

MyD88-NF- κ B Signalling Pathway Contributes to *Plasmodium Falciparum* Infected Malaria Severity: A Tribal Patient Based Study from Assam

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Abstract

Background: Severe *Plasmodium falciparum* malaria is characterized by dysregulated host inflammatory responses that contribute significantly to disease pathogenesis. The MyD88–NF- κ B signaling pathway plays a central role in Toll-like receptor-mediated innate immune activation, yet its contribution to malaria severity among ethnically distinct tribal populations of Northeast India remains inadequately explored.

Methods: This case-control study enrolled 64 *P. falciparum*-infected tribal patients (53 uncomplicated malaria and 11 severe malaria) and 88 age-, sex-, and community-matched healthy controls from Assam, India. The mRNA expression of MyD88, NF- κ B p65, and TNF- α was quantified by real-time PCR, while serum NF- κ B protein levels were determined by ELISA. Monocyte (CD14⁺) expression was analyzed by flow cytometry, and associations with disease severity were evaluated statistically.

Results: Malaria patients exhibited significantly elevated MyD88, NF- κ B p65, and TNF- α expression compared with healthy controls, with the highest levels observed in severe malaria cases. MyD88 expression correlated positively with NF- κ B p65 (Spearman's $\rho = 0.673$, $p = 0.048$) and TNF- α expression ($\rho = 0.714$, $p = 0.006$) in severe malaria, indicating activation of the MyD88-dependent inflammatory cascade. CD14⁺ monocyte expression was also significantly increased in malaria patients, particularly in severe disease.

Conclusions: Enhanced activation of the MyD88–NF- κ B signaling axis is strongly associated with inflammatory amplification and disease severity in *P. falciparum* malaria. These findings highlight the pathway as a potential prognostic biomarker and therapeutic target for severe malaria in endemic tribal populations.

Keywords: *Plasmodium falciparum*; severe malaria; MyD88; NF- κ B; TNF- α ; innate immunity; monocytes; tribal population; Assam.

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Introduction:

Malaria, a parasitic disease caused by the genus *plasmodium* still remains as major health problem across the world; with an estimated 282 million cases and 610 000 deaths been reported across 80 countries recently (1). Although the success of the present regime of drugs using artemisinin-based combination is undeniable; but several clinical and scientific concerns still persists including the 10-20% mortality on this regime and the spread of artemisinin-resistant parasites (2). Therefore the critical insights of disease pathogenesis still needs to be elaborately studied at distinct geographical population datasets levels for further development of probable adjunct treatment approaches for ameliorating the disease, especially for addressing the issue highlighted above, as well as for clinical interventions for patients progressing to severe disease state from the mild state.

The innate immunity plays a significant role in disease pathogenesis and responses against pathogens. Toll-like receptors especially TLR2, TLR4 etc., on immune cells especially those of myeloid origins like monocytes can potentially interact with key components of *Plasmodium falciparum* (3;4). The interactions can potentiate the monocyte expression and activation, phagocytosis, antigen presentation, anti-microbial peptide secretion, reactive oxygen/nitrogen species generation, and cytokine and chemokine production (5;6;7;8). Although a pre-requisite for pathogen clearance, but excessive production of this pro-inflammatory cytokines may cause immunopathogenesis in malaria (9).

The signal initiated by the microbial pathogen acting as pathogen-associated molecular pattern (PAMP) are transmitted through highly conserved Toll Interleukin receptor (TIR) that recruits a common adaptor protein MyD88 (10;11). This initiates the downstream inflammatory signalling cascade by activating NF- κ B pathway (12) and involves inflammatory cytokines induction (13.). The differential activation of the MyD88-NF- κ B axis and linked downstream cytokine and monocyte expression profile in *Plasmodium falciparum* needs exploration w.r.t disease susceptibility and severity; which was aimed to be explored through the present study involving malaria ethnically distinct tribal cases from Assam, India.

Materials and Method:

The present study involved enrolment of cases that were screened positive for malarial infection by RAPID test and blood smear examination from the Community Health Centre, Rani and Sonapur Civil Hospital, Assam (N=64) under the supervision of registered Medical Practitioners and with informed consent along with 88 age, sex and community matched healthy controls. The study was carried out between the year 2016-2023; and the patients were clinically stratified as uncomplicated malaria (UC-M, N=53) or severe malaria (SM, N=11) based on the WHO guidelines 2015 (14). Whole blood samples (3ml) were collected intravenously from OPD or on admission under clinical supervision. Patients with history of other inflammatory diseases, diabetes, viral or alcoholic liver diseases or pregnancy cases were excluded. The present study was approved by the IEC of participating institutions. The patient's age group was between 18 years to 50 years of age.

