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Toll-like receptor 2-mediated downstream cytokine levels as determinant of malaria pathogenesis

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***Background & objectives*: Toll-like receptors (TLRs) are transmembrane proteins that recognize specific molecular patterns and activate downstream cytokine production usually for the eradication of invading pathogens. The objective of this study was to evaluate the genetic polymorphism of *TLR2 Arg753Gln* (rs 5743708) and soluble cytokines and TLR2 expression levels in malaria disease cases.**

***Methods*: The study included prospectively collected 2 ml blood samples from 153 individuals clinically suspected for malaria and confirmed by microscopy and RDT from Assam. Stratification of the study groups was done as healthy control (HC, n=150), uncomplicated malaria (UC-M, n=128) and severe malaria (SM, n=25). The PCR-restriction fragment length polymorphism (RFLP) method was applied for the analysis of *TLR2 Arg753Gln* polymorphism and following the ELISA for soluble serum TLR2 (sTLR2) and its associated downstream cytokines, *viz*. tumour necrosis factor (TNF)-α and interferon (IFN)-γ levels.**

***Results*: Variation in *TLR2 Arg753Gln* gene showed no association with the susceptibility and the severity of malarial infection. Soluble TLR2 expression was significantly higher in uncomplicated malaria (UC-M) cases compared to healthy controls (*P*=0.045) and in terms of SM cases, the expression was also found to be higher in UC-M cases (*P*=0.078). The TNF-α expression was significantly higher in SM cases compared to both UC-M and control (*P*=0.003 and *P*=0.004). Similarly, significantly elevated expression of IFN-γ was noted in SM cases compared to both UC-M (*P*=0.001) and healthy controls (*P*<0.001).**

***Interpretation & conclusions*: The present study suggests the association of deregulated TLR2 pathway that leads to the deleterious downstream immune response in the development of malarial pathogenicity.**

Key words Expression - interferon-gamma - malaria - Northeast - polymorphism - toll-like receptor 2 - TNF-α

Malaria is a vector-borne disease caused by the *Plasmodium* species and major health crisis globally[1]. Assam, the major State of northeast (NE) India, is highly co-endemic to *Plasmodium falciparum* and *P. vivax*-mediated malaria infection contributing to 10 per cent of cases and 20 per cent of malaria-attributed deaths in India, in which the majority of the infections (77%) contributed by *P. falciparum*[2] with widespread distribution of chloroquine-resistant strains[3].

A large population gets infected with malaria each year and only a small subset of the infected individuals (1-2%) progress-to-severe life threatening forms, characterized by various clinical features including impaired consciousness, coma, difficulty in breathing, severe anaemia and multiorgan failure[4], raising the question as to why only a small group of the infected individuals develop severity, while the others remain asymptomatic. Only a deep insight into the role of molecular mechanisms involving the induction of the differential innate immune system and generation of inflammatory cytokines may lead to the probable justification of this query.

Toll-like receptors (TLRs) are the major pathogen recognition receptors present on the myeloid cells that play a crucial role in recognizing the type of microbial pathogens to elicit immune responses against them. In the context of malaria parasites, TLR2 mainly recognizes the *P. falciparum* derived toxic compound glycosylphosphatidylinositols (GPIs) and to some extent by TLR4 during the blood-stage level of the infection leading to the production of the pro-inflammatory cytokines through the activation of the macrophages[5]. Specific immune response is mediated *via* the production of inflammatory cytokines such as tumour necrosis factor (TNF-α), interferon (IFN-γ), interleukin (IL)-12, and IL-10[6]. Both the pro- and anti-inflammatory markers are also regulated by TLR2[7]. Enhanced expression of pro-inflammatory cytokines with the development of Th1 cells is essential, although excessive and/or prolonged production may lead to the development of severity of the disease[8].

