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PROTECTIVE IMMUNE RESPONSE IN *PLASMODIUM FALCIPARUM* MALARIA - A MOLECULAR IMMUNOEPIDEMIOLOGICAL STUDY

A THESIS SUBMITTED IN PART FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

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May 2011

Dedicated to my loving parents

Dorendra Lourembam

&

Chandralekha Chingangbam

ABSTRACT

Malaria is a major health problem with the disease burden mostly borne by economically productive ages. Recent studies indicate the disease is becoming more widespread in south east Asia with 10 of the 11 countries endemic for malaria and with 1.2 billion people at risk of exposure to the disease. India reported the highest malaria confirmed (1563344) and death (1133) cases in 2009 from this region (WHO). The north-eastern region of India (population 28.5 million) is highly endemic for malaria and has been declared as a 'high risk zone' by National Anti Malaria Program. Assam contributes to 64.7% of the malaria positive cases in the north-eastern region of which *P. falciparum* accounts for 58-75% of the cases and the remainder are due to *Plasmodium vivax.*

Though people living in endemic area develop immunity, which can be either anti disease or anti infection immunity, the factors delineating them are not clear. It is thus felt that an in depth understanding of the immune response leading to anti infection or to anti disease responses in population genetic studies and elucidation of parasite diversity is required for success of malaria control programs. The present study was designed against this perspective with the following objectives:

1) To study the clone multiplicity of the parasite genotypes existing in the study area
2) To elucidate the protective humoral immune response.
3) To elucidate the protective cell mediated immune response.

The study was conducted at Guabari village of Baksa district and Kondoli Tea Estate (KTE) of Nagaon district which are mesoendemic for malaria. The two sites differ in population demography and accessibility to health care.

Plasmodium falciparum diversity was assessed by typing polymorphic block 2 region of Merozoite Surface Protein 1(MSP-1) gene using primary as well as nested PCR cycles. A high degree of polymorphism in isolates from the two study sites was seen with 33 alleles of MSP-1 seen in Guabari village and 18

alleles at KTE. The mean number of alleles per isolate was however similar at the two sites. The Multiplicity of infection (MOI) was greater in high transmission season but was not dependent on age. The temporal variation in allelic composition and proportion of clones with an allelic family differed among the study sites.

Evaluation of antibody response against MSP-1_{19} variants and whole merozoite extract (WME) showed a relatively lower seropositivity to MSP1_{19} peptides derived from 3D7 and FVO strains in comparison to whole merozoite extract of local *P. falciparum* strain. Recognition of Q-KNG variant was markedly lower in TB ($p<0.0001$) indicating a role of ethnicity. The Tea tribes of Austro-Asiatic affinity had higher antibody response (E-TSR; $p = 0.038$ & Q-KNG; $p = 0.004$) and equally recognised the two variants. A reduced risk of clinical infection in high transmission summer season was seen in presence of anti MSP-1_{19} antibodies ($p = 0.013$) and antibody level was predictive of risk of clinical malaria (ROC=0.729). Anti E-TSR antibodies were inversely related to disease severity at KTE ($\lambda^2 p = 0.013$; t test $p = 0.032$).

As cytokines are postulated to play an important role both in malaria pathology and protection, gene expression of cytokines IL-2, IL-4, IL-10, IL-12, TGF-β and IFN-γ was analysed in relation to malaria symptomology. IL-10 levels were down regulated and TGF-β expression was higher in *Plasmodium falciparum* infected individuals in comparison to *P. falciparum* negative individuals. IL12α was seen to be negatively associated while IFN-γ was positively with complicated cases, indicating that high levels of proinflammatory cytokines predisposed to complicated malaria. The role of IL-12α, the cytokine that activates T and NK cells T and results in heightened Th1 and CTL response suggest a role for NK cell activation in protection from malaria.

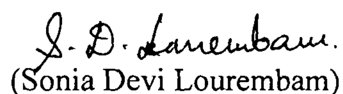
Based on the observation of strong correlation of humoral immune response with ethnic background of the population and cytokine analysis that suggested possible involvement of NK cells, we analysed the polymorphism of KIR genes and association with malaria in the Tibeto-Burman, Tea tribes and

Indo European speaker groups of north east India. Individuals were typed for 14 KIR genes and the two pseudo genes using sequence specific PCR protocol. Considerable heterogeneity in KIR gene frequencies and KIR genotypes was seen among the three ethnic groups that were reminiscent of their genetic background. KIR3DL1 frequency was positively associated with malaria severity (Pearson phi, R^2 = 0.297 p=0.006) and logistic regression modelling predicted KIR3DL1 as a risk factor in complicated malaria [Odds Ratio (95% C.I)] = [6.39 (1.34-30.60)]. An interaction between ethnicity and KIR3DL1 was also seen where higher proportion of KIR3DL1 positive and complicated malaria patients belonged to Tea tribes (p = 0.009). Notably, four activating genes protected from frequent malaria (p= 0.02) while six activating genes enhanced the risk of complicated malaria (p=0.05). Combination of KIR2DS4, KIR2DS4del, KIR2DS5 negatively influenced disease outcome in Tea tribes (p = 0.048) but not in Tibeto-Burman.

In conclusion, the high genetic diversity of circulating strains of *Plasmodium falciparum* in the study sites could be the reason for poor antibody response which was otherwise seen to be protective. Humoral response to different variants was seen to depend on genetic background of an individual. KIR3DL1/3DL1homozygosity predisposing to complicated malaria coupled with role of cytokine gene IL-12 in protection from complicated malaria indicates a role for NKT and NK cells in malaria immunopathology.

DECLARATION

I hereby declare that the thesis entitled "Protective immune response in *Plasmodium falciparum* malaria – A molecular immunoepidemiological study" being submitted to the department of Molecular Biology and Biotechnology, Tezpur University, is a record of original research work carried out by me. Any text, figures, methods or results that are not of own devising are appropriately referenced in order to give credit to the original author(s). All sources of assistance have been duly acknowledged. I also declare that the work has not been submitted to any other University or Institute for award of any other degree.



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This is to certify that the thesis entitled "Protective immune response in *Plasmodium falciparum* malaria – A molecular immunoepidemiological study" submitted to the School of Science and Technology, Tezpur University in part fulfilment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology is a record of research work carried out by Ms. Sonia Devi Lourembam under my supervision and guidance.

All help received by her from various sources has been duly acknowledged.

No part of this thesis has been submitted elsewhere for award of any other degree.

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ACKNOWLEDGEMENT

I am overwhelmingly indebted to several people who assisted, supported and encouraged me during the course of my study and without making a mention of them my work would be deemed incomplete.

First of all, my experimentation was made possible due to the visionary leadership of Prof. M. K. Chaudhuri, Vice-Chancellor, Tezpur University and the able supervision of Dr. (Mrs) S. Baruah, Dept. of MBBT, Tezpur University. Dr Baruah is instrumental from strategizing the study direction to persevering into its accomplishment. All the field trips, data collection, travel and accommodation were possible through her initiatives. It is to her credit that the data fittingly collated into a complete piece of work. Besides expressing my gratitude, I would like to say that no words will suffice to describe her contribution in completion of this thesis.

I would also like to make a mention of my deep appreciation to the HOD, Dept. of MBBT, Tezpur University, for all the facilities made available for my work and to Dr S.K. Ray, Dept. of MBBT, Tezpur University for rendering unconditional guidance in the form of suggestions and leads.

My experimentation would also not have shaped up into the desired status had it not been for the following personalities – Dr. Mainak Mazumdar (Applied Biosystem, Gurgaon) who assisted in real time PCR, Dr Raja Rajalingam (UCLA, USA) who never failed to give advices besides assisting in KIR typing, Dr P.K. Mahapatra (RMRC, Dibrugarh) who was more than willing to provide with the local parasite strain besides providing facilities for the parasite culture and Dr. Powdel (Lecturer, Darrang College, Tezpur) for his assistance in statistical analysis. I am also grateful to Malaria Research and Reference Reagent Resource Centre (MR4), Manassas, VA, USA, for providing the antigens.

Financial assistances by Defence Research Laboratory, Tezpur, Defence Research Development Organisation, Govt. of India, Jawaharlal Nehru Memorial

Trust, New Delhi and the University in the form of JRF/SRF and Scholarships were of immense help during the course of work.

Special acknowledgement to doctors of Primary Health Centres and Tea Gardens – Dr Kalita (PHC, Tamulpur), Dr Bora (PHC, Balipara), Dr Khound (Chief Medical Officer, Assam Tea Board), Dr Baishya (Tea Garden, Dimakuchi), Dr Ahmed (Tea Garden, Kondoli) and other medical staffs of the PHCs and the Tea Gardens, for exhibiting tireless enthusiasm during the sample collection. Also, special gratitude to Ms. Clara Sawain (Tezpur University) and Diganta Goswami (Defence Research Laboratory, Tezpur) for their constant support during field trips and experimentation.

Contribution of Bimla Basumatary, Sukumar Sarkar and Raju, Kondoli during data collection and slide examinations, were all memorable and valued.

Warm appreciation to department staffs and colleagues, Jetendra Kr Roy, Anusree Mahanta, Debashree, Ankita, Rituparna, Saswati K Deka, Anupam Dutta, Pradeep for their constant encouragement. Rajesh and Lucky your patience and trust in me are worth a lot. I would also like to thank all the study participants without whose cooperation, the study would not have happened.

I am also very much indebted to my family members who stood by me during the critical hours.

The outcome of my experiment is the manifestation of your assistance, support and encouragement. And, for that matter, I sincerely thank all of you.

I am also very thankful to God for all the blessings bestowed upon me in the form of your guidance and support.

Sonia Devi Lourembam

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LIST OF ABBREVIATIONS

WHO - World Health Organisation

SEAR – South East Asia Region

MSP – Merozoite Surface Proteins

RESA – Ring stage infected erythrocyte surface antigen

SERA – Serine Repeat Antigen

RBC – Red Blood Cell

CMI – Cell Mediated Immunity

IFN - Interferon

IL - Interleukin

NK – Natural Killer

NKR – Natural Killer Cell Receptor

NKC – Natural Killer Receptor Complex

KIR – Killer Immunoglobulin Receptors

HLA – Human Leukocyte Antigen

AMA – Apical Membrane Antigen

GLURP – Glutamate Rich Protein

IgG – Immunoglobulin

TNF – Tumor Necrosis Factor

TGF – Transforming Growth Factor

MHC – Major Histocompatibility Complex

KTE – Kondoli Tea Estate

TT – Tea Tribes

TB – Tibeto- Burman

IE – Indo – European

DNA – Deoxyribonucleic Acid

PCR – Polymerase Chain Reaction

TAE – Tris Acetate EDTA

ROC – Receiver operating characteristic

WME – Whole Merozoite Extract

EGF – Epidermal Growth Factor

HIV/AIDS – Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome

*Pf*EMP – *Plasmodium falciparum* Erythocyte membrane protein

CHAPTER I:

Introduction

1. INTRODUCTION

Malaria remains a major global health problem with 300 to 500 million clinical infections and more than a million deaths reported each year. In 2009, World Health Organization (WHO) reported 225 million malaria cases and 781000 deaths from 106 endemic countries. Majority of the cases were from African countries (78%) and the contribution from South East Asian and Mediterranean regions were 15% and 5% respectively. About 85% of the deaths caused by malaria were in children under 5 years of age[1]. It is commonly associated with poverty and is also an important cause of poverty slowing down economic growth by 1.3% per year in endemic areas. A 10% reduction in disease was associated with 0.3% higher growth[2]. In India, retrospective analysis of burden of malaria showed that disability adjusted life years lost due to it was 1.86 million years[3].

In the South East Asia Region (SEAR), approximately 70% of the total population is at risk of malaria, of which three countries, India (65%), Myanmar (20%) and Indonesia (12%) accounted for 94% of the reported cases[1]. 60.5% of all infection were caused by *Plasmodium falciparum*. Malaria is endemic in the Indian state of Assam contributing more than 5% of the total cases recorded in the country annually. Malarial outbreaks characterized by enhanced morbidity and mortality are common across the state[4]. The endemicity of malaria is not uniform across the state with many pockets along forest fringes, forest and foothill villages particularly along the inter-country/inter-state border vulnerable to malaria outbreaks[5]. These high risk zones are predominantly inhabited by indigenous tribal populations of distinct ethnic backgrounds. Most of these affected areas report widespread resistance to first line drugs like chloroquine, sulphodoxine-pyrimethamine and even quinine resistance is being reported. In fact, resistance to chloroquine was first reported from Karbi Anglong district of Assam in India[6].

Human malaria is caused by four distinct species of *Plasmodium* viz *P. falciparum, P. ovale, P. malariae* and *P. vivax.* The malarial parasite has a

complex life cycle (Fig 1.1) involving both female anopheline mosquito (sexual phase) and human (asexual phase) as host.

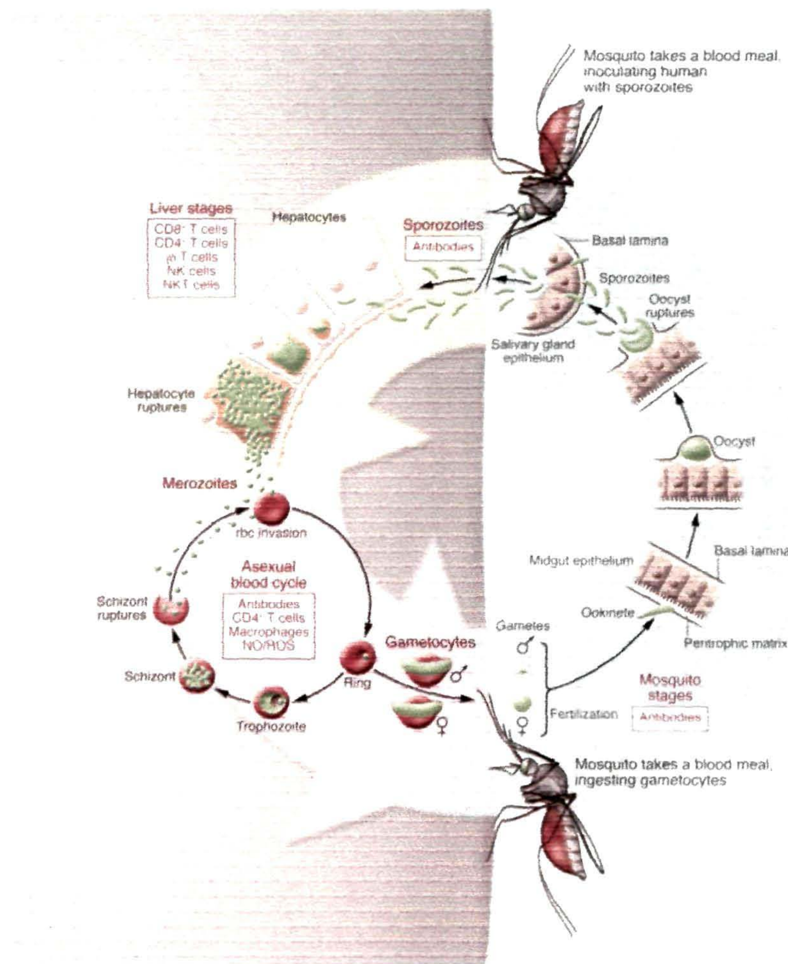


Fig 1.1: Life cycle of *Plasmodium falciparum*. (Adapted from Crompton *et al.* 2010).

Malaria causes a wide variety of symptoms ranging from asymptomatic infection to very mild symptoms to severe (complicated) disease and even death. Based on the clinical presentation, the disease may be divided into uncomplicated and complicated malaria as per WHO guidelines[7].

Strategies to control malaria have been redefined with effective diagnosis and treatment measures along with vector control interventions like Insecticide

Residual spray and use of insecticide treated mosquito nets. However, with emergence of insecticide resistant mosquitoes and the parasites developing resistance against conventional antimalarial drugs, it poses challenge to malaria control. Recently, artemisinin resistance has been reported from the Greater Mekong subregion and the threat of its spread to other areas is a serious issue[1]. Notably, chloroquine resistance in *P. falciparum* was first detected from South East Asia region[8].

Vaccination is considered an important control measure which has long been pursued but with little progress. Studies of various vaccine candidates including those of the liver stage and asexual blood stage candidate reported limited success in earlier attempts[9]. Combination B vaccine consisting of merozoite surface proteins 1 and 2 (MSP-1, MSP-2) and ring stage infected erythrocyte surface antigen (RESA) showed a 62% reduction in parasite density in vaccinees[10]. Recently a malaria vaccine candidate RTS, S, has entered phase III trial in Kenya[11]. The difficulties in development of a malaria vaccine may be attributed to the complex life cycles and antigenic polymorphism exhibited by the parasite and compounded by a poor understanding of protective immune mechanism[12]. Besides, the disease shows variation in epidemiology across different endemic regions[13].

The genetic structure of *P. falciparum* has been demonstrated to be highly diverse world over[14]. Major vaccine candidate genes exhibit antigenic diversity in the form of allelic polymorphism where the alternate forms of antigen coding genes exist. Mention can be made of the merozoite surface proteins (MSPs), the serine repeat antigen (SERA) genes families. Additionally diversity may be generated by clone multiplicity where a clonal lineage of parasite expresses alternate forms of an antigen without changes in genotype[15]. Variations in genes encoding antigens are also generated by non meiotic recombination such as strand slippage events, gene conversion as well as homologous recombination during meiosis[16]. This extensive diversity in *Plasmodium falciparum* antigens is seen as immune evasion mechanism of the

parasite. Repetitive sequences constitute immunodominant epitopes in parasite proteins, therefore sequence variation in allelic forms of the molecules may prevent the population from developing immunity to different strains of the parasite[17]. Elucidating the extent of genetic variation in the malaria parasite will therefore be central to decreasing the malaria disease burden[18]. The non uniform population structure of *P. falciparum* worldwide depends upon local factors related to parasite, vector and host factors[19]. Factors like transmission intensity and drug treatment too influence the genetic diversity of the parasite[14].

Allelic diversity and size polymorphism of merozoite surface protein 1(MSP-1) gene have been employed as a molecular marker in studies of malaria transmission dynamics and host immunity in *P. falciparum* malaria. MSP-1 gene has also been extensively studied as it has a role in invasion, disease and immune evasion[14]. MSP-1 gene is divided into 17 blocks, based on analysis of sequence diversity. Block 2 region near the N terminus and block 17 at the C terminus are major targets of host immunity. The block 2 of MSP-1 is described by 3 distinct allelic families MAD20, K1 and RO33 and contains antigenically unique tripeptide repeats with extensive diversity in the number of repeats[20]. Significantly, block 2 repeats are targeted by *P. falciparum*-inhibitory monoclonal antibodies and naturally acquired antibodies associated with clinical immunity in humans[15]. A comprehensive understanding of the immune responses lies in dissecting the antigenic diversity of *P. falciparum*.

Immune response to malaria is complex and is species and stage specific. Immunity to malaria can be either anti disease immunity which confers protection against clinical disease or anti infection immunity conferring protection against parasitaemia[21]. Naturally acquired immunity to malaria develops slowly and is rarely sterile. In holoendemic or hyperendemic areas older population generally develops immunity showing asymptomatic parasitaemia while in areas of low malaria transmission, individuals do not develop immunity in an age dependent manner. The infection and disease can therefore occur in both children and adults[22]. The picture that emerges from

human studies is that immunity to malaria infection is relatively slow to develop and incomplete, although immunity to disease is acquired more quickly and may be important after a single episode[23]. Acquired immunity involves both the antibody mediated and cell mediated immune response.

Malaria infection induces strong humoral immune responses, involving production of predominantly IgM and IgG but also other immunoglobulin isotypes. Species as well as stage specific antibodies against wide variety of parasite antigens have been reported while a large proportion of the antibodies are non-malaria specific[24]. The importance of antibodies as mediators of protective immunity to malaria is well established in both animal and human infections. Mice lacking B cells were unable to clear parasites from *P. chabaudi chabaudi* AS infection, rather such mice developed chronic parasitaemia[25]. Passive transfer of monoclonal antibodies against parasite antigens conferred protection in naive mice by reducing parasitaemia and clinical disease [26]. In humans, treatment of *P. falciparum*-infected patients from Thailand with IgG extracted from African immune adults, resulted in reduction of parasitemia and clinical symptoms[27]. Studies in humans and mice show that memory B cells are either poorly induced or short lived as a result of infection. Recent studies showed that memory B cells and antibodies increased gradually over many years and were dependent on cumulative exposure to malaria[9]. Despite the importance of antibody responses for protection against malaria, not all antibodies are reported to be protective. Polyclonal antibody specific to MSP-2 but not monoclonal specific to the same antigen enhanced invasion of multiple merozoites into RBC[28].

Protective antibodies primarily target the asexual stage antigens of which MSPs are leading blood stage vaccine candidate molecules of *Plasmodium falciparum*. MSP-1 is the major surface protein of the parasite, which is synthesized as a 190 kDa precursor protein[16]. It undergoes proteolytical cleavage into four fragments and remains attached to merozoite surface by glycoslyphosphatidylinositol anchor prior to invasion. During erythrocyte invasion, majority of the MSP-1 complex is shed of which only the 19 kDa C-

terminal fragment remains anchored on the merozoite surface[29]. Antibodies targeted to this fragment are shown to inhibit erythrocyte invasion and associated with protection from clinical malaria[30]. MSP-1_{19} fragment has a highly conserved sequence however at least six single nucleotide polymorphisms (SNPs) have been identified in its two epidermal growth factor like (EGF-like) domains[31]. Allele specific as well as cross-reactive antibody responses to variants of MSP-1_{19} fragment have been reported[32]. Further, certain MSP-1_{19} polymorphisms have been implicated as particularly important to immunity[31].

Cell mediated immunity (CMI) has crucial roles in protective immunity to malaria but also has the potential to cause tissue pathology and contribute to the development of severe malaria[33]. Vaccine formulations with T cell epitopes induce a strong memory response[9]. The CD4 T-cell subset is of major importance for the induction of blood-stage immunity in both murine and human malaria, while the CD8 subset has been shown to be cytolytic against liver stages of the parasite[28]. In experimental mice CD4 T cells could act independent of B cells in resolution of parasitaemia[34]. Humans lacking previous exposure to *P. falciparum* as well as malaria exposed individuals have CD4 T cells that proliferate and secrete IFNγ in response to parasite antigen and inhibit parasite growth in vitro[35]. Another subset of CD4+ T lymphocytes, T-regulatory (T-reg) cells is postulated to be involved in immunosuppression where effectors responses were enhanced in the absence of Tregs[33].

T cells also play a central role in the elimination of blood stage malaria parasites through the release of cytokines that activate other effectors cells[28]. Specific cytokine profiles are associated with different clinical manifestations. Th1 cells activated macrophages and other cells to produce mediators through release of inflammatory cytokine[36]. Th1 were seen to be responsible in the initial resolution of acute parasitemia through production of IFNγ while Th2 were required for eventual clearance of the parasites via T-B cell cooperation[37]. The balance between the Th1 and Th2 immune response may determine the level of parasitaemia and disease outcome. An early and sustained Th1 response was critically linked to IL-12 through IFN-γ production[38]. Recent evidence suggest

that rapid cell mediated immune responses are contributed by effector cells of NK and T cells lineages which contain the initial stages of malaria through IFN-γ production[39].

Natural killer (NK) cells are lymphocytes of the innate immune system that are involved in the early defense against foreign cells and autologous cells undergoing various forms of stress, such as microbial infection or tumor transformation. NK cells also influence adaptive immunity by modulating dendritic cell function and by inducing Th1 polarization via IFN-γ production[40]. NK cells and NK Receptor (NKR) positive cells are suggested to significantly control susceptibility and resistance to both malaria infection and severe disease syndromes. This was seen to depend on the receptors encoded within the Natural Killer Cell Receptor Complex (NKC) [41]. NK cells were seen to have direct contact with *P. falciparum* infected red blood cell via NK Cell receptors[42]. Further, NKR positive γδT cells were seen as the major source of IFN γ in response to *P. falciparum* infection in humans as well as in murine models which were in part controlled by NKR loci[41].

NKRs are largely encoded by two genetic loci in humans: the Natural Killer Complex (NKC) on chromosome 12 and the Killer cell Immunoglobulin-like receptors (KIRs) region on chromosome 19. Diverse set of inhibitory and activating NKRs controlled NK cell activation and the outcome of NK cell activity is the balance of signals from activating and inhibitory receptors[43].

The KIR gene complex is characterized by variation in gene content. Studies suggest that they are rapidly evolving genes which lack conservation among species and exhibit remarkable diversity as haplotypic variation in gene number and content and allelic polymorphism of individual genes[43, 44]. Two broad haplotype groups termed A and B are used to define variation in KIR genetic profile[44]. Frequencies of specific *KIR* haplotypes vary across different ethnic populations. The important role of KIR in the immune response and its genomic diversity coupled with its specificity for HLA ligands, affects resistance and susceptibility to pathogenesis of a number of infectious and autoimmune

diseases[43]. NK cells response to parasitized RBCs was noted to vary significantly between individuals and that the variation was associated with KIR genotype of an individual[42].

North East India is inhabited by people of various ethnic origins with a history of malaria endemicity. Malaria remains a major public health problem in the state of Assam. Nearly 65% of the total population of the state (26.6 million) is estimated to be living in high-risk areas[45]. Malaria transmission is perennial and continues to be uninterrupted supported by major vectors namely *An. minimus, An. fluviatilis* and *An. dirus*. The region is highly receptive to malaria transmission due to excessive and prolonged rainfall (2–3 m) promoting vector breeding and longevity due to high humidity (60–90%) and warmer climates (22–33°C) for most of the year[46]. *P. falciparum* is the major malarial infection and accounts for 58–68% of the cases and the remainder are due to *P. vivax*. Despite efforts to contain the disease mortality and morbidity are significantly high due to emergence of drug resistant *Pf* malaria, delayed diagnosis due to non-availability of facilities in interior villages, hilly areas and tribal belts[4]. Malaria in Assam is poorly investigated with respect to diversity of the circulating local *Plasmodium falciparum* and immune responses of the population. Besides it is populated by individuals of different ethnic origin. The present study was thus undertaken to understand the diversity of the *Plasmodium falciparum* of the region and the host parasite interactions with an aim to evaluate the protective immune mechanism and markers of protection if any.

Objectives:

1) ***To study the clone multiplicity of the parasite genotypes existing in the study area***
2) ***To elucidate the protective humoral immune response.***
3) ***To elucidate the protective cell mediated immune response***

CHAPTER II:

Review of Literature

2.1 Genetic diversity of *Plasmodium falciparum*

The complex life cycle of the parasite requires a sophisticated array of proteins encoded by a genome of 23 Mb distributed across 14 chromosomes and consisting of 5300 genes[47]. The parasite shows genetically structured populations varying even across local areas of the same region depending on the local epidemiological and demographic situations[48].

The variable genetic structure of *P falciparum* is contributed by the polymorphic nature of many of the proteins expressed during various stages of its life cycle. The polymorphism is often caused by variation in sequence of the short tandem repeats of the antigen which may constitute immunodominant epitopes[15]. MSP-1, which is a common molecular marker for assessing genetic diversity is divided into seven variable blocks that are separated by either conserved or semi conserved regions (Fig 2.1.1). There are two versions of each PfMSP-1 variable block, represented by the sequences of the isolates MAD20 and K1[16]. Most *Plasmodium falciparum* MSP-1 polymorphism may be generated by intragenic recombination between these two representative types at sites near the 5' end of the gene (blocks 3, 4, 5). Block 2 consists of direct repeats with three allele families of K1, MAD20 and RO33 identified in this region[14]. The various K1 and MAD20 type block 2 alleles differ in the number, sequence and relative arrangement of tripeptide repeats and in point mutation polymorphism of the flanking regions. While the non-repetitive RO33 alleles only differ by point mutations[49]. A fourth allele family in Block 2, called MR, which results from a recombination event between alleles of the Mad20 and RO33 families has also been identified[50].

Polymorphisms in repeats may enable parasites to evade immune responses elicited by past exposure to diverse forms of the same antigen. In addition, tandem repeats may stimulate T-cell-independent Bcell responses that fail to generate memory B cells or somatic hypermutation, leading to antibody affinity maturation [15]. Merozoite Surface Proteins (MSPs) are extensively studied to assess the genetic diversity of *Plasmodium falciparum* in different

transmission zones of *Pf* malaria as genetic diversity is an indicative of malaria transmission dynamics, complexity of infection and host immune response.

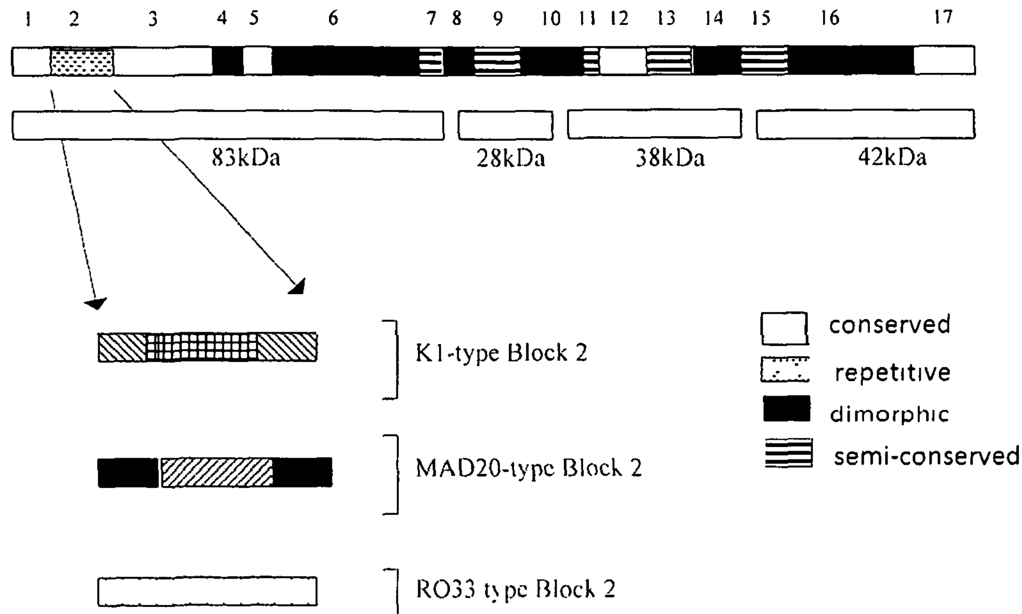


Fig 2.1.1: Schematic representation of MSP-1 of *P. falciparum.* (Adapted from Cavanagh *et al.* 1998)

2.1.1 Genetic diversity and transmission intensity

Studies in various transmission zones of malaria reveal a relation of *Plasmodium falciparum* genotypes from regions of differing endemicity and transmission characteristics.

