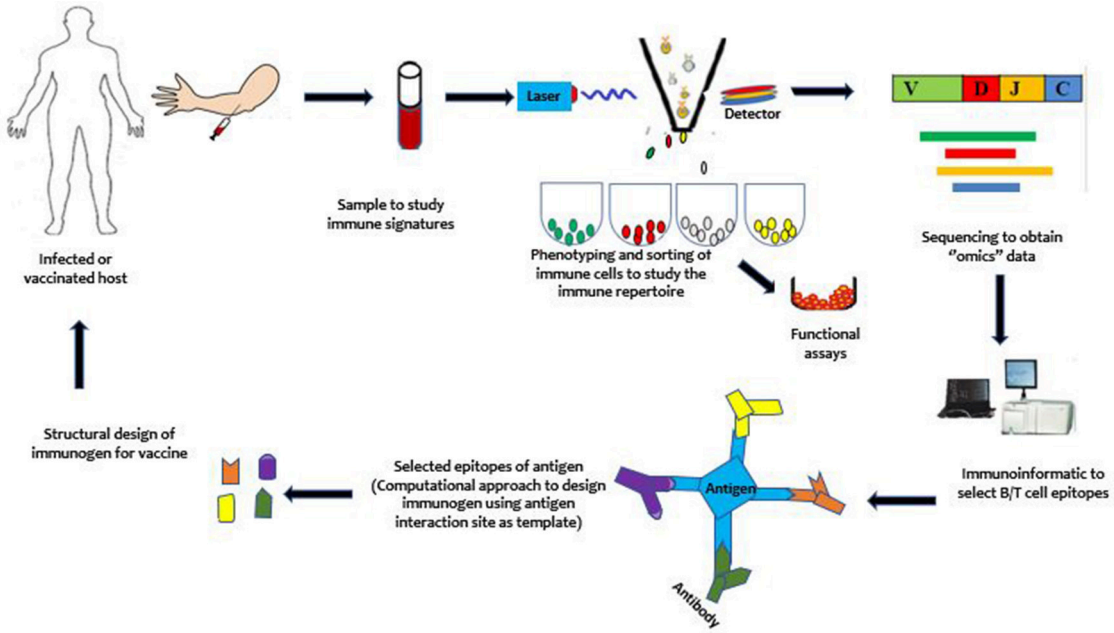

FIGURE 2 | An illustration of the immune-focused approach to vaccine development**.** Briefly, from top left, samples are obtained from an infected but protected or a vaccinated host. Immune cells are phenotyped and sorted using single cell sorting technologies such as the flow cytometry. The sorted cells may be sequenced directly or proliferated for functional studies before sequencing to study the immune repertoires (B/T cell receptor repertoire). With the use of computational approaches, the immunogen is designed and taken through further stages of development to yield a vaccine. After vaccination, mechanisms underlying vaccine efficacy and safety can be studied to further enhance the developed vaccine to achieve maximum protection against the targeted pathogen.

factors that may lead to the manifestation of clinical disease. However, these immune responses are not long-lasting and easily decay after infection has waned. T cell responses are hampered by the upregulation of negative immune regulatory receptors which may blunt or cause anergic responses (116, 120, 121). Our recent study found higher levels of T regulatory cells to be associated with higher blood levels of *P. falciparum* in children (118), suggesting less effective control of the parasite. Indeed, trying to understand these various aspects of the immune responses is a quite complex task (122).

Compared to natural infections, inducing sterile immunity in naïve individuals has been achieved through whole sporozoite immunization (29, 123) although similar outcomes have not been seen in individuals from malaria endemic areas (63, 64). Vaccination of volunteers with radiation-attenuated sporozoites has shown that both T cells and antibody responses play a significant role in protecting vaccinated cohorts against clinical challenge. It was observed that T cells from the periphery of these individuals, when stimulated with *P. falciparum* sporozoites *in vitro*, produced effector cytokines in a dose-dependent manner whereas antibody levels increased and prevented hepatocyte invasion (124–127).