The *TLR2* gene is located on chromosome 4q32, and is composed of three exons encoding 784 amino acids[9]. Several polymorphisms of *TLR2* have been identified previously and reportedly affect the host defence mechanism and disease progression[10]. *TLR2 Arg753Gln* polymorphism in the intracellular Toll-IL-1 receptor (TIR) domain abolishes TLR-2-mediated downstream signaling cascade and has been linked to increased risk of tuberculosis and other infectious diseases[11]. Data indicate that *TLR2* gene polymorphism may also have a role in the increased susceptibility to malaria infection[12]. The assessment of TLR2 polymorphism in malarial patients may hence provide information regarding risk stratification[13]. In this context, this study was aimed to evaluate the association of TLR2-mediated downstream cytokine alteration with the risk of malaria outcome in indigenous tribal populations residing in malaria-endemic districts of Assam to understand the influence of malarial infection in the selective engagement of TLRs.

Material & Methods

This study was conducted in the department of Life Science and Bioinformatics, Assam University Diphu Campus, Karbi, Anglong, Assam, for a period of four years from 2012 to 2016. The study was approved by the Institutional Ethical Committee. All participants were enrolled with informed consent.

Study population: This study included a total of 153 individuals with clinically confirmed diagnosis of *P. falciparum* related malaria (through rapid test and further confirmed by microscopic examination by a registered pathologist) from the Civil Hospital, Diphu, Karbi-Anglong, Assam. The sample size calculation for the current study was done by using Rao soft software (Raosoft Inc., Seattle, WA, USA). Depending on the clinical features and laboratory findings, patients were further stratified as uncomplicated malaria (UC-M, n=128) [patients who present the clinical symptoms of malaria with the positive parasitological test but with no features of severe malaria (SM)] and SM (n=25) that incorporated cerebral malaria, acute renal failure, respiratory failure and/or severe anaemia (<7 g/dl) patients[14].

The details of community wise breakup and case stratification are provided in Table I.

Blood samples (2 ml) were collected intravenously under clinical supervision. Participants with other inflammatory diseases, age less than 18 yr and above 60 yr, pregnancy and history of malaria were excluded from the current study. Whole blood (1 ml) was also collected from age- and sex-matched voluntary blood donors (n=150) with no history of clinically diagnosed malaria as controls.

Screening of toll-like receptors 2 (TLR2)Arg753Gln (rs 5743708) polymorphism: Analysis of *TLR2 Arg753Gln* (rs 5743708) genotype in the study cohort was determined by PCR-restriction fragment length polymorphism (RFLP) method. Genomic DNA was extracted from the collected whole blood sample using the standard phenol-chloroform method[15]. Extracted DNA samples were quantified on a Nanodrop (*Nano Vue plus,* Germany) spectrophotometer. Amplification of the *TLR2 Arg753Gln* gene was performed using a specifically designed set of primers (F: 5'- CTGTCTTTGTGCTTTCTGAAA-3', R: 5'-GAACCTAGGACTTTATCGCAGC-3') for

a total reaction volume of 20 µl each. Screening of the amplified PCR products (6 µl) was done for the presence of 264 bp amplicon by using 1.8 per cent agarose gel. The PCR products (14 µl) were then digested with the restriction enzyme AciI (Cat. No-R0551S, New England Biolabs, USA) at 37°C, incubated overnight, and were then analyzed based on band pattern on 2.5 per cent agarose gel electrophoresis. The presence of three bands at 161, 54 and 49 bp characterized the wild-type G allele, while the homozygous variant A allele was represented by the bands of 215 and 49 bp. Randomly selected 10 per cent of the total sample were re-genotyped in a blinded manner to validate the genotyping results.

Differential soluble TLR2 and downstream tumour necrosis factor (TNF)-α and interferon (*IFN)-γ expression analysis*: The differential soluble TLR2 and its mediated downstream cytokines, *viz*. TNF-α and IFN-γ expression were analyzed using specific sandwich ELISA kits for TLR2, TNF-α and IFN-γ, Abcam, UK as per manufacturer's instructions.

Statistical analysis: The statistical analyses were performed using SPSSv 13.0 software (IBM Corp., Chicago, IL, USA). Odds ratio and the corresponding 95 per cent Confidence Interval (CI) were calculated to determine the associative role between the given genetic variation and overall malaria development. $P<0.05$ was considered as significant.