Allelic diversity at MSP-1 locus was compared in three epidemiologically distinct malaria endemic areas: the hypo endemic Southwestern Brazilian Amazon, the mesoendemic southern Vietnam and the holoendemic northern Tanzania. MSP-1 diversity was relatively restricted in human hosts in holoendemic Tanzania when compared with the diversity found in mesoendemic Vietnam [14]. In a comparative study of parasite diversity in two Senegalese villages Dielmo and Ndiop 5 Km apart, complexity of infections was more than double in Dielmo where malaria was holoendemic, than in Ndiop where malaria was mesoendemic[51]. Characterization of *P falciparum*

populations in a hypo endemic Thai Burma Border area showed a higher number of genotypes per infected person than that predicted[52]. While relatively restricted number of PfMSP-1in a similar endemic area have also been reported[17]. Extensive polymorphism was identified in hypoendemic region of Myanmar in comparison to low endemic Asian countries like Thailand and Iran although it was less than other holoendemic areas of Senegal, Uganda and Gabon[53]. Similar to their observation, field isolates from eastern and north eastern regions of India showed high diversity of MSP-1 gene, diversity close to south east Asia, Papua New Guinea and Latin American countries, regions with low to mesoendemicity in comparison to African regions of hyper to holoendemicity[54]. Limited multiplicity of infection has also been reported from hyperendemic Solomon Islands in comparison to mesoendemic region of Thailand in South East Asia[55]. Thus, correlation between transmission intensity and number of genotypes detected has been found but the relationship may not be linear[52]. MSP-1 gene type was seen to distribute independently in the human host in lower endemicity such as Brazil and Vietnam but not in high endemicity such as northern Tanzania and the Gambia[14]. A study in Venezuelan Amazon argued that low diversity of parasite seen in the region may not be related to endemicity but to other influences like bottleneck effect[56]. The extent of allelic diversity is dependent on the number of alleles prevalent in the local parasite population and the extent of multiplicity of infections[57].

2.1.2 Genetic diversity and disease outcome

Studies have demonstrated association of genotypes of MSP-1 with the development of clinical disease and in severity of the disease. The likelihood to have a clinical episode of malaria was more in children carrying higher number of clones[58]. In a study, higher multiplicity of infection has also been correlated with reduced risk of contracting clinical malaria[59]. A higher mean number of genotypes were reported in Tanzania children with asymptomatic conditions than in the symptomatic case[60]. Comparing severe malaria and uncomplicated malaria

isolates, complexity of infections was found to be lower in severe malaria isolates[61].

Differential distributions of classes of *msp-1* alleles and association with clinical malaria outcome have been observed in studies. A study conducted in Orissa India, where malaria was hyper endemic observed overrepresentation of an allele of MAD20 family in severe malaria cases[62]. There are reports of association of genotypes of MSP-1 and disease severity where complete absence of an allele of MSP-1 was seen in severe malaria or studies failing to establish any such associations[57]. Presence of MAD20 allele was found to increase the probability of clinical disease[58, 63]. The presence of the K1 and MAD20 alleles was significantly associated with asymptomatic malaria and reduced risk of developing the symptomatic disease[64]. Studies have found RO33 allele in association with disease severity[61] and K1 as the major allelic family found in isolates from symptomatic cases[64, 65].

2.1.3 Genetic diversity and host factors

A number of molecular epidemiological field studies have shown that differences in multiplicity of infection (MOI) are age-dependent[51] while others did not observe any such relationship with age[52, 66]. In a cohort study in Ndiop village of Senegal, complexity of infection was seen to be least in children and remained stable with age[67]. A higher degree of complexity of infection was also seen in children greater than 2 years old in comparison to 0.5 to 1 year old[68]. But in adults measured multiplicity of infection decreased significantly with age[69]. Variation of multiplicity of infection (MOI) over age was highly affected by endemicity of malaria. The numbers of MSP-1 fragments were reduced in the older age group in highly endemic area of Dielmo but in Ndiop, a mesoendemic region, there was no influence of age on infection complexity[51].

The role of host genetic background on malaria infections has also been increasingly acknowledged. The genetic traits like haemoglobin variants type, a+ thalassemia may influence the multiplicity of *P. falciparum* and the presence of

distinct genotypes[57]. The multiplicity of the parasite was also seen to differ between two ethnically distinct African populations which may be attributed to their genetic differences[70]. The ability to clear anti malarial drug resistant parasites was also independently associated with ethnic background with the Fulani tribes of Africa less likely to clear infection by resistant parasites than Dogon or Malinke tribes suggesting a critical role of host factors in immunity[71].

2.2 Asexual blood stage antigens

Asexual blood stage of *Plasmodium falciparum* begins with the release of merozoites into the bloodstream from ruptured infected hepatocytes. Clinical illness occurs during blood-stage infection and studies have established that immune responses targeting blood-stage antigens protected against disease and facilitated control of parasitaemia[72]. The erythrocytic stage of malaria is mediated by the specific interaction of red cell receptors and parasite ligands. Invasion of erythrocytes involves initial attachment of the merozoite to the erythrocyte surface followed by apical reorientation, tight-junction formation, entry into the erythrocyte through an actin–myosin motor, and is completed by resealing of the erythrocyte membrane[73]. Various merozoite components expressed in different stages are involved in this process such as MSP-1, AMA-1and others in the initial, low-affinity interaction with the host cell (Fig 2.2.1).

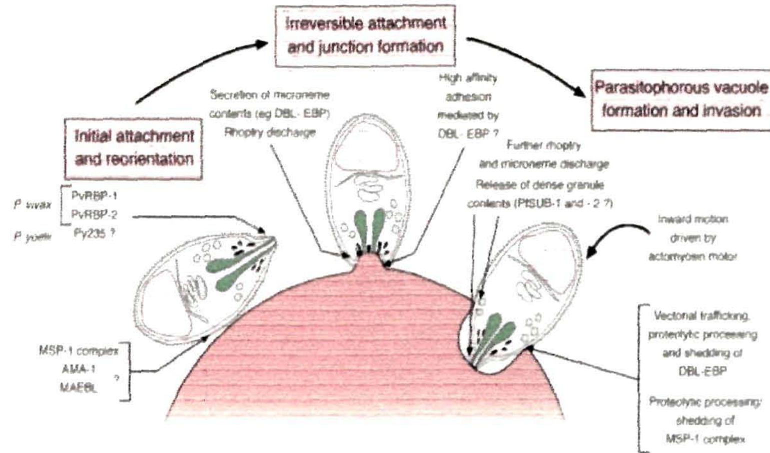


Fig 2.2.1: Merozoite components involved in attachment and initiation of penetration. (Adapted from www.malarianexus.com).

The leading blood-stage vaccine candidates are primarily merozoite proteins either located on the merozoite surface or contained within the apical organelle. These include apical membrane antigen 1 (AMA1), erythrocyte-binding antigen–175 (EBA-175), glutamate-rich protein (GLURP), merozoite surface protein 1 (MSP-1), MSP-2, MSP-3, MSP-4, MSP-5 and serine repeat antigen 5 (SERA5) all of which are highly expressed on the surface of the merozoite[74]. Another potential target for blood-stage vaccines is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) family, which is encoded by 60 or more *var* genes present in each parasite clone, with polymorphism between clones. They are responsible for cytoadherence a key player in the pathogenicity of *P. falciparum*[15].

2.2.1 Merozoite Surface proteins

Merozoite surface proteins play a role in RBC invasion and hence are important targets of immunity. Antibodies directed against merozoite proteins function by blocking RBC invasion, initiate parasite clearance by opsonisation

making the merozoite susceptible to phagocytic cells or complement mediated damage[28]. Merozoite surface proteins (MSPs) are likely to mediate the relatively weak and reversible initial interactions between the parasite and RBC[29]. Antibodies to MSP-1, MSP-2, and MSP-3 have been associated with protection from clinical malaria. Protection from clinical malaria was associated particularly with antibodies of the IgG3 isotype for both MSP-2 and MSP-3[75].

2.2.2 Merozoite surface protein-1

MSP-1 is a widely studied vaccine candidate molecule due to its important role in infection and immunity. Immunogenicity of MSP-1 has been shown with the whole molecule inducing protection against lethal *P. yoelli* and *P.falciparum* in mice and monkeys respectively[28]. MSP-1 is expressed initially as a ~ 200-kDa protein during the late stages of erythrocytic schizogony and is deposited in the merozoite membrane via a glycosylphosphatidylinositol anchor[29]. During invasion this 200 kDa precursor is proteolytically cleaved into two fragments, the 42-kDa ($MSP-1_{42}$) and a larger one which remained non-covalently bound forming a greater MSP-1complex along with two other peptides of 36 kD and 22kD.The $MSP-1_{42}$ is again cleaved into a 33 kDa and a C-terminal 19kDa fragment ($MSP-1_{19}$). The MSP-1 complex is released whereas $MSP-1_{19}$ remains membrane-bound and enters with the merozoite into the host cell signifying the crucial role of $MSP-1_{19}$ in invasion[76]

The regions of MSP-1 that are associated with protection include the C-terminal $MSP1_{19}$ and $MSP-1_{42}$ fragments and the N terminal fragment. A comparison of immunogenicity of conserved and polymorphic regions of MSP-1 found that immune responses mainly targeted the C terminal followed by block 2 region where the response were type specific[77]. Association of protection with antibodies to block 2 region of MSP-1 has been reported in different malaria settings. Presence of antibodies to recombinant proteins of the K1 and MAD20 types was negatively associated with clinical malaria contrastingly, anti MSP-1 block 2 IgG were positively associated with an increased risk of reinfection[49].

Role of C-terminal proteins in protective immunity was seen against *P. yeolli* infection and suppression of parasitaemia[78].

2.2.3 19Kda fragment of Merozoite surface protein-1

The MSP-1_{19} fragment consists of two epidermal growth factor (EGF) like domains. Each EGF like domain has six cysteines and presumably three disulfide bonds therefore proper disulfide bond formation is important in the structural integrity and antigenicity of MSP-1_{19}[79]. This 19-kDa fragment is carried into the newly infected erythrocyte during the process of invasion. MSP-1_{19} is relatively conserved but does contain six nonsynonymous single nucleotide polymorphisms (SNPs). One SNP corresponds to amino acid position 1644 (Q/E) in the first EGF domain of MSP-1_{19}. The second EGF domain contains the other five SNPs at positions 1691 (K/T), 1699 (S/N), 1700 (N/S), 1701 (G/R) and 1716 (L/F) respectively[31].

The 19kDa C-terminal fragment of MSP-1 is extensively studied to assess protective mechanism of MSP-1. Vaccination in animals models based on the C terminal fragments of MSP1, the larger fragment 42 kDa and its subfragment MSP1_{19} elicited protection against *Plasmodium falciparum* infection[80]. MSP-1_{19} specific monoclonal antibodies prevented the secondary processing of MSP-1_{42} and inhibited merozoite invasion in vitro[29].

Anti MSP1_{19} antibody has also been associated with lower incidence of malaria episodes[81], absence of clinical symptoms in parasetimic patients[82] and protection from parasitemia and febrile illness in infants and pregnant women[83]. Contrastingly, a few studies have reported no correlation of anti MSP1_{19} antibody and protection from malaria[84]. In a study high level of MSP-1_{19} specific invasion inhibitory antibodies reduced the risk of blood stage infection rather than the total IgG to MSP-1_{19}[85]. It has been suggested that protection is associated with the presence of antibodies to certain epitopes and that fine specificity of anti MSP-1_{19} antibodies is crucial in determining their function[86]. Antibodies to MSP-1_{19} have also been linked with severity of the disease.

Children with severe malarial anemia or uncomplicated malaria had a lower antibodies to MSP-1_{19} compared to those with cerebral malaria[87]. Similarly, individuals with severe malaria were seen to have lower anti MSP-1_{19} antibodies than those of uncomplicated cases[88].

The role of MSP-1_{19} in protective immunity is re-established in meta analysis of prospective cohort studies where MSP1_{19} antibodies were associated with protection from risk of infection[89]. Many of the MSP-1 vaccines currently in development are based on the MSP1_{19} fragment. A phase I trial of a vaccine based on the 19kDa fragment carried out at USA demonstrated that it was poorly immunogenic while another 19kDa based MSP-1/AMA-1 fusion antigen showed good immunogenicity in rabbits and non human primates[72].

Data on MSP-1_{19} and natural immunity towards falciparum malaria suggested that induction of B cell immunological memory of natural infections was not consistent in endemic areas posing a challenge for vaccine development[90]. Antibody response to MSP-1_{19} is known to be acquired slowly and antibodies increased gradually with time and were dependent on cumulative exposure to malaria[91]. Predictably, antibody prevalence was higher in areas with intense malaria transmission, compared with areas of lower transmission[92] and suggested that induction of a very high titre antibody response as a critical factor for protection[9].

Distribution of different alleles of MSP-1_{19} in different regions of the world has been observed. Presence of eight variants of MSP1_{19}, one of which was a novel variant (E-KYG-F) was reported from the Indian subcontinent[93]. In a cohort study at a vaccine testing site of Mali, it was observed that the prevalence of FVO (QKSNGL) and FUP (EKSNGL) strains whereas 3D7 (ETSSRL) strain was less prevalent[31]. The relevance of circulating alleles of MSP-1_{19} in vaccination has been emphasized recently in a review by Takala & Plowe[32].

Prevalence of antibodies to MSP-1_{19} alleles in malaria endemic region was reported to be as low as 4%[84], while high responses were also seen in some studies[85, 86]. Low prevalence of anti MSP-1_{19} antibodies was also reported from a study in African populations where too despite recurrent infections, 80% of children in Sierra Leone and 40% of adult Gambians did not possess antibodies to MSP1_{19}[87].

Antibodies to MSP-1_{19} fragment have been reported to recognize cross reactive as well as allele specific epitopes[96, 98, 99]. Study from malaria endemic region of India showed the presence of both variant specific and cross reacting antibodies to MSP-1_{19}[100]. Sutton *et al.*[95] in their study on Peruvian individuals observed that the MSP-1_{19} antibody targeted the conserved region, while differential recognition of MSP-1_{19} variants has also been reported[96, 98].

Particular changes in amino acid residues at position 1691 (K/T), 1700 (N/S) and 1701 (G/R) were associated with a shorter time to next clinical infection and development of clinical symptoms[31]. The predominant IgG sub-classes against MSP-1_{19} were IgG1 and IgG3 while IgG2 and IgG4 specific antibodies were less frequent[96]. Studies have demonstrated protective role of antiMSP1_{19} IgG1 and IgG3 subclasses in malaria[99]. The IgG subclass response against MSP-1_{19} was allele specific with predominance of IgG3 antibody response to Q-KNG and E-KNG alleles than to E-TSR[98]. IgG1/IgG3 class switching was seen to independently affect by the nature of the antigen, cumulative exposure to the antigen and the age of the individual[101].

2.3 Cytokines

Cytokines are low molecular weight regulatory proteins or glycoproteins secreted by various immune effector cells in the body in response to a number of stimuli. They bind to specific receptors on the membrane of target cells, which triggers the signal transduction pathways altering gene expression in the target cells. Cytokines regulate the immune response by stimulating or inhibiting activation, proliferation and or differentiation of various cells. They also regulate the secretion of antibodies or other cytokines. Cytokines rarely act alone, rather effectors cells are influenced by a certain cytokine milieu, consisting of several cytokines whose combined synergistic or antagonist effects can have very different consequences. A number of disease conditions result from overexpression or underexpression of cytokines or due to genetic defects of cytokine activity[102].

2.3.1 Cytokines in malaria

Cytokines have been demonstrated to play a role in protection as well as in the pathophysiology of *P. falciparum* malaria. The course of a malaria infection is highly dependent on the balance between the cytokines secreted by the various cells when activated[36]. Cytokine production appears necessary for the inhibition of parasitemia and stimulation of phagocytosis to enhance clearance of parasitized erythrocytes. Levels of endogenous pyrogens, such as IL-6, IL-1, and IL-8, are elevated in malaria disease and correlate with disease severity[103]. IFN-γ secreted by NK cells or γδ T cells induced low levels of TNF-α and cleared infection without clinical complications[41, 104]. With acquisition of immunity and decreased circulating parasite antigens, anti-inflammatory cytokines (TGF-β, IL-10) were seen to dominate[105]. Studies indicated that a delicate balance of anti-inflammatory and pro-inflammatory cytokines was required in induction and regulation of malaria[106]. Perkin *et al*[107] in their study suggested that the inflammatory cascade in severe malaria suppressed the protective effects of TGF-*β*1 and IL-12, and overproduction of TNF-*α* promoted deleterious effect, such as severe anemia. Panels of cytokines have been seen to discriminate

clinical malaria outcome. In a study from Central India, levels of IL-12, IL-5, and IL-6 discriminated severe forms of malaria from mild malaria while levels of IL-1β, IL-12, and IFN-γ were relevant for the discrimination of cerebral malaria from severe malaria[108]. In Malian children, concentrations of both proinflammatory and anti-inflammatory cytokines were higher in patients with cerebral malaria than in those with noncerebral malaria[103].

The roles of various cytokines which are of importance in the present study have been discussed.

2.3.1.1 Interleukin-2 (IL-2)

IL-2 has been observed to have little direct effectors function but it promoted expansion of T cells population and may be crucial for programming CD8+ T cells for improved capacity and effectors function[109]. Data on vaccination of malaria naive and malaria exposed human volunteers strongly indicated a role for IL-2 in memory cell differentiation[110]. Recently IL-2 was seen to regulate balance between natural T regulatory cells and effectors CD4+ Th1 cells in *P. chabaudi* infection[111] . T cell derived IL-2 is shown to be crucial for the induction of Foxp3 expression along with T cell derived IL-10 and TGF-β resulting in the generation of $CD25^{hi}Foxp3^{hi}CD4$ T cells[112].

2.3.1.2 Interleukin-4 (IL-4)

IL4 is a crucial mediator of CD4+ Th2 T-cell differentiation and suppression of IFNγ producing CD4+ Th1 cells[113]. Data indicates that induction of protective responses by merozoite surface protein based vaccines depends on IL4 and IFN-γ dependent pathways[114]. IL-4 production was also associated with a reduced in risk of developing anemia[115]. Polymorphism of IL-4 genes has been implicated in differential regulation of antimalaria antiisotype profiles and could alter malaria severity[116]. Investigation of IL-4 polymorphism in complicated and uncomplicated malaria patients in Thailand suggested that IL-4 590C/T polymorphism to be associated with control of parasitaemia[117].

2.3.1.3 Interleukin-10 (IL-10)

IL-10 plays a fundamental role in controlling the inflammatory response to malaria by inhibiting the production of IFN-γ and IL-12 production and of Tumor necrosis factor (TNF) secretion by macrophages[118]. Higher levels of IL-10 in Tanzanian children with acute uncomplicated malaria were associated with less effective clearance of the parasites[119]. Elevated levels of anti-inflammatory IL-10 have been reported in severe malaria[120]. Studies in mouse model and in vitro investigations have demonstrated ability of IL-10 to inhibit TNF production in response to malarial antigens[121]. Moreover, IL-10 was shown to prevent TNF associated lethal endotoxemia and inhibited antigen induced lymphoproliferation by downregulating major histocompatibility complex class II antigen expression on monocytes[103]. It has been proposed that the ability of the parasite to stimulate IL-10 production determined its relative virulence[122].

2.3.1.4 Interleukin-12 (IL-12)

IL-12 (p70) is a heterodimeric cytokine composed of IL-α (p35) and IL-β (p40) subunits. It is produced primarily by antigen-presenting cells and exerts immunoregulatory effects on T and natural killer (NK) cells[38]. IL-12 plays a major role in cell-mediated immunity against a variety of pathogens by rapid induction of IFN-γ production[39]. Levels of IL-12 has been seen both to increase and decrease in correlation to *Plasmodium falciparum* density and ratio of IL-12 to TGF-β and others anti-inflammatory was seen to correlate inversely with disease severity[123,124] . In experimental models, IL-12 administration decreased mortality in association with a reduction in peak parasitemia that was dependent on IFN-γ and partially dependent on nitric oxide[125]. Polymorphism in genes encoding the subunits of IL-12 has been associated with malaria outcome. Genetic variants of IL-12 related gene protected Kenyan children from severe malaria anaemia[126].

2.3.1.5 Interferon gamma (IFN-γ)

IFN-γ is mainly produced by CD8+ and CD4+ T lymphocytes and by NK cells. Levels of IFN-γ production were associated with symptomatic malaria

with a high level of the cytokine directly correlated with clinical pathology of the disease [127]. While lower concentration of IFN-γ was also seen in children with *P .falciparum* hyperparasitaemia in comparison to children with uncomplicated malaria[128]. IFN-γ responses to liver-stage and merozoite surface antigens were associated with resistance to reinfection[37]. IFN-γ has been seen to have a role both protection and pathogenesis with IFN-γ deficient mice unable to develop cerebral malaria while control of parasitaemia was also seen to depend on IFN-γ production[129]. It was seen to be essential for the resolution of primary infection by limiting the initial phase of parasite replication while overproduction of IFN- γ predisposed to severe pathology of malaria[38].

2.3.1.6 Transforming growth factor beta (TGF-β).

TGF-β is shown to have an important role for maintaining the balance between protection against and progression toward *P .falciparum* malaria[38]. Protective effect of Th1 cytokines can be downregulated or suppressed by regulatory response including TGF-β production[127]. TGF-β was seen to inhibit IFN-γ and TNF production while upregulating IL-10[130]. The production of TGF-β and the presence of CD4+CD25+*FOXP3*+ regulatory T cells were associated with higher rates of parasite growth in vivo[131]. Omer et al have reported that TGF-β has proinflammatory activity at low levels, whereas high levels of TGF-β are associated with anti-inflammatory activity[132]. Studies have shown contradictory results with a decreased of TGF-β production in severe malaria while some have reported increased in level of the cytokine with severity of the disease[133].

2.3.2 Role of Th1 and Th2 cytokines in protection and immunopathology

Studies have shown that both Th1 and Th2 responses are required to control the infection, but they need to be adequately tuned in intensity and time[37]. Infection with *P. yoelii* in mice showed that early activation of Th2 cells was deleterious, conferring susceptibility to infection but during peak parasitemia a shift from Th1 to Th2 in *P. chabaudi chabaudi* infection was

linked to clearance of parasites[37]. In acute uncomplicated *P. falciparum* malaria a more pronounced Thl regulated response successfully managed resolution of parasitaemia by IFN-γ production and IL-10 was a key molecule in the control of inflammatory response[134].

A protective role of Thl pathway involving IFN-γ and IL-12β in cerebral malaria has been suggested with levels of the two cytokines inversely related to malaria[135]. Switching from an initial Thl response to an essential Th2 response has been demonstrated in a study in Gabon where young children with acute malaria had high levels of IFN-γ, while decreased level of IFN-γ was associated with detectable IL-4[136]. Activation of Thl cytokines, IL-12 and IFN-γ was seen to initiate early elimination of *P. falciparum* in uncomplicated malaria[137]. More recently, early *P. falciparum* induced IFN-γ production from γδ T cells was seen to be associated with protection from clinical *P. falciparum* episodes in children from a malaria endemic region[138]. Similarly in murine experimental systems too, IFN-γ initiated inflammatory responses were needed to control infection[139].

Early protective Thl response was suppressed by Th2 cytokines like IL-4 and IL-10 otherwise it could lead to immunopathology[139]. In a study, susceptibility to the neurological complications of cerebral malaria and upregulation of adhesion molecules in brain endothelium was associated with predominant Thl response[140].

2.3.3 Cells specific cytokines response

Cytokines response to malaria is not only specific to T cells as the primary regulator but the secretion of cytokines from other cell types like dendritic cells, macrophages, granulocytes or platelets was suggested to play a significant role in determining the outcome of infection[127]. Recent investigation has shown that NK cells dominated the early IFN-γ response to *P. falciparum* and that T cells dominated the later response. IFN-γ response of NK cells to *P. falciparum* infected RBCs was crucially dependent on IL-2 secreted by CD4+ T cells in a MHC class II dependent manner[39]. Protection against malaria required early cellular response of monocytes/macrophages, B cells and others initiated by the release of IL-12[139].

2.4 The Killer Immunoglobulin-like Receptor (KIR)

The Killer cell Immunoglobulin like Receptors (KIRs) locus, containing a family of polymorphic and highly homologous genes, is encoded in the leukocyte receptor complex on human chromosome 19q13[141]. The KIRs locus has undergone expansion and contraction over time with gene duplication and unequal crossing over in the region exhibiting allelic variability and individual haplotypes differing in gene content. The structural characteristics of KIRs are presence of Immunoglobulin like extracellular domains and a cytoplasmic tail and the basic structure of the KIR genes entails a nine-exon unit[43] (Fig 2.4.1). The conventional nomenclature of KIRs is based on this structural features where it can have two (2D) or three (3D) Ig-like domains and either a long (L) or short (S) cytoplasmic tail. In addition, putative pseudogenes are denoted by the letter P[141].

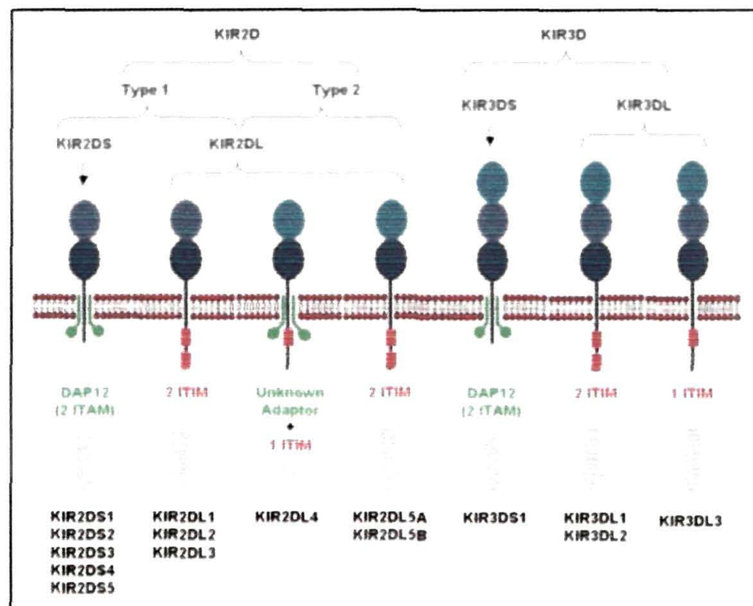


Fig 2.4.1: KIR protein structures. (Adapted from IPD-KIR database, www.ebi.ac.uk).

Despite their high degree of sequence identity, KIR genes encode proteins that confer opposing signals (activating or inhibitory) to the NK cell and have diverse recognition patterns (specific HLA class I allotypes) [43]. Inhibitory

KIR receptors have long cytoplasmic tails and contain Immune Tyrosine-based Inhibitory Motifs which transduce inhibitory signals to the NK cell. While activating KIR receptors have short cytoplasmic tail and possess a positively charged amino acid residue (lysine) in their transmembrane region, which allows them to associate with a DAP12 signaling molecule capable of generating an activation signal[142]. The HLA class I molecules serve as ligands for the KIRs. Two-domain KIRs recognise HLA-C allotypes while three domain KIRs recognise HLA-B allotypes. Allelic polymorphism has been described for all the *KIR* genes encoding inhibitory *HLA* class I specific receptors, and it appears most extensive for KIR3DL1 and KIR3DL2[44].

Segregation studies carried out in families have shown how KIR sequences previously thought to be different genes may represent alleles based on the inheritance behaviour observed . This was the case of KIR3DS1/ KIR3DL1 and also KIR2DL2/KIR2DL3 which segregate as alleles of a single locus[142].

2.4.1 Haplotypic variation

KIR genotypes can be grouped into two broad haplotypes termed A and B based on KIR gene content. The number of KIR genes present in a given haplotype ranges between 7- 16. The genes KIR2DL4, KIR3DP1, KIR3DL2 and KIR3DL3 are present on virtually all haplotypes and have therefore been termed framework loci[141]. The A haplotype is generally non-variable in its gene organization, using up to eight genes: those of the framework and KIR2DL1, KIR2DL3, KIR3DL1 and the only activating gene KIR2DS4, though they show extensive allelic polymorphism. The B haplotype exhibit extreme diversity both in terms of gene content and allelic polymorphism and is defined by the presence of one or more of the genes encoding activating KIRs, KIR2DS1/2/3/5, KIR3DS1 and the genes encoding inhibitory KIRs, KIR2DL5A/B and KIR2DL2[44]. Full haplotype-length sequencing has been performed for KIR haplotypes, showing the order of the genes on each haplotype to be KIR3DL3 at the centromeric end, KIR3DL2 at the telomeric end and KIR2DL4 in the middle (Fig 2.4.2). KIR3DP1 and KIR2DL4 divide the centromeric from the telomeric parts of the haplotype[44].

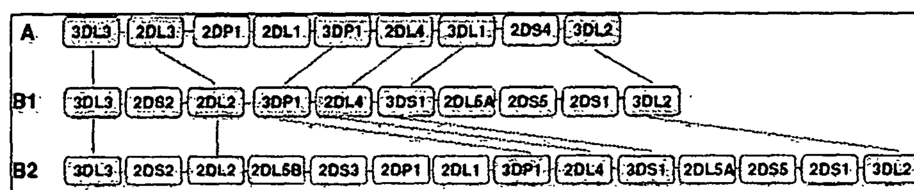


Fig 2.4.2: KIR haplotypes A & B. (Adapted from Bashirova *et al.* 2006).

Within each of these two regions there is extensive linkage disequilibrium. Several unusual haplotypes have been reported including truncated haplotypes that are missing some framework genes or elongated haplotypes that contain duplicated genes[43]. Frequencies of specific *KIR* haplotypes and the two major haplotypic groups, A and B, vary across ethnically defined populations. In Caucasian populations the A and B haplotype are shown to distribute approximately equally. B haplotypes have been shown to be more prevalent Australia Aborigines and Asian Indians. But in Japanese, Han Chinese and Koreans the A haplotype was more frequent[44].

