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ORIGINAL ARTICLE

MAD 20 alleles of *merozoite surface protein-1 (msp-1)* are associated with severe *Plasmodium falciparum* malaria in Pakistan

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Background: Various factors determine the outcome of *Plasmodium falciparum* infection such as parasite load, sequestration, adhesion molecules, and immune mediators. *P. falciparum merozoite surface protein-1* (*msp-1*) and *msp-2* genotypes were also found associated with severe disease. We investigated the association between *msp-1* and *msp-2* genotypes in patients with uncomplicated malaria (UM) and severe malaria (SM).
Methods: Twenty-two malaria patients with microscopy-confirmed *P. falciparum* infection and eight healthy endemic controls were selected for analysis. Nested polymerase chain reaction (PCR) was used to identify *P. falciparum* genotypes. The plasma concentration of cytokines [tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interferon-gamma (IFN-γ)] and chemokines [chemokine (C-X-C motif) ligand 9 (CXCL9) and CXCL10] were evaluated using enzyme-linked immunosorbent assay (ELISA).
Results: TNF-α levels were significantly higher in both UM (389 pg/mL, $p = 0.020$) and SM (771 pg/mL, $p = 0.004$) compared with healthy controls, while they were greater in SM ($p = 0.012$) as compared to UM. CXCL9 levels were significantly raised in SM as compared to UM and negative controls (NCs). CXCL10 levels were raised in UM (550 pg/mL, $p = 0.001$) and SM (1480 pg/mL, $p = 0.01$) as compared with NCs. Increased levels of IL-6 were found in patients carrying the FC27 allelic type of *msp-2*. A higher prevalence of MAD 20 and K1 *msp-1* alleles was observed in the SM group compared to UM.
Conclusion: Overall, a greater prevalence of MAD 20 alleles and increased serum TNF-α and CXCL9 levels were associated with severe outcome in malaria. Understanding the

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diversity of malaria genotypes is important for predicting disease-related outcomes of *P. falciparum* infection in endemic areas.

Introduction

Merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum* is involved in red blood cell (RBC) invasion and is a potential candidate for vaccine development.[1] The *msp-1* and *msp-2* genes are also reliable markers in studying genotypic diversity of *P. falciparum*.[2] In Pakistan, the distribution of *msp-1* and *-2* allelic types is similar to the diversity reported in regions of low to meso-endemicity of malaria in Africa.[3] The *msp-1* allelic types K1and RO33 have been shown to be associated with severe malaria (SM) in Africa.[4] However, MAD 20 and 3D7 genotypes of *msp-1* and *-2*, respectively, have been found to be predominant in SM cases in India.[5] In addition, allelic type FC27 of *msp-2* has been associated with increased morbidity.[6] Lower *P. falciparum* multiplicity of infection (MOI) was reported from India in mild malaria cases compared to severe cases.[5]

Human clinical and murine model studies support the role of immune mediators in pathogenesis of severe *P. falciparum* malaria. Plasma tumor necrosis factor-alpha (TNF-α) levels have been shown to be significantly higher in children suffering from *P. falciparum* malaria compared to children with gastroenteritis and mild respiratory infections.[7] Elevated levels of TNF-α, interleukin-1 (IL-1), and IL-6 in SM, particularly cerebral malaria in children, are correlated with increasing parasite load.[8] Increased levels of interferon-gamma (IFN-γ) were observed in patients with SM.[9]

Chemokines are small molecules known to mediate host immune response during protozoan infection. Chemokines such as chemokine (C-X-C motif) ligand 9 (CXCL9; monokine induced by IFN-γ, MIG) and CXCL10 (IFN-γ inducible 10 kDa protein, IP10) play a distinct role in pathogenesis of malaria in the murine model.[10] Elevated levels of CXCL10 are associated with cerebral malaria mortality in Ghana and India.[11] CXCL10 is known to have chemotactic and angiostatic activity in cerebral malaria and therefore may serve as a potential biomarker of severity and mortality.[11] Chemokine (C-X-C motif) receptor 3 (CXCR3) binds to various chemokines including CXCL9 and CXCL10 and is expressed on activated T cells, memory T cells, and natural killer (NK) cells, which are involved in immune responses to *P. falciparum* infections.[12]

In this study, we investigated the association between *P. falciparum msp-1* and *-2* genotypes with circulating levels of proinflammatory cytokines and chemokines in plasma from malaria patients.