Results

Demographic and clinical profile: The present study was undertaken in the State of Assam to determine the role of soluble TLR2 and its associated downstream cytokines in susceptibility and severity of malaria infection using case-control cohorts among major tribal groups of Assam. Majority (57.74%) of the patients enrolled were male (Table I).

Association of TLR2 Arg 753 Gln (rs5743708) gene polymorphism with malarial infection: The polymorphism distribution of the *TLR2 Arg753Gln* (*rs5743708*) gene revealed that majority of malarial cases [147/153 (96.07%)] and the healthy controls [144/150 (96%)] were of the wild type. No significant difference was observed in the specific TLR2 single-nucleotide polymorphisms (SNP) among malarial patients and case-control samples odds ratio [OR=1.102 (0.304-4.001), $P=0.883$] (Table II). *TLR2 Arg753Gln* homozygous was found to be absent in both the studied cohorts. Further, in the context of the severity of malarial infection, the presence of variant TLR2 genotype showed no significant association with the increased susceptibility to SM infection [OR=1.025 (0.115-9.169); $P=0.982$] compared to UC-M reflecting no association of *TLR2 Arg753Gln* polymorphism in malarial disease towards susceptibility and severity with respect to the populations under study.

Role of soluble TLR 2 expression in malarial pathogenesis: Analysis of the differential expression of soluble TLR2 in controls and malaria sub-cohorts revealed that the levels of expressions of the serum soluble TLR2 are significantly higher in UC-M (6107.23±1327.84 pg/ml) cases compared to healthy controls (4721.97±1006.32 pg/ml; $P=0.045$), as shown in Figure 1. In the context of severity of the disease, the soluble TLR2 levels were also found to be higher in UC-M cases as compared to that of the SM cases (4376.77±1196.46 pg/ml; $P=0.078$), although the difference in expression between SM cases and controls were found comparable ($P=0.713$).

Evaluation differential TNF-α and IFN-γ expression in malaria pathogenesis: The serum level analysis of TNF-α showed a significant increase in their expression in SM cases compared to healthy controls ($P=0.003$) and UC-M cases ($P=0.044$). The expression profile of IFN-γ showed a graded upregulation pattern: controls <UC-M<SM. The IFN-γ expression was significantly upregulated in SM cases compared to both UC-M ($P=0.001$) and healthy controls ($P<0.001$), as shown in Figure 2.

Discussion

The recognition of invading sporozoites is the first key event to initiate innate immune response against the sporozoite challenge[16]. The crucial role of TLRs in the recognition of *P. falciparum* derived toxic compounds and in the malaria, pathogenesis is supported by evidence several reports[6,17]. While encountering the parasitic ligand, TLRs mediate a complex signaling cascade activating the production of various pro-inflammatory cytokines, such as IFN-γ, IL-12 and TNF-α favouring the elimination of the infectious agent[6]. However, elevated serum levels of these pro-inflammatory cytokines can be frequently found in

Table I. Demographic and clinical profile of the enrolled participants

Tribes	n	Average age, mean±SD	Gender		Clinical manifestations	
			Male, n (%)	Female, n (%)	UC-M cases, n (%)	SM cases, n (%)
Karbi	64	37.2±13.96	37 (57.81)	27 (42.18)	52 (81.25)	12 (19.95)
Dimasa	38	33.82±12.14	23 (60.52)	15 (39.47)	35 (92.10)	3 (8.21)
Rabha	51	28.06±9.32	28 (54.90)	23 (45.09)	41 (80.39)	10 (20.00)

UC-M, uncomplicated malaria; SM, severe malaria

Table II. *Toll-like receptor 2 (TLR2) Arg753Gln* polymorphism distribution in different study groups