2.4.2 KIR diversity in world populations

The Killer cell immunoglobulin-like receptors (KIR) gene frequencies have been shown to be distinctly different between populations. Studies of KIR polymorphism in Caucasoid population showed higher frequencies of inhibitory KIR genes which were more homogenous compared to those for the activating genes[143, 144]. The Caucasian populations had predominantly the non expressed allele KIR2DS4*003 of KIR2DS4 as the predominant activating KIR receptor[145]. The African population consisted of significantly higher frequency of KIR3DL1 and KIR2DS4 but had least frequency of 3DS1 compared to other populations[146]. Selection against having KIR3DS1 had been reported in African populations with frequency of KIR3DS1 gene to be less than 7%. Amerindian populations exhibited very few haplotypes, but all the 14 KIR genes occurred in high frequency except for 3DS1[147]. Comparison of KIR gene frequencies of the Japanese with other populations suggested a relatively restricted diversity in Japanese KIRs which was consistent with their limited migration history[148]. Comparision in Chinese, Indians and Malay populations of Singapore also

showed the KIR gene frequencies to be consistent with their genetic origin in recent past[149].

Study of KIR frequencies in Indian population showed variation of genes according to their ethnic background. In a study on 72 members of the north Indian Hindus, all the members had a different KIR as well as HLA genotype and estimated that the frequency of unrelated individuals with identical *KIR* genes as <2% within this population. The North Indians had predominantly the B haplotype[150]. The KIR locus was seen to be more variable in the Maharastrians population of west India than the Parsis who migrated to western India in recent from Persia. Nonetheless, in comparison to North Indian they had more similar KIR frequencies emphasizing the locale specific selection pressures, with persistence of ancestral genotypes and genetic drift[151]. Study of KIR polymorphism in three Dravidian-speaking populations (Mollukurumba, Kanikar, and Paravar) from Tamil Nadu in southern India revealed that each of them had distinct KIR genotype profile and except for two KIR genes KIR2DS5 and KIR3DS1, the frequencies of KIR genes were comparable to North Indian Hindus[152].

KIR distribution in modern populations is shown to be determined by the waves of prehistoric migration and subsequent population admixture[151]. Principal component analysis of KIR genes frequencies in different populations worldwide showed that populations tended to group together according to a geographical gradient[153].

2.4.3 KIR and association with disease

A number of evidences have shown the influence of KIR polymorphism in human diseases. Abberant levels of KIR expression have been observed in a wide spectrum of diseases[43]. Different HLA/KIR genotypes can impart different thresholds of activation to the NK cell repertoire and such genotypic variation has been found to confer altered risk in a number of diseases. Analysis of over 1000 individuals infected with HIV demonstrated that KIR3DS1 in combination

with HLA-B alleles that encode molecules with isoleucine at position 80 (HLA-B Bw4-80Ile) resulted in delayed progression to AIDS after HIV infection than those individuals without this activating KIR-HLA combination. Indeed, KIR3DS1 in the absence of Bw4Ile80 was not protective[154]. The presence of activating receptor KIR3DS1 in the absence of ligand for the inhibitory KIR2DL1 and KIR3DL1 (i,e., HLA-C2 and HLA-Bw4 respectively) conferred susceptibility whereas absence of KIR3DS1 and presence of HLAC2 and HLABw4 conferred protection against cervical cancer associated with human papilloma virus[155]. In a single-center study of 150 patients, a synergistic association of KIR3DS1 in combination with Bw480Ile appeared protective against the development of Hepatocellular carcinoma[156]. The protective effect of KIR3DS1 and HLA-BBw4 against chronic Hepatitis C virus infection was also found in a study by[157]. In individuals exposed to Hepatitis C virus, a protective association of the inhibitory receptor KIR2DL3 in combination with HLA-CAsn80 was seen in those homozygous for both genes. A positive association of KIR2DL1 expression and development of recurrent infections from Cytomegalovirus was also reported[158].

A number of autoimmune disorders have been associated with specific KIR genes and a common theme of these studies is that specific activating KIRs are implicated in disease pathogenesis[159]. KIR2DS2 was significantly more prevalent amongst patients with rheumatoid vasculitis compared to either normal individuals or patients with rheumatoid arthritis, but no vasculitis[160]. Increased susceptibility to developing psoriatic arthritis amongst individuals with KIR2DS1 and/or KIR2DS2 was reported, but only when HLA ligand for their homologous inhibitory receptors, KIR2DL1 and KIR2DL2/3 were missing[161]. Genetic studies in both European and Japanese populations have demonstrated an increased frequency of KIR2DS1, both alone and in combination with HLACw6, in individuals with Psoriasis vulgaris compared to controls[159]. KIR2DS2 in combination with HLA-C1 and in the absence of HLA-C2 and HLA-Bw4 was associated with increased susceptibility to diabetes[162]. An

increased frequency of a specific combination of KIR, which included all the inhibitory receptors and only two activating receptors KIR2DS2 and KIR2DS4, was observed in patients with acute myeloid leukemia as compared to healthy controls[163].

An inverse correlation between the number of activating KIR genes present and the prevalence of pre-eclampsia was observed[164]. A study of 366 individuals with psoriatic arthropathy predicted a phenotype with more activating receptor–ligand interactions rendered an individual more susceptible to psoriatic arthropathy[165]. Study on Nasopharyngeal carcinoma showed the number of activating KIRs to be positively associated with having the carcinoma. In a study of patients with malignant melanoma, individuals homozygous for HLA-CLys80 were weakly protected from melanoma[166].

In malaria, the ability of NK cells to respond to parasitized RBCs was noted to vary significantly between individuals and that the variation was associated with KIR genotype of an individual where high malaria specific NK cell IFN-γ response was directly corresponded to an allele of KIR3DL2, namely KIR3DL2*002[42]. IFN γ^{+} production was also found to be associated with the expression of NKG2A, CD94 CD158a/KIR2DL1 genes. In a recent study, *P.falciparum* positive individuals were seen to have higher frequency of KIR3DL3 and KIR2DS4 genes[167]

CHAPTER III:

Temporal and Spatial variation in MSP-1 clonal composition of Plasmodium falciparum in districts of Assam, Northeast India

3. Temporal and Spatial variation in MSP-1 clonal composition of Plasmodium falciparum in districts of Assam, Northeast India.

3.1 ABSTRACT

Polymorphism in MSP-1 gene generated by insertion/deletion of repeats causing repeat length polymorphisms is widely used as a marker for parasite genotyping. Elucidating *P. falciparum* clonal composition in relation to transmission intensity and other epidemiological factors in endemic areas is crucial to understanding the dynamics of host-parasite relationship and the development of immunity in malaria. We have examined here the allelic diversity of *P. falciparum* and attempted to understand the polymorphism and distribution of alleles of MSP-1 with transition in transmission season and with differences in malaria epidemiology between sites. MSP-1 diversity expressed as number of alleles per isolate was 0.336 at Guabari and 0.45 at KTE. Size polymorphism of all the three allelic families at Guabari was distinctly different from KTE.

Infections in high transmission summer season tended to be more complex with higher number of alleles. At Guabari, an interaction between allele frequency and transmission season was noted for RO33 ($p<0.0001$) and K1 ($p=0.002$) allelic families and the clonal composition of alleles was also found to be different between the two transmission periods. A 380 base pair allele of RO33 was over represented in high transmission summer season and seen frequently in isolates with high parasitaemia raising the possibility of its association with *P. falciparum* virulence. At KTE allele distribution of only MAD20 was found to be different in each study year. The present study demonstrates clonal composition of *P. falciparum* varied with study site and between periods of high and low transmission as well as in different years of study. Other factors particularly the host factors were also indicated in allelic polymorphism of MSP-1.

3.2 INTRODUCTION

Malaria remains a huge health and economic problem globally. Given population growth and settlement patterns, it is projected that 50% of the world's population, that is approximately 3.5 billion people, will be living in areas with malaria transmission by 2010[168].

Antigenic diversity exhibited by *Plasmodium falciparum*[169, 170] remains a major challenge in vaccine development. World over the genetic structure of *P. falciparum* has been demonstrated to be highly diverse[14]. Studies in acquired immunity in endemic areas suggest that the variable genetic structure of the pathogen contributed significantly to the evasion of immune response leading to selection of certain clones, arguably those that do not evoke strong immune responses leading to dominance of some genotypes in certain populations or geographical areas[32]. Apart from immune status of the population, treatment and genetic background of the host have also been postulated to have significant bearing on the genetic structure of *P. falciparum*[14, 36, 171]. While some authors have reported direct correlation between transmission intensity and number of genotypes detected[20], others have found unexpectedly significantly higher number of genotypes in areas of low transmission. Paul and others[52] have hypothesised that genetic structure of *P. falciparum* is a function of transmission intensity in a given endemic area but this relationship may be non-linear.

Allelic diversity and size polymorphism of the Block 2 region of the merozoite surface proteins MSP-1 have been employed as molecular markers in studies of malaria transmission dynamics and host immunity in *P.falciparum* malaria. The Block 2 region of MSP1 gene is trimorphic and three allelic families: KI, MAD20 and RO33 have been identified and contain antigenically unique tripeptide repeats with extensive diversity in number of repeats[20]. We have studied here the genetic structure of *P.falciparum* and its genotypic variation in geographic areas that differ in malaria epidemiology and between dry winter months and high transmission summer months in Assam State of

Northeast India. The spatial distribution of malaria in Northeast is not homogenous and its transmission dynamics and intensity is governed by distinct epidemiological paradigms attributable to eco- geographical, ethnic and socio-cultural diversity[172]. Frequent epidemic outbreaks have been reported from several regions of the state in the summer months. Limited information is available on the genetic structure of *P. falciparum* strains from India, particularly from the northeast region[54,173]. We report here extensive diversity of *P. falciparum* in districts of Assam and the difference in composition of the clones with transmission season and site. MOI was not correlated with age but was greater in high transmission season. The confounding factors in *P.falciparum* diversity are discussed.

3.3 Material and Methods

3.3.1 Study sites

The study was conducted in the village of Guabari located in Baksa district along the foothills of Bhutan and at Kondoli, a tea estate in the Karbi Anglong foothills in Nagaon district of Assam. More than 85% of reported malaria cases in these sites are due to *Plasmodium falciparum*. Malaria in Guabari is perennial but highly seasonal with two peaks, the main peak in the summer from May-August varying with temperature and rainfall and a smaller peak from October-November. Slide positivity rate in Guabari varies from ≤15% in the dry winter months, when transmission is low, to 30-55% in the high transmission period spanning Mid March – Mid July. Kondoli tea estate (KTE) has seasonal malaria during the high transmission period and experiences negligible malaria in dry winter months. According to annual slide positivity rate and spleenomagaly index, these areas may be classified as 'mesoendemic'. However, sporadic malaria outbreaks occur at these sites. The two study sites differ mainly with respect to accessibility to health care and population demography among other factors. In Guabari accessibility to adequate health care was poor while in KTE, proper health care facilities were provided at garden

hospital and sub centres at each division. The population of Guabari comprised of the Bodo-Kachari tribes, Nepalese, Bengalis, non tribal Assamese, Tea tribes and Biharis. At KTE the population was predominantly the Mundari speaking Tea tribes community. An investigation into prevalence of K76T mutation at Kondoli and Guabari showed frequency to be 90 and 75 percent respectively.

3.3.2 Study population

The participants were recruited from northern area of Guabari village and from the Tapitsjuri subdivision of KTE. Participants were selected randomly irrespective of their *Pf* positivity status, sex, and ethnicity. Inclusion criteria were individuals with *Plasmodium falciparum* positivity or with history of *P. f* malaria. Exclusion criteria were children with age less than 1 year, pregnant women and individuals suffering from any other diseases. The study population was stratified into three ethnic groups namely the Tibeto-Burman (TB), the Tea Tribes (TT) and the Indo European (IE) according to their language affiliations and ethnic background for the purpose of statistical analysis. The TB consisted of Bodo-Kachari tribes like the Bodo, Hazong, Rabhas and Nepalese. The TT included tea tribes community mostly Mundari speakers of central and eastern India. The IE comprised of nontribal Assamese, Bengalis and few Biharis who are the speakers of Indo Aryan languages. All the participants had equivalent socio-economic status. The main occupation of the villagers in Guabari was farming and as migrant workers in other parts of Assam. In KTE, the participants worked as tea plantation labor. The research staff collected samples and data during the field visits as well as by trained field workers at the two sites. At Guabari, the field workers were trained community health workers of local primary health centre/community health centre and medication was dispensed by them as per the Government of India drug policy for North east region. Patients with complication were referred to the hospital, while at KTE, collection was mainly done by hospital staff.

A standardised patient information sheet had been developed and read to the participants and explained to them about the study by the field workers. Patients were enrolled into the study after they or their accompanying relatives were satisfied and gave full informed consent. The study was approved by Tezpur University Ethical Committee (TUEC).

3.3.3 Study design and sample collection

The study was designed as a longitudinal study in the two districts of Assam. The study was conducted during the malaria outbreak of 2006 and of 2007. Blood samples were collected by finger prick method and spotted on Whatmann 3MM and on FTA cards for parasite DNA extraction and PCR analysis. The samples were collected from patients who tested positive for *Plasmodium falciparum* infection using Rapid Diagnostic Kits and microscopy. Briefly, people with fever or feeling feverish with headache were asked to report to the field workers who checked for malaria and collected the samples which were transferred bi-weekly to the laboratory for investigation. Those positive for malaria were given medication and those with severe malaria were referred to the health centre doctors for evaluation and proper treatment.

3.3.4 Extraction of DNA

The DNA was extracted from the Whatmann paper using the Chelex extraction method[52]. The DNA was extracted from the FTA cards as per the manufacturer specified protocol.

3.3.5 Amplification of DNA

The extracted DNA of the positive samples was taken and the Block 2 of the MSP-1 was amplified by primary PCR followed by nested rounds of PCR using allele specific primers. The PCR cycles and the primer sequences used were as described by Paul *et al*[52]. Blood samples that were negative for *P. falciparum* and bacterial genomic DNA were used as negative controls.

3.3.6 Protocols for the Primary PCR

Template DNA (10μl of the extracted DNA) was amplified in a final reaction volume of 25μl in the presence of 12.5μl PCR mixture (Fermantas), 1.15μl of forward and 1.32μl of reverse primers and 0.03μl of Millipore water. The PCR cycle conditions were one cycle for denaturation at 95°C for 5 min,40 cycles at 92°C for 30 sec, 50 °C for 1min and 70°C for 1 min followed by one cycle at 70°C for 5 min.

3.3.7 Protocols for the nested PCR

Template DNA (10μl of the first PCR product) was amplified in a final reaction volume of 25μl in the presence of 12.50μl PCR mixture, 1.25μl of nested forward primer and 1.32μl of reverse primer. The PCR cycle conditions were one cycle for denaturation at 94°C for 1 min, 25 cycles at 94°C for 30 sec, 47°C for 30 sec, 64°C for 1 min, 64°C for 3 min followed by one cycle at 64°C for 4 min.

3.3.8 Detection of the PCR products:

The PCR products both of primary and nested PCR were detected by electrophoresis of 8μl from each reaction which was dyed using bromophenol blue. The amplicons were run on 1.8% agarose gels pre stained with Ethidium Bromide and using TAE buffer. 100 base pair and 50 base pair molecular markers were used to analyse the size of the bands (Fermentas).

3.3.9 Statistical analysis

The data was analysed using Excel Stat Software. Mean number of alleles per isolate was calculated by dividing the total number of alleles seen by the total no. of isolates examined. Variation in the frequency of the MSP1 allelic families MAD20, RO33 and K1 with respect to study sites and transmission season was analysed by Chi square (χ^2) test with Yates correction as required. *P. falciparum* diversity was analysed using Index of homogeneity or relative diversity, J`

(J`=H`\Hmax). Interaction of alleles with sites and also between transmission seasons was analysed using χ^2 test. The proportion of isolates with an allelic family of MSP-1 was compared using k-test for multiple proportions. Variance in the alleles in the three sites was compared using two-tailed z-test. The distribution of alleles was compared using Smirnov-Kolmogrov two-tailed test. The association between clones and age was analysed by Pearson's Correlation test. The effect of age, ethnicity and study site was analysed using logistic regression.

3.4 Results

Of the 796 and 546 persons screened for malaria at Guabari and KTE respectively 44.58% and 29.04% tested positive. Only 112 samples at Guabari and 50 samples at KTE (one in every three by random stratified sampling) were amplified. Care was taken to ensure that samples were representative of different transmission periods. Allelic diversity and size polymorphism were analysed with respect to transmission season and study site. The data was analysed for spatial as well as temporal variation between the two sites. At Guabari village, the study period spanned low transmission in the dry winter months to period of peak transmission in summer months for two consecutive years from December 2005 to June 2007 and at KTE the study was conducted for three consecutive summers from 2005 to 2007. A high degree of MSP-1 polymorphism was found in isolates from the two study sites with 33 alleles of MSP-1 seen in Guabari village and 18 alleles at KTE (Table 3.4.1).

Table 3.4.1: Distinct Alleles of MSP-1 and MOI

Site	Sample no.	MAD20	RO33	K1	Total MSP1 alleles	MOI
Guabari	98	9	10	14	33	3.306
KTE	40	6	5	7	18	3.52

MOI-Multiplicity of infection

The number of distinct alleles of the three allelic families of MSP1 seen at Guabari & Kondoli. No interaction between the number of distinct alleles of each of the families and study site existed ($\chi^2 = 0.948$, $p = 0.918$).

An allele was defined as amplification of a band of distinct size of the allelic family. The MSP-1 diversity expressed as mean number of alleles per isolate was was 0.336 at Guabari and 0.45 at KTE. Nesting of fragments of MAD20 was seen at Guabari (J` <0.05) (Table 3.4.2).

Table 3.4.2: Indices of homogeneity

Sites	MAD20	RO33	K1
Guabari	0.372	0.453	0.502
KTE	0.577	0.493	0.585
Guabari			
Season	MAD20	RO33	K1
W2005	0.9	0.793	0.754
S2006	0.483	0.6679	0.592
W2006	0.308	0.5142	0.504
S2007	0.4227	0.507	0.612

W=winter; S=summer

The fragment sizes were binned into 50 base pair bins and analysed for homogeneity employing Shannon's Weaver Index for analyzing diversity among the study sites and transmission seasons at Guabari and J` values obtained are presented.

Values < 0.50 were taken to indicate nesting while >0.70 were considered to be indicative of diversity / evenness. Nesting of fragments of MAD20 at Guabari may be noted but not at KTE. A decrease in diversity of fragments between summers of 2006 to 2007 at Guabari may be noted.

The number of alleles of each of the three allelic families, MAD20, RO33 and KI was similar at the two study sites (Table 3.4.1) indicating that the

polymorphism in the three allelic families was comparable. Though overall many size fragments were seen, 3–4 sizes were dominant and some fragments were seen in any one transmission period and were at low frequency and these minor fragments reflected the variations. We also analysed the proportion of isolates with an allelic family using k-test for comparing multiple proportions. Proportions of isolates either with MAD20, RO33 or KI did not differ among the study sites but were associated with transmission season (Table 3.4.3). At Guabari, proportion of isolates with only RO33 allelic family was found to be related to transmission season and year. Though MAD20 proportion varied during the study period but surprisingly these were not related to either summer or winter transmission season. It was RO33 and KI but not MAD20 that showed significant interaction with transmission season at KTE. Interestingly, proportion of RO33 was lower in winter months at Guabari and was found to increase as the transmission proceeded from dry winter months to peak transmission in summer months in both the study years. Similarly, at KTE too proportion of RO33 was much higher in 2006 and in 2007.

Table 3.4.3: The proportion of isolates with an allelic family of MSP-1

	Guabari				KTE			
Transmission season & year	No. of sample	MAD20	RO33	K1	Transmission Year	MAD 20	RO 33	K1
Winter 2005	11	0.818	0.636	0.545	Summer 2005	0.5	0.5	0.43
Summer 2006	40	0.675	0.75	0.625	Summer 2006	0.6	0.9	0.9
Winter 2006	20	0.65	0.65	0.65	Summer 2007	0.875	0.88	0.8
Summer 2007	27	0.962	1	0.629				
		p values				p values		
χ^2		0.025	0.01	0.951	χ^2	0.077	0.03	0.02
Monte Carlo		0.021	0.011	0.964	Monte Carlo	0.074	0.03	0.02

More than one allele can be present in a sample hence sum of the total alleles is greater than one

The proportion of isolates with an allelic family was analysed for temporal variation employing the two tailed k-test for multiple proportions at Guabari and KTE. At Guabari the variation was studied between the high transmission summer and the low transmission winter while at KTE between the three study years. Data analysis shows a distinct difference between the two study sites with respect to temporal variation in number of isolates.

3.4.1 Size polymorphism – temporal variation

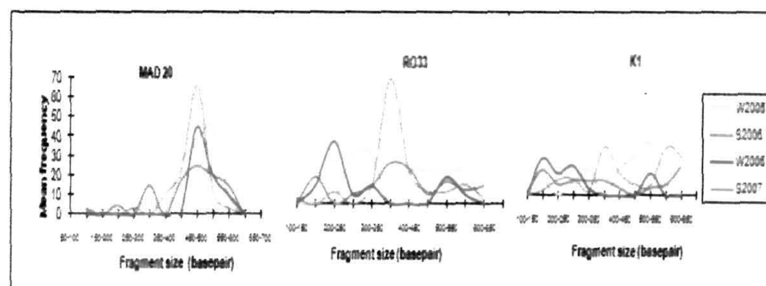
Frequency of alleles of RO33 and of KI was found to be positively associated with transmission season ($p < 0.001$, Pearson's Phi = 0.847 and p = 0.00 and Pearson's Phi = 0.760, respectively) at Guabari. Increased frequency of some alleles or novel alleles was seen in summer season (Fig. 3.4.1). Only a weak association was noted for MAD20 (Pearson's Phi = 0.587). Table 3.4.4 summarises the statistical analysis of comparison in allele distribution between the different seasons.

Table 3.4.4: Statistical comparison of distribution of alleles

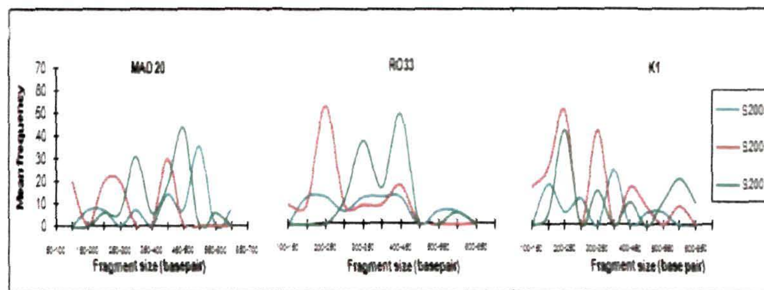
Comparison between transmission seasons at Guabari (*p values*)			
	MAD20	RO33	KI
W2005-S2006	0.282	0.938	0.227
S2006-W2006	0.007	0.008	0.118
W2006-S2007	0.001	<0.0001	<0.0001
W2005-W2006	0.121	0.004	0.009
S2006-S2007	0.041	0.195	0.004
Comparison between years at KTE (*p values*)			
	MAD20	RO33	KI
S2005-S2006	0.025	0.225	0.322
S2006-S2007	0.02	0.002	0.142
S2005-S2007	0.022	0.188	0.552

The temporal variation in allele distribution at Guabari was compared between the different transmission seasons i.e. between winter (W) and summer (S), between the two winters and between the two summers. At KTE comparison was done between the three transmission years by using two-tailed Smirnov-Kolmogrov test. Significant differences in allele distribution were noted with transition from winter of 2006 (W2006) to summer of 2007 (S 2007) but not with transition from winter of 2005 to summer of 2006. KI allele distribution was different between the two summers and the two winters, whereas MAD20 and RO33 distributions varied with transition of season between summer of 2006 and summer of 2007. At KTE, allele fluctuation of MAD 20 was markedly different in each year and KI was found not to vary.

Interestingly, there was marked variation in allele distribution with transition in transmission season being more pronounced in the period spanning summer of 2006 to summer of 2007 (Fig. 3.4.1). Progression from winter of 2006 to summer of 2007 was accompanied by changes in allele frequency of all the three allelic families. Surprisingly, allele composition of RO33 between the summer of 2006 and of 2007 was strikingly similar and was in contrast to MAD20 and KI where distribution of allele frequency as well as allele size was different between the two summers (Fig. 3.4.1 and Table 3.4.4). This observation neither was significant considering that allele composition of neither summer of 2006 nor of 2007 was similar with winter of 2006 the period between the two said summers.



(A)



(B)

Fig 3.4.1: Temporal variation in size polymorphism of the MSP1 alleles at Guabari (A) and KTE (B).

A remarkable finding was the appearance of the 380 base pair fragment of RO33 exclusively in the periods of peak transmission in the summers, particularly in 2007 when it accounted for more than 50% of all RO33 alleles. Complete absence of this allele of RO33 during the winter months of the two study years emphasised the effect of season on clonal composition. Further, the mean parasitaemia of infections having the 380 base pair allele was 9595.435 ± 23845.32 (range 680 to >100,000) parasites/ml against the summer season mean parasitaemia of 12178.2508 ± 22082.5967 parasites/ml. Unlike RO33 and KI allelic families, size polymorphism for MAD20 allelic family was limited and 450–550 base pair alleles of MAD20 were most dominant at Guabari and were seen uniformly in all transmission seasons. In order to understand the effect of transmission intensity on genotype frequency and clonal fluctuation, we pooled the observations of winter months and of the summer months of the two study years and analysed for transmission intensity associated variation. An interaction between allele frequency and transmission intensity was evident for RO33 ($p < 0.0001$) and KI ($p = 0.002$) allelic families of MSP-1 by Chi square analysis. The allelic frequency was also found to be different between the dry low transmission period and the wet high transmission period (MAD20, $p = 0.024$; RO33, $p = 0.001$ and KI, $p = 0.026$). Further, many of the alleles seen in summer months were not seen in the winter months for all the allelic families indicating that the

allelic composition as well as allele frequency was related to transmission season.

At KTE, association between frequency of alleles and transmission season was noted for MAD20 and KI allelic families of MSP-1 ($p = 0.004$ and $p = 0.002$, respectively). Allele composition of only MAD20 was found to be different in each study year while that of KI did not vary at all in the three summers. For RO33 interaction between sampling period and allele frequency was found to be weak ($p = 0.07$, C.I. = 93%) and its allelic composition differed between summer of 2006 and 2007 (Table 3.4.4). At KTE, it was not so much the appearance or disappearance of some genotypes but more consistent observation was that the not so dominant genotypes in 2005 became dominant in 2007 indicating a circulating genotype pool of *P. falciparum.*

3.4.2 Spatial variation

Significant interaction between size polymorphism and study site was seen for all three allelic families of MSP-1 by Chi square analysis (MAD20, $p = 0.000$; RO33, $p = 0.002$ and K, $p = 0.017$). Size polymorphism of the KI allelic families at Guabari village was distinctly different from that at KTE (Fig. 3.4.2). In addition, the allele frequencies were different for MAD20 ($p = 0.001$), RO33 ($p = 0.053$) and KI ($p = 0.013$) between the two sites. At the sites MAD20 alleles in the size range 400–550 base pair were found to be dominant and were seen in 55.33% at Guabari and 40% at KTE of the population respectively (Fig. 3.4.2). Though a large number of alleles were common to the sites, some site-specific alleles were also noted. Clearly, the clonal composition of *P. falciparum* in Guabari and KTE was different.

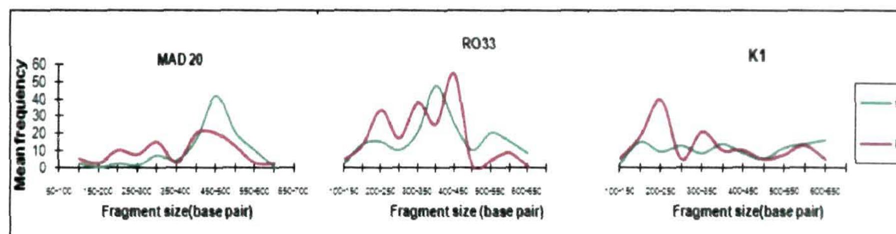


Fig 3.4.2: Spatial variation in size polymorphism of the MSP1 alleles at Guabari (G) and KTE (K).

It maybe noted from Table 3.4.5 that the median age, mean parasitaemia as well as the ethnic composition of the study population was different between KTE and Guabari. Study site ($p < 0.001$) and ethnicity ($p < 0.007$) were identified as risk factors in the probability of having >3 bands per infection by logistic regression analysis. Guabari and KTE were comparable in the probability of having more than three bands per infection. Analysis with respect to ethnicity showed Bengali ethnicity was positively correlated ($p < 0.064$, odds ratio 3.397, confidence interval 95%) with the probability of having >3 bands per infection. Adivasis and Nepalese tended to be negatively correlated but this correlation was not statistically significant. The odds ratio of 1.351 for the Bodo group also indicated the higher risk of this ethnicity having >3 bands per infection. The mean age of these three populations was comparable (Bengalis = 20.66 years, Nepalese = 22.09 years and Adivasis = 21.12 years).

Table 3.4.5: Patient characteristics:

	Guabari	KTE
Median Age	11	26
Febrile cases (> 98.6^{O} F)	91.48%	81.61%
Average parasitaemia	Summer 12178.2508 ± 22082.5967 Winter 2781.47 ± 4329.696	Summer 6066.471±5994.921
M/F ratio	0.925	3.33
Ethnicity (%):		
Bodos	47.42	-
Nepalese	11.34	3.846
Bengalis	29.89	-
Adivasis	10.3	96.15
Assamese	1.03	-

3.4.3 Complexity of infection

The infections tended to be complex both because of presence of more than one allelic family as well as more than one allele of the same family in an isolate. 75.6% and 72.5% of all infections were multiclonal at Guabari and KTE respectively. Multiplicity of infection (MOI) was comparable between the sites (Table 3.4.1). No association between the number of clones and age was noted at any of the study sites though 10–20 year olds at Guabari had the lowest mean number of clones but this was not statistically significant. MOI was higher in the summers at Guabari and in the summer of 2006 at KTE but was found not to be statistically significant.