Methods

Study group

All patients included in the study were recruited from the Aga Khan University Hospital and Medical College (AKUH) complaining of fever. Study subjects were stratified into three categories: uncomplicated malaria (UM), Sever malaria (SM), and healthy negative controls (NCs) with no recent history of malaria. Patients were considered UM if they presented with signs and symptoms of malaria and were slide-positive for *P. falciparum* mono-infection. Patients were defined as SM based on World Health Organization (WHO) criteria. Cerebral malaria (parasitemia $>$2% 100,000 parasites/μL) and severe anemia (Hb adults $<$7 g/dL, children $<$5 g/dL) were considered primary defining characteristics for recruitment in the SM group.[13] Asymptomatic individuals who were staff at AKUH with no recent history of malaria were enrolled as healthy NCs. All NCs were also slide-negative for *P. falciparum*. Plasma was obtained by centrifugation of whole blood collected in EDTA tubes and stored as small aliquots at -80°C until use.

Informed consent was obtained from all participants or in the case of children from their parents/legal guardians. The study was approved by the ethical review committee of AKUH, Karachi, Pakistan.

Blood collection and microscopy

An intravenous blood sample of 2 mL was collected in an EDTA tube in accordance with routine clinical practice. Thick and thin Giemsa-stained blood films were analyzed for species identification and parasite density. For patients with confirmed *P. falciparum* mono-infection, asexual parasites were counted against 200 white blood cells on the thick film. Parasite density was quantified (parasites/μL) by assuming an average of 8000 leucocytes per μL blood.

msp-1 and *msp-2* genotyping

Parasite genotypes based on *msp-1* and *msp-2* genes were determined using polymerase chain reaction (PCR) genotyping methodology. Briefly, genomic DNA was extracted from a total of 200 μL whole blood per patient using the Qiagen DNA extraction kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Nested PCR genotyping was performed both for the variable block 2 regions of *msp-1* and block 3 of *msp-2*, considered to be the two most informative genetic markers for assessment of *P. falciparum* MOI.[2] The initial amplification was followed by individual nested PCR reactions using family-specific primers for *msp-1* (KI, MAD 20, and R033), and *msp-2* (FC27 and 3D7/IC), respectively, as described previously.[14]

Cytokine and chemokine determinations

TNF, IFN-γ, and IL-6 were detected in plasma of controls and patients by using standards and enzyme-linked immunosorbent assay (ELISA) reagents obtained from Endogen (Rockford, IL, USA). Cytokines were measured using a

sandwich ELISA technique according to the manufacturer's instructions and as reported previously.[15] Recombinant human cytokine was used to obtain a dose—response curve with a range of detection from 3.9 pg/mL to 1000 pg/mL. All experimental samples were tested in duplicate. CXCL9 and CXCL10 standards and monoclonal antibody pairs for capture and detection were obtained from R&D Systems (Abingdon, UK). All measurements were carried out according to the manufacturer's recommendations and as described previously.[15] Recombinant human chemokine was used to obtain a dose—response curve with a range of detection from 6.25 pg/mL to 500 pg/mL for CXCL9 and from 3.9 pg/mL to 1000 pg/mL for CXCL10.

Statistical analysis

Data were entered and analyzed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using nonparametric Mann—Whitney *U* and Fisher's exact test as appropriate. Spearman's Rank Correlation was also performed. A p value ≤ 0.05 was considered to be statistically significant.

