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T cell and antibody responses in *Plasmodium falciparum* malaria and their relation to disease susceptibility

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SUMMARY

Malaria antigen-induced polarization of T cells into effectors Th1 and/or Th2 cells and their subsequent release of cytokines is known to affect antibody production. This thesis includes studies on early innate responses to the parasite, with a focus on γδT cells, and acquired specific responses in African sympatric ethnic tribes. In the last part of this thesis, a method for enrichment for the asexual blood stages of *P. falciparum* and their use in *in vitro* T-cell studies is presented.

To investigate mechanisms involved in parasite growth inhibition by γδT cells, an *in vitro* system was set up using blood stage parasites co-cultured with differently treated γδT cells. The results showed that Vγ9/δ2$^+$ γδT cells inhibited the *in vitro* growth of *P. falciparum* parasites whereas CD4$^+$ and CD8$^+$ T cells did not. This inhibition was positively correlated with the expression of cytolytic molecules in the cell lines tested. Anti-granulysin antibodies reversed γδT cell-mediated inhibition, suggesting a role for granulysin in the parasite growth inhibition. Thus, our data suggest that Vγ9/Vδ2$^+$ γδT cells inhibit the parasite growth by a granulysin-exocytosis dependent cytotoxic pathway that needs perforin.

To study the humoral responses and their relation to Th1/Th2 cytokine profiles, antibody levels, numbers of cytokine-producing cells and spleen rates were measured in two sympatric tribes living in Mali, the Fulani and the Dogon. Our results revealed significantly elevated malaria-specific IgG and IgE antibody levels and spleen rates in the Fulani compared to the Dogon. The Fulani exhibited elevated numbers of both IL-4 and IFN-γ-producing cells, a typical profile seen of CD1-restricted NKT cells. This together with the higher spleen rates and elevated anti-malarial antibodies suggests a role of CD1-restricted cells in the different responses seen between these tribes.

To investigate whether such responses were specifically confined to malaria or a reflection of a generally activated immune system, total levels of IgG and of IgM as well as IgG antibodies to non-malarial antigens were examined in the Fulani in Burkina Faso and Mali. The results showed that the Fulani consistently mounted stronger malaria-specific IgG, IgG1, IgG3 and IgM responses. Total IgM levels were significantly higher in the Fulani than the non-Fulani, whereas total IgG did not differ between the two tribes. While IgG levels to some non-malarial antigens were significantly higher in the Fulani, no such differences were seen in the responses to several other non-malarial antigens suggesting that the Fulani are not generally hyper-reactive and that other specific factors are of importance for their higher malaria resistance.

Finally, a new method to enrich for early and late asexual blood stages of *P. falciparum* parasite from a single parasite culture was developed, using a 3-step centrifugation procedure. Such enriched parasite fractions beside other malaria-parasite antigen preparations were used in an *in vitro* system to analyse T-cell responses in malaria-exposed and non-exposed donors. Such analysis revealed significant proliferative cell response and CD4$^+$ T cell expansion to whole-cell parasite antigens, but not to acellular parasite fractions, in the malaria-exposed as compared to the non-exposed ones. Our data suggest that natural infection preferentially leads to formation of memory cells against certain antigen expressed in live parasites.

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To the memory of my father, Farouk Bushra and my Aunt Shama Bushra

To my family, my mother Asia Bushra, my wife Hind and my son Mohamed

CONTENTS

ABBREVIATIONS

APC	Antigen presenting cells
CpG	Cytosine and guanine separated by a phosphate
D	Diversity segment of TCR gene
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ELIspot	Enzyme-linked immunospot
FCS	Fetal-calf serum
IFN	Interferon
Ig	Immunogloulin
IL	Interleukin
IPP	Isopentenylpyrophosphate
J	Joining
LPS	Lipopolysaccharide
MHC	Major-histocompatibility complex
MSP	Merozoite-surface protein
NK cells	Natural killer cells
PBMC	Peripheral-blood mononuclear cells
PCR	Polymerase-chain reaction
PDC	Plasmacytoid-dendritic cells
PHA	Phytohemagglutinin
RAG	Recombination-activating genes
RBC	Red blood cells
RT	Room temperature
SCID	Severe-combined immunodeficiency
TCR	T-cell receptor
TD	T-lymphocyte dependent
Th	T helper
TH	Tris-Hank's
TI	T-lymphocyte independent
TNF	Tumor necrosis factor
V	Variable segment of TCR gene

ORIGINAL PAPERS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I. Farouk, S. E., Mincheva-Nilsson, L., Krensky, A. M., Dieli, F., Troye-Blomberg, M. (2004). γδT cells inhibit the *in vitro* growth of the asexual blood stages of *Plasmodium falciparum* by a granule exocytosis-dependent cytotoxic pathway that requires granulysin. *Eur J Immunol.* ***34****:2248-56*

II. Farouk S. E., Dolo, A., Bereczky, S., Kouriba, B., Maiga, B., Färnert, A., Perlmann, H., Hayano, M., Montgomery, S. M., Doumbo, O. K., Troye-Blomberg, M. (2004). Different antibody and cytokine-mediated responses to *Plasmodium falciparum* parasite in two sympatric ethnic tribes living in Mali. *Microbes Infect (in press).*

III. [*]Bolad, A., [*]Farouk, S. E., Israelsson, E., Dolo, A., Doumbo, O. K., Nebié, I., Maiga, B., Kouriba, B., Luoni, G., Sirima, B. S., Modiano, D., Berzins, K., Troye-Blomberg, M. Distinct interethnic differences in IgG class/subclass and IgM antibody responses to malaria antigens but not in IgG responses to non-malarial antigens in sympatric tribes living in West Africa. *Manuscript submitted.*

[*]Ahmed Bolad and Salah E. Farouk contributed equally to this study.

IV. Farouk, S. E., Shen, J., Tangteerawatana, P., Bolad, A., Berzins, K., Troye-Blomberg, M. Analaysis of T-cell responses in malaria-exposed and non-exposed donors using *Plasmodium falciparum* asexual blood stages enriched by a simple centrifugation method. *Manuscript submitted.*

INTRODUCTION

THE IMMUNE SYSTEM

The immune system is classically defined as the body's response to foreign material (antigens). Immunity, the process by which memory and specificity are created, is divided into two parts, innate and adaptive immune responses. The innate immune system includes physical, chemical and microbiological barriers and encompasses elements of the immune system such as neutrophils, monocytes, macrophages, complement factors, cytokines and acute phase proteins, which provide immediate host defenses. Whereas the innate response is rapid and may damage normal tissues due to lack of specificity, the adaptive response takes several days or weeks to develop. The latter is characterized by specificity and memory that provide long lasting protection against specific antigens. The immune system is a complex network of specialized cells and organs that function in harmony to achieve protection against foreign invaders. Lymphocytes play a central role in the regulation of both cell-mediated and antibody-mediated responses against different antigens. It has been estimated that only 2% of all lymphocytes are circulating in the peripheral blood. Thus, the vast majority of the lymphocytes are in the lymphoid organs (tonsils, spleen, lymph nodes and Peyer's patches). This is crucial since it is here they exhibit their functional competence via encountering antigens in association with antigen presenting cells (APC), such as monocyte derived macrophages, dendritic cells and langerhans cells. The activated cells in the lymphoid organs communicate with the tissues using the lymphatic and the blood vessel systems.

Two classes of lymphocytes are recognized, the B- and T lymphocytes. The former are precursors of plasma cells that secrete antibodies while the latter are responsible for regulating the immune responses and effector functions. Precursors of both B- and T cells develop in the bone marrow. B lymphocytes mature in the bone marrow, whereas the precursor T lymphocytes migrate to the thymus where they undergo the process of maturation.

B lymphocytes

B cells constitute 15% of peripheral blood lymphocytes. They differentiate from haematopoietic stem cells in the bone marrow where their B-cell receptors (BCR) are assembled from genetic building blocks in a recombination-activating genes (RAG1)/RAG2-mediated process similar to that used for the production of functional T-cell receptors (TCR) [1]. There are four gene segments involved in BCR formation known as the variable (V),

diversity (D), joining (J) and constant (C) regions which exist on different chromosomes within the developing B cells. Developing B cells follow a course of sequential heavy (H) and light (L) chain gene rearrangement. The assembly of the heavy V region of the Ig chain occurs in two steps. First, the recombinase enzymes (RAG-1/RAG-2) catalyses the fusion of one of the D_H genes to a J_H gene, a process that occurs on both chromosomes. Next, the recombinase joins one of the V_H gene to the rearranged D_HJ_H gene. Light chain assembly, on the other hand, involves rearrangement of a V_L region gene to a J_L region gene. Several factors are involved in the BCR clonal diversity, such as the multiplicity of the V, D and J regions, junctional diversity and nucleotide addition. A greater repertoire of the BCR is generated when further Ig gene rearrangement occurs during B-cell division upon antigen stimulation, a process known as somatic hypermutation. Binding of an antigen by the BCR triggers B-cell proliferation, differentiation into antibody-producing plasma cells, memory formation, and antigen presentation to T cells. With regard to the capacity of antigens to induce antibody production, antigens have been classified as either T-lymphocytes dependent (TD) or T-lymphocyte independent (TI) [2]. The immune responses to TD antigens include T-cell help to B cells which is essential for the regulation of B-cell proliferation, antibody production, immunoglobulin class switching, rescue of B cells from apoptotic death, germinal center formation and generation of B-cell memory [3]. TI antigens can be further divided into TI-1, such as lipopolysaccharide (LPS) and TI-2 such as capsular polysaccharides of some bacteria [4, 5]. Unlike TD, the TI responses lack germinal center formation, no class switching, no generation of memory and of poor specificity.

Humoral immune responses to most protein antigens require collaboration between $CD4^+$ T cells and B cells in the secondary lymphoid tissues [6]. T-B cell interaction results in B cell activation via ligation of co-stimulatory molecules expressed on surfaces of activated T cells, such as CD40L and cytokines produced by them [7, 8]. Subsequent to this interaction, naïve B cells primarily produce IgM, and finally may differentiate into IgM-producing plasma cells or switching to production of other forms of antibody isotypes, having the same specificity to the antigen that initiated the primary response [9].

T lymphocytes

In the thymus, the T cells distinguish between self and nonself antigens through the expression on their surface of antigen-specific TCR [10]. TCR is comprised of $\alpha\beta$ or $\gamma\delta$ subunits and both are expressed noncovalently in association with several monomorphic proteins collectively called CD3 constituting the TCR/CD3 complex [11]. The TCR

generation is a complex process that creates a repertoire in the order of more than 10^{14} through combinatorial joining of V, D and J (β and δ chains) or V and J (α and γ chains) segments out of about 200 germline exons. During the combinatorial generation of the TCR self major-histocomatibility complex (MHC)-reactive and nonreactive TCRs are generated [12].

The αβ lymphocytes

The major class of T lymphocytes is defined by their expression of the αβ TCR, a disulphide linked heterodimer comprising α and β chains. Each of the chains is composed of a constant domain and a highly polymorphic variable domain. The TCR has evolved primarily to recognize peptide antigens presented in a complex with MHC class I or class II molecules on the surfaces of APC. The $\alpha\beta^+$ T cells differentiate into several subsets of which two major subsets are identified, $CD8^+$ T cells that act primarily to kill cells harbouring intracellular microbes [13], and $CD4^+$ T cells which regulate the cellular and humoral immune responses [14]. A minority of non-classical MHC molecule restricted double negative $CD4^-CD8^-$ αβ T cells has also been described [15].