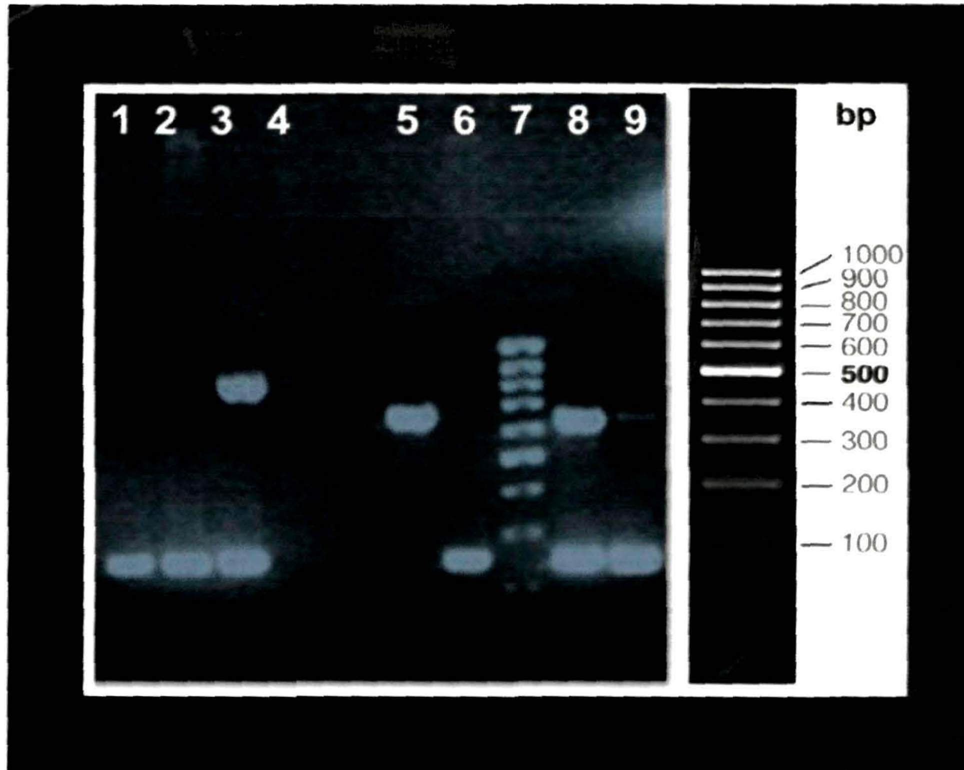


Fig 3.4.3: Nested PCR products:

Lane: 1, 2, 3, 4, 5, 6, 8 & 9:- amplified products of MAD20 allele of block 2 of MSP-1 gene. Lane 7:- 100 bp molecular marker (Fermentas).

3.5 Discussion

In this study we have examined the allelic diversity of *P.falciparum* and attempted to understand if the polymorphism and frequency of alleles of MSP-1 varied with (a) transition in transmission season and (b) differences in malaria epidemiology between sites. Extensive diversity of MSP-1 was seen at the study sites. The actual diversity however, could be much higher as we have not examined the sequence variations. The diversity with respect to size of RO33 seen in the present investigation was higher than the maximum of 4 reported earlier The *P. falciparum* diversity seen in our study was comparable to hyper endemic Gabon and Tanzania[170] but much higher than that reported for most mesoendemic areas[67] other than Ghana[174]. The extent of diversity seen in the present study is in agreement with the diversity reported from Kamrup region of Assam in a recent study[54]. Higher diversity than that expected in isolates from areas of intermediate and low transmission intensity has been reported by earlier investigators[14, 52] while some other authors[175] found no relationship between transmission intensity and parasite diversity. Nonetheless, the observed higher frequency than that expected for our mesoendemic study areas may be attributed in part to the malaria outbreak in the years 2006 and 2007 when transmission was high and could have resulted in the higher diversity. This rationale is consistent with our observation that more alleles of each of the allelic family were present in the high transmission summer season. On the contrary, a higher annual slide positivity rate but lower number of genotypes per isolate at Guabari as compared to Kondoli however, contradicts existence of direct relationship between transmission intensity and allelic diversity. It has also been proposed that allelic diversity of *P. falciparum* may not be entirely dependent on frequency of transmission but also on multiplicity of infection, immune status of the population and drug pressure among other factors[14, 32, 176]. In Guabari village access to health is poor and low socio-economic status of the population precludes effective and complete treatment leading to a level of immunity that allows carriage of some clones, arguably the more competent clones over time.

The observation that a significant percent of people in this region were found to be asymptomatic as observed by us and also by other[177] lends support to the contention that immune status would result in controlling some strains resulting in lower MOI at this site. Drug pressure as confounding factor in *P falciparum* diversity may also be operative in the region. Widespread prevalence of resistance to antimalarials has been reported from districts of Assam[178]. The resistance though widespread is not uniform and a difference in prevalence of K76T mutation between KTE and Guabari, was noted by us and this may have also contributed to the observed difference in extent of diversity seen among the sites. However, it was difficult to ascertain the level of drug use as most medication in these remote areas is provided by unqualified practitioners.

As reported by many other investigators we too did not find any correlation between number of clones and age. However, an indirect evidence for increased number of clones per isolates and propensity for clinical disease was noted as higher mean numbers of clones per isolate were present in summer season during the malaria outbreak period and is in agreement with an earlier study[58] where direct correlation was seen between number of clones and clinical illness due to *P. falciparum* infection was noted. In contrast, others found increased MOI to be related to asymptomatic *P. falciparum* infection[64]. Increased proportion of isolates with RO33 in high transmission season in both the years at KTE as well as Guabari indicated a role of RO33 in clinical malaria outcome. An interesting observation in our study at Guabari was the presence of 380 base pair allele of RO33 exclusively during peak transmission season for two consecutive years, raising the possibility of association of this allele with *P. falciparum* virulence at Guabari. Association of an allele or an allelic family of MSP-1 with disease severity has been demonstrated in earlier studies[58, 60, 66, 179, 180] but differs from this study where not only was an allele frequently seen in patients with high parasitaemia but also present only during high transmission period. However, unlike other studies where RO33 was seen to be dimorphic or up to four bands[175], the higher number of fragments seen in our study cannot be explained

as we neither sequenced these nor allele specific hybridisation was done. It may, however, be mentioned here that only 2–4 fragments were dominant at a site.

Our studies demonstrated a distinct difference in clonal composition as well as allele frequency with transition of transmission season. Our findings are in agreement with Mercereau- Puijalon and co-workers[67] who also noted a variation in frequency of the allelic families with transition in transmission season at Ndiop village, Senegal. Temporal variation in distribution ofMSP-1 genes has been reported from earlier studies in Brazil[181], Solomon Islands[55] and Tanzania[182]. The investigations at Guabari in the present study differ from the earlier studies in sampling in that at this site samples were drawn from a population regularly over a period of two years spanning periods with different transmission intensity. Complex infections with novel genotypes in summer tend to support the modification of *P. falciparum* genetic structure either because of sequestration or presence at undetectable levels during winter or introduction of new clones. Meiotic recombination as well as size polymorphism due to deletion/insertion of repeats leading to generation of novel genotypes particularly at high transmission has been reported by earlier workers[32, 182]. Reappearance of 380 base pair allele of RO33 in summer of 2007 after being completely absent in the intervening winter of 2006 suggests presence at undetectable level.

Evidence in our study indicated that allele distribution was also site dependent. Some alleles were common to all the three sites but some were unique to a site. Site-specific alleles is not an unexpected finding and has been reported earlier[183] and attributed to various factors. The ethnicity of our study groups at the study sites varied, while at KTE the population was tea tribes, at Guabari village it comprised of Bodo-Kachari group, Nepalese, Assamese and Bengalis and some Adivasis. The tea tribes are predominantly Adivasis and belong to the Austro-Asiatic tribal groups while the Bodo-Kacharis belong to the Tibeto- Burman tribal group[184]. The two groups differ among other factors in haemoglobin type[185]. As MSP-1 is involved in invasion of RBC it could be argued that some antigenic characteristics would better support *P. falciparum*

survival in a population and hence it is plausible that the observed variation in allele diversity at the study sites may in part be related to differences in ethnicities of study population. Ethnicity as a risk factor in the probability of having >3 bands per infection tends to favour this contention. Higher MOI as a factor in *P. falciparum* diversity has been reported earlier[182]. Further, Guabari with a mixed ethnic population where Bengalis and Assamese were likely to have more than three bands per infection could impact the MOI and the diversity.

In conclusion the present study indicated that allelic polymorphism was related to transmission season but not entirely dependent on it. The temporal variation in allelic composition and proportion of clones with an allelic family differed among the study sites. Other factors particularly the host factors appeared to influence allelic diversity. Appearance of an allele of RO33 exclusively in periods of high transmission and seen more frequently in isolates with high parasitaemia at Guabari raises the possibility of its association with *P. falciparum* virulence.

CHAPTER IV:

Antibody response to allelic variants of 19kDa fragment of MSP-1: recognition of a variant and protection associated with ethnicity in Assam, India

4. Antibody response to allelic variants of 19kDa fragment of MSP-1: recognition of a variant and protection associated with ethnicity in Assam, India

4.1 Abstract:

Evidence suggests association of anti MSP-1_{19} antibodies with protection from clinical malaria. However, the target epitope was reported to vary with respect to response to conserved or variant epitopes in different studies. We have investigated here humoral response of naturally exposed individuals of Tibeto-Burman and Austro-Asiatic ethnic groups to E-TSR and Q-KNG variants of MSP1_{19} in comparison to whole merozoite extract (WME) of local strain of *P. falciparum* in a longitudinal prospective cohort study. The association of antibodies in relation to risk of infection and disease severity was determined.

A relatively lower seropositivity to MSP-1_{19} peptides derived from 3D7 and FVO strains in comparison to whole merozoite extract of local *P. falciparum* strain was observed. Recognition of Q-KNG variant was markedly lower in TB ($p<0.0001$) indicating a role of ethnicity. The Tea tribes of Austro-Asiatic affinity had higher antibody response (E-TSR; $p = 0.038$ & Q-KNG; $p = 0.004$) and equally recognised the two variants. A reduced risk of clinical infection in high transmission summer season was seen in presence of anti MSP-1_{19} antibodies ($p = 0.013$) and antibody level was predictive of risk of clinical malaria (ROC=0.729). Anti E-TSR antibodies were inversely associated to disease severity at KTE ($\lambda^2 p = 0.013$; t test $p = 0.032$).

The present study demonstrated antibody response to MSP-1_{19} was associated with protection from frequent episodes of malaria and disease severity and that the host genetic background was important factor in response to MSP-1_{19} allelic variant.

4.2 INTRODUCTION

Merozoite Surface Proteins (MSPs) are leading blood stage vaccine candidate molecules of *Plasmodium falciparum*. MSP-1 a major surface protein of the parasite, is synthesized as a 190 kDa precursor and is proteolytically cleaved to yield four fragments of which only the 19 kDa C-terminal fragment remains anchored on the merozoite surface during erythrocyte invasion[76]. Antibodies targeted to this fragment are shown to inhibit erythrocyte invasion by preventing secondary processing that releases this fragment from the rest of the MSP-1 complex[30]. MSP-1_{19} fragment is relatively conserved with few single nucleotide polymorphisms (SNPs) identified in its two epidermal growth factor like (EGF-like) domains[186]. At least six SNPs have been reported, one in the first EGF like domain at amino acid position 1644(Q/E) and five in the second EGF like domain corresponding to positions 1691(K/T), 1699(S/N), 1700(N/S), 1701(G/R) and 1716(L/F). Polymorphisms at positions 1691, 1700 and 1701 have been implicated as particularly important in determining allele specificity of anti MSP-1_{19} immunity[31]. Antibodies to MSP-1_{19} fragment have been reported to recognize cross reactive as well as allele specific epitopes[98, 99]. Further, other investigators observed that certain MSP-1_{19} polymorphisms might be more relevant to cross reactive immunity[31].

In context of MSP-1_{19} based vaccine molecule, Takala and Plowe[32] emphasized on the need for a polyvalent MSP-1 vaccine that incorporates allelic variants or focuses on residues more relevant to cross protection. Nonetheless, it is important to understand the circulating alleles of MSP-1 and the immune response to them as illustrated by FMP1 vaccine trial in Kenya. Antigen based on C-terminal 42 kDa MSP-1 fragment from 3D7 strain of *P. falciparum* showed lack of clinical efficacy in phase II pediatric trial conducted in Kenya[187] and the low prevalence of 3D7 allele at this site[188, 189] was considered as the reason for failure of the vaccine[32]. Further, Ferreira *et al*[15]. reported vaccine induced selective effect resulting in selection for clinical infections with nonvaccine type parasites in vaccinated individuals. Considering the role of natural acquired

immunity in antigen polymorphism, it is important to understand the immune responses that confer protection.

Assam in northeast India is endemic for malaria. The endemicity of malaria is not uniform with many pockets along forest fringes, forest and foothill villages particularly along the inter-country/inter-state border are vulnerable to malaria outbreak[5]. Interestingly, most of these affected areas report widespread resistance to anti- malarial drugs[178]. Evidence indicates presence of artemisinin resistant *Plasmodium falciparum* parasites on the Thai-Cambodian border and on the Myanmar-Thailand border[190]. Considering the proximity of Myanmar to Northeast India, it poses a major threat to malaria control in the state of Assam and thus it is anticipated that malaria vaccine could be deployed in this region as and how it becomes available. However little is known about the antigen structure of the local parasite and the immune responses of the local population and hence it was considered important to analyze these. In our earlier study on genetic diversity of block 2 of MSP-1 we reported extensive diversity of MSP-1 in this region, which showed spatial and temporal variation between the transmission seasons[191]. In the present study, we have investigated the antibody response to 3D7 (E-TSR) and FVO (Q-KNG) allotypes of MSP-1_{19} which are components of vaccine molecules under trials with the aim to 1) determine the recognition of these variants by the local population 2) relation of response to these variants in comparison to whole merozoite extract (WME) of local strain with risk of infection and disease severity to evaluate the response as immune correlate of protection.

4.3 Materials and Methods:

4.3.1 Study sites and study population

The study was conducted at Guabari village and Kondoli Tea Estate. The details of the sites and the study population have been described in Chapter 3.

4.3.2 Study design & Sample Collection

The study was designed as a prospective cohort study with samples collected during winter of 2006 to winter 2007 i.e. period spanning pre and post high transmission summer season of 2007. For the purpose of stratifying

individuals according to clinical episodes of malaria and clinical manifestations, our data of three years from January 2006 to December 2008 was taken into account. On the basis of number of clinical episodes of malaria in the last 3 years, the participants were classified as infrequent (≤ 2 episodes) or frequent malaria (> 2 episodes) groups. A malaria episode was defined as having fever >38°C and blood smear positivity. Multiple episodes within 28 days were counted as recrudescence. Further, recrudescence was confirmed by genotyping for MSP-1 and MSP-2 genes of the parasite. Based on the clinical manifestation of malaria, the participants were stratified into complicated and uncomplicated malaria cases. Complicated malaria was defined following WHO guidelines with parasitaemia >5000/μl of blood and at least one of the following conditions: unrousable coma, convulsions (more than two episodes in the preceding 24 hr), severe anemia (hemoglobin<7.0 g/d), respiratory distress and prostration. Uncomplicated malaria included parasitaemia ≤ 5000/μl of blood, fever, headache, body ache and other mild symptoms or asymptomatic cases of the disease. Sample size was calculated on the basis of variance observed in the pilot study, keeping α_1 as 0.05 and α_2= 0.02 and a power greater than 95 percent.

Blood samples were obtained by finger prick method and collected in tubes containing EDTA. Serum was separated from the blood samples by centrifugation and stored at -20°C until used. Individuals were tested for *Plasmodium falciparum* infection using Rapid Diagnostic Kits followed by microscopic examination of thin and thick blood smears. The study was approved by Tezpur University Ethical Committee (TUEC).

4.3.3 Preparation of crude antigen

A local strain of *Plasmodium falciparum* which was in vitro cultured in O+ human RBCs up to 10% parasitemia was used to prepare the antigen. Antigen preparation was as per Blomberg *et al*[192]. Erythrocytes from the culture were pelleted, parasitized red blood cells (pRBC) separated on 60% Percoll and lysed using Tris NH_4Cl (pH 7.6). After centrifugation at 2000g for 10 min, the pellet was resuspended in minimal PBS and sonicated for 2 minutes in pulses of 30°C on ice. Protein estimation was done using BSA (Bovine serum albumin) as

standard following Lowry's method. *Plasmodium falciparum* uninfected erythrocytes preparation following the same procedure was used as control.

4.3.4 Recombinant peptides

Recombinant peptides for the conserved C- terminal domain of MSP-1_{19} were obtained as a kind gift from MR4 (Malaria Research Center, Massachusetts, USA). MRA 49 represents the E-TSR version of the carboxy terminus of MSP-1 from *P. falciparum* 3D7 strain fused to P30P2 and MRA 53 represents the Q-KNG variant of the carboxy terminus of MSP-1 from *P. falciparum* FVO strain fused to P30P2.Two other antigens, MRA 56 and 58 also corresponding to the Q-KNG and E-TSR variants were used for validation of the results.

4.3.5 Elisa

Antimalarial antibody was assessed using ELISA technique[193]. Briefly, 96-well microtitre plates (Maxisorb, Nunc, Denmark) were coated at 4°C overnight with 50 µl of recombinant proteins (1µg/ml) or crude extract (101µg/ml) in carbonate coating buffer (pH 9.6). Plates were blocked using 0.5% casein in coating buffer for 3 hours. 50 µl of 1:250 diluted serum samples in PBS (P^H 7.4) were added to duplicate wells and incubated for 1h at 37°C. The plates were subsequently developed with 50 µl of protein G-HRP conjugate (Sigma Aldrich ,USA) for 1h at 37°C followed by 3,3'55'-tetramethylbenzidine (Sigma Aldrich, USA) for 30 min. The reaction was stopped with 1N H_2S0_4 and read at 450 nm with an Ascent Multiscan reader. Twenty individuals who had never been exposed to malaria served as the negative control. O.D was taken in duplicate readings. The mean optical density (O.D) plus three standard deviations of the 20 negative controls was used as the cutoff reading. For determination of IgG subclasses (IgG1 to IgG4) monoclonal mouse anti human IgG subclasses (Sigma Aldrich, USA) were used. The total IgG and IgG subclasses antibody level were expressed as ratios (arbitrary units) which were calculated by dividing the mean optical density of a test sample by the mean optical density plus 3 standard deviations for the 20 negative controls.

4.3.6 *Statistical analysis*

Statistical analysis was performed using Excel Stat Software, 2010 version. Data was analyzed with respect to study sites, ethnicity, disease status and frequency of disease using Chi square (χ^2) test. Proportion analysis was done using z test for two proportions and k proportion. Pearson's correlation coefficient was used to correlate antibodies titre with age. Mean titre differences were determined by Student's t test. Logistic Regression modeling was done to identify the risk factors in disease outcome and frequency of malaria episode.

4.4 *Results*

A total of 300 individuals were examined for their antibody response against Q-KNG and E-TSR variants of MSP-1_{19} and to whole merozoite extract (WME). Approximately 61% of the study subjects were from Guabari village and adjacent areas and 39% were from Tapitjuri subdivision of KTE (Table 4.4.1).

Table 4.4.1: Characteristics of the study participants

	Guabari (n=183)	**KTE (n=117)**
Median Age (min-max) years	18(1.11-70)	25(0.4-60)
Male/Female ratio	1.05	1.58
Ethnic group (%)		
Tibeto-Burman (TB)	68.8	8.6
Indo European (IE)	18.6	4.3
Tea tribe (TT)	12.6	87.1
% complicated malaria	21	26
% uncomplicated malaria	73.08	74.01
% Frequent malaria	62.05	49
% Infrequent malaria	32.2	51

Episodes of malaria with P.vivax were not considered while determining the frequency of episodes. Parasitological cutoff for complicated malaria was

>5000/μl; for uncomplicated malaria it was ≤ 5000/μl. 11 individuals from Guabari were lost to follow up due to migration or withdrawal from the study.

Antibodies to E-TSR, Q-KNG and WME could be detected in 38%, 33% and 88.3% of the study population respectively. The mean antibody titre was noted to be 4.858 ± 0.315 for E-TSR variant, 5.231 ± 0.484 for Q-KNG variant and 2.2 ± 0.060 against WME. Though a higher number of individuals were positive for anti WME but the titre of antibody to it was lower in comparison to the two MSP-1_{19} peptides. Intriguingly, only 15% of the population recognized both the MSP-1_{19} variants and 30% of the population could not recognize any of the MSP-1_{19} variants. Antibody recognition and antibody titre was seen to increase with age though a small correlation coefficient indicated weak correlation and gradual acquisition of the antibodies (Fig 4.4.1).

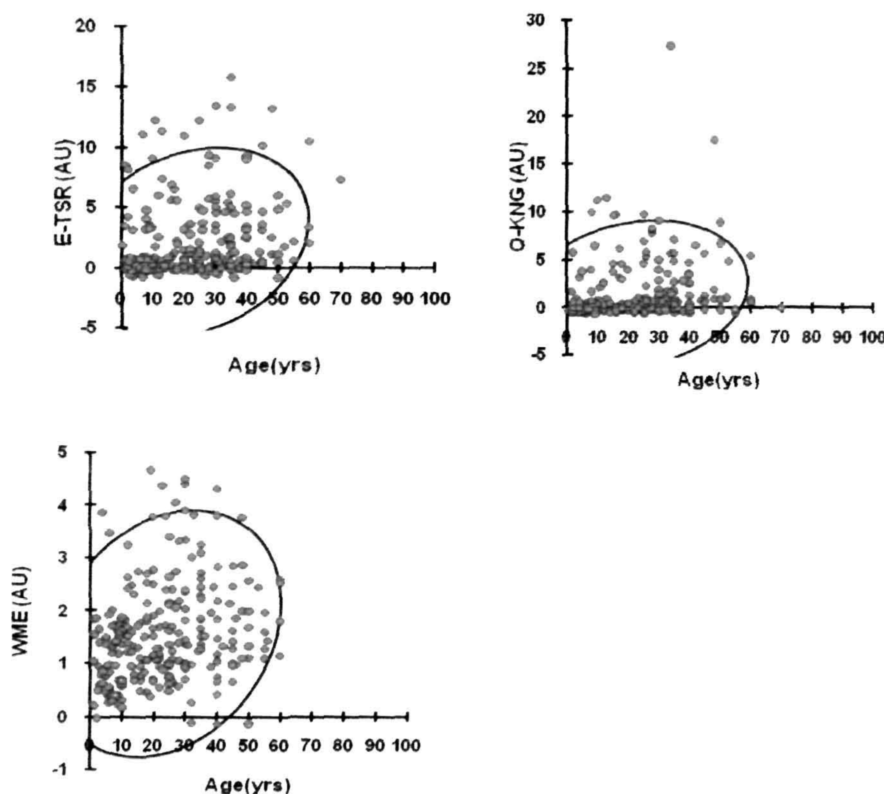


Fig 4.4.1: Scatter plots depicting correlation of antibody titre with age.

Pearson's correlation coefficient (R^2) for WME = 0.060, p<0.0001, E-TSR = 0.055,p=0.0002, Q-KNG = 0.034,p=0.003.

Comparison of paired samples collected at six months interval showed a decrease in antibody titre with time but it was not statistically significant (Fig 4.4.2). Recognition of E-TSR variant was also associated with transmission season with an increase in number of positive responders in the high transmission summer season (p = 0.020).

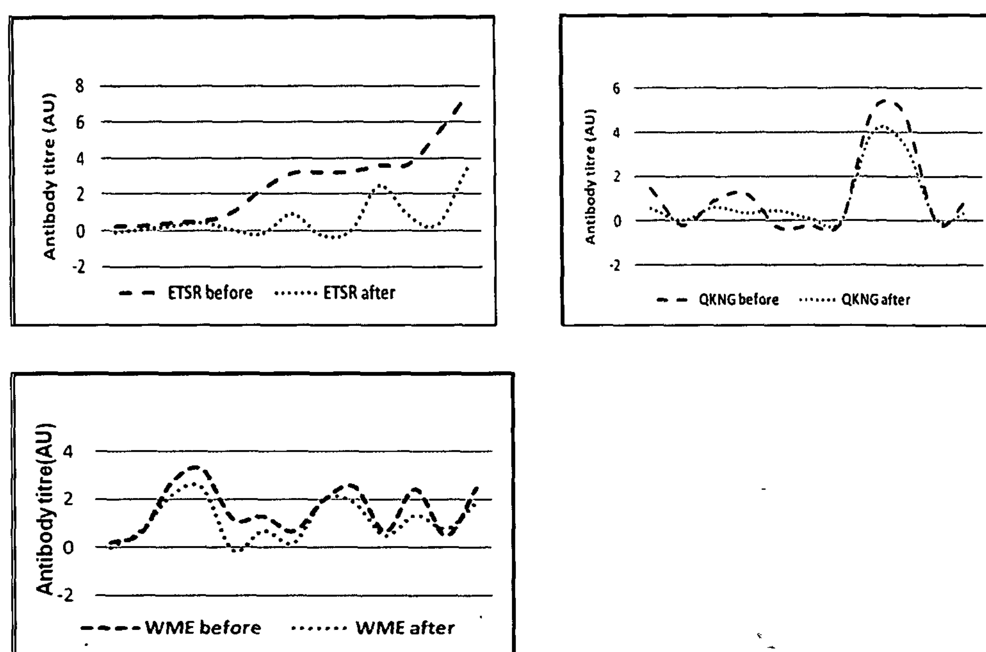


Fig 4.4.2: Comparison of antibody titres in repeat samples taken at six months interval.

4.4.1 Comparison of antibody response at the two sites

At KTE, recognition of Q-KNG and E-TSR variants was comparable (QKNG = 45.26% & ETSR = 50.54%). Anti WME antibody was seen in 92 % of the participants with a mean titre of 1.945 ± 0.789. The proportion of positive responders and titre of antibodies to E-TSR and to Q-KNG increased with age (ETSR p = 0.015; QKNG p = 0.0003, Table 4.4.2). At Guabari, recognition of Q-KNG variant was markedly lower (17.45%) in comparison to E-TSR (37.43%) though the titre of antibodies to the two variants was comparable. Anti WME antibody could be detected in 85 % of the participants with a mean titre of 2.44 ±

0.872. Recognition as well as antibody titre to WME and E-TSR variant was noted to increase with age but not for Q-KNG at this site (Table 4.4.2).

Table 4.4.2: Antibody response with respect to age at the study sites.

Antigen	**Guabari** 0-14 n=81	15-29 n=42	≥ 30 n=60	p values (k proportion)	Pearson's correlation coefficient(R^2)
Q-KNG	0.11 (9)	0.21 (9)	0.22 (13)	0.225	0.025
E-TSR	0.28 (23)	0.34 (14)	0.58 (35)	**0.005**	0.046
WME	0.5 (41)	0.62 (26)	0.85 (51)	**0.003**	**0.107**
	KTE				
Antigen	0-14 n=37	15-29 n=33	≥ 30 n=47	p values (k proportion)	Pearson's correlation coefficient (R^2)
Q-KNG	0.27 (10)	0.44 (15)	0.62 (29)	**0.015**	0.049
E-TSR	0.21 (8)	0.6 (20)	0.7 (33)	**0.0003**	0.08
WME	0.65 (24)	0.73 (24)	0.77 (36)	0.574	0.018

Numbers in the brackets denote case number for each group

Proportion of individuals positive for each tested antigens in the different age groups was compared by k proportion test. At Guabari, the positive responders increased significantly with age against E-TSR and WME while at KTE it was against both the MSP-1_{19}peptides

As Guabari has a heterogeneous population with people of different ethnic origin, data was analysed in context of ethnicity of the population. Low seropositivity to the two $MSP1_{19}$ peptides, particularly for Q-KNG variant (p = 0.033) was seen among the TB.

In comparison to Guabari a relatively higher proportion of subjects from KTE had anti WME as well as anti MSP-1_{19} variants antibodies (p = 0.002). Though the recognition of Q-KNG was low at Guabari but the mean antibody titre was comparable between the two sites (Fig 4.4.3).

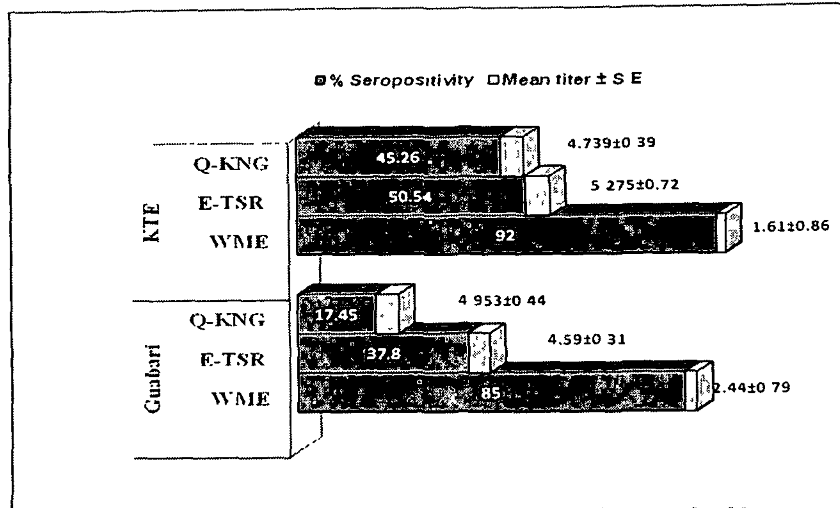


Fig 4.4.3: Comparison of antibody response between the two sites.
Though the seropositivity was much lower at Guabari for Q-KNG variant, the anti Q-KNG titre between the sites was comparable.