Study groups	Sample number	*TLR2* (Arg753Gln)			Variant allele, n (%)	*P*	Odds ratio (95% CI)
		Wild type, n (%)	Heterozygous, n (%)	Homozygous			
Healthy control	150	144 (96)	6 (4)	0	6 (4)	Reference	1.102 (0.304-4.001)
Overall malaria cases	153	147 (96.07)	6 (3.92)	0	6 (3.92)	0.883	
UC-M	128	123 (96.10)	5 (3.91)	0	27 (18)	0.892	1.098 (0.287-4.191)
SM	25	24 (96)	1 (4)	0		0.918	1.125 (0.120-10.520)

UC-M, uncomplicated malaria; SM, severe malaria; CI, confidence interval

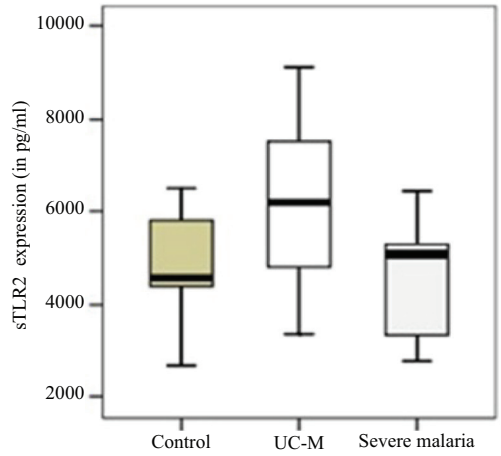

Fig. 1. Box-plot analysis showing the differences in the sTLR2 level in different malaria cohorts. TLR2, toll-like receptor 2

most of the SM cases[18]. SNPs of different TLRs have been reported to be associated with modulation of either susceptibility or resistance to several infectious and inflammatory diseases, specifically TLR2 and TLR4 in case of malaria[12].

Previous studies have shown that out of two common polymorphisms (*TLR2*Δ *22* insertion/deletion and GTn dinucleotide repeats) present in 5'untranslated region of *TLR2*, only Δ 22 is associated with protection against cerebral malaria[17]. The functional *TLR2 Arg753Gln* variant comprises the substitution of arginine with glutamine at nucleotide 2257 at position 753. Transfection of the given variant showed decreased cellular response towards the lipoprotein and has been found in septic patients[19]. The frequency of this variant allele was reported to be 4.7 per cent in the Turkish population. Furthermore, its association with the increased risk of developing tuberculosis was also reported[20]. However, conflicting results with no association of the given SNP was observed in increased susceptibility towards tuberculosis in Indian patients[21]. The complete absence of variant allele was reported in malaria-endemic Indian population[22] and in African children from malaria-endemic regions[23]. Northeastern States of India including Assam comprise a diverse ethnic population in comparison to the other parts of India. Due to the widespread distribution of chloroquine-resistant strain of malarial parasite and limited information is available from these regions, especially in the context of *TLR2 Arg753Gln* polymorphism that signifies the importance of host genetic factor alteration on host immune modulation in disease outcome, were undertaken in this study. However, no significant association of the TLR2 SNP within the TIR domain either with the susceptibility or the severity of malaria was observed in this study corroborating previous findings[22]. This discrepancy of the occurrence of the variant allele may be explained by the different phenotypes and different populations examined.

The TLR-mediated signal cascade is crucial for the DC maturation, cytokine production and upregulation