Selection of cells carrying functional TCR genes occurs in the thymus which contains three compartments. First, in the subcapsular zone, the prothymocytes rearrange their TCR β chains. The cells then move to the thymic cortex where they rearrange the α chains, potentially forming a functional, mature αβ TCR. In the cortex, a process of positive selection occurs that involve interactions between the developing lymphocytes and the specialized cortical epithelium [16]. The positive selection ensures that TCR have sufficient affinity to self-MHC molecules that enables them ultimately to recognize antigen-MHC complexes. Lymphocytes that fail to recognize self MHC molecules undergo apoptosis and are cleared by thymic cortical macrophages. Finally, in the thymic medulla, cells are tested for potential autoreactivity. Self-reactive cells, which have very high avidity to self-MHC molecules, are removed by apoptosis (negative selection), and cells with moderate avidity for self-peptides can survive and further differentiate. In the cortex the developing lymphocytes initially express neither CD4 nor CD8 (double negative) and later they express both (double positive) [17]. During the positive selection in the thymic cortex, the double positive cells, selected based upon their interaction with MHC class I, become $CD4^-CD8^+$, and those selected based upon interaction with MHC class II molecules, become $CD4^+CD8^-$ T cells, which then move to the thymic medulla to undergo the negative selection. The surviving cells are exported to

the periphery. Two models have been described to explain the transformation of double-positive cells into one of two single positive ($CD4^+$ or $CD8^+$): 1) The instructional model proposes that it is the multiple interactions between the TCR, $CD8^+$, $CD4^+$ coreceptors, and MHC class I or class II molecules that instruct the cells to differentiate into either $CD8^+$ or $CD4^+$ single-positive cells, respectively; while, 2) The stochastic model, on the other hand, postulates that the CD4 or CD8 molecules are switched off randomly regardless of the TCR specificity to a certain MHC molecule.

$CD4^+$ T lymphocytes

The $CD4^+$ T cells represent 60–70% of the T cells in the blood and in the secondary lymphoid organs. They recognize exogenous peptides presented within the cleft of MHC class II molecules. Upon antigen encounter, they differentiate to functionally distinct subsets as indicated by transition of the naïve $CD4^+$ T cells to effector populations [18]. Resting naïve $CD4^+$ T cells are designated as T helpers (Th) and release very little amounts of cytokines. Early in the stimulation phase, Th cells begin to produce IL-2 and hence designated as Th0. As the antigen stimulation continues, the Th cells are polarized towards either Th1 or Th2 subsets depending on the nature of the cytokines present at the site of activation [19]. In addition to Th1 and Th2, some investigators revealed another subtype designated as Th3 [20]. The $CD4^+$ Th1 cells are characterized by the production of IL-2, IFN-γ and lymphotoxins. In general the Th1 cells mediate cellular immunity, enhance the microbicidal activity of monocytes and macrophages, promote the differentiation of cytotoxic lymphocytes and regulate certain B-cell responses. On the other hand, the Th2 cells produce IL-4, IL-5, IL-10, IL-13 and GM-CSF cytokines, mainly involved the induction of B cells to develop into antibody-producing cells and to induce isotype switching from IgM/IgG to IgE [19].

T-regulatory cells

T-regulatory cells (Treg) constitute 5-10% of peripheral $CD4^+$ T cells in humans and are characterized by their expression of the IL-2Rα chain (CD25) [21-25]. The broad repertoire of these $CD25^+CD4^+$ Treg enables them to recognize various self and non-self antigens [26, 27]. The Treg in both human and mice have been shown to suppress the proliferation and cytokine production by $CD4^+$ T cells in response to a variety of polyclonal stimuli. Furthermore, Treg can downregulate the responses of $CD8^+$ T, NK cells and antigen-specific responses of $CD4^+$ T cells [21, 28]. Treg have been shown to be involved in downregulating a variety of autoimmune diseases [29], tolerance to allografts [25, 30], and in

suppressing immune responses to infectious diseases, such as malaria [31] and Leishmaniasis [32].

Factors that influence Th1/Th2 phenotypes

The balance between Th1- and Th2-producing cells plays a key role in the development of immunity and/or pathogenesis to different pathogens. The factors that may influence $CD4^+$ Th phenotype include presence or absence of cytokines such as IL-12, IFN-γ, or IL-4, the nature of the encountered pathogen, antigen type, infecting dose and route of administration, the nature and the type of APC and/or the co-stimulatory signals involved and host genetic factors [18, 33, 34].

Microbial products such as endotoxins and uncharacterised viral antigens, intracellular bacteria (*Mycobacterium tuberculosis*) and protozoa (*Toxoplasma*), stimulate IL-12 production by macrophages and steer the immune response towards Th1 [33, 35]. Pathogen-derived signals or factors that are produced by infected cells in the surrounding tissues can induce development of immature DC into polarized mature DC with a capacity to express molecules that promote Th1, Th2 or regulatory T cell development [36, 37].

Development of Th2 phenotype is induced upon exposure of naïve $CD4^+$ T cells to produce IL-4 early in an immune response [38]. Besides differentiated T-helper cells, several other cell types are known to produce IL-4 early in the course of an immune response, including the $NK1^+$ subset of $CD4^+$ and double negative (DN) T cells [39, 40], *Leishmania* homolog of receptors for activated C kinase (LACK)-specific $CD4^+$T cells expressing Vβ4/Vβ8 TCR [41], mast cells, basophils and esinophils [42].

$CD8^+$ T lymphocytes

The $CD8^+$ T cells represent 30–40% of the T cells in the blood and the secondary lymphoid organs. They show major cytotoxic activity against cells infected with intracellular microbes and tumour cells. $CD8^+$ T cells recognize antigens in the context of the MHC class I molecule. They also contain a subset that can downregulate immune responses (suppressor cells). These suppressor T cells are $CD8^+$, but they are MHC class II restricted and lack the expression of the costimulatory receptor CD28 [43].

$CD8^+$ T cells have cytolytic granules in their cytoplasm where the pore-forming proteins, perforin, and granzymes reside that mediate killing of target cells [44]. The $CD8^+$ T cells also can be divided into type 1 and type 2 cytokine producing cells, hence designated T cytotoxic cell type 1 (Tc1) and Tc2 [45].

The γδ$^+$T lymphocytes

The γδT cells represent a relatively rare type of T cells in both mice and humans whose function appears to be distinct from that of αβT cells and B cells (reviewed in [46]). While the αβ$^+$ TCR represent more than 90% of peripheral blood T lymphocytes, the γδT cells represent less than 6% of peripheral blood T lymphocytes [47]. They recognize small non-peptidic antigens in MHC-independent manner [48-53].

γδ$^+$T cells development and subsets

Compared to αβT TCR, there are fewer γ and δ genes available for recombination. Much of the γδ repertoire is achieved through TCR junctional diversity. Early in the development of T lymphocytes, the TCR-γ and TCR-δ genes rearrange before the TCR-α and TCR-β genes. The expression of αβ or γδ heterodimer is mutually exclusive [54-56]. At least six γδT cell subsets have been designated (Vδ1 – Vδ6) based on the usage of the δ-chain variable region. In humans, the most commonly used genes are δ1 or δ2. In human blood and tissue more than 97% of γδT cells belong to the Vδ1 and Vδ2 subsets. The majority of the circulating γδT cells, the Vδ2 have preference for pairing with Vγ9 and the disulphide linked Cγ1, whereas the Vδ1 seems to have no preference to a particular Vγ chain [57]. Based on the Vδ-chain usage and the anatomical distribution, the γδT cells can be roughly divided into two groups: (i) circulating γδT lymphocytes comprising 1-10% of the peripheral blood mononuclear cells in healthy individuals, using Vδ2 chains in their receptor and (ii) resident γδT cells in the mucosal surfaces and epithelia of the digestive-, respiratory and urogenital tracts, using Vδ1chains (reviewed in [58-60]). Only a few Vδ1$^+$ γδT cells are present in peripheral blood.

Features of γδ$^+$T cells

Unlike the conventional αβT and other cell types, γδT cells have unique characteristic features. Some of these are listed below:

a. Expression of a unique TCR
b. Specialized anatomical distribution
c. Characteristic cell phenotypes
d. A distinct developmental pathway
e. Unique antigen specificity

f. A broad spectrum of cell-cell interactions
g. A unique ability to recognize and protect host against specific pathogen
h. Immunoregulatory capacity in a non-redundant fashion
i. Unique age dependent activities

Antigen recognition by human Vγ9/Vδ2 cells

Early investigations have indicated that the Vγ9/Vδ2 expressing γδT cells recognize mycobacterial antigens [61, 62] and a variety of other pathogens, including *Plasmodium falciparum* (*P. falciparum)* [60], *Toxoplasma gondii* [63], *Yersinia enterocolitica* [64], *Francisella tularensis* [65] in an MHC-independent manner. Further studies revealed that the γδT cell-stimulating components in microbial extracts were not proteins, but rather consisted of nonpeptidic, phosphatase-sensitive, low molecular weight compounds [66]. Some of these phosphorylated antigens have successfully been isolated from *M. tuberculosis* (TUBag) [50] and isoprenoid pyrophosphates such as isopentenyl pyrophosphate (IPP) [51]. Collectively, these ligands have been termed phosphoantigens [67]. In addition, phosphorylated sugars and alkyl phosphates such as monoethyl phosphate have also been reported to stimulate Vγ9/Vδ2 bearing T cells [49, 68]. Phosphoantigens extracted from some plants with a γδT cell-stimulating capacity have also been reported [69]. A large variety of pathogens can produce phosphoantigens [50, 51, 65, 70].

Effector functions of γδ$^+$T cells

There is increasing evidence that γδT cells play important roles in the immune defense against a variety of microorganisms and certain tumour cells. The γδT cells expressing the Vγ9/Vδ2 TCR represent the majority (95%) of the circulating γδT cells [71] and as a part of this thesis, the focus will be on this subset. Similar to αβT cells, γδT cells display two broad types of effector functions: cytokine production and cytotoxic activity. Most activated human γδT cells, produce cytokines and/or mediate cytotoxic effector activity (reviewed in [57, 60]). This effector function of δ2$^+$ γδT cells includes production of TNF-α, IFN-γ and CC chemokines and cytotoxic activity against pathogen-infected macrophages [60, 72-75]. Given their ability to produce various cytokines, Vγ9/Vδ2 T cells might have regulatory functions of other immune cells such as dendritic cells and B cells [76, 77].