4.4.2 Antibody response and association with Ethnicity.

As the two study sites differed in population demography amongst other factors, the response to MSP-1_{19} peptides and WME was also analyzed in context of ethnicity of the population. Recognition of MSP-1_{19} peptides and WME was associated with ethnicity of the study population ($p = 0.013$), with a higher proportion of TT recognizing WME and the MSP-1_{19} peptides (Table 4.4.3). The negative responders particularly with respect to Q-KNG variant predominantly belonged to the TB irrespective of the study site. The 8.6% TB population at KTE also could not recognize the Q-KNG variant. Of the total negative responders, 59.16% of them belonged to TB. The antibody titre to both the MSP1_{19} variants was higher in TT (ETSR; $p = 0.038$ & QKNG; $p = 0.004$).

In order to differentiate between the interactions with site from interaction with ethnicity, we analyzed the pattern of response of TT belonging to Guabari as opposed to TT of Kondoli and found that recognition and titre of antibody to an antigen by TT was independent of the study site emphasizing the contribution of ethnicity in antigen recognition (Table 4.4.4).

Table 4.4.3: Comparison of antibody response between the two ethnic groups

Antigen	Proportion of seropositive individuals			Mean antibody titre (AU) ± S.E		
	Tea tribe (TT)	Tibeto-Burman (TB)	TT& TB (p)	Tea tribe (TT)	Tibeto Burman(TB)	TT& TB (p)
E-TSR	0.43 (52)	0.32 (45)	0.081	2.600±0.326	1.724±0.267	0.038
Q-KNG	0.44 (53)	0.14 (20)	<0.0001	2.072±0.344	0.874±0.188	0.004
WME	0.71 (86)	0.51 (70)	0.001	1.613±0.082	1.499±0.096	0.366
no. of samples	122	138				

Numbers in the brackets denote case number for each group.

The table depicts the Z proportion analysis of seropositive individuals among the ethnic groups and the mean titre differences between them as by t test. IndoEuropean (IE) were not considered for the analysis due to small sample size (n=40).

Table 4.4.4: Comparison of antibody response of Tea tribes of Guabari and KTE.

Proportion of seropositive TT individuals			
Antigen	Guabari (n=23)	KTE (n=86)	p value
E-TSR	0.478 (11)	0.511(44)	0.776
Q-KNG	0.4(9)	0.47(40)	0.335
Mean antibody titre (AU) ±S.E			
Antigen	Guabari	KTE	p value
E-TSR	2.25±0.34	2.7±0.38	0.497
Q-KNG	1.97±0.095	2.1±0.24	0.894

Numbers in the brackets denote case number for each group.

The table depicts the comparison of seropositivity and antibody titre to the two antigens of Tea tribes belonging to Guabari and KTE. It may be noted that there was no difference in either the seropositivity or the titre between the Tea tribes of two sites.

4.4.3 Antibody responses and association with clinical malaria outcome and frequency of malaria episodes.

At KTE, proportion of positive responders as well as mean antibody titre to E-TSR variant was higher in uncomplicated cases in comparison to complicated malaria (Table 4.4.5A). Anti WME antibody titre was negatively associated with frequent episodes of malaria (p = 0.04) at Guabari (Table 4.4.5B). Antibody response to MSP-1_{19} peptides was positively associated with frequent malaria at KTE (Q-KNG p = 0.005, E-TSR p = 0.016) and 83.3% of these persons did not have clinical malaria at the time of sample collection. Increased titre may be seen as an outcome of frequent antigenic stimulation.

Table 4.4.5: Antibody response in relation to frequency of malaria episodes and disease severity at KTE (A) and Guabari (B).

	Antigen	Frequent Cases (n = 57)	Infrequent case (n= 60)	p values	Complicated cases (n= 30)	Uncomplicated Cases (n= 87)	p values
Seropositivity	E-TSR	0.619	0.4	0.049	0.29	0.578	**0.013**
	Q-KNG	0.526	0.34	0.087	0.375	0.484	0.359
	WME	0.77	0.66	0.293	0.75	0.716	0.79
Mean titre ± S.E	E-TSR	3.52± 0.23	1.73± 0.02	**0.016**	1.54± 0.26	3.19± 0.28	**0.032**
	Q-KNG	2.85± 0.21	1.058±0.09	**0.005**	1.91± 0.02	2.076± 0.04	0.83
	WME	1.71± 0.16	1.2 ± 0.12	0.06	1.36± 0.05	1.64± 0.02	0 225

(A)

	Antigen	Frequent cases (n = 114)	Infrequent cases (n= 59)	p values	Complicated cases (n= 38)	Uncomplicated cases (n= 134)	p values
Seropositivity	E-TSR	0.4	0.43	0.701	0.47	0.412	0.581
	Q-KNG	0.191	0.2	0.909	0.227	0.176	0.572
	WME	0.63	0.619	0.906	0.66	0.597	0.58
Mean titre ± S.E	E-TSR	2.12±0.05	1.53±0.018	0.56	2.19±0.09	1.88±0.05	0.92
	Q-KNG	1.27±0.019	0.89±0.012	0.53	1.05±0.02	1.12±0.06	0.53
	WME	1.01±0.04	1.82±0.0.8	**0.04**	1.39±0.08	1.49±0.02	0.69

(B)

Proportion of individuals positive for an antigen was checked for association with disease using chi test, while mean antibody titre between the groups were compared using Student's t test. Significant values are indicated in bold.

Logistic regression modeling of data from the two sites with clinical episodes of malaria as the outcome predicted the high transmission summer season to be positively correlated with frequent malaria episodes (O.R = 2.488, p = 0.003) but in presence of antibody to the two MSP-1_{19} variants, the probability of frequent episodes of malaria in summer season decreased in older age group individuals (>14 years) (O.R = 0.969, p = 0.013, Table 4.4.6) suggesting antibody mediated protection from risk of malaria. Also, in the younger age group (< 14 years) the probability of having a clinical infection in high transmission season decreased in presence of antibodies to both the E-TSR and Q-KNG variants but it did not reach statistical significance. Interestingly, comparison of antibody titre between the young and the old age groups showed a lower mean antibody titre in the younger group against the two MSP-1_{19} peptides (E-TSR p = 0.002, Q-KNG p = 0.0027). Though at KTE antibodies to E-TSR were associated with protection from disease severity, but in the overall data age (p = 0.002) and ethnicity (p = 0.023) were seen as confounding factors in disease severity with the TB at a greater risk of complicated malaria (p =

0.047) by logistic regression modeling of data with disease status as the outcome.

Table 4.4.6: Logistic regression analysis showing the association of parameters with malaria outcome and frequency of malaria episodes

Complicated vs Uncomplicated cases					
Parameter	Standardized Coefficient	Wald (χ^2)	p value	Odds ratio (O.R)	95% CI (O.R)
Age	0.046	9.416	0.002	1.047	1.016-1.078
Ethnicity		7.505	0.023		
Tibeto-Burman	0.776	3.785	0.047	2.173	0.994-4.750
Frequent vs Infrequent cases					
Parameter	Standardized Coefficient	Wald (χ^2)	p value	Odds ratio (O.R)	95% CI (O.R)
Transmission season		9.403	0.002		
Summer season	0.912	8.743	0.003	2.480	1.359-4.552
E-TSR AU* Q-KNGAU*Season		6.869	0.008		
[a]E-TSRAU* Q-KNGAU*Summer	-0.410	6.214	0.012	0.960	0.945-0.992
[b]E-TSRAU* Q-KNGAU*Summer	-7.459	0.916	0.339	0.469	0.099- 2.212

The TT population was taken as reference for the TB and winter season against summer season. ([a]) indicates the analysis in the older age group (>14 years) while ([b]) in the younger age group ($\leq$ 14 years).

4.4.4 Isotyping

The contribution of any subclass of immunoglobulin was checked for positive samples against the E-TSR and the Q-KNG variants respectively (Fig 4.4.4). The frequency of positive responder to E-TSR variant with IgG2 and IgG3 was higher (p = 0.015). Further, the mean titre of anti E-TSR IgG3 was lower in IE in comparison to TT (t = 2.101, p = 0.044). No such significant difference was found for the Q-KNG variant even though the frequency of IgG4 subtype was more than the other subclasses. No significant association in

distribution of the IgG subclasses was found with frequency of malaria episodes and disease complication. Age was not seen to contribute towards the isotype response.

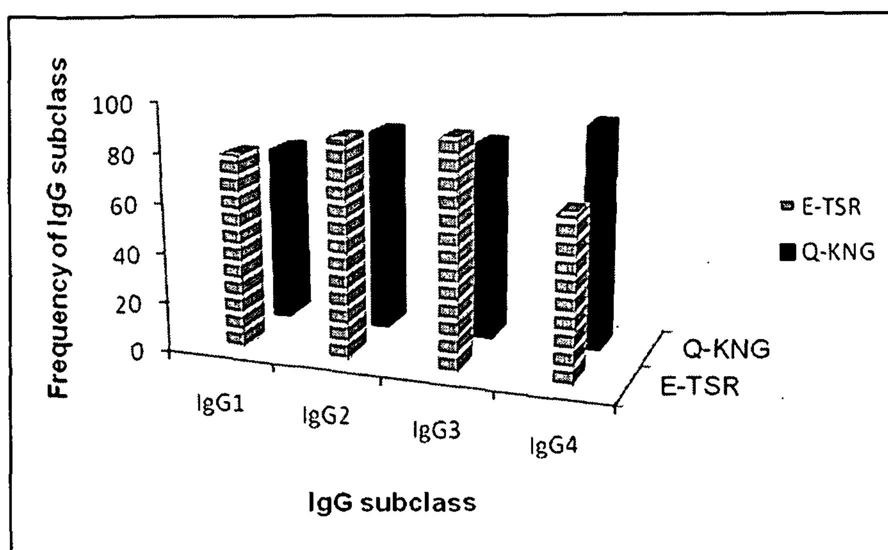


Fig 4.4.4: Frequency of IgG subclass of antibody to the $MSP1_{19}$ peptides .
A higher IgG3 and low IgG4 response was seen to E-TSR variant (p = 0.015).

4.5 Discussion

The aim of the present study was to evaluate the antibody response to 3D7 and FVO allotypes of $MSP\text{-}1_{19}$, a vaccine candidate molecule of *P. falciparum* in reference to WME of local strain and the relation to protection from malaria. The frequency of antibody responders to the two $MSP\text{-}1_{19}$ peptides was similar to that reported in earlier studies from some malaria endemic regions[94, 97] but low as compared to others [95, 98]. The study population showed an age dependent increase in antibody response though acquisition of the antibodies was gradual. Similar observations on age related acquisition of antibody response have been reported by earlier investigators[91, 194].

A striking observation in the present study was marked difference in recognition of the antigens between the two sites with low seropositivity of Q-KNG at Guabari and a relatively higher seropositivity for WME and $MSP1_{19}$

peptides at Kondoli. Difference in antibody response to the two variants of MSP-1_{19} could be related either to host genetics, difference in allotypes of MSP-1 prevalent at the two sites or to transmission dynamics. The population of Guabari consisted of the Bodo-Kachari tribes and Nepalese of the Tibeto-Burman, Tea tribes of Austro-Asiatic, Bengalis and non-tribal Assamese of Indo European affinities respectively. At KTE, the population belonged predominantly to the Mundari speaking Tea tribes community of Austro-Asiatic affinity. We observed a distinct difference in the antibody response between the ethnic groups to the two MSP-1_{19} peptides where a majority of the negative responders to Q-KNG at Guabari belonged to the Tibeto-Burman ethnic group. Further, comparison of antibody response of TT from the two sites was seen to be independent of site, emphasising ethnicity dependent antibody response to Q-KNG variant. Ethnicity was also predicted as a confounding factor in disease severity with TB at greater risk by logistic modelling of data ($p = 0.047$, Table 4.4.6). Based upon the genetic variations found on the Y-chromosome, mitochondrial and microsatellite DNA markers, the Tibeto-Burman and Austro-Asiatic population are distinct groups[195]. They also differ in their haemoglobin type with TB having hemoglobin E[185]. Difference in antibody response has been reported for two African tribes, the Fulani and Dogon where the Fula populations made stronger antibody responses to malaria[70]. Okech *et al*[96]. in a study of antibody specificity and resistance to malaria in two unrelated populations suggested genetic regulation in fine specificity of naturally acquired anti MSP-1_{19} antibodies. Genetic basis to malaria susceptibility and clinical symptomology has been demonstrated recently where chromosome 10p15 and 5q31 encoding cytokine genes was linked with malaria fever episodes [196]. Detailed investigations are required for a better understanding of relation of host genetics and antibody response in the TB and TT population, including the HLA profile of these groups.

Alternatively, difference in *Plasmodium falciparum* diversity at the two sites may also contribute to differential recognition of Q-KNG. Our observation

that the Tea tribes population of Guabari was seropositive for Q-KNG variant does not support this argument (Table 4.4.4). Further, in our earlier study on diversity of block 2 of MSP-1, both MAD20 and K1 allelic families were prevalent at the two sites[191].

Logistic regression analysis of data demonstrated relation between antibodies to anti MSP-1_{19} variants and protection from frequent clinical episodes of malaria in the older (>14 years) age group (Table 4.4.6). Presence of anti MSP-1_{19} antibodies was seen to reduce the risk of frequent episodes of malaria in the high transmission season. In fact antibodies titre was seen as a fairly good variable for predicting the odds of having an infection. Though negative association between antibody titre and malaria episodes was seen in the younger age group, but it did not reach statistical significance. This is not surprising as mean antibody titres to the peptides was lower in the young age group and gradually acquired as seen by us and others[101,194]. Our data implies that boosting of anti MSP-1_{19} by vaccination could reduce the risk of infection in younger age group. MSP-1_{19} has been shown to have a critical functional role in invasion of RBC and several studies have demonstrated a role of MSP-1_{19} in protection from clinical episodes of malaria both in mouse model[30] and in field studies[81, 197]. Nevertheless contrasting findings exist for MSP-1_{19} and protection from clinical episodes of malaria[84]. Recently meta analysis of 33 prospective cohort studies established that antibodies to MSP-1_{19} were indicative of protection and that participants with antibodies to MSP-1_{19} had a risk of symptomatic *P. falciparum* that was 18% less than those without detectable IgG[89].

Differences were found between the two sites with respect to the target antigens associated with antibody mediated protection. At KTE, antibodies to E-TSR variant were negatively associated with complicated malaria where seropositivity and the mean antibody titre were inversely realated to disease severity. Consistent with this finding, Elbashir *et al.*[88] reported that the prevalence of antibodies to MSP-1_{19} was lower in severe malaria as compared to

uncomplicated malaria while contrasting results too have been reported[87]. Our observation that anti MSP-1_{19} antibody titre was positively associated with frequent episodes of malaria at KTE would tend to suggest lack of antibody mediated protection. Examination of data however revealed that of the individuals with history of frequent malaria and high antibody titre only 16.7% had clinical malaria at the time of sample collection and the others were asymptomatic indicating the role of antibody in protection. At Guabari, anti WME antibodies could be linked to protection from the risk of infection with a higher mean antibody titre in individuals who suffered less frequent episodes of malaria. Osier *et al.*[198] in a study on Kenya children suggested that natural immunity to malaria resulted from exposure to multiple antigenic targets. In this line protection from clinical malaria episodes could be expected as the response to WME was observed to be a reflection of the total response to many individual antigens in the malarial parasite in an earlier study[199]. Previous studies also indicated that antibodies against single parasite proteins were insufficient to confer protection[200]. Our data suggested the immunodominant epitopes of the local strain could be different and warrants detailed antigenic analysis of the local strain of *P. falciparum.*

Anti E-TSR antibody in our study was predominantly of IgG2 and IgG3 isotype. In contrast to our observation, Shi *et al.*[98] found predominance of IgG3 antibody response to Q-KNG than to E-TSR. Nonetheless, IgG1/IgG3 class switching was seen to independently affected by the nature of the antigen, cumulative exposure to the antigen and the age of the individual[101]. IgG1 and IgG3 isotypes of anti-malarial antibody have been associated with protection in several studies[98,99] however we did not find association of any of the IgG subclasses with frequency of malaria episodes or disease severity. Interestingly our isotyping data indicated ethnicity as one of the factors in differential IgG subclass response with anti E-TSR IgG3 antibodies significantly higher in TT suggesting genetic link to the subclass of antimalarial IgG.

In conclusion, we observed a relatively lower seropositivity to MSP-1_{19} peptides derived from 3D7 and FVO strains in comparison to whole merozoite extract of local *P. falciparum* strain emphasising the need to analyse the local allotypes. The negative responder particularly with respect to Q-KNG predominantly belonged to Tibeto-Burmans population. Among the various factors seen to influence the immune response, the ethnic background of the individuals was strongly correlated to the recognition of MSP-1_{19} variants and to immune response with Tea tribes of the Austro-Asiatic affinity having a better immune response than the Tibeto-Burmans. The antibody response to MSP-1_{19} antigenic peptides could be correlated with protection from risk of infection and disease severity in our population. Considering anti WME antibodies were associated with protection at Guabari and low seropositivity of MSP-1_{19}, warrants detailed analysis of the antigenic structure of the local strain of *P. falciparum* to determine the immunodominant epitopes. Overall, our data supports MSP-1_{19} peptides based vaccines.

CHAPTER V:

Cytokines mediated cellular immune response in Plasmodium falciparum infection

5. Cytokines mediated cellular immune resposne in Plasmodium falciparum infection.

5.1 Abstract

Cytokines are important molecular markers of cell mediated immune response and are associated with clinical complications and protection from malaria. A delicate balance between pro-inflammatory and anti -inflammatory cytokines response was suggested to contribute to the variable outcomes of malaria. However, the outcome of the infection and its association with cytokines is suggested to depend on the balance of the host-parasite interaction. .We have examined here the cytokines gene expression levels in individuals with different symptomology of malaria in comparison to healthy controls and patients with non- malaria fevers to determine the correlation of cytokines with clinical outcome of the disease.

Our data showed an increased expression of IL-2, IL-12α and TGF-β and decreased levels of IL-10 in *Pf* positive individuals. In complicated malaria, cytokines levels of IFN-γ and TGF-β levels were higher while IL-2 and IL-12α showed lower levels of expression in comparison to uncomplicated malaria. Further, modeling of data with complicated malaria as the outcome showed that expression of IL-12α was negatively associated with disease severity. An interaction of the cytokines combination IL-10*IL-12α*TGF-β in presence of IFN-γ was positively associated ($p= 0.038$) while the same combination IL-10*IL-12α*TGF-β along with IL-2 was associated negatively with complicated malaria ($p= 0.046$). We observed an interaction between age and disease severity in expression of TGF-β where levels of TGF-β were negatively associated with age. The expression of TGF-β was significantly different between the TT and TB population ($p=0.048$) with an elevated level of the cytokine among the TT.

In conclusion, our result tends to suggest induction of balanced cytokine response with activation of both Th1 and Th2 cells in *Pf* infection while in complicated malaria suppression of T cell function was indicated. A role of IL-12α in protection and of IFN-γ in disease severity was observed. Increased IFN-γ expression associated with disease severity might indicate role of other arms of immunity namely the innate immune response in pathogenesis of the disease.

5.2 INTRODUCTION

Cytokines have been shown to play an important role in protection as well as in the pathogenesis of malaria[37,38,201]. The optimal immune response to malaria infection has been suggested to be an early and intense pro-inflammatory cytokines with a rapid suppression of this response by anti-inflammatory cytokines[202].

An early and effective pro-inflammatory cytokine response mediated by IFN-γ in the IL-12 and IL-18 dependent manner was associated with resolution of parasitaemia and control of malaria infection [38, 129]. Low levels of IFN-γ induced TNF-α which caused inhibition of parasitaemia and stimulation of phagocytosis to enhance clearance of parasitized erythrocytes[103, 104]. However, the proinflammatory cytokines were also seen to contribute to adverse disease outcome with a strong response of cytokines like TNF, IFN-γ associated with severe disease syndromes in both human and experimental models[38,106,201]. Their production was seen to induce an increased cytoadherence of infected erythrocytes to venular endothelium through upregulation of adhesion molecules[106]. Anti- inflammatory cytokines like IL-10 and TGF-β have been shown to inhibit overproduction of these pro- inflammatory cytokines[33, 38, 103]. TGF-β is known to stimulate T regulatory cells which in turn inhibited IL-2 production and cell proliferation of CD4+ T lymphocytes[201]. However, elevated levels of IL-10 have also been reported in patients with severe falciparum[37, 38, 103].

Studies have shown cytokines to be associated differentially with appearance of disease symptoms[37], levels of parasitaemia[103]and with disease severity and complications[103, 108]. Panels of cytokines have been demonstrated to associate with different clinical manifestations of the disease. In a study in endemic region of central India levels of IL-12, IL-5, and IL-6 discriminated severe forms of malaria from mild malaria while levels of IL-1 β, IL-12, and IFN- γ was relevant for the discrimination of cerebral[108].

However, the outcome of the infection and its association with cytokines is suggested to depend on the balance of the host-parasite interaction[201]. The

immune status of the individuals, parasite load, virulence of the parasite strains, host genetic factors were seen to actively modulate the immune response[201, 203,204] In a study, TGF-β production was associated with early resolution of parasitaemia in mice infected with the nonlethal strain of *Plasmodium yoelii* while in lethal strain infection early TGF-β response failed to clear the parasites[204]. Differences in cytokine response and immunity to malaria have been reported between the Fulani and Mossi tribes of Africa with the Fulani having better immune response[205].

The state of Assam in North East India is endemic for malaria with reports of high malaria related morbidity and mortality[45,46]. The disease is unevenly distributed across the state and associated with varying intensity of malaria transmission and risk factors[46]. Most of the highly affected areas are populated by indigenous tribal populations of different ethnic backgrounds. Studies have reported high genetic diversity of *Pf* existing in the region with development of drug resistant strains of parasite[5,6,54] It is therefore pertinent to study the cytokines mediated cellular immunity in differentiating protection from pathogenesis to understand the host pathogen interaction at play in the population. The present study was thus designed to understand the role of cytokines and its association with disease outcome and to determine immune correlate of protection if any in the population.

5.3 Material and Methods

5.3.1 Study Sites and study population

The study was conducted at Guabari village and adjacent areas and at Kondoli Tea Estate, the details of the sites and the study population has been described in Chapter 3.

5.3.2 Study design

The study was designed as a case control study and conducted during the winter of 2008 and continued till summer of 2009. A total of 58 *Plasmodium falciparum* positive individuals and 30 *Plasmodium falciparum* negative individuals were included in the study. *Pf* negative individuals were further

classified into groups of individuals having non malaria specific fever and healthy controls. Individuals were included in the study irrespective of age, sex and their ethnic backgrounds. *Plasmodium falciparum* positivity was assessed using Rapid Diagnostic Kits and confirmed by microscopy. On the basis of clinical manifestation of malaria, the *Pf* positive individuals were stratified into complicated and uncomplicated cases defined as per WHO guidelines and described in details in Chapter 4. Individuals were also classified into symptomatic and asymptomatic cases according to the presence or absence of clinical sign and symptoms of the disease. Proper medical treatments were given to the study participants. The study was approved by Tezpur University Ethical Committee (TUEC).

5.3.3 Blood collection and RNA isolation

1 ml of intravenous blood was collected in tubes containing EDTA, of which 500 μl was mixed thoroughly with 1.3 ml of RNA later solution (Ambion) and stored at -20°C for further used.

Total RNA was extracted from whole blood using Ribopure blood Kit as per manufacturer protocol (Ambion). Briefly, the samples in RNA later solution were centrifuged for 1 min and the supernatant was removed. To the cell pellet 800 μL lysis solution and 50 μL sodium acetate solution was added to lyse the blood cells followed by extraction using 500 μL Acid-Phenol: Chloroform. To the recovered aqueous phase containing RNA, 600 μL of 100% ethanol was added and passed through a filter cartridge provided with the kit. The filter was then washed with wash solutions. Finally the RNA was eluted with 50 μl of preheated elution solution and stored at –20°C.

5.3.4 Quantitative real time RT-PCR

Total RNA (1μg) was reverse-transcribed into cDNA using high capacity cDNA Reverse Transcription Kit (Applied Biosystems) using random hexamers. The reactions were carried out at 25^0C for 10 min, 37^0C for 120 min followed by 85^0C for 5 sec. Cytokine gene expression was analyzed by quantitative real time PCR on an Applied Biosystems 7900 Real Time PCR System (Applied Biosystems, Foster City, CA). cDNA was amplified in duplicate for the

following cytokines IL-2, IL-4, IL-10, IL-12α, IL-12β, INF-γ and TGF-β using Assay on Demand (AOD), Applied Biosystems (AB) and Taqman Master mix, Applied Biosytems (AB). The conditions for the PCR were as follows: 1 cycle at 50^0C for 2 min followed by 40 cycles at 95^0C for 10 min, melting at 95^0C for 15 s and final annealing at 60^0C for 1 min. The endogenous control gene 18s (pre-Developed TaqMan Assay Reagents, Applied Biosystems) was used as a reference gene to normalize the expression levels of target genes by correcting differences in the amount of cDNA loaded between samples. Relative amounts of the target genes were calculated by using the comparative CT method.

5.3.5 Statistical Analysis

The Statistical analysis of the data was done using *XLSTAT* software 2010 version. Cytokine transcriptional expression was measured in duplicate and presented as mean ± standard error of the mean. Comparison of the cytokines genes expression between groups was performed using student's t test. Correlation of cytokines expression was checked using Pearson's correlation coefficient test. Interaction of age and cytokines genes expression was determined by ANOVA. Relationships between cytokine genes expression levels and variables like age, sex and ethnicity was predicted using logistic regression models.

5.4 Results

Of a total of 88 individuals examined for cytokines gene expression, 89% of them had *P. falciparum* malaria related fever and headache as the most common symptoms among others and 67% of the control participants had non malaria specific fever (>98.6^0 F). Individuals with *Pf* positivity were classified into complicated and uncomplicated malaria cases. A 10% of the *Pf* cases were asymptomatic for the disease whose *Pf* positivity was confirmed by microscopy. Individuals who had not suffered from *Pf* malaria and hailed from other nonendemic areas were also included as healthy controls unexposed to malaria. Standardization procedure for selection of an endogenous control included used of the genes GAPDH, beta- actin and 18s gene for which both inter and intra group variances were checked.

Table 5.4.1: Patient characteristics

Group	No. of cases	Age mean (range) yrs	Sex ratio (M/F)
Healthy controls	10	31.28 (3-40)	0.75
Non malaria fever controls	20	14.23 (1.3-40)	3
Complicated cases	12	19.88 (12-29)	0.5
Uncomplicated cases	46	22.31 (3-70)	0.66
Total	88		

5.4.1 Cytokines expression in disease and controls

The expression of the cytokines - IL-2, IL-4, IL-12α, IL-12β, IFN-γ and TGF-β could be detected in the samples studied. For the cytokines IL-4 and IL-12β, Ct values could be determined in only 26 and 12 samples respectively of which duplicate readings could not be obtained for majority of the samples (Fig 5.4.1). The mean cytokine level was highest for IL-2 while that of TGF-β was lowest in the study population. Statistical dispersion of Ct values was maximum for IFN-γ (range = 12.94) in the diseased group.

Relative quantification of the mRNA levels of the cytokines showed that expression of IL-10 and IFN-γ were downregulated whereas IL-2, IL-12α and TGF-β were upregulated in the *Pf* positive individuals (Fig 5.4.2). Between the controls and disease a significantly lower levels of IL-10 ($p = 2.3 \times 10^{-7}$) and higher expression of TGF-β ($p = 0.013$) was seen in the *Pf* infected individuals. No significant differences were seen in the levels of the other cytokines.

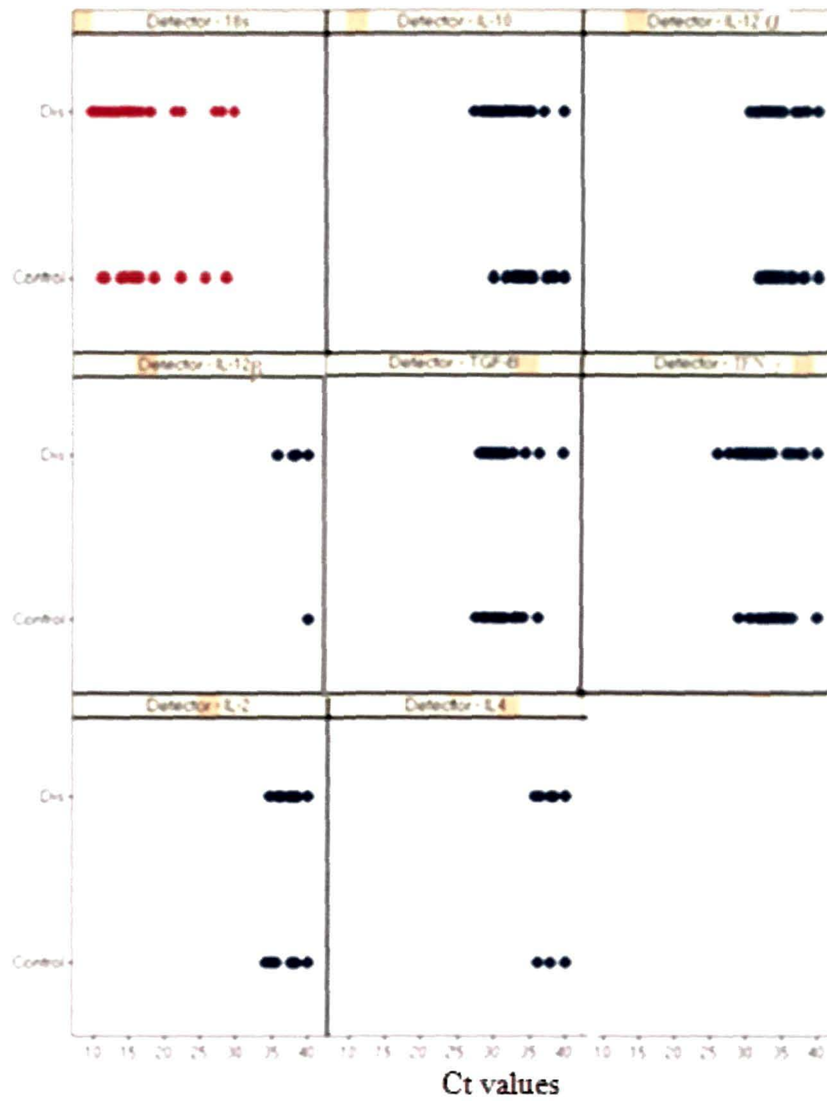


Fig 5.4.1: Distribution of detectors values in control and disease groups.