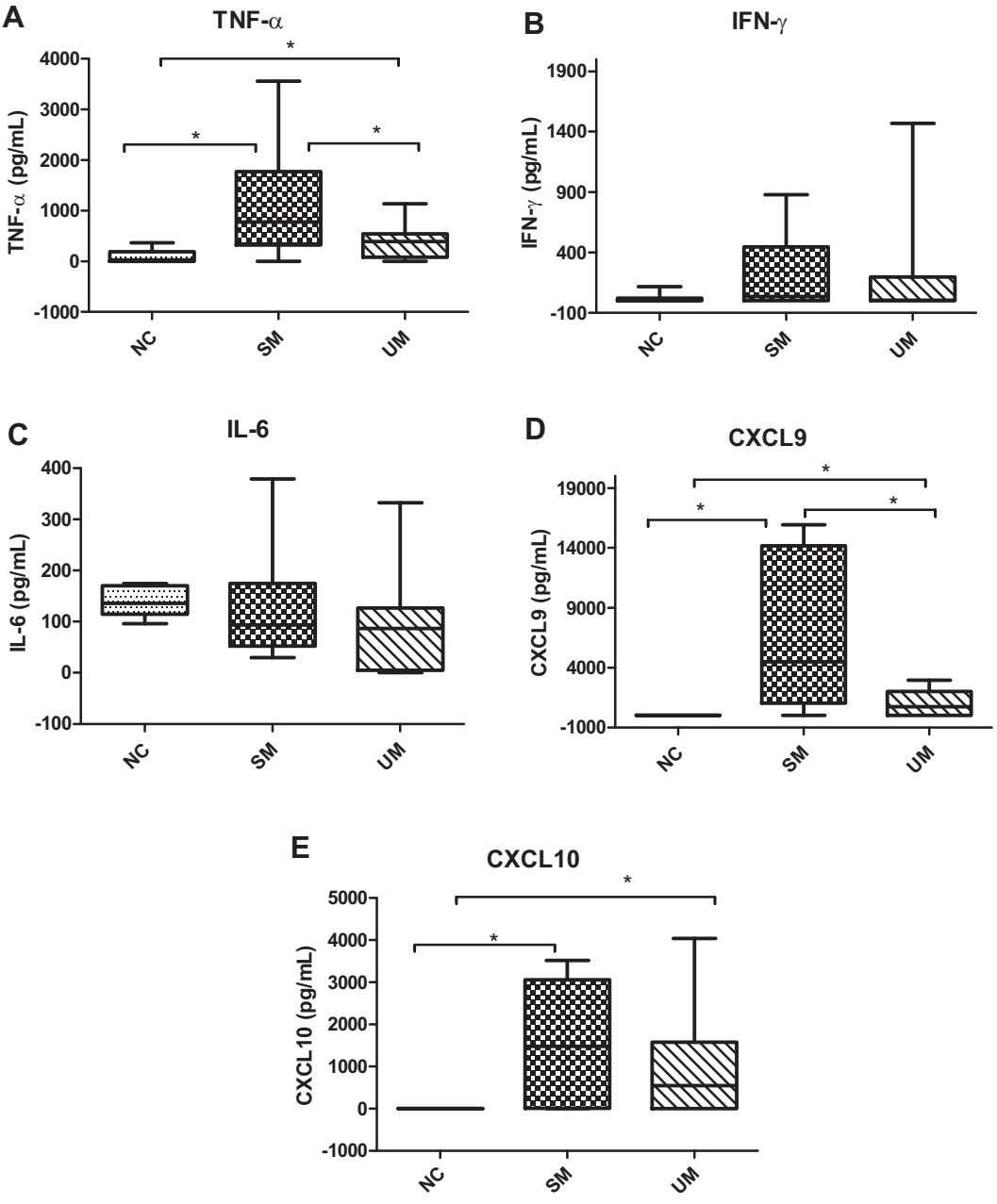

Figure 1. Plasma levels of cytokines (TNF-α, IFN-γ, IL-6) and chemokines (CXCL9, CXCL10) in NC ($n = 8$) and malaria severity groups [SM ($n = 10$) and UM ($n = 12$)]: (A) TNF-α; (B) IFN-γ; (C) IL-6; (D) CXCL9; (E) CXCL10. Levels from study subjects are depicted as box plots presenting the median 25th and 75th quartiles of cytokine levels. * Denotes significant difference ($p \leq 0.05$) between groups on the nonparametric Mann—Whitney *U* test. CXCL9 = chemokine (C-X-C motif) ligand 9 (monokine induced by IFN-γ, MIG); CXCL10 = chemokine (C-X-C motif) ligand (IFN-γ inducible 10 kDa protein, IP10); IFN-γ = interferon-gamma; IL-6 = interleukin-6; NC = negative controls; SM = severe malaria; TNF-α = tumor necrosis factor-alpha; UM = uncomplicated malaria.