Dendritic cells

Dendritic cells (DC) are generated in the bone marrow and migrate as precursor cells to sites of potential entry of pathogens. Human peripheral blood has three different populations of dendritic cell subsets (DC), which include $CD11c^+$ myeloid precursor DC (expressing CD1b/CD1c, CD16 or BDCA3), $CD34^+$ and $CD11c^-$ plasmacytoid DCs that express CD123, BDCA2 and BDCA4 [78, 79]. DCs recognize pathogens through pattern recognition receptors (PRRs) that directly recognize conserved microbial molecules, termed as pathogen-associated molecular patterns (PAMPs), many of which are shared by a variety of pathogens [80]. Recently, these PRRs have been shown to include molecules that mediate opsonization, endocytosis, activation of complement and coagulation cascades, activation of inflammatory signaling pathway and/or induction of apoptosis [81]. An important group of PRRs are the toll-like receptors (TLRs), members of the IL-1 receptor superfamily. The two well characterized DC subsets, the myeloid and the plasmacytoid DCs, show a mutual exclusive expression profile of TLR. The myeloid DCs express TLR2 and TLR4 whereas the plasmacytoid DCs selectively express TLR9 [82]. Adaptive immunity to pathogens starts with the initiation of DCs maturation after ligation of TLRs, which, therefore TLRs are vital proteins that link innate and adaptive immunity [83, 84].

NK/NKT cells

NK cells are critical to host defense against virally infected cells and neoplastic transformation through release of cytokines and cytotoxic activity [85]. Phenotypically, NK cells are characterized by the expression of the CD56 surface antigen and the lack of the CD3. NK cell functions include production of immunomodulatory cytokines, including IFN-γ, TNF-α, IL-10, GM-CSF and various other chemokines capable of generating an immediate immune response [86]. They have spontaneous cytotoxic activity against virus-infected and tumour cells. In addition, NK cells can mediate antibody-dependent cellular cytotoxicity through FcγRII (CD16), a receptor molecule that specifically binds the Fc part of antibodies [87]. Subsets of NK cells can be distinguished by the surface density expression of the CD56 antigen ($CD56^{bright}$ and $CD56^{dim}$) as well as the presence or absence of CD16. Resting $CD56^{dim}$ cells comprise 90% of total NK cells, whereas the $CD56^{bright}$ cells represent around 10%. Upon activation, the $CD56^{bright}$ cells produce IFN-γ, TNF-α, TNF-β, GM-CSF, and IL-10, while the $CD56^{dim}$ cells produce few of these cytokines [88]. The $CD56^{bright}$ cells express high levels of the CD94/NKG2 receptor and only a small fraction expressing the killer-cell

immunoglobulin-like receptor (KIR), whereas the $CD56^{dim}$ cells express both KIR and CD94/NKG2 receptors at high surface density [89].

Natural killer T (NKT) cells are a subpopulation of lymphocytes that coexpress the CD56 and CD3-TCR complex [40]. NKT cells usually express a biased TCR, containing predominantly Vα24Jα18 and Vβ11 chains in humans and homologous Vα14Jα18 and Vβ8 chains in mice [90, 91]. These cells recognize glycolipids such as α-galactosylceramide (α-GalCer) and glycocylphosphatidolinositol (GPI) in a CD1-restricted manner [92, 93]. NKT cells comprise two functionally distinct phenotypes with significant differences in their profile of cytokine secretion as well as pattern of expression of chemokine receptors, integrins and NK cell receptors. $CD4^+$ NKT cells exclusively produce Th2 cytokines (IL-4 and IL-13), whereas the $CD4^-CD8^-$ NKT cell subsets (DN NKT) have a strict Th1 profile, producing TNF-α and IFN-γ [94]. Differently from conventional T lymphocytes, NKT cells have the ability to promptly release high amounts of IFN-γ and IL-4 upon primary TCR stimulation [95-97]. The NKT cells are regarded as actors of the innate immune response [95], playing a major adjuvant-like role during an immune response [40, 98], spanning from NK-cell activation [99], helper-T cell differentiation [100], to the control of autoimmune diseases [101, 102], tumour growth [103] and infections [104]. Interaction of human NKT cells with CD1d expressing B cells, provides direct help for B cell proliferation and antibody production through CD1d-restricted mechanisms [105].

Antigen presentation

Antigen processing and presentation are prerequisites for T-cell antigen recognition, activation and function. A major role of T cells is to identify and destroy infected cells which require recognition of both self-components and microbial determinants. These self-components are the MHC molecules expressed on the surface of APC and encoded by the MHC gene located on chromosome 6 in humans. MHC molecules bind antigenic fragments and display them to various cells of the immune system. TCRs recognize small linear peptides of 8–25 amino-acids residues in length that are processed and presented in the antigen-presenting groove of MHC molecules [106]. MHC molecules bind peptidic fragments that have been processed in different cellular compartments. MHC class I molecules bind to peptide antigens that have been synthesized within APC (endogenous pathway), whereas MHC class II bind peptidic antigens that have been ingested by APC (exogenous pathway).

$CD8^+$ and $CD4^+$T cells show preferential restriction to MHC class I and II molecules, respectively [107, 108].

Non-conventional T cells like $CD4^-CD8^-$ T lymphocytes do not recognize antigens in the context of MHC class I or class II, but instead in the context of class I-related protein CD1, which is adapted to present glycolipids from mycobacteria and other microbes [109]. A subset of γδT cells recognizes the MHC class I-related chain (MIC) [110].

The human CD1 locus on chromosome 1 encodes five distinct MHC-related proteins, designated as CD1a, -b, -c, -d and –e [111]. Unlike the classical MHC class I and II molecules, which are characterized by extensive allelic polymorphism, the CD1 protein have been reported to be monomorphic [112]. The CD1 gene comprises two groups, group 1 comprising CD1a, -1b, -1c and –e, which are present in humans but not in mice. Group 2, which comprises CD1d, is generally conserved in mammalian species [113] and presents antigens to NKT cells [95]. CD1 molecules have a hydrophobic antigen-binding pocket [114], and are hence specialized for binding and presentation of lipid antigens derived from exogenous or endogenous pathways [115].

Cytokines

The cells of the immune system are widely distributed in the human body, unlike other cells which are confined to certain organs or tissues. Therefore, cells of the immune system need network communication. Such communication is achieved by molecules, which were recognized as lymphokines and monokines, to identify lymphocytes and monocytes as the cellular sources, respectively [116]. When it became evident that many of these lymphokines and monokines were actually produced by a wide range of cell types, the nomenclature was designated as cytokines [117]. The term interleukin (IL) refers to cytokines which are produced by leukocytes [118]. Cytokines are a group of regulatory proteins consisting of low molecular weight proteins, usually less than 30 kDa, which are secreted by a variety of cells in response to different stimuli. The actions exerted by cytokines are diverse, including induction of growth, differentiation, cytolytic activity, apoptosis and chemotaxis. Actions could be autocrine, when a cytokine binds to receptors on the same cell that secrets it, paracrine when it binds to a receptor on another cell in close proximity, and endocrine when it binds to a receptor on a distant cell. However, most cytokines act in a paracrine manner. Cytokines regulate and coordinate immune cell activities by acting in a pleiotropic, redundant, synergistic, antagonistic or in a cascade inducing fashion. A variety of cells can secrete cytokines, among these are the Th cells and macrophages. Cytokines secreted

by Th1 subset induce cell-mediated responses, whereas those secreted by Th2 cytokine are involved in B cell activation and humoral responses.

MALARIA

Malaria remains a major public health problem in many developing countries. Worldwide, an estimated 300-500 million contract malaria each year, resulting in 1.5-2.7 million deaths annually [119, 120]. More than 90% of the worldwide malaria cases and deaths occur in sub-saharan Africa [121]. Among the Plasmodium species, the causative agents of malaria, the *P. falciparum* is particularly lethal and causes cerebral malaria.

Parasite and life cycle

The four etiological agents of malaria disease in humans belong to the protozoan parasites, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is the most virulent form of the four species causing severe disease in humans. The life cycle of *P. falciparum* parasite is accomplished in two hosts, the human and the female *Anopheles* mosquito (Fig. 1). The life cycle includes asexual reproduction in the human host and sexual reproduction in the mosquito. The parasite reproduces asexually in the human hosts through a pre-erythrocytic (in the liver) and erythrocytic (in the red blood cells) stages. The sexual forms of the parasite, the gametocytes, emerge as a result of the asexual reproduction. These gametocytes are taken by the mosquito from an infected individual as part of the blood meal. In the mosquito, the gametocytes undergo a sexual reproduction which finally generates sporozoites, which can be stored in their salivary glands.

Sporozoites are inoculated in a human host via bite of an infected mosquito. The circulating sporozoites enter hepatocytes shortly after inoculation into the blood stream. The intrahepatocytic sporozoites develop into pre-erythrocytic schizonts, each containing 1×10^3-30×10^3 merozoites. Merozoites released upon rupture of these pre-erythrocytic shizonts enter the circulation and invade red blood cells (RBC), where the erythrocytic asexual cycle is started. The intra-erythrocytic cycle includes development of merozoites into rings, trophozoites and schizonts which rupture in about 48 hours releasing merozoites (15 to 30/schizont) that can infect other RBC. Some merozoites develop into sexual forms (gametocytes), which can be taken by the Anopheles mosquito to start another cycle. Exponential growth of intraerythrocytic parasites, the modification of infected RBC (expression of the parasite proteins on their surface) and the concomitant immune response to the parasite are the main determinants of the disease manifestations of clinical malaria.