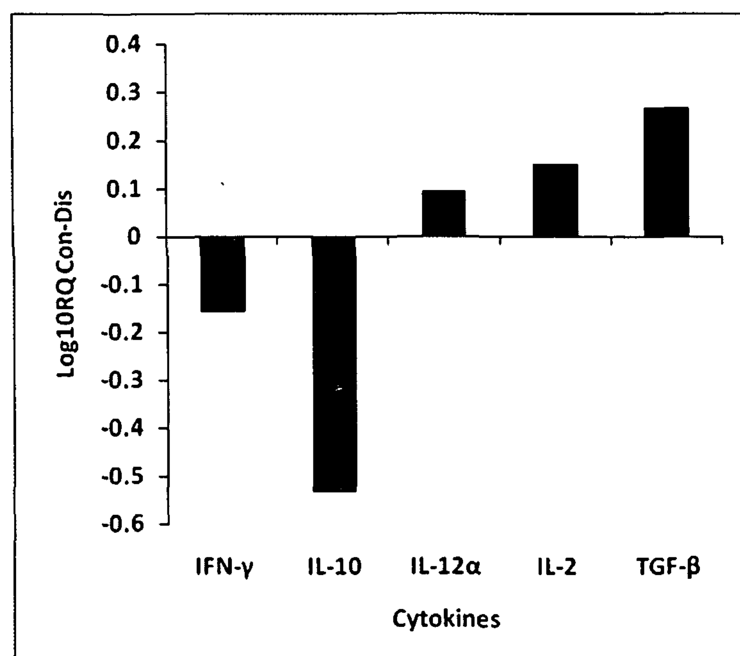


Fig 5.4.2: Relative quantification of cytokines expression between the disease and control groups taking disease as the calibrator.

When the controls were stratified into healthy controls and controls with non- malaria specific fever and compared separately with diseased participants we observed a significantly lower level of IL-10 expression in the *Pf* positive individuals irrespective of status of the controls individuals. Whereas the higher level of TGF-β seen in the *Pf* individuals was significant only in comparison with controls having non malaria fever (Table 5.4.2).

Table 5.4.2: Comparison of mean values of cytokines levels between the groups.

Cytokines	Disease mean ± S.E	Healthy control mean ± S.E	Disease vs Healthy control (p value)	Non malaria fever control mean ± S.E	Disease vs non malaria fever control (p value)
IFN-γ	3.82±0.92	4.18±0.21	0.521	4.42±0.2	0.145
IL-10	4.066±0.69	5.63±0.62	**0.005**	5.91±0.7	**$1.5x \cdot 10^{-6}$**
IL-12α	5.155±0.70	4.71±0.65	0.625	4.89±0.54	0.712
IL-2	8.17±0.55	6.9±0.85	0.305	8.08±0.79	0.92
TGF-β	3.23±0.51	2.45±0.22	0.151	2.3±0.27	0.026

Hierarchical clustering revealed that expression of IL-2 was most similar between the control and the diseased.

A positive correlation between the levels of expression of the various cytokines was also seen in the study population (Table 5.4.3). Analysis of data with respect to ethnic background of the population showed that the expression of TGF-β was significantly different between the TT and TB population (p=0.048) with an elevated level of the cytokine among the TT.

Table 5.4.3: Pearson's correlation between cytokines.

Cytokines	TGF-β	IL-10	IL-12α	IFN-γ	IL-2
TGF-β	1	0.37 (0.0018)	0.216 (0.001)	0.408 (0.0001)	0.12 (0.07)
IL-10		1	0.14 (0.065)	0.529 (0.00008	0.189 (0.06)
IL-12α			1	0.23 (0.04)	0.439 (0.0004)
IFN-γ				1	0.10 (0.077)
IL-2					1

Pearson's correlation coefficient (r) and p values in brackets are shown.

5.4.2 Association with Age

Expression levels of cytokines were compared between the younger age group (≤ 10 years) and older age group (> 10 years). Comparison of cytokines levels between disease young and control young showed a significantly lower level of IL-10 (p=0.0019) and IFN-γ (p=0.018) and elevated levels of IL-2 (p= 0.016) and TGF-β (p=0.014) in the disease. While between the old age group of disease as well as control only a significant decreased of IL-10 level was seen in

disease individuals (Table 5.4.4). But when we compared the cytokines profile of diseased young and old, expression of all the cytokines were upregulated in the young age individuals though only IL-2 showed a significance difference in expression between the groups (Fig 5.4.3).

Table 5.4.4: Differences in cytokine expression with age in disease and control

Cytokines	Disease Young Mean±S.E	Control Young Mean±S.E	p values	Disease Old Mean±S.E	Control Old Mean±S.E	p values
IFN-γ	3.82±0.12	5.03±0.17	0.018	3.82±0.27	3.28±0.16	0.236
IL-10	4.23±0.45	6.29±0.62	0.0019	4.01±0.18	5.52±0.32	0.0004
IL-12α	5.67±0.34	4.9±0.74	0.168	5.01±0.56	4.58±0.42	0.570
IL-2	10.01±0.56	7.65±0.44	0.016	7.46±0.54	7.32±0.61	0.888
TGF-β	3.82±0.15	2.19±0.11	0.014	3.07±0.12	2.38±0.25	0.135
No. of cases	12	10		46	20	

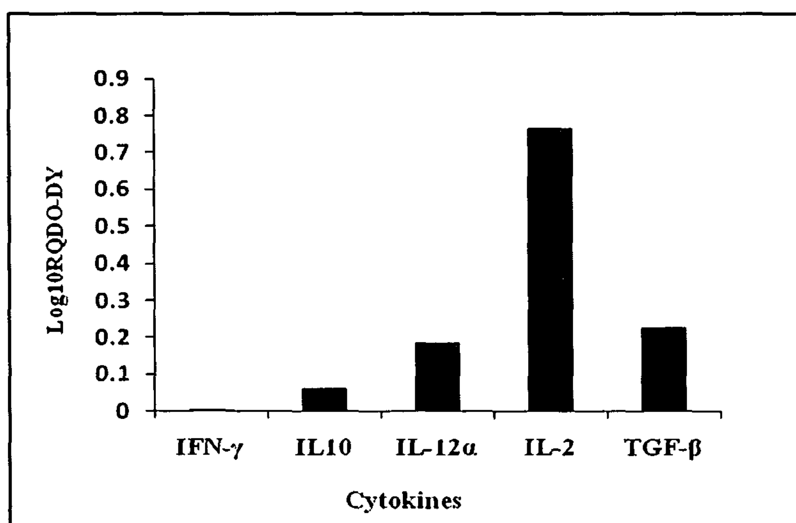


Fig 5.4.3: Comparison of cytokines expression between young: DY (<10 years) and old age group: DO (>10 years) of disease (Calibrator: DY).

Within the control group, though the expression levels of all the cytokines were upregulated in the younger age except for TGF-β, only IFN-γ showed a significant difference in expression between the age groups (p = 0.005).

5.4.3 Cytokines and disease outcome

On the basis of severity of malaria symptoms, the *Plasmodium falciparum* infected individuals were stratified into complicated and

uncomplicated cases and data was analysed. Comparison of cytokines levels between the groups showed that IFN-γ and TGF-β levels were upregulated while IL-2 and IL-12α were downregulated in individuals suffering from complicated malaria. Significant differences in the expression of cytokines genes were for IL-2 ($p= 0.021$) and IL-12α ($p= 0.049$) with an increased expression in the uncomplicated malaria patients whereas over expression of TGF-β ($p= 0.007$) and IFN-γ ($p=0.05$) were seen in the complicated participants (Table 5.4.5). Further, the increased levels of TGF-β in complicated malaria was maintained when compared with both healthy and non- malaria fever controls (Table 5.4.6). Additionally, in uncomplicated malaria the expression of IL-2 and IL-12α were elevated as compared to the controls (Table 5.4.7).

Table 5.4.5: Comparison of cytokines expression between complicated and uncomplicated cases.

Cytokines	Uncomplicated Cases mean± S.E	Complicated Cases mean± S.E	p values
IFN-γ	3.66±0.07	4.486±0.17	0.05
IL-10	4.025±0.71	4.225±0.67	0.648
IL-12α	5.454±0.61	3.99±0.32	0.04
IL-2	8.76±0.58	5.72±0.82	0.02
TGF-β	2.975±0.31	4.266±0.54	0.007
No. of cases	46	12	

Table 5.4.6: Comparison of cytokines expression in complicated malaria versus different groups of controls.

Cytokines	Healthy control mean ± S.E	Non malaria fever control mean ± S.E	Complicated cases mean± S.E	p values complicated vs healthy control	P values complicated vs non malaria fever
IFN-γ	4.18±0.21	4.42±0.2	4.486±0.17	0.83	0.86
IL-10	5.63±0.62	5.91±0.7	4.225±0.67	0.02	0.005
IL-12α	4.71±0.65	4.89±0.54	3.99±0.32	0.79	0.41
IL-2	6.9±0.85	6.08±0.79	5.72±0.82	0.48	0.24
TGF-β	2.45±0.22	2.3±0.27	4.266±0.54	0.01	0.009

Table 5.4.7: Comparison of cytokines expression in uncomplicated malaria versus different groups of controls.

Cytokines	Healthy control mean ± S.E	Non malaria fever control mean ± S.E	Uncomplicated Cases mean± S.E	p value Uncomplicated cases vs. healthy control	p value Uncomplicated cases vs. Non malaria fever control
IFN-γ	4.18±0.21	4.42±0.2	3.66±0.07	0.29	0.073
IL-10	5.63±0.62	5.91±0.7	4.025±0.71	0.007	1.4×10^{-6}
IL-12α	4.71±0.65	4.89±0.54	5.454±0.61	0.06	0.098
IL-2	6.9±0.85	6.08±0.79	8.76±0.58	0.06	0.139
TGF-β	2.45±0.22	2.3±0.27	2.975±0.31	0.081	0.188

Stratification of data on the basis of malaria symptoms into asymptomatic and symptomatic cases, showed no differences in cytokine profiles between the groups. Nonetheless, the expressions of the cytokines IL-10, IL-12α were up regulated whereas those of IL-2, TGF-β were down regulated in the asymptomatic patient (Fig 5.4.4)

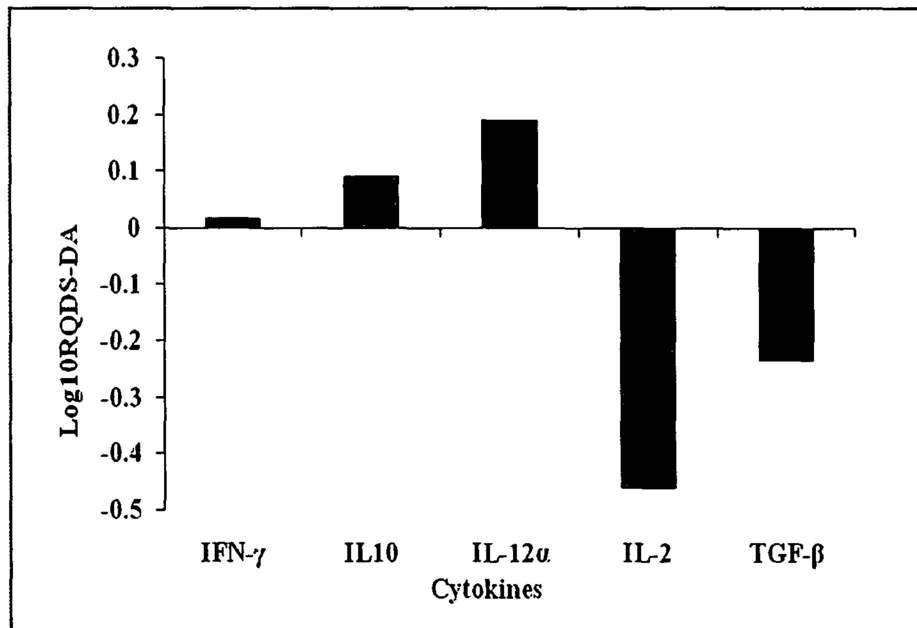


Fig 5.4.4: Comparison of cytokines expression between asymptomatic (DA) vs. symptomatic cases (DS) (Calibrator : DA).

Further, modeling of data with complicated malaria as the outcome showed that expression of IL-12α could predict disease severity of malaria (ROC = 0.916). The expression of IL-12α was negatively associated with complicated malaria outcome (p=0.015) while taking into account factors like age, ethnicity and sex. An interaction of the cytokines combination IL-10*IL-12α*TGF-β in presence of IFN-γ was positively associated (p= 0.038) while the same combination IL-10*IL-12α*TGF-β along with IL-2 was associated negatively with complicated malaria (p= 0.046). When data was modelled for asymptomatic malaria as outcome, the combination of the cytokines IFN-γ*IL-10*IL-12α with IL-2 was predicted to increase asymptomatic malaria (p = 0.045) whereas the IFN-γ*IL-10*IL-12α combination in presence of TGF-β was negatively associated with asymptomatic disease (p= 0.049).

Simultaneous analysis of effect of age on cytokines expression in *Pf* positive participants and healthy controls by ANOVA and multiple regression showed no association of age and cytokines expression with disease status of an individual (Table 5.4.8). When effect of age and disease severity on cytokines expression was analysed, we observed an interaction between the variables in expression of TGF-β where levels of TGF-β was negatively associated with age (Table 5.4.9). A weak interaction was also seen for TGF-β when age and symptomatic status of an individual was taken into account (Table 5.4.10).

Table 5.4.8: Interaction of age with disease status

Cytokines	R^2 Goodness of fit coefficient	p value Disease status	p value age
IFN-γ	0.06	0.968	0.006
IL-10	0.176	<0.0001	0.156
IL-12α	0.013	0.237	0.762
IL-2	0.038	0.209	0.458
TGF-β	0.079	0.003	0.323

Table 5.4.9: Interaction of age with disease severity

Cytokines	R^2 Goodness of fit coefficient	p value Disease severity	p value age
IFN-γ	0.128	0.070	0.005
IL-10	0.036	0.728	0.08
IL-12α	0.051	0.045	0.44
IL-2	0.158	0.022	0.50
TGF-β	0.139	0.010	0.019

Table 5.4.10: Interaction of age with disease symptoms

Cytokines	R^2 Goodness of fit coefficient	p value Disease symptoms	p value age
IFN-γ	0.09	0.807	0.005
IL-10	0.028	0.669	0.159
IL-12α	0.008	0.519	0.664
IL-2	0.036	0.449	0.541
TGF-β	0.095	0.089	0.011

5.4.4 Cytokines ratios

The ratio of Th1 & Th2 cytokine was compared between the controls and *Pf* positive individuals. The ratios of IL-10/IFN-γ ($p = 0.01$) and IL-10/IL-12α ($p < 0.0001$) were significantly higher in the controls than the disease group.

Comparison of ratios of TGF-β to the cytokines did not show any significant differences. (Table5.4.11). Cytokines ratios also showed no differences between the groups with respect to disease outcome.

Table 5.4.11: Comparison of cytokine ratios in disease and control

Cytokine ratio	Disease Mean ± S.E (n= 58)	Control Mean±S.E (n= 30)	p value (t test)
IL-10 ratio			
IL-12-α	0.73±0.09	1.45±0.03	<0.0001
IFN-γ	1.057±0.01	4.438±0.07	0.010
TGF-β	1.557±0.04	1.363±0.2	0.708
TGF-β ratio			
IL-12-α	1.687±0.3	1.168±0.2	0.539
IFN-γ	1.183±0.05	0.903±0.06	0.672

5.5 Discussion

Cytokines play an important role in immune response to malaria but need to be finely tuned to mediate antiparasite immunity. Disturbance in this fine balance of cytokines leads to either immune suppression whereby Th1 cytokines responses are compromised or an increased pro inflammatory cytokine response which contributes to disease pathology[127, 201].

We observed qualitative and quantitative differences in cytokines between *Plasmodium falciparum* positivity and complicated malaria. TGF-β mRNA level was upregulated in both conditions as compared to healthy controls and to non malaria fevers. However levels of IL-10 were lower in *Pf* positivity along with an increased expression of IL-2 and IL-12α. In contrast, an increased IFN-γ with no change in IL-10 levels along with decreased levels of IL-2 and IL-12α was seen in complicated malaria in comparison to uncomplicated malaria.

Increased TGF-β levels in *Pf* infected individuals have been reported in earlier studies[38,131]. However, unlike Walther *et al.*[131], where enhanced production of TGF-β was associated with a decreased levels of IFN-γ, IL-12, IL-6, we observed increased levels of IL-2, IL12α. Though the levels of IFN-α were low in *Pf* infection, but it did not reach statistical significance (p= 0.06). TGF-β is shown to be essential for maintaining the balance between protection against and progression toward *P falciparum* malaria[127]. TGF-β has been demonstrated to be a pleiotropic, immune-suppressive and anti-inflammatory cytokine that has a central role as regulator in T regulatory cells (Treg) proliferation and function[129,132,201]. In a recent study it was seen that two subsets of CD4+CD25+Foxp3+ T regulatory cells Foxp3 ^{hi} and Foxp3^{int} regulated parasite infection differentially where Foxp3^{hi} cell mediated inhibition of T cell cytokine response with the help of TGF-β and IL-10 whereas Foxp3^{int} produced the inflammatory cytokines IFN-γ, IL-4 and IL-17[112]. Further, TGF-β derived from monocytes favoured differentiation of naive T cells to T reg subsets in human malaria infection[131]. In mice models, it was seen that the ability to survive infection with either *P. berghei* or *Plasmodium chabaudi* was negatively correlated with TGF-β secretion[204]. Virulence of malaria infections was dependent upon the cellular source of TGF-β and the timing of its production[201, 204]. In mouse models infected with nonlethal strain of *Plasmodium yoelii,* TGF-β was produced from 5 days postinfection and correlated with resolution of parasitaemia while lethal strain induced high levels of TGF-β within 24 h associated with delayed and blunted IFN-γ and TNF-α responses and failed to clear the parasites[204]. Higher TGF-β gene expression observed in our study both in *Pf* infected cases and in complicated malaria, indicates a role for the regulatory cytokine in disease pathogenesis in the study cohort. Association of severe malaria with an increased as well as decreased TGF-β production have been reported in earlier studies[132, 204]. A difference in TGF-β expression was also seen between the Tibeto-Burman and Tea tribes ethnic group of the study population with the level of cytokine significantly higher among the Tea tribes. In an earlier study, variation in TGF-β response and disease outcome has been

attributed to difference in genetic components of the participants. The Fulani tribes of Africa showed better immunity with reduced frequencies of T reg cell along with up regulation of immunity associated genes as compared to the Mossi tribes[205].

The level of IFN-γ expression was seen to be higher in complicated malaria. A linear correlation of levels of IFN-γ mRNA and disease was also observed though the correlation coefficient was weak ($r = 0.112$). Further, modelling of data using logistic regression analysis revealed that the combination of IL-10*IL-12α*TGF-β with IFN-γ was positively associated while the combination IL-10*IL-12α*TGF-β in presence of IL-2 was negatively associated with complicated malaria indicating a positive role of IFN-γ in predisposing to disease severity. Consistent with our finding, earlier investigators have also reported an increased IFN-γ levels in patients with complicated malaria in comparison to uncomplicated malaria [206]. In mouse models too, role of IFN-γ in cerebral malaria has been reported where production of IFN- γ lead to increase apoptosis in the brain of cerebral malaria susceptible mice[207]. Overproduction of IFN- γ was seen to predispose to severe malaria pathology with regulation of IFN-γ secretion involving IL-18, another cytokine which induced severe malaria[206]. However, contrary to our observation, some reported lower concentrations of T cells secreting IFN-γ in children with *P. falciparum* hyperparasitemia than with uncomplicated malaria[128]. High levels of malaria specific IFN-γ were associated with decreased risk of development of fever or of clinical malaria[127, 133]. In humans an increased expression of IFN- γ has been shown to be important during initial resolution of parasitaemia[115]. A role for IFN-γ in protection as well as in pathogenesis has been seen[38]. An early and efficient amount of IFN-γ production was shown to exhibit antiparasitic immune response while an exaggerated and uncontrolled production of IFN-γ was related to pathologic complications of the disease by several investigators with overproduction of IFN-γ predisposing to severe disease[206, 208].

The levels of IL-10 were down regulated in *Pf* infected individuals but remained unchanged in complicated malaria. IL-10 is suggested to be involved both in protection and immunopathology during malaria where high levels of IL-10 may be beneficial by reducing the inflammatory response, but also detrimental by decreasing antiparasitic cellular immune responses[38]. Low levels of IL-10 as well as low IL-10/TNF-α ratio were seen to be inversely associated with severe anemia in previous studies[37]. A significant increase in IL-10 expression within CD4+ T cells during the course of disease with higher level of IL-10 in malaria cases as compared to healthy control has been reported[128]. In another study, high levels of IL-10 were seen associated with less effective clearance of *Pf* parasites[119]. In our study elevated levels of TGF-β were associated with depressed in IL-10 level which was different from previous studies. This might be explained by the observed high IL-12 levels which counterregulate IL-10[209].

In complicated malaria, we also observed lower expression of the cytokines IL-2 and IL-12α. Further by logistic modeling, expression level of IL-12α was negatively associated with complicated malaria and could predict severity of the disease. Increase in serum level of IL-12 in uncomplicated malaria cases compared to complicated participants had been shown in earlier studies carried out in endemic regions suggesting a protective role of IL-12 from severity of the disease[123]. In the present study a higher IL-10/IL-12α ratio was seen in the control groups with a higher expression of IL-12α in the diseased cases together with lower level of IL-10 in them which indicates differential macrophage activation. Reduced IL-12 concentration in severe *Pf* malaria was suggested to be related to the reduced T cell mediated IFN-γ activity[38]. In our study, however, it was found that IFN- γ level was elevated in patients with severe malaria. Decreased IL-2 and IL-12 α along with increased expression of TGF-β and no change in IL-10 mRNA levels in complicated malaria would indicate T cell suppression. This implies that the role of T_H arm in malaria is impaired as has been suggested in earlier studies[201]. It also implies that T cells

are unlikely to be the source of the observed elevated levels of IFN-γ. Higher level of IFN-γ seen in complicated malaria could then be produced by NK cell activation but a decreased IL-12α mRNA levels seen in this condition does not tend to support NK mediated IFN-γ secretion. Notably, the receptors for IL-12α are mainly expressed on T cells, NK cells, and NKT cells. Nonetheless, NK cells have also been shown to directly contact iRBC via NK receptor complex leading to NK activation resulting in IFN-γ secretion[42]. Alternatively γδ T cells could also be the source of IFN-γ as seen in other studies[210]. Our data suggests the relevance of other arms of cellular immunity to malaria other than T helper cells in cellular immunity particularly in complicated malaria[201]. Interestingly, higher TGF-β response was observed in Tea tribes and this ethnic group was shown to have a higher antibody response with decreased risk of severe malaria in our study. In *Pf* positivity decreased IL-10, and high TGF-β, IL-2 and IL-12α with no change in mRNA level of IFN-γ suggests induction of balanced cytokine response with activation of both Th1 and Th2 cells while in complicated malaria a dysregulation of cytokine response was evident.

Correlation of a cytokine levels with disease symptoms was not seen in present study, contrary to other studies where some cytokines were associated with symptoms of malaria[103,208]. However, by logistic regression modelling, the combination of the cytokines IFN-γ*IL-10*IL-12α with IL-2 was predicted to increase the probability of asymptomatic malaria whereas the IFN-γ*IL-10*IL-12α combination in presence of TGF-β was negatively associated with asymptomatic disease outcome. Earlier investigators have associated asymptomatic malaria with downregulation of inflammatory cytokines in high endemic areas and suggested that it may be a component of acquired clinical immunity to malaria[208].

In conclusion our result tends to suggest a role of Th cytokine IL-12α in *Pf* infection which was protective and the differences in disease outcome between complicated and uncomplicated malaria lay in balanced cytokine response in the later. The cytokines TGF-β predisposed to complicated malaria

with a depressed T cell function which might be an immunoevasion strategy of the parasite. Increased IFN-γ expression was additionally associated with disease severity and T cells appeared to be the source of IFN-γ, it rather hints for involvement of other arms of immunity namely the innate immune mechanism. Identification of cell type(s) responsible for exaggerated IFN-γ response is crucial to understanding and addressing immunopathology of cerebral malaria.

CHAPTER VI:

Differential association of KIR gene loci to risk of malaria in ethnic groups of Assam, Northeast India

6. Differential association of KIR gene loci to risk of malaria in ethnic groups of Assam, Northeast India

6.1 Abstract

Receptors encoded within the Natural Killer Cell (NKC) complex and Killer Immunoglobulin like (KIRs) genomic regions has been suggested to influence malaria pathogenesis and infection susceptibility. We have examined KIR locus in relation to risk of infection and disease in Tea tribes (TT) of Austro Asiatic affinity and Tibeto-Burman (TB) populations from malaria endemic regions of Assam. Consistent with differences in their genetic background, KIR gene loci frequencies differed in studied groups. Surprisingly, KIR3DS1 frequency in TT was low (17%) and comparable to that reported from African populations.

KIR3DL1 frequency was positively associated with malaria severity (Pearson phi, R^2 = 0.297 p=0.006) and logistic regression modelling predicted KIR3DL1 as a risk factor in complicated malaria [Odds Ratio (95% C.I)] = [6.39 (1.34-30.60)]. An interaction between ethnicity and KIR3DL1 was also seen where higher proportion of KIR3DL1 positive and complicated malaria patients belonged to Tea tribes (p = 0.009). Notably, four activating genes protected from frequent malaria (p= 0.02) while six activating genes enhanced the risk of complicated malaria (p=0.05). Combination of KIR2DS4, KIR2DS4del, KIR2DS5 negatively influenced disease outcome in Tea tribes (p = 0.048) but not in Tibeto-Burman.

In conclusion our data indicates KIR gene loci differentially influenced malaria outcome in Tea tribes and Tibeto-Burman and that four activating genes appeared to provide optimal activation that protected from frequent episodes of malaria. Our data also indicated KIR3DS1 to be an ancestral genotype, maintained at low frequency possibly by malaria in the Austro Asiatic tribes.

6.2 INTRODUCTION

Natural killer (NK) cells constitute a crucial component of innate immunity and have also been suggested to participate in acquisition of adaptive immunity. Recent evidence indicates a role for NK cells in malaria as regulatory and as effector cells in pathology of malaria in humans and in mice models[33, 165]. In mouse model, NK cells and NK Receptor (NKR) positive cells were suggested to significantly control susceptibility and resistance to both malaria infection and severe disease syndromes and this was seen to depend on the receptors encoded within the Natural Killer Cell Receptor Complex (NKC) [41]. These cells were seen to respond to *Plasmodium falciparum* infected RBC (iRBC) either by production of IFNγ or by cytotoxicity[211]. Further, NKC loci positively controlled proinflammatory T_H1 and counter regulatory T_H2 cytokine production in mouse model. NK cells were also seen to be the early source of IFN γ in humans and it was observed that activation of human NK cells by blood stages of *P. falciparum* appeared to depend on at least two signals i.e. cytokines released by bystander cells such as monocyte-macrophages or dendritic cells and direct recognition of the infected red blood cell by NKC receptors[40]. Some studies have suggested NKR mediated cytotoxicity of *P. falciparum;* but it remains poorly demonstrated[212]. The ability of NK cells to respond to parasitized RBCs was noted to vary significantly between individuals and that the variation was associated with killer cell Ig-like receptors genotype of an individual where high malaria specific NK cell INFγ response directly corresponded to 3DL2*002 allele of KIR3DL2[42]. IFN γ^+ production was also found to be associated with the expression of NKG2A, CD94 CD158a/KIR2DL1 genes. In a recent study, *P. falciparum* positive individuals were seen to have higher frequency of KIR3DL1 and KIR2DS4 genes[167].

NK cells sense infection by the activation of diverse group of receptors known as Natural Killer Cell receptors (NKRs) that include the killer cell Ig-like receptors (KIR), the lectin like CD94:NKG2 encoded by genetic loci on chromosome 19 and on chromosome 12 in humans respectively. The NKRs occur both as activating and inhibitory receptors and it is the balance of signals

from activating and inhibitory receptors that determines the outcome of NK cell activity[43]. The KIR gene family consists of 15 distinct KIR gene loci (including two pseudogenes KIR2DP1 and KIR3DP1) which vary with respect to their presence or absence on different KIR haplotypes, creating considerable diversity in the number of KIR genotypes observed in a population. Variation in the KIR genetic profile has been described by two broad haplotype groups termed A and[44]. Frequencies of each gene can also vary remarkably across world populations, which might reflect differential selection pressures as well as persistence of ancestral genotype[151].

The KIR locus has been suggested to be fast evolving and under positive selection pressure with pathogen pressure as the driving force. KIR distribution in modern populations is shown to be determined by the waves of prehistoric migration and subsequent population admixture[152]. It has been hypothesized that populations with different genetic backgrounds and length of period of exposure to pathogens could have a bearing on the KIR profile[146]. Malaria is a known evolutionary driving force in selection of several genes involved in malaria resistance, including the Duffy antigen protein (DARC) and Glucose-6-phosphate dehydrogenase (G6PD) deficiency as well as rarer mutations in the alpha- and beta-globin genes that can lead to sickle cell anaemia or thalassemias[213]. Little is known about interaction of malaria with the KIR receptors or malaria driven selection of KIR genes.

Assam in northeast India is a malaria endemic state, with frequent malaria epidemics[214]. This region is peopled by different ethnic groups that include the speakers of Tibeto-Burman, the Indo-European and Austro-Asiatic languages among others[215] and belong to different time periods of settlement[195]. Evidence based on mitochondrial DNA (mt DNA) and Y-chromosomal markers suggested considerable genetic heterogeneity among these ethnic groups that was indicative of their diverse origin and of racial admixtures[216]. In this context, we have examined the KIR profile of these genetically distinct populations and its correlation with altered risk or severity of falciparum malaria. We report here considerable variation in KIR genes frequencies of the three ethnic populations

and that KIR genes were differentially associated with malaria in the studied population. Our data suggests low KIR3DS1 frequency to be an ancestral genotype maintained by similar pathogen pressures possibly malaria in the Austro Asiatic tribes. Four activating genes appeared to provide optimal activation as this protected from frequent episodes of malaria.