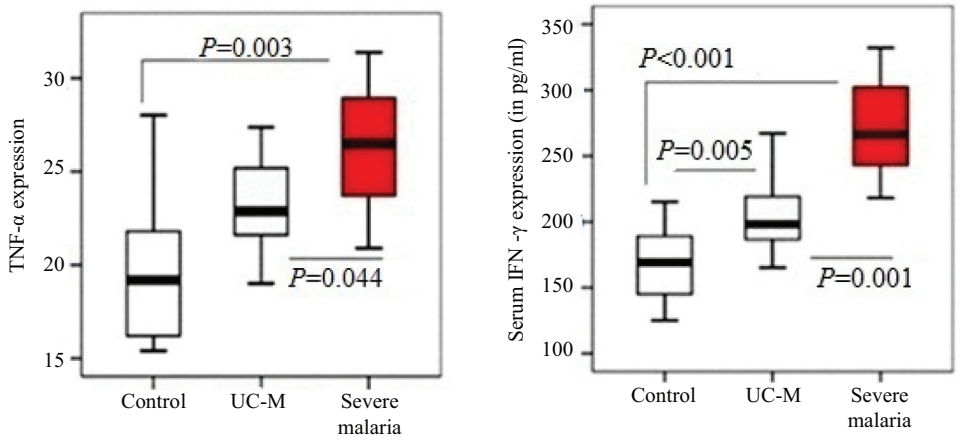

Fig.2. Box-plot analysis showing the differences in serum TNF-α and IFN-γ level in different malaria cohorts compared to controls. TNF-α, Tumour necrosis factor-alpha; IFN-γ, interferon-gamma

of the co-stimulatory molecules[24]. Soluble TLR2 that act as a decoy receptors, restrain the cellular TLR-PAMP interaction, and thus play an important role in the regulation of TLR2-mediated immune response[25]. Evaluation of the expression of soluble TLR2 on the different study cohorts carried showed an enhanced TLR2 expression in patients having UC-M as compared to healthy controls, possibly due to the binding of the malarial toxic compound, GPI to the TLR2, as suggested by previous studies[26]. Down regulation of serum TLR2 in SM cases compared to UC-M cases also indicated its association with the severity of the disease similar to earlier studies reporting the crucial role of TLR2 expression in both mice models and human malarial infection[7]. These findings further support the opinion that the extracellular negative regulatory mechanism involving the production of sTLR2, acts as a decoy receptor, and binds with the PAMP in the extracellular space, leading to the attenuation of the TLR signaling pathway to maintain the immunological balance[27]. Although the human assessment of the expression of the TLR protein in terms of malaria disease outcome is limited, our data are in line with a Thailand based study[28] reporting a significant increase in the expression of the TLR2 protein on antigen presenting cells in malaria-infected individuals. Therefore, the present data points to the possible role of soluble TLR2 as a prognostic marker and its association with malaria infection including SM linked to high morbidity and mortality rate.

Pro-inflammatory cytokines such as TNF-α and IFN-γ play the protective role of an immune mediator against the early liver-stage malarial parasite[29]. However, deregulation and imbalance of inflammatory markers may causes an ineffective immune response or tissue damage in the host[30]. An increased concentration of serum TNF-α has been correlated with *P. falciparum* inducing malaria pathogenesis[31]. Findings of the present study also corroborated the same as significantly higher TNF-α protein level in SM cases was observed. The inhibitory effect of IFN-γ on parasite multiplication after the treatment with human recombinant IFN-γ on *P. Berghei* sporozoites-infected murine hepatocytes was revealed[32]. *In vitro* studies have confirmed that IFN-γ secreted in primary *P. yoelii* sporozoite infection is a crucial innate immune component that restricts liver-stage parasite growth in the secondary infection[33]. A population based study in Papua New Guinea also revealed that high and early IFN-γ response appears to be a protector in children from symptomatic malaria[34]. However, one of the major limitations in the present study was that participants were sampled at a single point of time. No follow up cases were taken to determine if any of the UC-M cases further progressed to the severity of the disease and to check the cytokine profile at different stages of the disease in the same cases. Nevertheless, the present study noted a significant increase in the expression of IFN-γ in both UC-M as well as SM cases.

To conclude, the present study provided a crucial insight into the role of deregulated TLR2 signaling leading to the deleterious downstream hyper Th1 immunomodulation in susceptibility and the severity of malaria. Our data suggest that a lower level of soluble TLR2 in SM cases in turn leads to the faulty TLR2 mechanism that plays a crucial role in disease outcomes. The differential levels of TNF-α and IFN-γ also possess prognostic significance and can be used

as a prognostic marker for stratifying patients with predisposition to malaria. However, more elaborate studies including the role of cofactors would be required to establish the association of deregulated TLR2 pathway in malaria pathogenesis.

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***Conflicts of Interest*:** None.

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