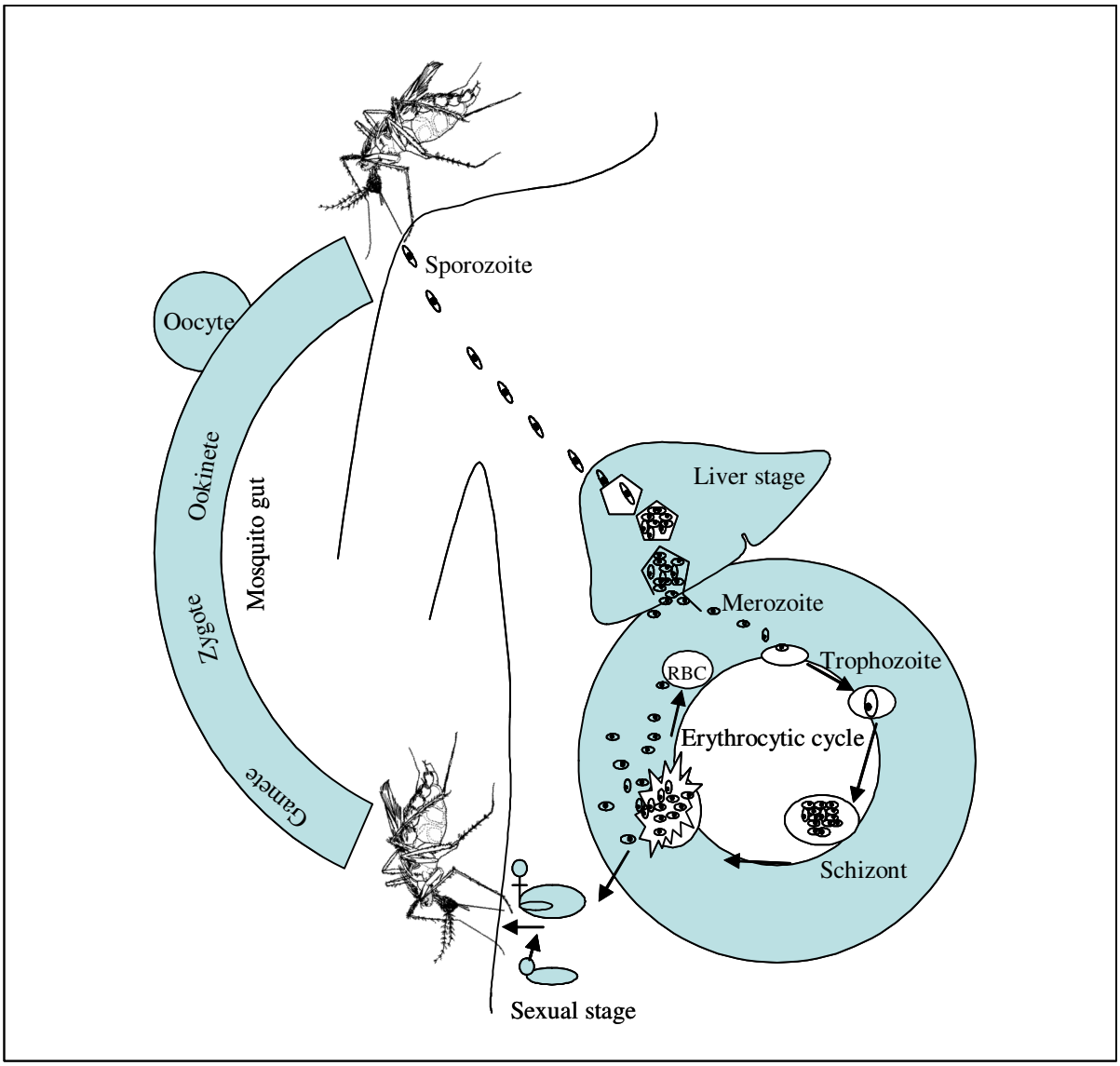

Fig 1. Life cycle of *P. falciparum*

Clinical features and pathogenesis of *P. falciparum* malaria

The typical cyclic fevers, the hallmark of malaria, arise shortly or at the time of infected red blood cells rupture, which occurs every 48 hours in the case of infection with the *P. falciparum* parasite. This intense fever is accompanied by nausea, headaches and muscular pain amongst other non-specific symptoms of a systemic pro-inflammatory cytokine response, which is believed to originate from cells of the innate immune system [122]. Renal failure, hypoglycaemia, hepatic dysfunction, severe anaemia, pulmonary oedema, convulsions and shock are complications in severe malaria. A frequent presentation of severe malaria is cerebral malaria, which has been attributed to the ability of the parasite to modulate the surface of infected RBC so that they bind to endothelial surfaces, hence leading to obstruction

of cerebral blood flow [123]. Further reports have suggested that pro-inflammatory cytokines and nitric oxide induced by parasite material also contribute to the pathogenesis of cerebral malaria [124]. Malaria can complicate pregnancy and result in miscarriages, fetal death, low birth weight and premature delivery. In children, severe malarial anaemia is the most important determinant of survival that leads directly to respiratory distress syndrome [125], which is predominantly caused by a lactic acidosis [126]. This lactic acidosis is due to increased production of lactic acid by parasites (through direct stimulation by cytokines), decreased hepatic clearance and the combined effects of other factors that reduce tissue oxygen supply [127]. Inflammatory mediators, resulting from the concomitant immune response to the parasite have been implicated in the severity of the disease [128, 129]. This has led to the hypothesis that severe malaria is an immune-mediated disease. Blood parasitemia increases exponentially so that almost all RBCs are infected leading to an inevitable death, unless controlled by anti-malarial drugs or by the immune system. Innate or the adaptive immune effector mechanisms can limit the peak of parasitemia and prevent severe pathology.

Innate immunity to malaria

Accumulating evidence support the concept that macrophages, DCs, NK, γδ, and NKT cells are important effectors of the innate immunity against malaria. The innate immune mechanisms have been shown to operate when parasite density crosses a predefined threshold [130].

NK cells have been shown to be the first cells to respond to *P. falciparum* infection by increasing in number and the ability to lyse infected RBC *in vitro* [131]. The production of IL-12 and IL-18 by monocytes/macrophages and DC in response to many infectious agents, activates NK cells [132]. In the case of NK-cell activation by *P. falciparum*, IL-12 and IL-18 are required but not sufficient for optimal IFN-γ production [133], unless direct contact between NK cells and parasitized RBC is achieved [134]. Thus, IFN-γ produced by NK cells activates macrophages to eliminate infected RBC. Evidence for the role of macrophages in the innate immunity is their ability to phagocytose infected erythrocytes in absence of cytophilic or opsonizing malaria-specific antibodies [135, 136], and thus contributing to the reduction of the initial parasitemia. Evidence for the role of NKT cells have emerged from their capacity to inhibit the liver-stage parasite replication in a murine model *in vitro* [137]. A possible role of the NKT cells in the human malaria could be

speculated from their simultaneous production of high levels of both IFN-γ and IL-4 upon primary TCR stimulation as shown in other systems [138].

Activation of DC and macrophages might be one of the earliest events in the innate response to malaria. Plasmacytoid dendritic cells (PDC), a unique subset of DC, have earlier been shown to have a key role in the innate immunity because of their ability to produce high levels of IFN-α in response to viral [139, 140] or microbial DNA or CpG DNA stimulation [141, 142]. The relevance of such PDC as innate effector function has recently been investigated in malaria, showing that soluble products of the late stages of the parasite can activate PDC in a TLR-9-dependent manner [143]. IFN-α has also been shown to activate γδT cells [142]. Thus, the resulting IFN-α from activated PDC can activate γδT cells. A marked increase in circulating γδT cells has been reported in acutely infected malaria patients [60, 144-149] which, strongly suggests their involvement in the innate immune responses to malaria. This has been shown by the capacity of γδT cells to directly inhibit the growth of blood stage parasite *in vitro* [150]. This increase may be due to the PDC-induced activation of γδT cells and/or to the nonprotein component of schizont lysate-containing phosphate groups [151]. A later study has identified a role of the phosphoantigen, IPP, synthesized by intracellular parasites, including *P. falciparum*, in the activation of γδT cells [152]. The pathway of IPP synthesis by intracellular parasite is illustrated in Fig. 2. Taken together, the innate immune responses may therefore function to limit the initial phase of parasitemia. However, acquired adaptive mechanisms are required for complete parasite elimination.

Adaptive immunity to malaria

Naturally acquired immunity to malaria takes as long as 10-15 years of exposure to develop [153]. However, this acquired immunity is non-sterile, and is species-, stage-, strain-, and variant-specific [154-156]. Residents in malaria endemic areas frequently have premunition (parasitemia and antibodies without symptoms) [153]. Acquired immunity to malaria involves both antibody-mediated and cell-mediated immunity.

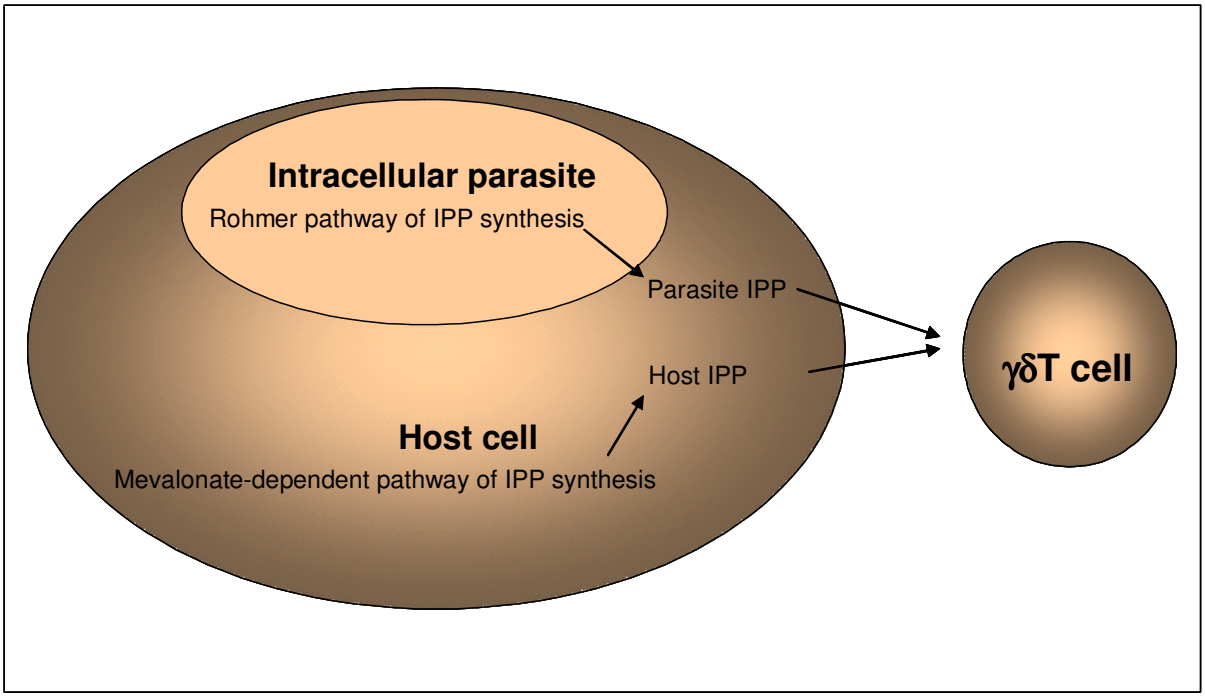

Fig. 2. IPP synthesis by intracellular parasites (Adapted from Sicard et al, 2000)

Antibody-mediated responses to asexual blood stages of P. falciparum

It is well established that B cells and antibodies play a crucial role in immunity to malaria. It has been shown that mice lacking B cells are unable to clear parasites from *P. chabaudi chabaudi* AS infection, rather such mice developed chronic parasitemia [157, 158]. It has been demonstrated that passive transfer of monoclonal antibodies against parasite antigens may confer protection in naïve mice [159, 160]. In humans, treatment of Thai *P. falciparum*-infected patients with IgG extracted from African immune adults, resulted in reduction of parasitemia and clinical symptoms [161].

Antibodies protect against malaria by a variety of mechanisms. They may mediate their effector functions against malaria parasites on their own or in collaboration with other effector cells. On their own, antibodies against merozoite surface-associated proteins may block RBC invasion [162] and by blocking merozoite release from schizonts either by binding to surface exposed antigens or by entering the infected RBC through leaky membrane at the time of rupture [163]. Moreover, antibodies may block cytoadherence preventing infected RBC to being sequestered in the periphery, and allowing them to be removed by the spleen [164]. They may also inhibit spontaneous binding of uninfected RBC to infected RBC (rosetting) [165] and consequently may guard against cerebral malaria. In collaboration with other effector immune cells, parasite antigen–specific antibodies play an

important role via antibody-dependent cellular inhibition (ADCI), whereby binding of antibodies to phagocytes via Fc receptors lead to inhibition of parasite growth [161, 166-168]. Alternatively, antibodies may initiate parasite clearance by opsonization, thus enhancing the activity of phagocytic cells or initiating complement-mediated damage [169, 170].