Results

Cytokine and chemokine analysis was performed on 22 malaria patients and eight healthy NCs (Fig. 1). Table 1 compares the age, sex, parasitemia, and hemoglobin (Hb) in patient groups and NCs. The number of male patients (17/22) was higher than the number of females. Hb and parasite densities (number of *P. falciparum* sexual stages per microliter of blood) were compared between UM and SM groups. Peripheral parasitemia was significantly higher in SM cases ($p = 0.025$) compared to UM cases.

All allelic families of *msp-1* (RO33, K1, MAD 20) and *msp-2* (3D7, FC27) were detected in both UM and SM groups. The prevalence of MAD 20 and K1 alleles of *msp-1* was higher in the SM group compared to the UM group ($p = 0.04$). The *msp-2* allelic families 3D7 and FC27 were equally distributed in both groups (Table 2).

Cytokines (TNF-α, IFN-γ, and IL-6) and chemokines (CXCL10 and CXCL9) were measured in plasma from UM and SM groups and compared with NC as control group. Significantly higher levels of TNF-α were observed in UM (median: 389 pg/mL, $p = 0.020$) and SM (median: 771 pg/mL, $p = 0.004$) compared to NC (median: 28 pg/mL). No significant differences were observed in IL-6 and IFN-γ levels between groups. CXCL9 levels were significantly raised in the SM group (median: 4479 pg/mL) compared to the UM group (median: 734 pg/mL, $p = 0.012$). CXCL9 levels were significantly higher in UM ($p = 0.005$) and SM ($p = 0.001$) compared to NC. Similarly, CXCL10 levels were significantly higher in UM (median: 550 pg/mL, $p = 0.001$) and SM (median: 1480 pg/mL, $p = 0.01$) compared to NC. CXCL10 levels were low in the UM group compared to the SM group, however the difference was not statistically significant.

The relationship between *P. falciparum* genotypes and cytokine and chemokine levels was assessed. IL-6 levels were higher in the NC group when compared with UM and SM. However, when all malaria patients were stratified based on *P. falciparum* genotypes, IL-6 levels were significantly higher in all malaria patients infected with the FC27 allelic type as compared with patients infected with other genotypes ($p = 0.03$). IL-6 levels were also found higher in SM cases carrying the FC27 allele of *msp-1* compared to UM cases ($p = 0.03$). No significant difference in CXCL10 and CXCL9 levels and *msp-1* and *-2* genotypes in malaria patients were observed. Parasite densities were significantly higher in patients infected with *msp-1* multiclonal infections ($p = 0.039$) in both UM and SM groups. There was strong positive association observed between parasite density and CXCL9 ($\rho = 0.673$, $p = 0.03$) and CXCL10 ($\rho = 0.745$, $p = 0.013$) levels observed in the SM group. In the UM group, strong positive correlation was observed between parasite density and CXCL9 level ($\rho = 0.787$, $p = 0.002$). Interestingly, no significant association between parasite density and TNF-α, IFN-γ, and IL-6 levels was observed in both UM or SM groups.

Discussion

In this study, we evaluated levels of inflammatory mediators (TNF-α, IFN-γ, IL-6, CXCL9, and CXCL10) in plasma of *P. falciparum* malaria patients and explored the role of particular genotypes of *Plasmodium* in the severity of disease.