6.3 MATERIAL AND METHODS

6.3.1 Study sites and study population

The study was conducted at Guabari village and Kondoli Tea Estate, the details of the sites and the study population has been described in Chapter 3.

6.3.2 Study design

The study was initiated during the winter months of 2005 and carried till 2008 as a cohort prospective study. 135 individuals were enrolled into the study after they or their accompanying relatives gave informed consent. Blood samples were obtained from individuals irrespective of their *Pf* + status at the time of collection. 25 individuals who had no known history of malaria were included inn the study. Exclusion criteria were children with age less than 1 year, pregnant women and individuals suffering from any other diseases. Individuals were categorized according to their disease symptoms into complicated and uncomplicated cases and with frequency of malaria incidence as described in chapter 4. Samples of 30 Khasi individuals of Austro-Asiatic affinity were also typed for KIR gene loci. The study was approved by Tezpur University Ethical Committee (TUEC).

6.3.3 Molecular typing of KIR genes

Genomic DNA was extracted from 200 μl of whole blood using QIAamp DNA Mini Kit (Qiagen, Germany) and quantified by spectrophotometry. Typing of KIR genes was performed by polymerase chain reaction based sequence specific primers[217]. PCR was carried out in 15μl volumes consisting of 200 μM dNTPs, 150 ng of DNA, 1μM of each gene specific primer, 0.1μM of the control primer, 1X buffer and 0.625U of Klen taq LA (Sigma Aldrich, USA) and nuclease free water to make up the volume. Presence or absence of amplicon was visualized by gel electrophoresis wherein 10 μl of the amplified product was

loaded with 6x loading dye on 1.8% gel prestained with ethidium bromide. In addition to the polypopsis coli gene, the framework genes (KIR2DL4, KIR3DL2 and KIR3DL3) served as a positive control for PCR amplification. All negative reactions were repeated twice to confirm non amplification by PCR.

6.3.4 Statistical Analysis

Carrier frequencies (F) for each KIR gene was determined by direct counting. KIR gene frequencies (f) were calculated using the formula $f = 1-\sqrt{(1-F)}$ [145]. Data was analyzed using Excel Stat 2010 version. Comparison of KIR gene frequencies was done by Fisher's exact test and confounding factors checked by logistic regression. The odds ratio (OR) was calculated by the cross product ratio and exact confidence intervals of 95% were obtained. Multivariate analysis was performed to compare frequencies of KIR genes of the study population with other world population. Classification of genotypes on the basis of centromeric and telomeric gene clusters was according to Ashouri *et al*[218]. On the basis of the KIR genes frequencies, the population was divided into A or B haplotype group. Haplotype group A consisted of KIR2DL1, KIR2DL3, KIR2DS4 and KIR3DL1 besides the framework genes. The B haplotype group is defined by the presence of one or more of the genes encoding activating KIRs, KIR2DS1/2/3/5, KIR3DS1 and the genes encoding inhibitory KIRs, KIR2DL5A/B and KIR2DL2[44]. Linkage disequilibrium was analyzed and significance checked by Chi square test (Yates correction). The λ^2 test was used for testing of Hardy–Weinberg equilibrium by comparing the observed number of subjects for each genotype with the expected number of subjects, assuming the existence of Hardy–Weinberg equilibrium.

6.4 Results

6.4.1 KIR carrier frequencies in the three ethnic populations

The 14 KIR genes and the two pseudo genes could be detected in the study population, with the framework genes KIR2DL4, KIR3DL2, KIR3DL3 and KIR3DP1 seen at 100% frequency (Table 6.4.1). Of the KIR genes, KIR2DL2 of inhibitory genes and KIR3DS1 of activating genes were seen at lowest frequencies in our population. A significantly higher carriage of

KIR3DL1 (p<0.0001) and KIR2DL3 (p=0.001) was detected than of their alleles KIR3DS1 and KIR2DL2 respectively. Distinct differences in KIR genes frequencies were noted between the Tea tribes (TT), the Tibeto-Burman (TB) and the Indo-European (IE) populations. The TT population differed from the other two populations in having the lowest frequency of KIR3DS1. In the TB population, frequencies of activating genes were lower as compared to both TT (KIR2DS1 and KIR2DS4) and the IE (KIR2DS2, KIR2DS3, KIR2DS4 and KIR3DS1) and the frequency of KIR2DL3 was higher than that of KIR2DL2. Overall, the inhibitory genes occurred at higher frequency in TT but activating genes in IE.

Table 6.4.1: Comparison of KIR genes frequency in three ethnic groups of Assam

KIR genes	Tea tribe (n= 53)		Tibeto-Burman (n=57)		IndoEuropean (n=25)		TT vs TB	TT vs IE	TB vs IE	TT vs TB vs IE
	%F	f	%F	f	%F	f	p values			
2DL1	64	0.40	56	0.34	64	0.40				
2DL2	77	0.52	42	0.24	56	0.34	0.0005	0.066		0.017
2DL3	76	0.50	86	0.63	64	0.40			0.037	
2DL5	76	0.50	74	0.49	68	0.43				
3DL1	77	0.52	70	0.45	84	0.60				
2DS*001	8	0.03	44	0.25	52	0.31	<0.0001	<0.0001		0.0001
2DS1	64	0.40	32	0.17	48	0.28	0.001			0.003
2DS2	59	0.36	56	0.34	80	0.55		0.077	0.048	
2DS3	47	0.27	37	0.21	60	0.37			0.05	
2DS4	72	0.47	47	0.27	72	0.47	0.012		0.05	0.017
2DS4full	53	0.31	33	0.18	48	0.28	0.05			
2DS4del	32	0.18	40	0.23	56	0.34		0.05		
2DS5	66	0.42	65	0.41	60	0.37				
3DS1	17	0.09	40	0.23	64	0.40	0.011	<0.0001	0.05	0.0003
2DP1	77	0.52	65	0.41	72	0.47				
2LD4	100	1	100	1	100	1				
3DL2	100	1	100	1	100	1				
3DL3	100	1	100	1	100	1				
3DP1	100	1	100	1	100	1				

6.4.2 Comparison of carrier frequencies with world populations.

The carrier frequencies of variable KIR genes of our population were compared with other Indian and some world population. On the whole, gene frequencies of TT were similar to Indian population except for KIR3DS1, which was markedly lower in them. The TT were similar to African population in low carriage of KIR3DS1.The low prevalence of KIR2DS4 among the TB could be compared with that from populations of Taiwan, Costa Rica, Australian aborigines and the Yucpa of Venezuela. Comparison of gene frequencies of our populations with those of world populations by Principal Component Analysis (PCA) and by Agglomerative Hierarchical Clustering (AHC) revealed each of the three ethnic groups of our study population was different. The TB plotted between the native Americans (Waro), the South East Asian (Indonesian, E.Timor) and the Chinese (Han) populations on the PCA plot (Fig 6.4.1). However, in the Agglomerative Heirachial Clustering (AHC) the TB clustered with the Taiwanese and was again positioned between the Austronesian and Chinese populations (Fig 6.4.2). The TT mapped close to the North Indians both on PCA plot as well as on AHC dendogram. The gene frequencies of the IE of Assam were distinct from other world populations and were seen as an outlier on PCA plot while on AHC, this group was seen to cluster with the South Indians.

6.4.3 KIR genotypes and haplogroups

A total of 103 genotypes were noted in 135 individuals typed for the KIR genes. The proportion of genotypes was higher in the Tibeto-Burman (50/57) and the IE population of Assam (22 /25) in comparison to the Tea tribes (41/53). Apart from the framework gene, the maximum and minimum number of genes in a genotype was 12 and 3 respectively. Majority of the population belonged to the B haplogroup with a frequency of 78.15%. The predominance of the homozygous BB genotype was seen in the study population with a frequency of 58.7% followed by the AB genotype with 39.7% and only 1.6% of the

population had the AA genotype. The distribution of AA, AB and BB genotypes between the ethnic groups was found to be similar.

Only a few genotypes were common between the three ethnic groups. The TB and IE shared four genotypes while the TB and TT shared 6 genotypes, of which two were novel to our study. Genotype ID 382 was common to all three ethnic groups. Genotypes ID 382, 11 and 6 constituted the most frequent genotypes, with 382 and 11 detected in six individuals each and genotype ID 6 in four individuals. The Genotype ID 6 which is characterized by having all 16 genes was most frequent in the IE population.

A total of 23 genotypes were common between our population and other Indian populations. Surprisingly, the TB had 13 genotypes in common with other Indian populations, while, the IE shared seven and the TT shared eight genotypes.

Forty two of the genotypes detected in our study population were common with other world populations (Fig 6.4.3). Approximately 37 % of genotypes of each TT and of TB and 52 % of IE were common with other world population's genotypes. 61 genotypes were novel to our population (Fig 6.4.4). The TT, TB and IE contributed to 35.93%, 48.43% and 15.62 % of the novel genotypes respectively. The high number of genotypes was due to diverse combination of activating genesin the population and that 84 genotypes (62.22 % of both unique and common) could be detected in an individual each only.

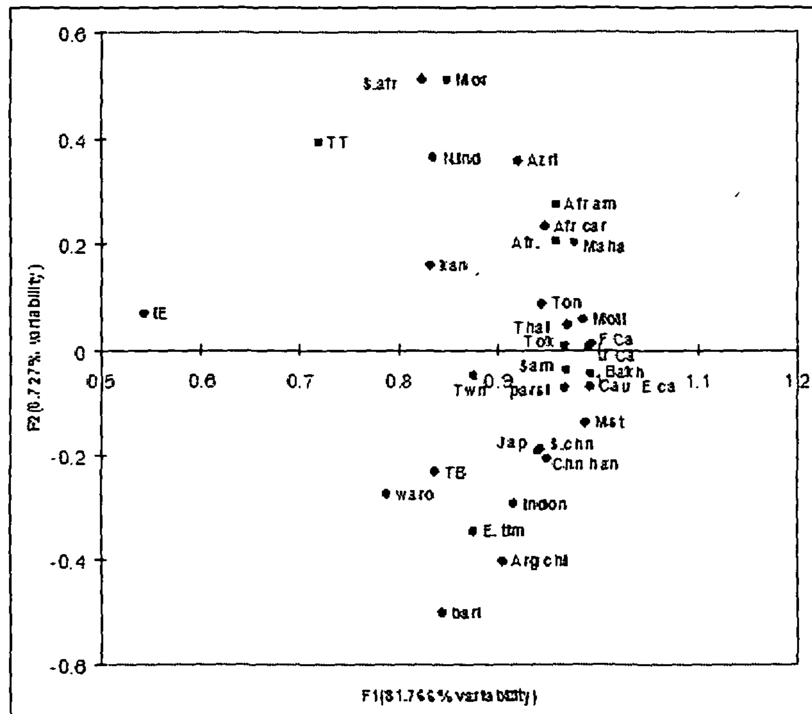


Fig 6.4.1: Principal Component Analysis (PCA) of KIR gene frequencies of TT, TB and IE along with 30 numbers of selected world populations.

PCA was performed for nine variable KIR genes (KIR2DL1, KIR2DL2, KIR2DL3, KIR3DL1, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR3DS1). TT Tea tribes, TB Tibeto Burman, IE Indo European, Chn Chinese han, Sam Samoan, Tok Tokelau, Ton Tongan, moll Mollukurumba, N:ind North Indian, Kan Kanikar, Waro Warao, Bari Bari, Afr.am African American, Afr.car Afro-Caribbean, S.afr South African, mor Moroccan, Azri Azeri, Afr African, Bakh Bakhtiari, Parsi Parsis, Mst Mestizo, S.chn Singapore Chinese, E.tim East Timor, Arg chi Argentina chiri, indon Indonesian,Maha Maharashtrian, Ir ca Ireland Caucasian, F ca France Caucasian, Twn Taiwanese, Cau Caucasian, E ca England Caucasian, Jap Japanese. KIR genotype frequencies obtained from http://www.allelefrequencies.net.

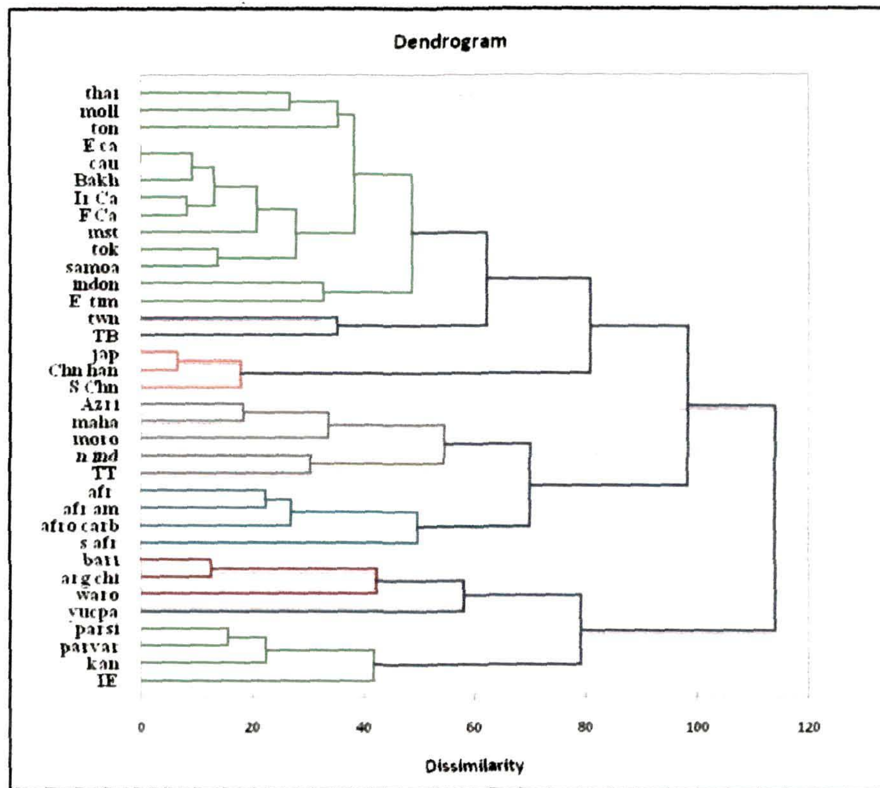


Fig 6.4.2: Agglomerative Hierarchical Clustering (AHC) based on KIR genotype frequencies for the three ethnic groups TT,TB and IE in the study along with selected world populations.

KIR genotype frequencies obtained from www.allelefrequencies.net. TT Tea tribes, TB Tibeto Burman, IE Indo European, Chn han Chinese, Samoa Samoan, Tok Tokelau, Ton Tongan, Moll Mollukurumba, N.ind North Indian, Kan Kanikar, Parvar Paravar, Waro Warao, Bari Bari, Yucpa Yucpa, Afr.am African American, Afro.carb Afro-Caribbean, S.afr South African, moro Moroccan, Azri Azeri, Afr African, Bakh Bakhtiari, Parsi Parsis, Mst Mestizo, S.chn Singapore Chinese, E.tim East Timor, Arg chi Argentina chiri, indon Indonesian, Maha Maharashtrian, Ir ca Ireland Caucasian, F ca France Caucasian, Twn Taiwanese, Cau Caucasian, E ca England Caucasian, Jap Japanese, Thai Thailand.

No.	2DL1	2DL3	3DL1	2DS4	2DS2	2DL2	2DS3	2DL5	3DS1	2DS5	2DS1	2DP1	Genotypes	C4T4 clusters	Genotype ID	No. of individuals
1	■	■	■	■	■	■	■	■	■	■	■	■	AB	C4T4	6	4
2	■	■	□	□	■	■	■	■	■	■	■	■	BB	C4T4	70	2
3	■	□	■	■	■	■	■	■	■	■	■	■	BB	C4T4	73	1
4	□	□	■	■	■	■	■	■	■	■	■	■	BB	C4T4	82	1
5	■	■	□	■	■	■	■	■	■	■	■	■	BB	C4T4	87	1
6	□	□	■	■	■	■	■	■	■	■	■	□	BB	C4T4	262	1
7	■	■	■	■	■	■	■	■	□	■	■	■	AB	C4TX	382	6
8	■	■	■	■	■	■	■	■	□	■	□	■	AB	C4TX	25	3
9	■	■	■	■	■	■	■	■	□	□	■	■	AB	C4TX	11	6
10	■	□	■	■	■	■	■	■	■	□	■	■	BB	C4TX	90	2
11	■	■	■	■	■	■	■	■	□	□	□	■	AB	C4TX	5	1
12	■	□	■	■	■	■	■	■	□	□	□	■	BB	C4TX	71	1
13	■	□	■	■	■	■	■	■	□	■	■	■	BB	C4TX	91	1
14	■	□	□	■	■	■	■	■	□	■	■	■	BB	C4TX	314	1
15	■	■	■	■	□	□	□	■	■	■	■	■	AB	CXT4	2	2
16	■	■	■	■	■	■	□	■	■	■	■	■	AB	CXT4	3	2
17	■	■	■	■	□	■	□	■	■	■	■	■	AB	CXT4	18	1
18	■	■	□	□	■	■	□	■	■	■	■	■	BB	CXT4	68	2
19	■	■	□	□	□	□	□	■	■	■	■	■	BB	CXT4	69	1
20	■	■	□	■	■	□	□	■	■	■	■	■	BB	CXT4	96	1
21	■	■	□	□	■	□	■	■	■	■	■	■	BB	CXT4	166	1
22	■	■	□	□	□	■	□	■	■	■	■	■	BB	CXT4	167	1
23	□	■	■	■	□	□	□	■	■	■	■	■	BB	CXT4	359	2
24	■	■	■	■	■	■	□	■	■	■	□	■	AB	CXTX	24	1
25	■	■	■	■	■	■	□	■	□	■	■	■	AB	CXTX	9	3
26	■	■	■	■	□	□	□	□	□	□	□	■	AA	CXTX	1	2
27	■	■	■	■	□	□	□	□	□	■	□	■	AB	CXTX	23	3
28	■	■	■	■	□	□	□	■	□	■	■	■	AB	CXTX	35	1
29	■	■	■	■	■	□	■	■	□	□	□	■	AB	CXTX	48	1
30	■	■	■	■	■	■	□	□	□	□	□	□	AB	CXTX	55	2
31	□	□	■	■	■	■	□	□	□	□	□	□	BB	CXTX	72	1
32	□	■	■	■	□	□	□	■	□	■	■	■	BB	CXTX	170	1
33	■	□	■	■	■	■	□	□	□	■	□	□	BB	CXTX	172	1
34	■	■	■	■	□	□	□	□	■	■	■	■	AB	CXTX	202	2
35	□	■	■	■	■	■	□	□	□	□	□	■	BB	CXTX	293	1
36	■	■	■	■	■	■	■	□	■	■	■	■	AB	CXTX	319	1
37	□	■	■	■	□	□	□	□	□	□	□	■	BB	CXTX	332	1
38	■	■	■	■	□	■	■	■	□	□	■	■	AB	CXTX	339	2
39	■	■	■	■	■	■	■	□	□	□	■	■	AB	CXTX	373	1
40	□	■	■	■	■	■	□	□	□	□	■	■	BB	CXTX	391	1
41	■	■	■	■	□	■	□	■	□	■	■	■	AB	CXTX	46	1
42	■	■	□	□	■	■	□	■	□	■	■	■	BB	CXTX	164	1

Fig 6.4.3: KIR genotypes in study population of Assam common with other worldpopulations.

The genotype IDs are as in www.allelefrequencies.net. Black box indicates presence of KIR gene and white box, the absence of KIR gene. The number of individuals bearing each genotype is indicated. Genotypes limited to few populations (IDs 166,167, 314, 319, 332,359, 382) or reported as unique from North Indians (ID 55), South Indians (ID 172), Singapore Chinese (ID 339), Macedonia (ID 373), Palestian Jordan (ID 96), France Reunion (ID 293),

South African (ID 170), Iran Bakhtiari (ID 391) were detected in our population. 37% of genotypes of each TT and of TB and 52% of IE were common with earlier studies. Genotypes 382 common to TT, TB and IE whereas ID 339 & 18 seen in TT and 373 and 319 seen in TB.

No.	2DL1	2DL3	3DL1	2DS4	2DS2	2DL2	2DS3	2DL5	3DS1	2DS5	2DS1	2DP1	Genotypes	C4T4 clusters	No. of individuals
1													BB	C4T4	1
2													BB	C4TX	1
3													BB	C4TX	1
4													BB	C4TX	1
5													BB	C4TX	1
6													BB	C4TX	1
7													AB	C4TX	1
8													BB	CXT4	1
9													BB	CXT4	1
10													BB	CXT4	1
11													AB	CXT4	1
12													BB	CXT4	1
13													BB	CXTX	1
14													BB	CXTX	1
15													BB	CXTX	1
16													BB	CXTX	1
17													BB	CXTX	2
18													BB	CXTX	1
19													BB	CXTX	1
20													AB	CXTX	1
21													AB	CXTX	2
22													BB	CXTX	1
23													BB	CXTX	1
24													AB	CXTX	1
25													BB	CXTX	1
26													BB	CXTX	1
27													BB	CXTX	1
28													BB	CXTX	1
29													BB	CXTX	1
30													BB	CXTX	1
31													BB	CXTX	1
32													BB	CXTX	1
33													AB	CXTX	1
34													BB	CXTX	1
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41													BB	CXTX	1
42													BB	CXTX	2
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44													BB	CXTX	1
45													BB	CXTX	1
46													BB	CXTX	1
47													BB	CXTX	1
48													BB	CXTX	1
49													BB	CXTX	1
50													BB	CXTX	1
51													AB	CXTX	1
52													BB	CXTX	1
53													BB	CXTX	1
54													BB	CXTX	1
55													BB	CXTX	1
56													AB	CXTX	1
57													BB	CXTX	1
58													BB	CXTX	1
59													AB	CXTX	1
60													AB	CXTX	1
61													BB	CXTX	1

Fig 6.4.4: KIR genotypes novel to the study population.

49 of the novel genotypes belonged to CXTX clusters as indicated Each genotype was observed in a single individual except for genotype numbers 17, 21 and 42.

The presence of C4 and T4 clusters were also evaluated in the B haplogroup population (Bx genotype). 59% of the population lacked both the clusters while only 8 % was detected with both C4 and T4 clusters. The C4 cluster was more prevalent in the TT while T4 was seen at higher frequency in TB (p=0.002). The proportion of C4T4 clusters was highest in the IE (Fig 6.4.5)

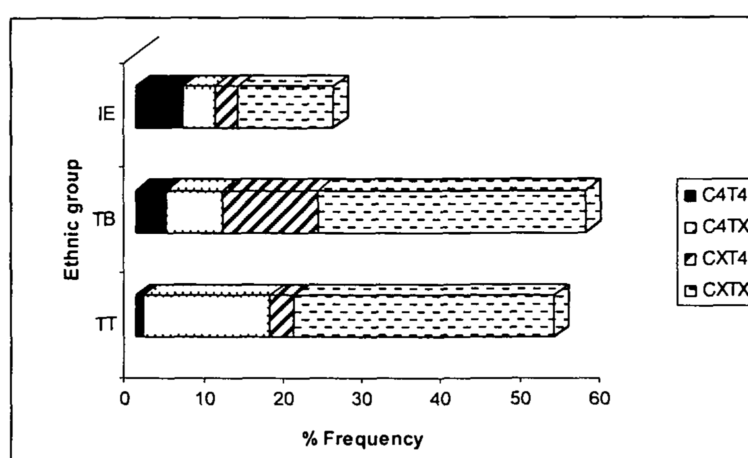

Fig 6.4.5: The C4T4 clusters frequency among the three ethnic groups.

6.4.4 Association of KIR genes loci with malaria

The frequency of KIR genes in relation to malaria and its severity and with frequency of malaria episodes was analysed. Comparison of KIR genes frequencies between the malaria and non malaria group showed that the frequency of the activating KIR3DS1 was higher in the non malaria group ((p= 0.022) while that of inhibitory gene KIR 2DL3 was lower in this group (p = 0.03) (Fig 6.4.6).

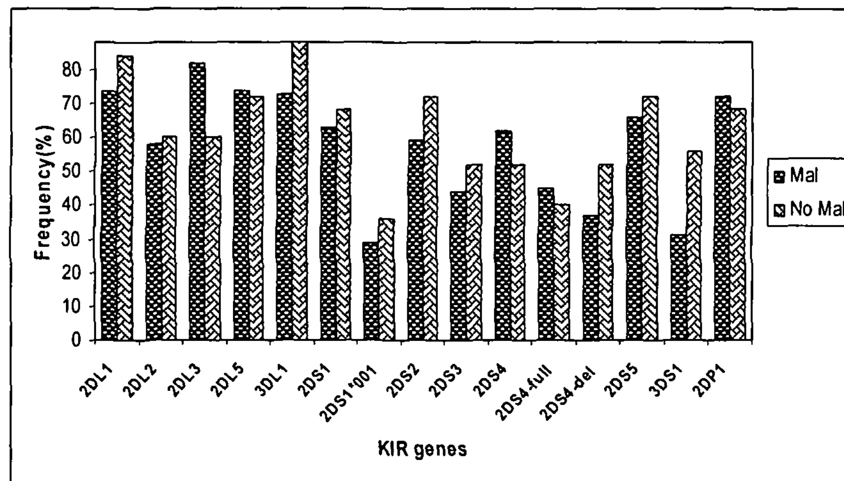

Fig 6.4.6: KIR genes frequencies in malaria (n=110) and non malaria (n= 25) groups.

KIR2DL3 frequency was higher in the malaria group (p = 0.03) while that of KIR3DS1 frequency was higher in the non malaria group (p = 0.02).

Analysing KIR genes frequencies with respect to malaria severity revealed that the frequency of KIR3DL1 was higher in the complicated malaria as compared to uncomplicated malaria group (Fisher's Exact p = 0.006) (Table 6.4.2).

Table 6.4.2: Comparison of KIR genes frequencies between the complicated vs uncomplicated malaria cases and between the frequent vs. infrequent malaria cases.

KIR gene loci	Complicated cases (n=30)	Uncomplicated cases (n=77)	p values	Frequent cases (n=67)	Infrequent cases (n=37)	p values
2DL1	70	55.8	0.196	56.3	76.5	0.084
2DL2	63	57.1	0.663	56.2	55.88	1
2DL3	86.7	79.2	0.581	79.7	82.3	0.795
2DL5	80	70.1	0.343	70.3	70.59	1
3DL1	93.3	66.2	0.006	75	61.8	0.354
2DP1	83.3	67.5	0.221	68.8	70.6	0.819
2DS1	43.3	46.8	0.83	40.6	52.9	0.297
2DS1*001	33.3	26	0.478	21.9	38.2	0.104
2DS2	70	53.2	0.19	54.7	58.8	0.528
2DS3	56.7	38.96	0.129	40.6	47	0.526
2DS4	66.7	62.3	0.826	62.5	64.7	1
2DS4 - full	53.3	42.9	0.397	40.6	52.9	0.145
2DS4 - del	50	33.8	0.185	40.6	23.5	0.079
2DS5	63.3	62.3	1	62.5	67.6	0.829
3DS1	30	31.2	1	29.7	32.4	1

The table depicts the comparison of KIR genes frequencies between the complicated vs uncomplicated cases and between the frequent and infrequent cases. Frequency of KIR3DL1 was higher in complicated malaria (Fisher's exact p = 0.006). The frequency of malaria episodes was not related to changes in genes frequency though there was a weak association of the gene KIR2DL1 and KIR2DS4-del allele of KIR2DS4 with frequency of malaria episodes. Clinical malaria data on severity and no. of malaria episodes could not be obtained for 3 and 6 individuals respectively due to loss to follow up.

Further, KIR3DL1 frequency was positively associated with malaria severity (Pearson phi, R^2 = 0.297) and logistic regression modelling predicted KIR3DL1 as a risk factor with KIR3DL1 positive individuals seen to be at greater risk of complicated malaria [Odds Ratio (95% C.I)] = [6.39 (1.34-30.60)] (Table 6.4.3).

Table 6.4.3: Logistic regression analysis showing the association of parameters with disease outcome.

	Complicated vs. Uncomplicated cases				
Parameter	Standardized coefficient	Wald ($\chi 2$)	p values	Odds ratio (O.R)	95% CI (O.R)
KIR3DL1	0.459	5.393	0.02	6.39	1.34-30.60
KIR2DS1*001	0.238	3.085	0.079	2.54	0.89- 7.18
KIR2DS4*KIR2DS4del * KIR2DS5*TT	-3.52	3.907	0.048	0.03	0.001-0.91
KIR2DS4*KIR2DS4del * KIR2DS5*TB	2.345	2.097	0.148	10.43	0.44-249.11

Logistic regression was performed with disease status as the outcome variable using XLSTAT 2010 version.

An interaction between ethnicity and KIR3DL1 was also seen where higher proportion of KIR3DL1 positive and complicated malaria patients belonged to Tea tribes (Table 6.4.4).

Table 6.4.4: Comparison of KIR3DL1/3DS1 zygosity between the TB and TT groups in relation to clinical malaria outcome.

	TT			
Genotypes	CNC (n=14)	UNC (n=34)	p values	O.R (95% C.I)
$3DL1^+/3DS1^-$	13 (92.85)	18 (52.94)	0.009	11.55 (1.7-74.86)
$3DL1^+/3DS1^+$	1 (7.14)	3 (8.8)	1	0.795 (0.11- 6.23)
$3DL1^-/3DS1^+$	0	3 (8.8)	0.546	0 (0- 3.12)
	TB			
Genotypes	CNC (n=12)	UNC (n=36)	p values	O.R (95% C.I)
$3DL1^+/3DS1^-$	6 (50)	18 (50)	1	1 (0.28- 3.55)
$3DL1^+/3DS1^+$	4 (33.3)	7 (19.44)	0.43	2.07 (0.52- 8.51)
$3DL1^-/3DS1^+$	2 (5.5)	8 (22.22)	0.704	0.58 (0.13- 2.87)

Significant differences between the complicated (CNC) and uncomplicated (UNC) for $KIR3DL1^+/3DS1^-$ genotypes in TT was seen ($p = 0.009$). But in TB no difference was seen. The values in the parenthesis ( ) indicate the genotype percentage.