Cell-mediated responses to asexual blood stages of P. falciparum

It is now evident that T cells play a major role in the acquisition and maintenance of protective immune responses to malaria infection. Available evidence in both animal models and humans points to a major role of the $CD4^+$ T cell subsets. It has been shown that $CD4^+$ T cells alone are able to confer protection against malaria. Mice with severe combined immunodeficiency (SCID) and reconstituted with T cells from immune donors suppress parasite growth, suggesting a protective role of T cells against malaria parasites [171]. B cell-deficient mice are also able to suppress parasitemia at the same rate as normal mice [171]. Depletion of $CD4^+$ T cells from such mice lead to a loss of the mice to suppress parasitemia. This indicates that $CD4^+$ T cells can act independently of B cells in the resolution of the parasites.

In humans, direct studies of the responding T cells during malarial infection are difficult, as these cells may leave the peripheral circulation and sequester in the spleen or other tissues [172, 173]. $CD4^+$ T cells play a central role in regulating the immune responses to the asexual blood stages of *P. falciparum* via cytokine production and B-cell help [174]. It has been shown that $CD4^+$ T cells from individuals naturally exposed to malaria, respond to blood stage antigens of *P. falciparum* by proliferation, production of IFN-γ and/or IL-4 secretion *in vitro*. Such production of IL-4 was neither associated with proliferation nor with IFN-γ production, but was well correlated to serum antibodies to the peptides used to activate the T cells [175]. This is in line with the finding that malaria-specific $CD4^+$ T cells can provide help for B cells to produce *P. falciparum*-specific antibodies [176, 177]. A correlation between resistance to fever and high parasitemia and *in vitro* T cell responses to *P. falciparum* blood stage antigens has been reported [178]. In contrast, other studies failed to demonstrate such correlations [179, 180]. The role of $CD4^+$ T cells has been questioned as there is no evidence that the advent of AIDS has exacerbated malaria [181]. This should not be conclusive with regard to the role of the $CD4^+$ T cells, rather this may reflect the complexity of the *in vivo* immune responses and the lack of reliable *in vitro* systems to elucidate cellular responses involved in protection against malaria.

Available evidence indicates an important role of MHC class 1-restricted $CD8^+$ T cells in the pre-erythrocytic immunity [182, 183] and contribution to protection against severe malaria [184, 185]. However, no available evidence for a protective role of $CD8^+$ T cells against *P. falciparum* blood stage has been reported. This is supported by the fact that RBC do not express classical MHC class 1 molecules, hence lacking the antigen processing machinery, suggesting that RBC do not represent a target for $CD8^+$ T cells.

In contrast to the MHC-restricted αβ T cells, γδT cells recognize schizont-derived phosphorylated molecules [151], which are not recognized by αβ T cells. *P. falciparum* antigen-activated γδT cells produce primarily, but not exclusively, proinflammatory cytokines [150], suggesting both regulatory and cytotoxic functions. Taken together, a major role of γδT cells is to enhance the cellular immune responses towards antigens that are not activating/recognized by αβ T cells.

RELATED BACKGROUND

Functions of γδT cells in *P. falciparum* infection

It has well been established that γδT cells must be considered as important effectors in the host defense against infections with the *P. falciparum* parasite. γδT cells may contribute to the outcome of the disease by mediating anti-parasitic responses, induction of pathology and/or performance of immunomodulatory functions.

It is known that during the first few days of a primary *P. falciparum* infection, γδT cell populations, particularly the Vγ9/Vδ2 subsets, expand in peripheral blood [144, 146, 149, 186]. Such expansion has also been reported following an *in vitro* stimulation of PBMC from naïve donors by *P. falciparum* antigens [187-189]. The expanded γδT cells express TNF and IFN-γ and can inhibit parasite growth *in vitro* [147, 150, 190]. IFN-γ has a protective role in inducing parasite killing by monocytes/macrophages and other effector immune cells [191, 192]. In addition, the presence of IFN-γ and/or TNF-α promotes the synthesis of nitric oxide (NO) [135, 193, 194], that has been shown to exert anti-parasitic effects on the various stages of the malaria parasite [195]. However, several studies indicate a pathogenic role of NO as well as of TNF-α [196, 197] in cerebral malaria [195, 198].

Beside the protective and possibly pathogenic potentials, γδT cells can also exert immunoregulatory functions during *P. falciparum* infections. An interesting aspect of the γδT cells response to malaria is the fact that activated γδT cells continue to circulate after the parasite clearance and disappearance of symptoms [186, 199]. This suggests a downregulatory function of the surviving γδT cells on the αβT cells as has been shown in a murine influenza model [200]. The finding that γδT cells require $CD4^+$ T cells for their expansion, implies that αβT-cell responses may influence the extent of the γδT cell responses. A considerable proportion of human $CD4^+$ αβ T-cell responses to malarial antigens in naïve subjects may be the result of T cells cross-reacting with different common bacterial, viral and fungal antigens [201]. Such cross-reactivity with a subsequent IL-2 production may drive the γδT cell responses which in turn may influence the development of the adaptive immunity. Taken together, γδT cells are considered to be crucial in protection and may be in the pathogenesis of malaria, as well as having immunomodulatory functions in this disease.

Granule-exocytosis cytolysis of *P. falciparum*-infected RBC

Cytotoxic granules are secretory lysosomes that are present only in cells with cytolytic potential [202]. When a cytotoxic T cell recognizes its target, these cytotoxic granules migrate from their scattering locations in the cytosol towards the formed immunological synapse [203, 204]. This allows a directional release of the granular contents into the immunological synapse. These granules contain various molecules, such as granzymes, perforin [205-209] and granulysin [210-212] that mediate the toxic effects on their targets. Considerable data point to the ability of γδT cells and NK cells to inhibit the *in vitro* growth of the asexual blood stages of *P. falciparum* parasite [131, 147, 150]. Granule-mediated killing of the malaria parasite has only been reported for NK cells [213] and not in other cytotoxic T cells including γδT cells. γδT cells have been shown to kill intracellularly residing *M. tuberculosis* via a granule-exocytosis cytotoxic pathway involving granulysin [214]. Granulysin has recently been suggested as a novel serum marker to evaluate the Th1/Th2 balance, especially Th1 response in pre-eclampsia [215]. In view of the fact that *M. tuberculosis* antigens share some homology with those of *P. falciparum* antigens [70], a role of such pathway may be anticipated in *P. falciparum* parasites.

Th1 and Th2 cytokine network in *P. falciparum* infection

A critical balance between Th1 and Th2 immune responses is of vital importance in determining the level of parasitemia and disease outcome [216, 217], otherwise overproduction of both Th1 and Th2 cytokines can lead to severe disease and mortality [218, 219].

Evidence for a protective role of IFN-γ against malaria was based on the finding of higher levels of this cytokine in protected individuals as compared in non-protected ones living in a malaria endemic areas in Madagascar [220]. *P. falciparum* blood stage antigen-induced production of IFN-γ by $CD4^+$ T cells has also been associated with protection against malaria reinfection in Africa [221]. These findings are in line with the fact that IFN-γ produced by T cells in response to malaria antigens can help in the induction of malaria-specific cytophilic antibodies (IgG1 and IgG3) which mediate antibody-dependent cellular inhibitory mechanisms against the parasite [166]. IFN-γ seems to be essential for the resolution of the primary infection by limiting the initial surge of parasitemia. However, IFN-γ can also contribute to the acute symptoms of malaria through the induction of TNF-α and IL-1 which are cytokines predisposing to the severe pathology seen in the disease [217]. The

detrimental effects of IFN-γ over-production are mostly under the control of the IL-12, which is a key cytokine that initiates Th1-effector mechanisms by triggering IFN-γ production from NK and $CD4^+$ T cells [222]. Children infected with mild *P. falciparum* malaria have higher levels of plasma IL-12 compared to those with severe disease, and these levels are inversely correlated with parasitemias and numbers of malaria pigment containing neutrophils [223, 224]. These findings indicate that IL-12 plays a crucial role in the protection against the blood stage malaria by inducing IFN-γ production by NK and $CD4^+$ T cells. Not only IL-12 has an inverse correlation with parasitemia, but also the pro-inflammatory cytokine, TGF-β [225]. Early in malaria infection, TGF-β has been shown to promote Th1-effector mechanisms that, in turn, control parasite growth, and later in the infection, downregulates the Th1 responses and thereby reducing their possible detrimental effects [226]. In addition to its ability to downregulate IFN-γ production, TGF-β can also upregulate IL-10 [227]. Lower IL-10/TNF ratios in anaemic children living in malaria endemic areas than that in children with uncomplicated disease, suggest an inhibitory capacity of IL-10 on TNF-induced anaemia [228]. The anti-inflammatory cytokine IL-4 has also been shown to be inversely correlated with parasitemias in residents in a malaria endemic area in Gabon [229]. In contrast, IL-4 has been shown to suppress macrophage-mediated killing of the *P. falciparum* parasite [230]. In individuals naturally exposed to *P. falciparum* malaria, increased ratios of *P. falciparum*-induced IL-4/IFN-γ producing cells have been shown to be associated with elevated malaria-specific IgE antibodies [231]. Therefore, a critical balance between pro-inflammatory and anti-inflammatory cytokines is important in determining the disease outcome. It may also determine the quantity and quality of antibody-mediated immune responses which are responsible for the final elimination of the parasite.

Naturally acquired antibody responses to *P. falciparum* antigens and disease susceptibility

In regions where malaria is endemic, it has been observed that acute disease is a feature of children, and though adults can be infected, they acquire, after several years, a state of relative resistance, known as premunition [232]. The effector mechanisms mediating such resistance are poorly understood.

The antibodies of the IgG class are important component of acquired immunity as has been shown by passively transferred Africans' IgG antibodies [233]. Despite the finding that total IgG against malaria antigens is a poor predictor of immunity [234], several

studies have investigated the different roles played by each of the four IgG subclasses (IgG1-4) in the acquisition of naturally acquired immunity to malaria. Substantial differences in the distribution of these IgG subclasses between clinically protected and nonprotected individuals have been reported. The cytophilic isotypes (IgG1 and IgG3) have been found to predominate in protected adults having low parasite rates and reduced risk of malaria pathology [235], while the non-cytophilic antibodies (IgG2 and IgG4) predominate in non-protected children and in adults with primary attack [235-237]. This role of the cytophilic antibodies was further supported by the finding that the parasite-specific IgG3, but not total IgG, was inversely correlated to susceptibility to clinical malaria [238]. These observations have led to the suggestion that the development of naturally acquired immunity in residents of malaria endemic areas may be associated with an age-dependent switch from IgG2 and IgG4 to IgG1 and IgG3 subclasses [235]. The cytophilic antibodies have been shown to bind to Fc receptors on monocytes and mediate antibody-dependent cellular inhibition in African immune adults [235, 239]. Conversely, the non-cytophilic antibodies have been suggested to antagonize the protective activity of the cytophilic IgG1 and IgG3 antibodies [235]. However, others have shown that IgG2 to RESA and to MSP2 are associated with resistance to malaria [167]. In contrast, levels of IgG4 to the same antigens were shown to be lower and positively correlated with the risk of infection.