IFN-γ levels were elevated in malaria patients compared to NC corroborating with previous reports.[16] We did not observe a difference in IL-6 levels between UM and SM groups, which may be due to the smaller sample size of this study. The imbalance in IL-6 and TNF-α levels in an untreated patient may serve as a marker for severity of disease; low levels may promote parasite clearance in early stages of the disease,[8,17] suggesting a protective role of IL-6 in malaria. Previously, lower levels of TNF-α, IL-6, and IFN-γ have been reported in cerebral malaria patients in contrast with other complicated falciparum malaria.[18] We found higher TNF-α and CXCL9 circulating levels in patients with SM compared to the UM group, which may suggest their association with severity of malaria. Increased levels of CXCL9 and CXCL10 levels correlated with higher parasite density. Previous reports suggested a role of IFN-γ and IL-6 in parasite clearance; by contrast, chemokines CXCL9 and CXCL10 have not been evaluated in terms of parasite density.[17] CXCL9 and CXCL10 knockout mice were found to be partially protected from *P. berghei* ANKA-induced malaria.[10]

We found a higher frequency of MAD 20 ($p = 0.04$) and K1 allelic types of *msp-1* in SM compared to UM. Because MSP-1 is associated with RBC invasion, infection with MAD 20 and K1 alleles may favor high parasitemia ($p = 0.039$) leading to severe outcomes in malaria.[5,19] The FC27 type of *msp-2* was significantly associated with increased levels of IL-6 in the SM group. In Amazonia, the population remains susceptible to infection with FC27-type parasites despite having high levels of antibodies, therefore, in such cases, increased levels of IL-6 may serve as a marker of disease severity.[20]

Table 1 Characteristics of the study group

Groups	NC	AM	UM	SM	*p*
N	8	22	12	10	
Age (y)[a]	27 (17)	34 (25)	29 (16)	44 (35)	0.57
Male/female	5/3	17/5	9/3	8/2	
Parasitemia[a]	No parasites	19,280 (69,570)	6620 (38,350)	67,060 (91,520)	0.025*
Hb[a]	NA	10.4 (6)	12.45 (4.5)	8.9 (5.4)	0.083

[a] Data are presented as median (IQR).

* Denotes significant difference ($p \leq 0.05$) between the UM and SM groups on the nonparametric Mann–Whitney U test.

AM = all malaria; Hb = hemoglobin; IQR = interquartile range between 25th and 75th percentile; NA = not applicable; NC = negative controls; SM = severe malaria, group, UM = uncomplicated malaria.

Table 2 Number of *Plasmodium falciparum msp-1* and *-2* genotypes detected in UM and SM cases

Groups	*msp-1*				*msp-2*		
	RO33	K1	MAD 20	MOI[a]	3D7	FC27	MOI[a]
UM (n = 12)	6 (50)	3 (25)	4 (33)	1.08	5 (41.5)	8 (66)	1.08
SM (n = 10)	1 (10)	5 (50)	8 (80)	1.40	4 (40)	6 (60)	1.00
p*	NS	NS	0.04		NS	NS	

[a] Mean multiplicity of infection (MOI) = number of genotypes per infection.
* Numbers of allelic types in UM and SM groups were compared using Fisher's exact test.
Data in parentheses are percentage frequencies of genotypes in each group.
NS = no significant difference between groups; SM = severe malaria; UM = uncomplicated malaria.

Several factors may contribute to varying levels of cytokines and genotype detection, such as sample size, numbers of previous malaria episodes, duration of the current clinical acute syndrome, period of time between the previous malaria and the current episode, and treatment duration, therefore plasma levels may not reflect events taking place in the microvasculature. However, this study provides valuable information regarding the role of immune modulators in the pathogenesis of malaria in Pakistan.

Although this study has a relatively small sample size, the trend of increased MAD 20 alleles in the SM group correlates with data from neighboring India[5] and the increased association of TNF-α and CXCL9 with SM may be a consequence of this. This is the first report regarding *P. falciparum* genotypes in association with circulating cytokine levels in patients with UM and SM in Pakistan. Understanding the diversity of malaria genotypes is important for predicting disease-related outcomes of *P. falciparum* infection in endemic areas.

Conflicts of interest

The authors declare that they have no competing interests.

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