The challenges of inducing immunity to malaria by natural or artificial means are compounded by the sophisticated immune-evasion strategies of the parasite. The parasite has a large genome consisting of about 5,300–5,500 possible antigenic targets (70). This extensive gene repertoire coupled with the parasite's high mutation rate allows for extensive variation of antigens that can be potential vaccine targets. Moreover, the epitopes targeted by the immune system exhibit a hierarchy of immunogenicity, with immunodominant epitopes that induce large amounts of antibodies, not all of which are neutralizing and may mask sub-dominant epitopes bound by neutralizing antibodies (73). In addition, the parasite switches off antigenic phenotypes, associated with the variant antigens resulting in functional diversity. Consequently, infections are mostly characterized by successive parasitemia waves caused by different parasite variants, making the development of long-lived immunity to the parasite very challenging (36, 128). Furthermore, key antigens such as CSP contain tandem repeats that have been implicated in immune evasion by suppressing antibody responses against adjacent antigens (129).

High Throughput Identification of Immune Correlates of Protection

The age and genetics of a person may modulate the immune responses elicited during infections and vaccinations (130, 131). Nonetheless, these responses that modulate infection may help to systematically define factors associated with protection from disease. Conventional approaches to understanding immune correlates of protection against *P. falciparum* includes, but is not limited to, ELISA, Elispot and Western blots. However, recent advances in high throughput assays have allowed in-depth analysis of immune correlates of protection to multiple *falciparum* antigens. Individuals in malaria-endemic areas

generate antibodies to different *P. falciparum* proteins which may be protective or serve as a serological marker for exposure.

High throughput assays that probe the genomic, proteomic and transcriptomic data of immune responses are useful means of determining correlates of protection in exposed and vaccine trial cohorts. Independent studies using library expression and protein microarray has characterized host immune reactivity to different *P. falciparum* antigens. Using these approaches, Doolan et al. (132) were able to identify stage-specific *P. falciparum* antigens associated with protection in naturally exposed individuals, vaccine protected and non-protected individuals using a protein microarray chip with 250 proteins. They observed distinctive antibody profiles in the various groups to these antigens. Also, in an independent study, involving a large cohort of children naturally exposed to malaria (≤10 years old) in Kenya, it was observed that responses to fewer proteins from the 39 *P. falciparum* antigens analyzed were significantly associated with protection, and these included AMA1 and MSP2. Also, antibodies to the top 10 proteins provided an additive effect whereas most antibody responses to the other antigens were markers of malaria exposure (133).

A similar study conducted in Mali probed sera from malaria-exposed children and adults against 1204 proteins. Among these proteins, 91 were associated with sexual stage-specific immunity with specific-IgG responses culminating during the transmission season. It was further observed that immunity to these sexual stage vaccine candidates (Pfs48/45 and Pfs230 but not Pfs25) can be boosted in natural infections (134). These studies showed evidence that the breadth and magnitude of the antibody response is a better correlate of immune protection. Furthermore, in analyzing PBMCs for non-humoral immune responses associated with protection using DNA microarrays, qRT-PCR and flow cytometry, it was observed that repeated exposure to malaria in children was associated with the upregulation of genes involved in immune regulation (such as IL-10 secretion from CD4+Foxp3-), phagocytosis and activation of adaptive immune system. In contrast, gene expression levels of chemokines and cytokines associated with fever and inflammation (such as IL-1β, TNF, CXCL2 and IL-8) were downregulated (101).

Interestingly, the application of next-generation sequencing techniques such as lymphocyte immune repertoire sequencing, including T cell receptor (TCR), membrane-bound B cell receptor (BCR) or secreted BCR can allow an in-depth analysis of host factors associated with pathogen recognition, identification and protection from disease. The TCR structure is heterodimeric with two protein subunits; an alpha and beta chain or gamma and delta chain with both a constant and variable region. Similarly, the BCR consists of two heavy and light chains which are joined together by disulphide bonds to form a Y shaped immunoglobulin together with a variable and a constant region. The lymphocyte receptors (TCR/BCR) have similar structures including a variable, diversity and joining regions that enable diversification in identifying different host pathogens. In the generation of receptor diversity, there is a recombination of a V, D, and J segment of a beta or heavy chain, and a V and J segment for the alpha or light chain. For the BCR, this process helps expose very potent neutralizing antibodies that may be public in protecting against clinical disease. The generation of the variable regions may help guarantee higher levels of somatic mutation at the antigen binding site which may be shared or unique to an individual(s).