Also antibodies of other than the IgG class have been shown to play different roles in the immunoprotection and/or pathogenesis of malaria. Previous studies have reported higher levels of IgE in patients with complicated malaria than those with un-complicated malaria [240, 241]. Conversely, elevated levels of malaria-specific IgE in asymptomatic residents in a holoendemic area in Tanzania were associated with reduced risk of developing clinical episode of malaria [242]. Little information on the involvement of IgM antibodies in protection against malaria is available. However, it has been shown that IgM antibody levels, but not IgG antibody levels, had a weak negative correlation with parasitemias in children and adults living in Liberia [243]. Further evidence for the involvement of malaria-specific IgM in the protection against malaria was the *in vitro* observation that IgM antibodies collaborate more efficiently with monocytes in the *in vitro* killing of the parasite than IgG antibodies [244]. Therefore, for an individual to attain an appropriate level of relative resistance against malaria, relevant antibodies of the right Ig-class are important variables that determining the outcome of the disease.

Enrichment for different blood stages of *P. falciparum* parasites and their role in lymphocyte activation

The fact that *in vivo P. falciparum* parasite-infected RBC rupture every 48 hours is indicative of a state of synchronization. This synchronization has been suggested to be mediated by the innate immune responses [130], which are not present in *in vitro* conditions. Thus, such synchronization of *P. falciparum in vitro* cultures is not possible, unless induced by different physical or chemical agents. Therefore, it is of vital importance to synchronize parasite cultures to be able to investigate what antigens are expressed during the different developmental stages and to define the major targets of the immune responses. Crude malaria parasite extracts have been shown to induce proliferation of lymphocytes from malaria naïve individuals [245-248] as well as from malaria exposed ones [174, 249]. This similar response seen in both groups has been attributed to mitogenic components of *P. falciparum* parasite [250]. However, others suggest that the parasite does not contain a mitogen, but rather that such responses are attributed to classical parasite antigens [251]. Whether there is a difference between stimulation of lymphocytes with intact live parasites and crude parasite antigens in individuals with different exposure to malaria, has not been well investigated.

AIMS OF THE PRESENT STUDY

This thesis addresses the analysis of cellular and humoral factors involved in the killing/growth inhibition of the *P. falciparum* parasite and their relation to disease susceptibility and/or resistance.

Specific aims:

- To investigate immune-effector mechanisms involved in the killing/growth inhibition of *P. falciparum* parasite by human γδT cells
- To investigate mechanisms involved in the interethnic differences in cell- and antibody-mediated responses to *P. falciparum* infection and their relation to protection or susceptibility to malaria.
- To develop a simple method to enrich for specific developmental stages of *P. falciparum* cultures and use such fractions for analysis of T-cell responses in differently malaria-exposed donors.

METHODOLOGY

The methods are described here in general, for details refer to each individual paper (paper I-IV).

Parasites (study I, II, III and IV)

The strain F32 of *P. falciparum* was maintained in continuous cultures as described by Jensen [252]. Sonicates of late stage infected erythrocytes, enriched by 60% percoll gradient centrifugation were used as antigen, and similarly treated cultures of normal red blood cells (RBC) were used as control antigen and prepared as described earlier [253]. Alternatively, the parasite cultures were synchronised by treatment with 5% D-sorbitol in distilled water as described [150]. The cultures were adjusted to 1% parasitemia of early parasite stages in 2% haematocrit and used in parasite reinvasion inhibition assays.

Isolation of peripheral blood mononuclear cells (PBMC) (study I, II, III and IV)

Human PBMC were obtained from healthy donors with or without a previous history of malaria infections. PBMC were separated by Ficoll-Hypaque density gradient centrifugation according to the manufacturers' instructions. Washed PBMC were resuspended in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2mM glutamine, 10mM HEPES and 50 μg/ml gentamycin (TCM).

Activation of PBMC (study I)

PBMC were plated into 24-well plates at a concentration of 1×10^6/ml together with 30 μg/ml IPP for two weeks with addition of 20 U/ml of rhIL-2 every three days. At day 14, cells were washed twice in TCM, counted, phenotyped by immunoflow cytometry and used as effector cells in the parasite reinvasion/growth inhibition assays. $CD4^+$- and $CD8^+$T cells, were purified from PBMC, and were further expanded by stimulation with 10 μg/ml PHA for one week and two weeks for $CD4^+$ and $CD8^+$ T cells, respectively. Selection for $CD4^+$- and $CD8^+$ T cells was then carried out by Dynabeads according to the manufacturer's instructions. Further expansion of $CD4^+$ and $CD8^+$ was then continued by IL-2 (20 U/ml). $CD4^+$, $CD8^+$ and γδT cells were used as effector cells three days following the last stimulation with the IL-2.

***In vitro* parasite reinvasion/growth inhibition assay (study I)**

The parasite reinvasion/growth inhibition *in vitro* assay was performed as described by Wåhlin *et al* [162]. Briefly, 100 µl of the *P. falciparum* cultures diluted to 2% haematocrit in P-TCM were cocultured with 100 µl P-TCM and the different effector cells (γδ, $CD4^+$ and $CD8^+$ T cells) at ratio of 2:1 (effector cells: iRBC). In some experiments, γδT cells, intact, degranulated or preincubated with antibodies to $\delta1^+$, $\delta2^+$ or pan-$\gamma\delta^+$ TCR for 4 hours, were used in the assays. In other assays, γδT cells were cocultured with the parasite in the presence of varying concentrations of anti-granulysin antibodies. The plates were then incubated at 37°C for 36-42 hours in a candle jar [252]. After incubation, duplicates of parasite and effector cell mixtures were harvested and washed in Tris-Hank's solution (TH) and finally diluted to 1% haematocrit by TH. Eight-well multitest slides were incubated with a coating buffer for 30 minutes and then washed in TH. Monolayers were made in duplicates and incubated for another 30 minutes, washed in PBS, fixed in 1% glutaraldehyde in PBS and washed with water and air dried. Slides were stained by acridine orange before analysed in a fluorescence-microscope using 100 × magnification. Parasitemias were calculated from a total of 40,000 RBCs/culture, and the % invasion/growth inhibition of the parasite was calculated according to the following equation: % parasite invasion/growth inhibition = (% parasitemia in control - % parasitemia in test)/(% parasitemia in control) × 100.

Assay of cell contact requirement (study I)

To study cell contact requirement, parasite reinvasion inhibition assays were performed using transwell plates. Briefly, 600 µl of the parasite cultures were added to the lower compartment and 200 µl of γδT cells at 5×10^6 were added to the upper compartment of the transwell plates. The two compartments were separated by 0.4 µm pore sized semipermeable membrane. The plates were then incubated at 37°C for 36-42 hours in a candle jar and percentages of parasite growth inhibitions were calculated as stated above.

Total RNA extraction and real-time quantitative RT-PCR (study I)

Lysates from IPP-expanded $CD4^+$- and $CD8^+$ T cell lines were used to extract total RNA by the acid guanidinium thiocyanate-phenol-chloroform method [254]. Briefly, first strand cDNA copies were made from 1 µg of total RNA using random hexamers and murine leukaemia virus reverse transcriptase. Reverse transcription was performed at 42^o C for 15 min followed by denaturation at 99^o C for 5 min. PMA/ionomycin stimulated PBMC

from healthy donors and freshly isolated decidual mononuclear cells [255] were used as a positive control. The real-time quantitative (RT-PCR) was performed as previously described [256, 257]. The values of the cytolytic molecules expressed in the different T cell lines were presented as quantities related to the cytolytic molecule expression (=1) in the $CD4^+$ T cells and presented as an *n*-fold difference.

Study population (study II and III)

Mali

Four villages in Mali in Mopti area, only few kilometers apart and populated by the sympatric ethnic tribes, the Fulani and Dogon, were identified for the study. Malaria transmission is mesoendemic in the area with *P. falciparum* as the main parasite species. The dry season extends from October to May and the rainy season from July to October. Entomological inoculation rate (EIR) is similar in both tribes [258]. Thirty six and 47 asymptomatic individuals of the Fulani and Dogon, respectively, participated in the study. During the end of the transmission season, PBMC and plasma samples were obtained from venous blood collected into EDTA-treated tubes. Fifty µl whole blood were collected in filter papers for genotyping of parasite DNA.

Burkina Faso

The study area included villages in the vicinity of Ouagadougou, the capital of Burkina Faso. The rainy season in the area lasts from June to October, and corresponds to high transmission period. The area is populated by the sympatric ethnic tribes, the Mossi and the Fulani. The EIR is similar between these tribes. PBMC and plasma samples were obtained from venous blood collected from 92 and 88 Fulani and Mossi, respectively, during the peak transmission period.

DNA extraction and PCR of *msp-2* (study II)

Parasite DNA was extracted from the blood collected in filter papers using a fast methanol-based DNA extraction as described earlier [259]. Briefly, 3 × 5 mm cuts of the filter papers corresponding to 20 µl blood were placed into micro centrifuge tubes containing 125 µl methanol for 15 minutes at RT. Methanol was removed and the filter papers were dried and 75 µl distilled sterile water were added and incubated at 95-100°C for 15 minutes. Thereafter, DNA samples were stored at -20°C until used in the PCR. The polymorphic regions of block

3 of msp2 were amplified by nested PCR specifically targeting the two allelic types of *msp-2* block 3 denoted, Indochina (IC) and FC27 using nested PCR as described earlier [260]. The PCR products were visualized by UV transillumination following electrophoresis on 2% MetaPhor agarose gels and staining with ethidium bromide. The total number of alleles per sample determines number of concurrent clones.

ELISA (study II and III)

P. falciparum specific IgG and IgE and total IgE antibodies were determined using ELISA as previously described [240]. Briefly, ELISA plates were coated with 50 µl of crude parasite antigens (10 µg/ml). The coated plates were incubated at 4°C overnight. Sera were diluted 1:50 for determination of *P. falciparum* IgE antibodies and 1:1000 for *P. falciparum* IgG and total IgE antibodies. The sera were then added in duplicates into the plates and incubated for 1 hour at 37°C for anti-malarial IgG and overnight for anti-malarial IgE determination, respectively. Bound IgE and *P. falciparum* specific IgG antibodies were assayed by ALP-conjugated goat anti-human IgE or IgG antibodies. For total IgE detection, ELISA plates were coated with affinity purified goat anti-human IgE. Total IgE were detected with biotinylated goat anti-human IgE. The concentrations were calculated from standard curves obtained by incubating serial dilutions of a purified standard of human IgE or human IgG antibodies using a kinetic micro plate reader.

Antibodies to the endotoxin-free recombinant PstS-1 protein of *M. tuberculosis*, crude measles antigen and IgG subclasses were determined by the same ELISA procedure as above.

Alternatively, ELISA kits (DSL, Texas, USA) precoated with purified and inactivated Rubella, *Toxoplasma gondii* or *Helicobacter pylori* antigen were used to determine levels of IgG antibodies against these antigens. The ELISA assays were performed according to the manufacturer's instructions. Briefly, 100 µl/well of the plasma samples diluted 1:100 in an assay buffer were added to the ELISA plates and incubated for 45 minutes at 37°C. The well were washed and treated with the peroxidase-conjugated anti-human IgG monoclonal antibodies and incubated for 45 minutes at 37°C. After washing a substrate (Tetramethylbenzidine) was added and incubated for 15 minutes at RT. The enzymatic turnover of the substrate was determined by absorbance at 450 using the ELISA plate reader.