The zygosity of KIR3DL1 and its allele 3DS1 was examined in the TT and TB population. KIR $3DL1^-/ 3DS1^+$ genotype was markedly lower in TT ($p = 0.044$) (Table 6.4.5). Analysis of KIR3DL1/3DS1 zygosity between the TB and TT, with respect to malaria outcome showed that the relative risk of having complicated malaria in TT was higher in $KIR3DL1^+/ 3DS1^-$ genotype (Table 6.4.4). In contrast, $KIR3DL1^-/3DS1^+$ individuals were more likely to have uncomplicated malaria [Odds Ratio (95% C.I)] = [0.352 (0.084-1.504)]. No such interaction was observed in TB. Further, the KIR3DL1 and 3DS1 alleles were not in Hardy Weinberg Equilibrium in TT as well as in overall study population ($\lambda^2 = 84.89$, $p < 0.001$), indicating the alleles were under selection.

Table 6.4.5: Comparison of KIR3DL1/3DS1 zygosity between the TB and TT groups.

Genotypes	TT (N=53)	TB (N=57)	P values	O.R (95% C.I)
$3DL1^{+}/3DS1^{+}$	11.32	21.05	0.204	0.479 (0.171-1.346)
$3DL1^{+}/3DS1^{-}$	62.26	49.12	0.184	1.704 (0.803-3.638)
$3DL1^{-}/3DS1^{+}$	5.66	19.29	0.044	0.251 (0.071-0.897)

KIR3DL1/3DS1 zygosity compared using Odds ratio (O.R), it may be noted that KIR3DS1 homozygosity was significantly lower in the TT, while KIR3DL1 homozygosity was higher in them though it was not significant.

In order to understand that if low KIR3DS1 frequency in TT was related to their genetic background, we examined the KIR gene frequency of Khasis, another Austro-Asiatic population of North east India. The frequency of KIR3DS1 gene in Khasis was 22% which was comparable to the TT population.

Considering that KIR3DL1 is characteristic of A haplotype, individuals of AB genotype could be expected to be at greater risk of complicated malaria. Indeed, the frequency of AB genotype was higher in complicated malaria patients (50%) than in uncomplicated malaria (36.36%) but it was not statistically significant ($p = 0.272$). No significant difference was noted in frequency of KIR3DL1 between individuals of AB and BB genotypes.

In addition, KIR2DS1*001 allele of KIR2DS1 was also predicted as a risk factor in complicated malaria by logistic regression [Odds Ratio (95% C.I)] = [2.54 (0.89-7.18)]. Interestingly the frequency of KIR2DS1*001 was negligible in the TT. The combination of KIR2DS4 and its deleted variant (KIR2DS4del) and of KIR2DS5 gene along with the ethnicity of the population was seen to influence the disease outcome. Increased frequency of this combination was likely to decrease the risk of complicated malaria in TT (p =

0.048), but in TB it tended to increase the probability of complicated malaria though it was not significant (Table 6.4.3). Though the frequency of activating genes was higher in individuals of complicated malaria group particularly of KIR2DS2 and KIR2DS3, but these differences were not statistically significant. No significant differences in KIR genes frequencies was seen between the frequent and the infrequent cases though KIR2DL1 and KIR2DS4-del were weakly associated but it did not reach the significant level (Table 6.4.2)

6.4.5 No. of activating genes and malaria outcome.

Activating genes have been postulated to influence the outcome of infectious diseases. The relation between numbers of activating genes ranging from one to six in an individual with that of malaria severity and frequency of malaria episodes was analysed (Fig 6.4.6 & 6.4.7). Individuals with six activating genes were more prone to suffering from complicated malaria ($p = 0.05$) suggesting that overrepresentation of activating genes tended to predispose to complicated malaria. Indeed, a linear relationship between number of activating genes and increased percent of complicated malaria cases was noted with more than four activating genes (Pearson Correlation coefficient, $R^2 = 0.611$) (Fig 6.4.6). Interestingly, presence of four activating genes was positively associated with infrequent malaria ($p = 0.025$) where individuals having four activating genes were seen to be protected from frequent episodes of malaria (Fig 6.4.7).

6.4.6 Linkage Disequilibrium

Linkage disequilibrium of genes pair was calculated and KIR genes pairs which showed positive and negative association are given in Table 6.4.6. Genes pair which were in strong positive linkage were KIR2DL2 with KIR2DS2 and KIR2DS3 ($p < 0.0001$), KIR3DL1 with KIR2DS4 ($p < 0.0001$) and KIR2DS2 with KIR2DS3 ($p < 0.0001$). Among the genes pair in strong negative linkage were KIR2DS5 with KIR3DL1 ($p = 0.006$) and KIR2DS5 with KIR2DS4 ($p = 0.003$). Distinct differences in LD were seen between the two ethnic groups with TT showing strong linkage disequilibrium of KIR2DS4 with KIR2DS3 and of KIR2DL2 with KIR2DL5, KIR2DS2, KIR2DS3. A higher number of genes loci in linkage

disequilibrium were seen in the TB (KIR2DL2 with KIR2DS1, KIR2DS2 and KIR2DS3 but not with KIR2DL5).

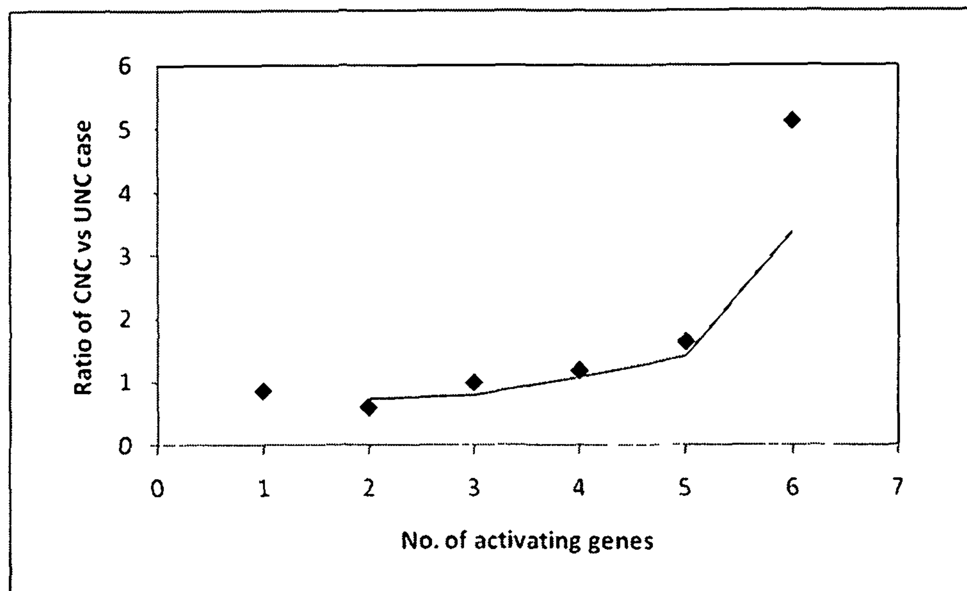

Fig 6.4.6: Number of activating genes and disease severity.

Complicated (CNC) vs uncomplicated (UNC) malaria. The line and the dot depict the moving average and ratio of cases respectively. A linear correlation was noted with the increased in number of activating gene. ($y = 0.703x-0.7349$, $R^2 = 0.6011$) and it attained statistical significance with genotypes having six activating genes ($p = 0.05$).

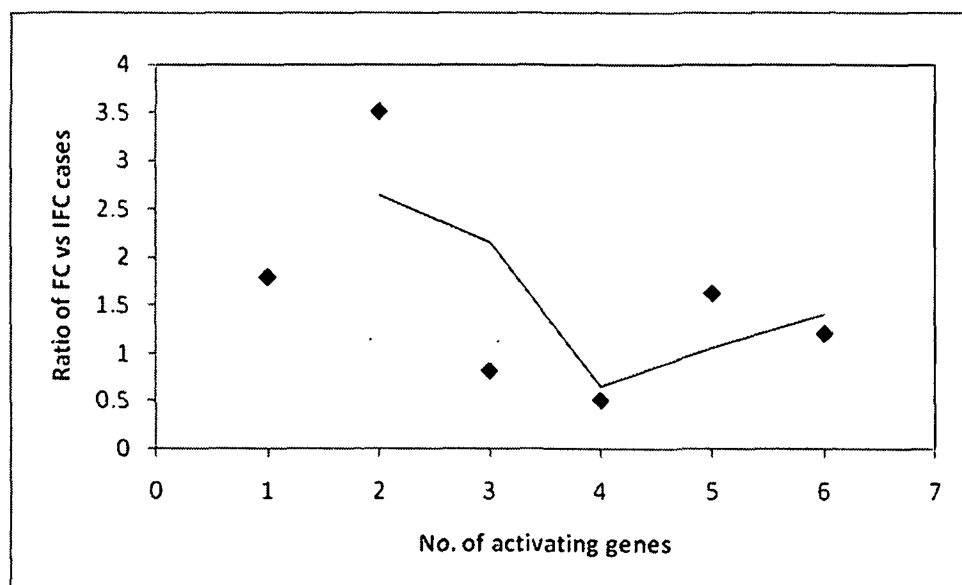

Fig 6.4.7: Number of activating genes and frequency of malaria episodes.

Frequent (FC) vs Infrequent (IFC) malaria cases. The line and the dot depict the moving average and ratio of cases respectively. A linear correlation was noted with the increased in number of activating gene though the correlation was weak ($y = -0.2534x + 2.4607$, $R^2 = 0.1989$). The genotypes with 4 activating genes were associated with protection from frequent episodes of malaria ($p = 0.025$).

Table 6.4.6: Pair of KIR loci with significant linkage disequilibrium ($p < 0.05$) in the study population and between the TT and TB populations.

	KIR Locus									
Total population (n= 135)	**2DL1**	**2DL2**	**2DL5**	**3DL1**	**2DS1**	**2DS2**	**2DS3**	**2DS4**	**2DS5**	**3DS1**
Positive LD	3DL1 2DS2	2DL5 2DS1 2DS2 2DS3 2DS4	2DS1 2DS2 2DS3 2DS5	2DS4	2DS3 2DS4 3DS1 2DP1	2DS3	2DS4 2DP1	3DS1	3DS1	2DP1
Negative LD		2DL3 2DP1	3DL1	2DS5 3DS1				2DS5		
	Ethnic LD									
TB (n= 57)	2DL2 2DS2	2DS1 2DS2 2DS3 2DP1		2DS4 2DS5	2DS2 2DP1 3DS1				3DS1	2DP1
TT (n= 53)		2DL5 2DS2 2DS3	2DS3			3DS1	2DS4			

LD, Linkage disequilibrium; Pairs of KIR loci with $p < 0.0001$ (KIR2DL2 KIR2DS2, KIR2DL2-KIR2DS3, KIR3DL1-KIR2DS4, KIR2DS2-KIR2DS3.

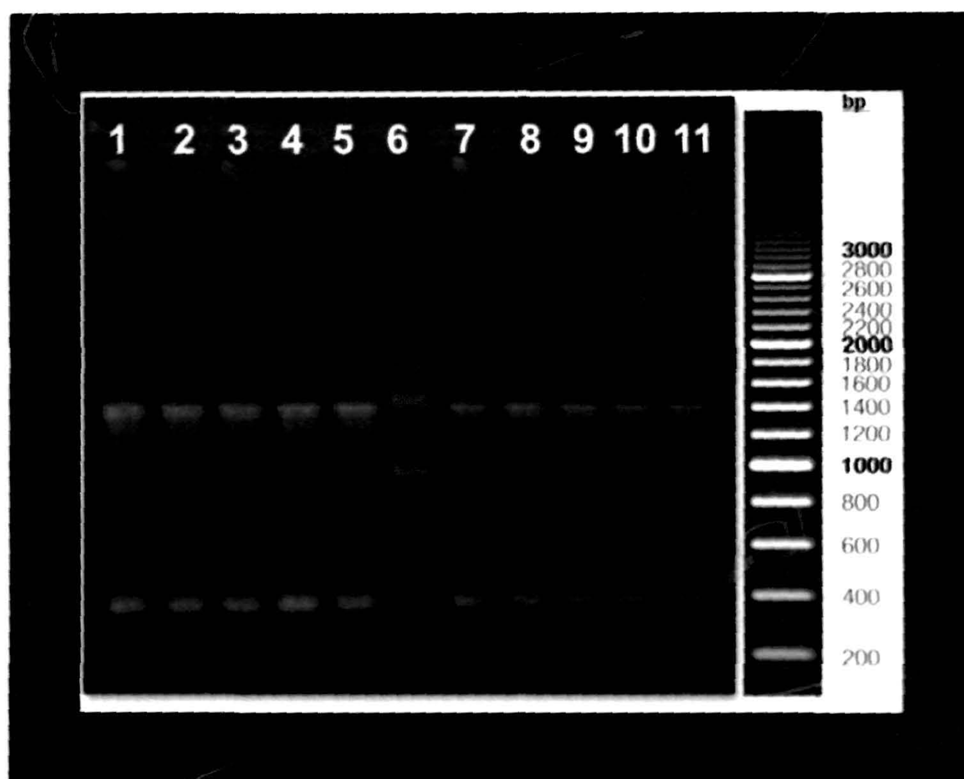

Fig 6.4.9: PCR products:

Lane: 1, 2, 3, 4, 5, 7, 8, 9, 10, 11:- amplified products of KIR3DL1 gene (1905 bp) and of internal positive control (256 bp). Lane 6:- 200 bp molecular marker (Fermentas).

6.5 DISCUSSION

The present study examined the premise that KIR gene frequencies and genotypes of two ethnic groups namely the Tea tribes (Mundari speakers) of Austro-Asiatic origin and of the Tibeto-Burman from malaria endemic regions could be shaped by malaria infection and could therefore translate to altered risk of disease or of infection with Plasmodium species. Not surprisingly, considerable variation in KIR genes frequencies was seen between the groups, which differ in their genetic backgrounds with respect to mitochondrial DNA and NRY haplogroups and in migration history[195,215,216]. This is consistent with differences in KIR genes frequencies in ethnically distinct groups in other world populations[219]. The TT and IE were closer to Indian populations in their KIR gene frequencies and may be explained on the basis of their genetic history[220,221]. The Tea tribes of our study population were Mundari speakers of tribes like Bhumij, Munda, Mura etc and belong to the Austro-Asiatic ethnic group that is believed to be one of the earliest settlers of Indian plains that entered India in prehistoric migrations of humans out of Africa[221]. The Indo-European group of our study belonged to the caste populations of India[222]. Majumdar[221] has argued that although there is significant genetic differentiation between caste and tribal groups of India, the contribution of Central Asian migration on South Asian gene pool was minor and that genetic patterns of the caste groups contain some predominant genetic signatures found in central Asia (Indo-European speakers) with signatures that are indigenous to India. Though, the IE was more similar to South Indian populations in prevalence of C4T4 clusters, shared genotypes with TB, but they mapped as an outlier group on PCA plot. Their unique KIR gene frequencies may either be representative of their origin, extensive racial admixture or local differentiation of their KIR locus. Intriguingly, the TB was seen to cluster with the Chinese, Austronesian and native American populations on PCA plot and on AHC dendograms. While genetic similarity with the Chinese could be expected in line with their migration history[195, 216], the affinity to Austronesians and native American populations could be related to early expansion of populations in East Asia and to Americas[221]. Interestingly, the TB

were similar to the American Natives and Mexicans in higher prevalence of T4 cluster[218]

The TT were however distinct from other Indian populations in having markedly lower frequency of 3DS1. The markedly low frequency of KIR3DS1 in Tea tribes of our study population was comparable to that reported from African populations[146], suggesting it to be an ancestral genotype. Low KIR3DS1 frequency also seen in Khasis, another Austro-Asiatic tribe of Northeast India[215], supports this contention. Notably, the Mundari speaking Austro-Asiatic to which the TT belong have been postulated to be one of the earliest settlers of Indian plains that migrated out of Africa[221]. Dominance of B haplotypes seen in the present study is in accordance with other populations from the Indian subcontinent and other Asian populations[150,151,152]. It has been hypothesized that diverse B haplotype seen in tropical countries may give an upper edge in protection against the circulating local pathogens[150].

Our results showed a significant interaction of frequency of KIR gene loci with disease status as well as frequency of malaria episodes. Presence of KIR3DL1 gene was associated with higher risk of complicated malaria. To the best of our knowledge, this is the first study where in complicated malaria and interaction of KIR genes with risk of disease has been demonstrated. Interestingly, KIR3DL1 gene frequency was also seen to be higher in *P. falciparum* positive Melanesian individuals of malaria endemic region[167]. The association of KIR3DL1 with complicated malaria in the present study was ethnicity dependent where a significantly higher proportion of KIR3DL1 positive individuals having complicated malaria belonged to Tea tribes. This observation together with low KIR3DS1 frequency in TT raises the moot point if low KIR3DS1 frequency in TT was maintained by malaria. Our data indicates a role for malaria as (1) the alleles KIR3DS1 and KIR3DL1 were not in Hardy Weinberg equilibrium and neither KIR3DL1 nor KIR3DS1 was in linkage with other genes in TT suggesting these were under selection. But in Tibeto-Burman, linkage of KIR3DL1 with two activating genes; KIR2DS4 and KIR2DS5 could

be one of the reasons for weak association of KIR3DL1 with complicated malaria in this population. Further, KIR2DS5 was in linkage with KIR3DS1 and this probably accounted for higher KIR3DS1$^+$ genotype frequency in TB population. 2). KIR3DS1 frequency was higher in no malaria group in comparison to the malaria group and the relative risk of having complicated malaria was increased in KIR3DL1$^+$/3DS1$^-$ genotypes in the TT whereas KIR3DL1$^-$/3DS1$^+$ individuals were more likely to have uncomplicated malaria. It may be argued that low KIR3DS1 frequency with higher probability of complicated malaria would be an advantage to the parasite as cytoadherence and sequestration seen in complicated malaria favour the parasite since it helps to evade immunity and increases the chances of its transmission to the mosquitoes[223]. An ongoing selection on KIR3DL1/ 3DS1 in Africans has been suggested in a recent study[146] and the authors argued for KIR3DS1 having a biological function of persisting value. (3) Though, low KIR3DS1 frequency in TT and Khasis, suggests that it could be an ancestral genotype in these two populations, however, role of pathogen pressure in maintaining this low frequency, possibly of malaria, which is a common denominator in all these populations cannot be ruled out. (4) KIR3DS1 homozygosity was also reported to be higher in *Plasmodium falciparum* negative individuals as compared to increased KIR3DL1 homozygosity in the *P f* positive individuals in Melanesian populations from malaria endemic Solomon Islands[167], clearly indicating a role for KIR3DS1 in malaria status. Nonetheless, detailed studies are required to understand the interaction of malaria with KIR genes as higher KIR3DS1 frequency has been reported in populations from other malaria endemic regions[219]

Further, KIR3DS1 appears to exert its effect by modulating KIR3DL1 mediated inhibition, probably by competing for HLA-Bw4, which serves as a ligand for both KIR3DL1 and 3DSI, such that KIR3DL1$^+$/3DS1$^+$ mediated less inhibition. This argument is supported by our observation that 33 of the 39 KIR3DS1 positive individuals showed prevalence of four or more activating genes and that six activating genes were associated with risk of complicated

malaria. The observed difference in association of KIR3DL1 with complicated malaria between Tea tribes and Tibeto-Burman may also be related to the difference in the allelic variants of KIR3DL1 or of HLA in the two populations. The correlation of 3DL2*002 allotype of 3DL2 with the likelihood of making a strong NK response to *P. falciparum* infected RBC observed by Riley *et al.*[43], is consistent with the differential activation by allotypes of KIR genes in malaria. We have not examined the allotypes of KIR3DL1 and hence exact influence of allotypic variants cannot be commented upon and warrants a high resolution typing. Nonetheless, we noted marked differences in distribution of allotypes of KIR2DS1 in the three ethnic groups, where the 2DS1*001 allotype was nearly absent in TT indicating the prevalence of different allotypic variants in the three ethnic groups of our study. KIR2DS1*001 allotype was identified as risk factor for complicated malaria and was positively associated with it, indicating the influence of allelic variants on disease outcome. The low prevalence of KIR2DS1*001 allele but higher frequency of KIR2DS1 in TT could be seen as positive selection in this ethnic group conferring protection from complicated malaria. Notably, KIR2DS1 has been associated with protection from other diseases by earlier investigators[224,225]. The observed higher frequency of KIR2DS4 in this ethnic group could be considered as favorable as combination of KIR2DS4 with KIR2DS4-del and KIR2DS5 was negatively associated with complicated malaria in TT ($p < 0.048$). In contrast, this combination was positively though weakly associated with complicated malaria in TB and the observed lower frequency of KIR2DS4 in Tibeto-Burman could thus be an advantage. These observations emphasize the polygenic adaptation to malaria and that different populations adapt differently to malaria. It also tends to suggest that particular combination of activating genes to be important in delivering the signals.

In the present study, presence of 4 activating genes was associated with decreased malaria episodes, whereas six activating genes were identified as risk factor for complicated malaria. Our seemingly contradictory observations may be explained on the basis of differential NK signaling, where lower activation

mediated by four genes probably elicited appropriate immune response whereas six might be responsible for over activation of NK cells leading to exaggerated inflammatory response that contributed to pathogenesis of malaria[203]. In a recent study in Papua New Guinea, IFNγ was seen to protect children from frequent episodes of clinical infection[105]. However, lack of correlation between number of activating KIRs expressed and magnitude of NK-iRBC response or activating genes with *P. falciparum* infection was noted by earlier investigators[42, 167].

In conclusion, considerable heterogeneity in KIR gene frequencies and KIR genotypes was seen among the three ethnic groups that were reminiscent of their genetic background. KIR genotype and more than one KIR gene was noted to influence disease outcome suggesting polygenic adaptation to malaria. Combination of KIR2DS4, KIR2DS4del, KIR2DS5 negatively influenced disease outcome in Tea tribes ($p = 0.048$) while in Tibeto-Burman the association was positive though it was not significant. Four activating genes appeared to provide optimal activation as this protected from frequent episodes of malaria while six genes were seen to predispose to complicated malaria. $KIR3DL1^{-}/ 3DS1^{+}$ was associated with uncomplicated malaria while $KIR3DL1^{+}/ 3DS1^{-}$ was linked to complicated malaria in TT ($p = 0.009$). The low KIR3DS1 frequency seen in Tea tribes was similar to that reported from African populations. Overall, our data suggests low KIR3DS1 frequency to be an ancestral genotype maintained by similar pathogen pressures possibly malaria in the Austro Asiatic tribes. Though it is tempting to speculate that it could be related to their longer exposure to disease as Austro-Asiatic speaking tribals, the group to which the tea tribes of our study belong are believed to be the earliest inhabitants of Indian plains however, it warrants detailed studies with high resolution typing, particularly of relevant genes to fully understand the footprint of malaria on human KIR locus.

CHAPTER VII:

Conclusion

7. CONCLUSION

The study was designed to assess the clone multiplicity of *P falciparum* genotypes existing in the study area and to elucidate the humoral and cell mediated immune response of the population to identify an immune correlate of protection.

A high *P falciparum* diversity with more alleles of each of the allelic family in the high transmission summer season was noted. Complex infections with novel genotypes in summer seen in the study tend to support the modification of *P. falciparum* genetic structure. Our data indicated that allele distribution was site dependent with some alleles common to the two sites but some were unique to a site. Multiplicity of infection could not be directly related to transmission intensity but rather host factors like ethnicity and the immune status of the individuals were implicated in complexity of infections seen in the study sites.

Anti MSP-1_{19} antibody response of the study population was seen to protect both from risk of clinical infections and severity of the disease. A lower antibody response to MSP-1_{19} peptides in comparison to whole merozoite extract of local *P. falciparum* strain was seen. A gradual acquisition of antibodies with age was also noted. This might be explained by the temporal variation of the *P. falciparum* structure seen in the study sites. The immune response was seen to be site dependent with a marked difference in recognition of the antigens between the two sites. A low seropositivity of Q-KNG at Guabari and a relatively higher seropositivity for WME and MSP1_{19} peptides at KTE were observed. This was seen to be appropriately explained by the ethnicity dependent antibody response with the TT having a relatively higher immune response than the TB. A striking observation was that majority of the negative responders to Q-KNG belonged to the TB group. This observation taken together with a lower MOI in Guabari, correlation of host factors with complexity of infection suggests a strong

influence of host genetics in immune response to malaria. Anti-E-TSR antibodies were also negatively associated with complicated malaria.

When cytokines response was measured in the study population with respect to different outcome of malaria infection we observed a higher TGF-β, IL-2 and IL-12α genes expression and depressed level of IL-10 with no change in mRNA level of IFN-γ in *Pf* infected cases suggesting induction of balanced cytokine response with activation of both Thl and Th2 cells. In complicated malaria our data indicates suppression of T cell function in the study population with decreased levels of IL-2 and IL-12 α along with increased expression of TGF-β and no change in IL-10 mRNA levels. TGF-β levels were elevated both in *Pf* infection as well as in complicated malaria. Further, TGF-β levels were negatively associated with age. A difference in TGF-β expression was also seen between the TB and TT with the level of cytokine significantly higher among the Tea tribes.

In addition, increased IFN-γ was directly associated with disease severity. But an increased TGF-β along with decreased IL-12α levels is not consistent with hyper activation of T cells leading to increased IFN- γ expression in complicated malaria. Therefore, we suggest that T cells are unlikely to be the source of elevated IFN-γ secretion and alternatively cells like NK cells, γδ T cells might play a role in the pathogenesis of the disease. The proposed model of the cytokine network is explained as follows.

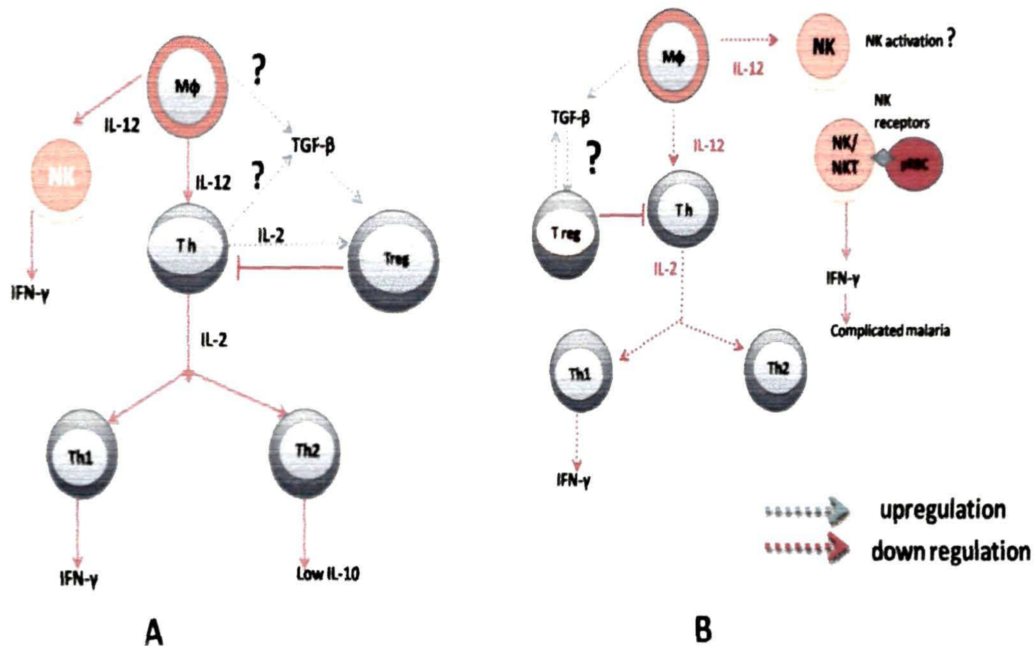

Fig: Proposed model to explain regulation of cytokine network in uncomplicated (A) and in complicated (B) malaria. *IL-12 secreted by Mφ activates Th cells which differentiate into Th1effector cells. IL-12 also activates NK cells. TGF-β produced by T cells or Mφ or both counter-regulates Th activation to give balanced cytokine response. In complicated malaria high TGF-β production by Mφ and T reg cells coupled with decreased IL-12 production downregulates Th cell activation resulting in lower levels of IL-2 and decreased Th function. NK activation is also downregulated by lower IL-12, however NK receptor ligation with pRBC/parasite molecules results in increased NK cell activation and IFN-γ secretion leading to complicated malaria.*

A possible role of NK cells in pathogenesis of the disease was supported by our observation of KIR profiles in the population where the presence of the inhibitory KIR3DL1 was associated with higher risk of complicated malaria. Presence of six numbers of KIR activating genes was seen to predispose to disease severity. The increased levels of IFN-γ relating to complicated malaria might be explained by over activation of NK cells through its activating receptors signalling. Importance of a balance immune response has been

reaffirmed from our study where KIR genes profiles with four numbers of activating genes were related to protection from frequent episode in malaria.

Consistent with their history of migration, the TT and TB differed distinctly in their KIR genes frequencies. Further, a significantly higher proportion of KIR3DL1 positive individuals having complicated malaria belonged to the TT. Also, the TT was found to have a low frequency of KIR3DS1.

In conclusion, we observed a high *P falciparum* diversity which was seen to vary with transmission seasons and between years. This could contribute to the low anti $MSP1_{-19}$ antibody response of the population. Anti- $MSP1_{-19}$ antibodies could confer protection from risk of infection as well as disease severity. IL-12α was seen to be an important marker of protection from complicated malaria. Risk to complicated malaria was associated with increased levels of IFN-γ, TGF-β and presence of KIR3DL1 gene. Genotypes with six activating genes were also seen to be at higher risk of complicated malaria. A strong role for genetic background was indicated in immune response to malaria. The TT and TB ethnic groups seemed to have different adaptation mechanism with KIR genes frequencies differentially associated with protection in them and the TT having a higher antibody response which conferred protection from risk of infection and severity.

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List of publications

1) Baruah, S., Lourembam, S.D., Sawian, C.E., Baruah, I., Goswami, D. Temporal and spatial variation in MSP1 clonal composition of *Plasmodium falciparum* in districts of Assam, Northeast India. *Infect.Genet.Evol*, **9**, 853–59 (2009).
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