The plasma/serum component was separated and stored at -80 degrees before use; and the 200 μ L whole blood was used for total RNA extraction. The myd88 {primer seq: F: 5'GCTTGCTGTTCTCAAAGTGATTTTGGGAGAA3' and R: 5'GGAAATCCGTGTTCTAGTTGTTCTAAGCC3' and NF- κ B-p65 {primer seq: F: 5'CCTATGTGGAGATCATTG3' and R: 5'GGTGGGTCTTGGTGGTAT3'} differential mRNA expression profiles were studied at transcript levels utilizing Realtime PCR and SYBr green chemistry using the cDNA prepared from the extracted RNA from the cases and the controls as templates and β -

actinas an internal normalization control The serum NF- κ B levels were evaluated using simple step ELISA kit (ab176648, Abcam, UK). The downstream differential TNF- α expression was also analysed using Realtime PCR and correlated statistically with myD88 and NF κ Bp65 expression. The monocyte expression profile change was analysed using the MUSE based flowcytometry analysis using freshly collected blood samples stained with monocyte specific marker CD14 (PECy7 mouse anti-Human CD14, cat no. 557742, BD). All data were analysed using the SPSS25.0 software.

Results:

Demographical profile:

The majority of the cases were males and belong to the middle age group of 31-40 years. The cases were from different ethnic tribal groups including Karbis (N=18), Dimasas (N=8), Bodo-Kacharis (N=24), Tiwa or Lalungs (N=10) and Rabhas (N=4). All the cases were characterized to be *Plasmodium falciparum* pathologically; and were stratified as UN-C or SM cases based on clinical and pathological examinations.

Myd88-NF- κ B pathway activation:

The myd88 mRNA expression was found to be increased in malaria cases compared to controls (3.089 ± 1.671 folds). Importantly, the fold change was significantly higher in SM cases compared to UC-M cases ($p=0.024$) (Figure 1).

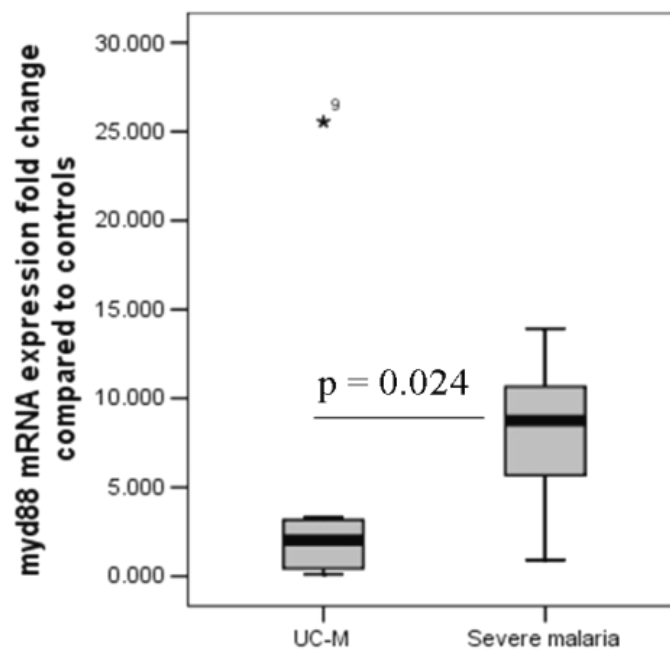


Figure1. Box-plot representing the difference in differential mRNA expression of *myd88* based on fold change compared to controls in UC-M and severe malaria cases, indicating a significant change in *myd88* mRNA expression in severe malaria cases.

The differential expression of NF- κ B-p65 at mRNA and protein levels was studied by Realtime PCR and ELISA respectively. The NF- κ B-p65 was increased in malaria cases compared to controls at both mRNA and protein levels. Importantly the expression was prominently increased in the severe malaria case cohort compared to both UC-M and healthy controls (Figure 2). Moreover, the *myd88* mRNA expression correlated significantly with the NF- κ B-p65 mRNA expression in the severe malaria sub-cohort (Spearman's $\rho=0.673$, $p=0.048$), suggestive of a MyD88 dependent NF- κ B-p65 modulation in severe malaria cases.