Immunodiffusion assay (study III)

The concentrations of total IgG and IgM in the plasma samples were determined using immunodiffusion plates according to manufacturer's instructions. Briefly, 20 µl/well of the diluted plasma samples (1:500 for IgG and 1:50 for IgM) were added to the plates and allowed to diffuse at RT. After the end of the diffusion period, diameters of the formed precipitin rings were measured. Concentrations of IgG and IgM were determined from a standard curve (Abscissa: antigen concentration, ordinate: mm^2).

ELISPOT (study 11)

Precoated and dried ELISPOT plates with anti-human IL-4, IFN-γ, IL-10 and IL-12 antibodies were used for enumeration of cytokine producing cells. Prior to experiments, the precoated plates were incubated for 1 hour at 37°C in TCM medium. After aspirating the media, 100 µl/well of unstimulated 200.000 cells/well for IL-4 and 20.000/well for IFN-γ, IL-10 and IL-12 were added to in triplicates. Cells were either unstimulated or stimulated with parasite antigen (10 µg/ml), RBCs (10 µg/ml) and the plates were then incubated for 40 – 42 hours at 37°C in 5% CO_2 atmosphere. The plates were then washed 6 times with sterile filtered phosphate buffered saline (PBS) to remove the cells away. Thereafter, 100 µl of the biotinylated MoAbs at 1 µg/ml were added per well and incubated for 2 hours at RT. After a series of washes, 100 µl of streptavidin alkaline phosphatase diluted 1:1000 were added per well and incubated for 90 min at RT. Unbound conjugates were removed by another series of washings and finally 100 µl color developing buffer were added and incubated at RT, until dark spots emerged. The colour development was stopped by adding tap water. The number of spots was enumerated using an automatic AID reader.

Enrichment of different developmental blood stages of *P. falciparum* parasites (study IV)

Parasite cultures of the laboratory F32 strain or the N40 isolate were allowed to grow and parasitemias were adjusted to different levels. Four mL of the cultures composed of ring (R), trophozoite (T) and schizont (S) stages were then collected in 5 ml sterile tubes and centrifuged at 500 × g for 1 minute at RT three times using the DiaCent centrifuge. Supernatants were collected into separate 10-mL tubes and the resulting pellets were resuspended in MCM and hematocrits were adjusted to 5%. The erythrocytes in the supernatants were concentrated by centrifugation at 500 × g, for 1 minute, RT at low break and were then resuspended in MCM to 5% hematocrit. The resulting parasite fractions as well

as the original parasite cultures were then stained either with acridine orange or with 5% Giemsa and the percentage of the different parasite stages (rings, trophozoites and schizonts) in the parasite culture fractions were determined by normal light microscopy.

Proliferation assay (study IV)

PBMCs obtained from 5 malaria-exposed or non-exposed donors were cultured in triplicate at 1 x 10^5 cells/well in 96-well flat-bottomed microtiter plates in a volume of 100 µl of TCM. The cells were stimulated with 100 µl of appropriate antigens (30 µg/ ml isopentenyl pyrophosphate (IPP), 10^5 schizonts/ml, 10^5/ml early stages (rings), F32-S, F32-IS, and 10^5 un-infected RBC). Un-stimulated cultures were negative controls and those stimulated with phytohemmagglutinin (PHA) served as positive controls. Cell culture plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. On day five of incubation all plates were pulsed with ^{3}H-thymidine (1 µCi/well) and incubated for 16–18 hours. Cells were harvested onto filter mats using a cell harvester (Tomtec), and radioactive uptake was measured using a microbeta counter. Proliferative responses were expressed as stimulation indices (SI).

Flow cytometry (study I and IV)

Purified PBMC were plated in triplicates at 1×10^5/well into 96 V-shaped microtiter plates and incubated with the appropriate antigens as described in the proliferation assay above. For characterization of cells from differently malaria-exposed donors, 10^5 of the unstimulated or antigen-stimulated cells were suspended in PBS supplemented with 0.2% FCS, plated in V-shaped microtiter plates and incubated with predetermined concentrations of anti-CD3-PE, anti-CD4, anti-CD8 or anti-TCR δ2-FITC antibodies. The cells were incubated on ice in darkness for 30 minutes and washed three times in PBS. Analysis of cells was performed with a FACScan flow cytometer. Ten thousands viable cells were acquired within each population and the results were analysed using CellQuest software.

Ethical approval

Informed consent was obtained from all individuals or their guardians included in study II and III, and human subjects guidelines for the institutional Ethical committee of the Faculty of Medicine and Pharmacy of Mali, Ethical committee of Karolinska Institute, Sweden and The Ministry of Health of Burkina Faso were strictly followed.

RESULTS AND DISCUSSION

γδT cells-mediated growth inhibition/killing of *P. falciparum* parasite requires granulysin (study I)

Several reports have shown the expansion of γδT cells in acutely malaria-infected individuals [144, 146-148]. The predominating *P. falciparum*-responding γδT cells belong to the subset that expresses Vγ9/Vδ2 heterodimer [261]. Such expansion suggests role played by these cells in the host defense against malaria [150]. However, little information of mechanisms mediated by this cell subset is available.

The aim of this study was to investigate the effector mechanisms responsible for the observed parasite growth inhibition by human γδT cells. The Vγ9/Vδ2$^+$ γδT cells represent only a small fraction of the peripheral T lymphocytes. We have set up an *in vitro* culture system to enrich and expand for this scarce population using IPP. In line with previous findings [262], we found a preferential expansion of Vγ9/Vδ2$^+$ γδT cells. Earlier findings revealed that γδT cells inhibit the *in vitro* growth of the asexual blood stages of the malaria parasite which required cell-to-cell contact, suggesting TCR involvement in this inhibition [147, 150]. The present study supported these findings by using a transwell system. Further analysis of the active cells revealed the involvement of δ2$^+$ TCR in the *in vitro* parasite growth inhibition, because anti-δ2 and -pan-γδ TCR chain antibodies abrogated this inhibition. The study also demonstrated that this inhibition required intact granules, suggesting an effector role of the granule in the parasite inhibitory process. Both induction and blocking of degranulation experiments abrogated γδT cell-mediated inhibition of the *P. falciparum* parasites. The correlation of granulysin expression in γδ, CD4$^+$, and CD8$^+$ T cells, with their capacity in inhibiting the *in vitro* parasite growth, led us to further test if this molecule was directly involved in this inhibition. Antibodies directed against granulysin indicated its involvement in the process of *in vitro* parasite inhibition. This granulysin-mediated inhibition of the parasite growth required the presence of perforin as shown by degranulation blocking experiments. These findings are in line with ability of a similar γδT cell subset to killing *M. tuberculosis* by a granulysin-exocytosis dependent cytotoxic pathway [60, 263]. This is in agreement with our previous finding that γδT cells exert their inhibitory effect on the parasite at the time of reinvasion [150]. Phosphoantigens such as IPP synthesized by the parasite [152] and a recently reported soluble products which are released upon

shizonts rupture [143] may synergistically act in activating γδT cells, thus leading to induction of their inhibitory machinery against the parasite.

Taken together, this study reports a novel mechanism by which γδT cells may contribute in reducing the acute parasitemias that occur early in malaria infection. Thus, γδT cells may play an important role in the host defense against *P. falciparum* infection.

Antibody levels and Th1/Th2 cytokine profiles in response to malaria in sympatric ethnic tribes of Mali (study II)

It has earlier been well-established that the Fulani individuals of Burkina Faso were relatively resistant to clinical malaria, had higher antibody levels to malaria antigens and had less parasite rates as compared to an other ethnic group, the Mossi, despite similar entomological inoculation rates (EIR) [264-267]. The aims of this study (study II) were to investigate whether such distinct interethnic differences could also be seen in Mali (Fulani/Dogon), and to examine if the Fulani of Mali were more polarized towards Th2 as reflected by higher numbers of IL-4 and IL-10-producing cells and lower numbers of IFN-γ and IL-12-producing cells as compared to the other ethnic tribe, the Dogon. The results of this study revealed higher *P. falciparum*-specific IgG and IgE antibodies in the Fulani as compared to the Dogon, and in line with earlier findings reported in Burkina Faso [265]. The IgE antibodies against malaria antigens have been shown to be associated with malaria pathology [241, 268, 269]. This in contrast to what was seen in the present study where significantly elevated levels of malaria-specific IgE were found in the asymptomatic individuals of the Fulani, and thus suggesting a protective role of the anti-malarial IgE antibodies. This is in line with a previous finding in Tanzania where asymptomatic individuals with elevated levels of anti-malaria IgE were at lower risk in developing clinical malaria [242].

Our study also showed higher spleen rates in the Fulani compared to the Dogon, concurring with an earlier study in the same population in Mali [258]. Such high spleen rates may reflect a hyper-responsive immune system (both humoral and cellular) in the Fulani. Elevated levels of IgG and IgE are usually the hallmark of Th2-mediated responses. Conversely, the present study revealed that the Fulani are not polarized toward Th2 type of response, but instead they had higher numbers of IL-4 and IFN-γ-producing cells as compared to their neighbours, the Dogon. IFN-γ induces cytophilic anti-malarial antibodies (IgG1 and IgG3), which are important in antibody-dependent cellular inhibition of the malaria parasite

[166]. The simultaneous production of both IL-4 and IFN-γ is a characteristic of CD1-restricted NKT cells upon stimulation of their TCR [95-97]. These cells have been shown in a murine model to contribute to the splenomegaly associated with malaria and to help B cells to produce malaria-specific antibodies [138]. Thus, the simultaneous production of both IL-4 and IFN-γ, elevated malaria-specific antibody levels and higher spleen rates, suggest a similar mechanism in the Fulani. A possible polymorphism in the CD1 genes [270, 271] may explain these distinctive responses between the Fulani and the Dogon.

In conclusion, the findings of elevated malaria-specific IgG and IgE antibody levels and the elevated numbers of both IL-4 and IFN-γ-producing cells, may explain the lower parasite persistence in the Fulani. In addition, our findings may also explain the lower susceptibility to clinical malaria and indicate a hyper-responsive immune system in the Fulani and not in the Dogon.

Malaria-specific IgG class/subclass, IgM antibodies and the non-malarial antigen-specific IgG responses in the Fulani and non-Fulani of Mali and Burkina Faso (study III)

High levels of *P. falciparum*-specific IgG have been shown to be poor predictors of protection to malaria [234]. However, several studies in residents of malaria endemic areas indicated an association between the cytophilic IgG subclasses, IgG1 and IgG3, and protection against malaria [238, 272, 273]. Accordingly, the humoral immune responses seen in Mali were further dissected in this study. The aims of this study were to compare levels of malaria antigen-specific IgG, IgM, and IgG subclasses (IgG1-4) in the Fulani/Mossi and Fulani/Dogon in Burkina Faso and Mali, respectively. Furthermore, total IgG and IgM and IgG antibody levels against a panel of different viral and bacterial antigens were analysed to see whether the hyper-responsiveness in the Fulani is restricted to malaria or not. The results revealed that besides malaria-specific IgG, the Fulani had higher IgM antibody levels as compared to the non-Fulani in both countries. Similarly, higher levels of malaria-specific IgG1, IgG3 were seen in the Fulani as compared to Mossi and Dogon in Burkina Faso and Mali, respectively. While no differences were observed in the levels of total IgG, significantly higher levels of total IgM were observed in the Fulani as compared to the non-Fulani. While the IgG against some non-malarial antigens showed consistently higher levels in the Fulani residing in both countries, no such responses were seen for the other non-malaria antigens tested.