Despite the documented importance of lymphocyte receptors for antigen recognition and, hence, for the initiation of adaptive immune responses, the specific TCRs/BCRs that determine immunity to particular pathogens remain poorly understood. To our knowledge, no previous study has comprehensively mapped these receptors and analyzed how their expression profiles may correlate with individual variations in immune protection against malaria. In addition, the application of machine learning algorithms such as random forests, support vector machines may allow the identification of patterns on immune correlates that may predict protection against disease (118, 135).

Moreover, these approaches generate huge amounts of data that can be computationally analyzed to generate new, experimentally testable hypotheses. These may yield novel insights into the mechanisms underlying vaccine safety and efficacy. Importantly, data from such studies will inform pathways to which vaccine strategies should focus.

The B Cell Response and Vaccine Design

Effective vaccines are supposed to elicit and provide long-term protection as well as require both B and T cells to produce effective antibodies to neutralize surface-expressed antigens. B cell lineage vaccine design is an immune-focused approach that combines human immunology, structural biology, and computational protein design to develop a vaccine. The aim is to identify in both naïve and memory B cell receptors, paratopes (antigen binding sites) that interact with immunogens of interest. For a vaccine to be designed through this approach, memory B cell clones from the same lineage (or clone) are first identified and isolated from patients that produce broadly neutralizing antibodies or protective antibodies. These clones are then sequenced to obtain the V(D)J and VJ gene pairs that make up the B cell receptors in order to identify the paratope. Computational approaches are used to design an immunogen that interacts with the identified paratope (95). For *P. falciparum,* neutralizing antibodies produced by activated B cells are required to prevent the infection of new RBCs. By isolating such protective B cells from malaria patients and sequencing and analyzing their antigen receptors, it might be possible to identify immunogens able to induce protective immunity in susceptible individuals.

In malaria, the identification of broadly neutralizing antibodies remained elusive partly due to the high polymorphic nature of *P. falciparum* antigens. In addition, malaria vaccine candidates tend to induce antibodies with weak neutralizing ability, low breadth and strain-specific. However, Tan et al. (136) have recently identified monoclonal antibodies that can recognize *P. falciparum*-infected RBCs (iRBCs) from different strains of parasites. These antibodies recognize and bind to iRBCs through the RIFIN proteins, a group of variant antigens that are extracellularly expressed on the surface of iRBCs and have been associated with immune evasion (128) to initialize opsonization.

Another remarkable example is the identification of the novel antigenic target NPDP (part of the sequence in the N-terminal

junction peptide) that is found between genes for the CSP and NANP and NVDP tandem repeats (137, 138). Independent studies in 2018 by Tan et al. (137) and Kisalu et al. (138) identified and isolated neutralizing antibodies from memory B cells and plasmablasts that could inhibit hepatocyte infection by PfSPZ. Through structural information, they were able to identify that these antibodies bind to conserved epitopes in the N-terminus of the CSP that is not found within the RTS,S vaccine.

Furthermore, mAbs that can inhibit parasite replication to about 97% have been isolated from CHMI donors immunized with RTS,S. Deciphering the structure and functionality of these antibodies have provided an informed overview on the structure of the CSP *in vivo*. Thus provides positive implications in the design of CSP immunogens against *P. falciparum* (139). However, there are still unsolved questions on the antibody responses to the PfCSP which have been described to be protective (139) and non-protective (129) as well as more structural information is needed to induce such potent neutralizing antibodies during vaccination Nonetheless, it is very interesting since they have implications in designing immunogens that can target specific immune responses and probably improve the efficacy of the RTS,S vaccine.

T Cell Response and Vaccine Design

Protective immunity to malaria liver-stage infection has been attributed to T cells in both human and rodent models. In studying immune responses to malaria such as cerebral malaria, murine models have provided significant understanding of various immunological properties that have impacted our understanding of the immune activity in humans.