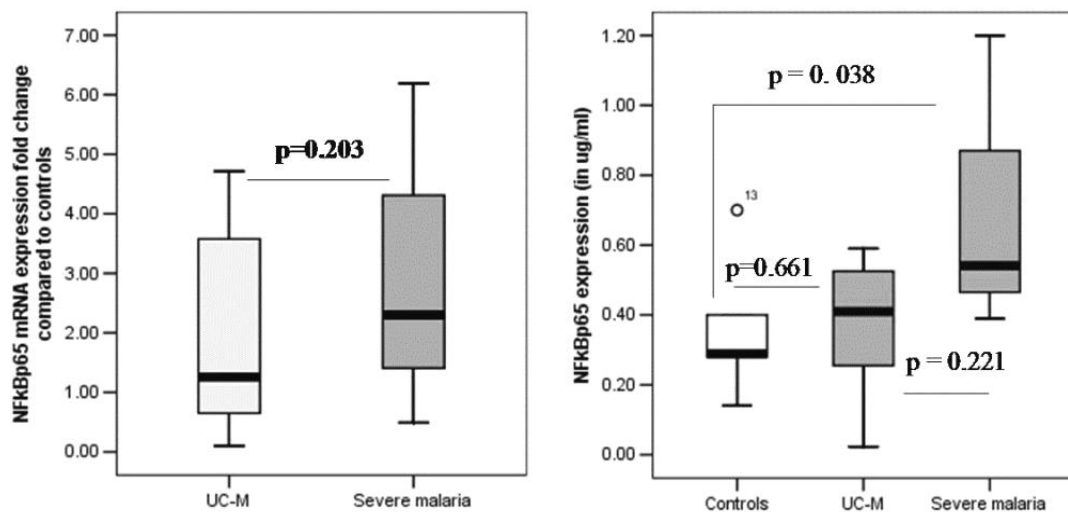


Fig 2. Box plots showing difference in expression profile at [left] mRNA level fold change compared to controls and [right] protein level between enrolled sub-cohorts of malaria cases compared to controls.

Effect on downstream inflammatory modulation:

A mark increase in $TNF-\alpha$ mRNA expression (9.28 ± 4.19 folds) was observed in malaria cases; with significantly higher expression in SM cases compared UC-M cases ($p < 0.001$) indicating a pro-inflammatory biased state in SM cases. The increased $TNF-\alpha$ mRNA expression correlated positively with increased myd88 expression, significantly so in severe malaria cases (Spearman's $\rho = 0.714$, $p = 0.006$), suggesting the role of myd88 dependent NFkBp65 - $TNF\alpha$ axis activation in the pathogenesis of severe malaria. The monocyte expression based on monocyte specific marker CD14 indicated a significant increase in malaria cases compared to healthy controls ($p = 0.029$); more so in SM cases compared to both UC-M cases ($p < 0.001$) and healthy controls ($p < 0.001$).

DISCUSSION:

Malaria still remains a global health issue especially in Africa, south-east Asia and the resource limited countries of the tropics. Moreover, the existing prescribed drugs are showing clinical limitations which challenges the success achieved at global levels in bringing down the number of malaria cases significantly. The pathology of involve involves the pathogen at one end and the host responses especially the immune response at the other. Understanding the key parameters governing the pathogenesis especially the progression of the disease is pertinent both clinically and scientifically. Therefore, the present study tried to underline the role of myd88-NFkB axis activation and linked downstream effectors in disease susceptibility and severity. The study involved subjects from ethnically distinct tribal population in the south bank of river Brahmaputra in Assam, India who had been historically suffered from the disease of the geography conducive to *Plasmodium*'s vector.

Plasmodium falciparum malaria is a major cause of morbidity and mortality in northeast India (15, 16); and all the malaria cases of the present study were *Plasmodium falciparum* positive. Published *in vitro* studies have shown that haemozoin and GPI can stimulate monocytes and macrophages to synthesize pro-inflammatory cytokines via TLR linked NF- κ B pathways (17) mediated through the major adaptor MyD88 (18), which was also clearly indicated through the data from the present study.

The NF- κ B activation leads to its nuclear translocation and regulation of gene expression. The NFkB levels have been demonstrated to be a critical factor for malaria pathogenesis and development of severe malaria (19) and was substantiated through the present study. The activation of MyD88-dependent pathway is known to activate subsequent release of pro-inflammatory cytokines e.g. $TNF-\alpha$, which contributes to an effective and robust host defence response to microbial infection, but can be detrimental if uncontrolled (9). The $TNF-\alpha$ mRNA expression was significantly higher expression in SM

cases compared UC-M cases, and correlated with myd88 expression. Elevated serum concentrations of TNF- α have been previously documented in *P. falciparum* malaria patients, and their levels correlated with disease severity (20, 21) similar to our data. Moreover, the MyD88-dependent pathway activation correlated with increase in the expression of monocytes in malaria cases especially in SM cohort. It holds significance because it is known that increased monocytes expression and activation is a key activator of pro-inflammatory Th1 cytokines like TNF- α .

To conclude, the present study accentuates the significance of differential modulation of Myd88-NFkB axis and linked downstream events in the *P. falciparum* malaria especially in severe stages. The data therefore holds prognostic significance and promise serve as a target for clinical intervention in the disease.

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