The elevated levels of both *P. falciparum*-specific IgG and IgM antibodies in the Fulani as compared to the non-Fulani in both countries, suggest a role of these antibodies in the lower susceptibility to malaria seen in the Fulani. This is in line with the role of malaria-specific IgG and IgM in the defense against malaria [233, 274]. The fact that long lasting memory IgM^+ B lymphocytes can persist after malaria transmission seasons [243, 274], may explain the consistently elevated levels of this antibody class in the Fulani. Possible interethnic differences in such persistence of these lymphocytes may exist which need further investigations. The fact of persistence of IgM secreting cells, is supported not only by the finding in our study of higher malaria-specific IgM, but also by consistently higher levels of total IgM in the Fulani of both countries as compared to the non-Fulani.

The elevated levels of the cytophilic IgG1 and IgG3 in the Fulani of both countries, indicate that these antibodies may contribute in the lower susceptibility to malaria seen in this tribe. This is in line with the findings reported by others, that IgG1 and IgG3 were found to predominate in malaria-protected individuals as compared to non-protected ones [235]. In African immune adults, the defense mechanism mediated by IgG1 and IgG3 has been proposed to require the binding of these antibodies to Fc receptors on monocytes, thus leading to antibody-dependent cellular inhibition of parasite replication [235, 239]. The IgG subclass responses against different malarial antigens including crude *P. falciparum* antigens in people living in exposed areas are, at least partly, determined by host genetic factors and are age dependent [275]. Therefore, it is plausible that the higher responses of the cytophilic antibodies against malaria could be attributed to distinct regulatory genetic factors in the Fulani, but not in the non-Fulani.

For the non-malarial antigens our results revealed that the Fulani had consistently higher IgG levels against measles and *T. gondii* antigens compared to the non-Fulani in both countries. Significantly different levels of IgG against *M. tuberculosis* (PstS-1) antigens were only seen in Mali where the Fulani exhibited higher levels as compared to the Dogon. No significant interethnic differences in the levels of IgG antibodies against Rubella, *H. pylori* were observed in either country, and against PstS-1 antigen in Burkina Faso. These findings suggest that the Fulani are not generally hyper-responsive to other pathogens but also that the higher IgG responses are not exclusively specific for malaria. The higher IgG responses to the mycobacterial antigens in the Fulani of Mali may indicate higher prevalence of this disease in the Fulani individuals or they are more frequently vaccinated as compared to the Dogon tribes. The consistent responses to measles and *T. gondii* in the Fulani may be explained by antigens signalling through TLR9 [141, 276, 277] a possible cross-reacting with

soluble schizont antigenic structures of the parasite and that also has been shown to signal through TLR9 [143]. Thus, a polymorphism in TLR9 [278] may be responsible for the differences in responses to malaria seen between these sympatric tribes.

In conclusion, this paper reports findings that are in concordance with previous published data with regard to the stronger IgG responses in the Fulani who show relative resistance to clinical malaria. In addition, the present study further dissected the IgG responses in this tribe and suggested an association of the IgG subclasses, IgG1 and IgG3, with protection against malaria. Furthermore, this study suggested a protective role of IgM antibodies.

Enrichment for blood stages of *P. falciparum* cultures to analyse T-cell responses (study IV)

Enrichment of the different developmental blood stages of malaria parasite is important for studies where there is a need for *in vitro* systems aiming at understanding the immune responses to the parasite. Isolation methods for blood stages malaria parasites have been described in several studies where most of the methods described used certain chemicals or drugs to enrich for the different stages. These methods used one of the following drugs or chemicals; colchicines [279], aphidicolin [280], DL-alpha-difluoromethylornithine [281], and sorbitol [282]. Only a few studies used physical procedures [283, 284]. The majority of these methods enrich or synchronize for a single developmental stage. The aim of this study was to simultaneously enrich for early and late blood stages of the *P. falciparum* parasite using a simple physical method. In addition was to use such enriched fraction to analyse T-cell responses in malaria exposed and non-exposed donors.

A simple 3-step centrifugation method was developed that yields two fractions, supernatant and pellet, which consist of late and early blood stages of *P. falciparum* parasite, respectively, from a single parasite culture. The advantage of this method as compared to most of the others methods, is that it is a chemical- and drug-free procedure. Such chemicals or drugs may interfere with the antigens of the specific blood stages or may interfere with the normal *in vitro* growth of the parasite. The magnetic enriching method is one of the few chemical free procedures and efficient in enriching for blood stage parasite, but being expensive, which may limit its use in some laboratories. The present procedure was applied on a new adapted isolate obtained from Burkina Faso, N40, and it proves its effectiveness in enrichment of both early and late blood stages from this culture. Thus, the method is applicable on newly adapted parasite isolates as well as established cultures. In this study, the

proliferative responses and expansion of different cell from malaria exposed and non-exposed donors were investigated and compared in response to the whole-cell malaria parasite antigens enriched by this method and acellular parasite antigens as well as to a phophoantigen, IPP. In concordance with earlier findings [245-249, 253], all antigens tested induced proliferation and expansion of cells from both group of donors. While there was a tendency of higher proliferative responses of lymphocytes from malaria non-exposed than from exposed donors to IPP, F32-S and F32-IS, lymphocytes from malaria-exposed donors showed higher proliferative activity in response to whole-cell parasite antigens. This indicates that whole-cell parasites and the acellular parasite fraction presented different antigens and that natural infection preferentially results in formation of memory cells against live-parasite antigens. Therefore, this suggests that such responses to intact live parasites may differentiate mitogenic from specific responses in such group of donors. The schizont fraction induced higher proliferative response in both group as compared to the ring-stages parasites, in line with the fact that schizonts express more parasite antigens than ring stages. This further supported the efficiency of this method in enriching for these asexual blood stages of the parasite. Phenotypic characterization of the responding cells in both group of donors, revealed higher $CD4^+/CD8^+$ T-cell ratios in the malaria exposed donors than the non-exposed ones in respose to live parasite antigens, whereas γδ T cell showed similar expansion in both groups. These findings suggest that presence of memory $CD4^+$ T cells in malaria-exposed individuals can be detected by whole-cell parasite antigens and not by acellular parasite antigens. This is in contrast to earlier findings that showed crude parasite extracts (acellular antigens) lead exclusively to expansion of $CD4^+$T cells in PBMC from naïve donors [201, 285-287]. The similar expansion of γδT cells in both groups may reflect the non-specificity of the phosphoantigen components in all antigens tested [151, 187].

Taken together, the present study describes, a simple, cost-effective enrichment method suitable for application for newly adapted parasite isolates and also in the fields where such simple methods can be affordable. The use of the enriched parasite antigen fractions, revealed that memory $CD4^+$ T cells can be detected by stimulation of PBMC from malaria exposed individuals by whole-cell parasite antigens rather than the use of acellular parasite antigens in such stimulation.

CONCLUDING REMARKS

In conclusion, our results revealed that IPP-expanded human Vγ9/Vδ2$^+$ γδT cells inhibit the *in vitro* parasite growth. This inhibition required cell-cell contact and δ2$^+$ TCR involvement. The correlation of perforin and granulysin in the T cell lines tested with their ability to inhibit the *in vitro* parasite growth, suggests a role of these molecules in the parasite inhibition. Anti-granulysin antibodies abrogated the *in vitro* parasite growth mediated by γδT cells, suggesting a role of granulysin-exocytosis dependent cytotoxic pathway in such inhibition. The exact nature of the parasite antigens targeted by γδT cells are not known. Thus future studies should aim at elucidating the nature of such antigens. The findings may reflect a potential role of granulysin in reducing the initial surge of parasitemias seen in malaria infection, which may have a therapeutic implication in malaria.

The Fulani had lower parasite rates, higher anti-malarial antibody levels (both IgG and IgE) and fewer episodes of clinical malaria, despite similar entomological inoculation rates. However, little or no data on cellular-mediated responses, IgG subclasses or other antibody classes than IgG and IgE are available in such settings. In this study, we investigated both humoral and cellular immune responses in the Fulani/Dogon in Mali. The results revealed that the Fulani had elevated levels of both IgG and IgE antibodies as well as higher spleen rates as compared to the Dogon. The Fulani exhibited higher numbers of both IL-4 and IFN-γ. The association of such intense IL-4 and IFN-γ production with higher spleen rates in the Fulani, suggests a role of CD1-restricted NKT in the different immune responses to malaria seen between the Fulani and the Dogon. Therefore, this study highlights a new gene candidate such as the CD1 gene as a target for future studies in these tribes.

The distribution of the IgG subclasses were investigated and compared in the Fulani/Mossi and Fulani/Dogon in Burkina Faso and Mali, respectively. In addition, total and specific IgG and IgM were also investigated in these tribes. Furthermore, the IgG responses to non-malarial antigens were also investigated in these tribes. The finding that the Fulani had significantly elevated levels of anti-malaria IgG1, IgG3 and IgM antibodies as compared to non-Fulani, suggests a protective role of these antibodies against malaria. The findings that malaria-specific IgM levels were significantly elevated in the Fulani and not in the non-Fulani of both countries, strongly support the hypothesis that surface IgM$^+$ B lymphocytes with long-lasting memory can persist after malaria transmission season. Thus, identification of molecules or factors that favour the dominance of cytophilic IgG subclasses or those that lead

to persistence of long-lasting IgM^+ B lymphocytes may have important implications on vaccine development against malaria. Our finding that the IgG responses to some of the non-malarial antigens were not consistently higher in the Fulani than the non-Fulani indicates that the Fulani are not generally hyper-responsive to infecting pathogens. This conclusion may be supported by exploring the IgG subclasses to non-malarial antigens. The IgG responses to measles and *T. gondii* antigens were, however, consistently higher in the Fulani than the non-Fulani. This could be due to the fact that CpG components of these viral antigens and a soluble fraction of the late stage of *P. falciparum* parasite can both activate certain subset of dendritic cells through TLR9. Therefore, this study suggests that a polymorphism in TLR9 may be involved in the distinct immune responses to malaria seen between the Fulani and the non-Fulani tribes. This hypothesis should be addressed in future studies.

This study, reports on a new simple method where early and late asexual blood stages of *P. faciparum* parasite cultures can simultaneously be separated in a good yield. The enriched parasites can immediately be used for different experimental purposes, without the need to add any other chemical or drugs. Employment of whole-cell parasites in *in vitro* systems aiming at investigating differential immune responses to malaria parasite, has not been frequently used. In this study, stimulation of PBMC with such enriched whole-cell parasite fractions, but not acellular parasite antigens, resulted in significant lymphocyte proliferation as well as expansion of $CD4^+$ T cell in the malaria exposed donors as compared to the non-exposed ones. Thses findings imply that naturally-occurring malaria infections preferentially result in formation of memory cells against antigen presented by live parasites, suggesting the importance of including whole-cell parasites in analyses of malaria immune responses. Thus, this study indicates that the whole-cell parasites exert different lymphocyte activation as compared to the acellular antigen preparations. Such difference between the whole-cell fractions and the acellular-parasite antigens in the *in vitro* activation of lymphocyte from individuals differently exposed to malaria, need further investigation.

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