For instance, Lau et al. (140) characterize MHC-restricted TCR that have potential in enhancing antigen presentation to T cells to enhance T cell immunity. They developed a novel CD8+ T cell receptor to *P. berghei* termed PbT-I from transgenic mouse with immune specificity for liver-stage and blood-stage infections. Isolated TCR genes from Vα8.3 and Vβ10 were isolated from a restricted hybridoma T cell line generated from *Plasmodium berghei* ANKA (PbA) blood-stage infection. Despite been developed for PbA, this transgenic MHC-I restricted T cell line was cross-reactive to *P. chabaudi* and *P. yoelli*. This implies that they may recognize conserved regions in rodent *Plasmodium spp*. Functional analysis revealed that the PbT-I cells produced effector cytokines (IFNγ, TNFα) and was positive for the degranulation marker (CD107a) showing their involvement in immune activity during the PbA infection. Using PbT-I CD8+ T cells, the peptides responsible for their activation were elucidated.

In a subsequent research, they identified and developed PbT-II CD4+ T cells from mouse transgenic line using the TCRα (Vα2.7, Jα12, Cα) and TCRβ gene (Vβ12, Dβ2, Jβ2.4) segments to blood-stage PbA infection (141). These cells were cross-reactive to rodent parasites (*P. berghei, yoelli and chabaudi*) and to *P. falciparum.* These MHC-II restricted PbT CD4+ T cells enhanced both humoral activity of B cells and cytotoxic activity of CD8+ T cells. In addition, the study confirmed that immunity to antigens in both blood stage and liver-stage development can restrict parasite replication in the hepatic stage and characterize the impact of blood stage antigen presentation to T cells that can enhance such T-cell immunity during infection. The uses of these target antigens may delineate protective immune responses and possibly circumvent pathologic outcomes. More importantly, further work should be focused on identifying and understanding such broadly reactive *Plasmodium*-specific T cells in host infections.

Structure-Based Immunogen Vaccine Design

The structure-based vaccine approach can be employed in both the parasite and immune-focused approach. However, in the immune-focused approach, the principle is based on understanding the structural properties of the immune cell providing the desired response. Here, the properties of the antigenic binding site on the immune cell is studied at the atomic level (80, 81). By understanding these properties, the approach seeks to design and develop immunogens to target the protective response or develop these immune cells for use as interventions.

Structural-based vaccine design has aided in unmasking immunodominant epitopes in the haemagglutinin-stem of the influenza virus (142), the fusion protein in the respiratory syncytial virus (143) and CD4 binding site in HIV-1 virus. For example, identifying conserved immunogenic epitopes in HIV has been quite challenging. However, elucidating the structure of broadly neutralizing antibodies (bNAbs) has been very useful. Using NAb, subdominant epitopes in the CD4 binding site by the gp120 viral protein were identified. Probing the structure of the antigenic binding site on CD4, the structural properties helped in the development of a recombinant protein (RSC3) with specificity to the NAb. The RSC3 was further used to identify and isolate B cells that expressed broadly neutralizing antibodies with increased breadth. VRC01 and 3BNC117, highly potent monoclonal bnMAb with reactivity to about 91% to HIV-1 isolates were developed (144). Phase I clinical trials of the VRC01 were reported as safe with no allergenicity (145, 146). It is currently being evaluated in a Phase IIB trials with a projected overall efficacy of 53 and 82% (147). These observations indicate that using structural properties, subdominant epitopes can be uncovered to design immunogens to target a specific immune response.

Currently, there are few examples of the successful use of these approaches in malaria vaccine design. For instance, using invasion-inhibitory monoclonal antibodies, the novel structure of PfRh5 in complex with basigin was characterized, together with novel protective epitopes found in the complex (148). Similarly, for *P. vivax* infections, bNAbs that confer strain-specific immune responses (149) were isolated. These bNAbs, enabled the characterization of protective epitopes in the duffy binding protein that can be included in the design of a potent *P. vivax* vaccine (150).

CONCLUSION

The development of a highly efficacious malaria vaccine faces many challenges, both technical and biological. Partly because

the parasite is equipped with a variety of evasion mechanisms allow it to co-exist with the host. With the recent advent of high throughput approaches such as lymphocyte repertoire sequencing and structural design of immunogens, the breadth of protection of previous and current vaccine candidates may be enhanced as well as the identification of new candidate vaccines. In addition, vaccinologist may be able to design vaccines that drive the immune system through unusual yet protective pathways. Likewise, the application of mathematical modeling and computational approaches to the data thus obtained will open new pathways toward designing highly effective vaccines against malaria and aid in achieving the targets set by the malaria vaccine technology roadmap for 2030.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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