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Original Research Article

Assessment of chemokine responses involved in the cerebral and mild malaria in murine model

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Abstract

Background: The role of chemokines on the integrity of Central Nervous system (CNS) during malaria infection remains unclear; we therefore, in this study investigated the alternation in gene expression of chemokines in the Brain samples of *P. yoelii 17XL* infected mice with Cerebral Malaria (CM) as well as Mild Malaria (MM).
Objective: To evaluate the pro-inflammatory chemokine expression in the brain samples of murine CM and MM
Methods: mRNA levels of IFN γ, IP-10, RANTES, MIP1-α, MIP1-β and MCP were measured by qRT-PCR in Brain samples of *P. yoelii 17XL* infected mice. GAPDH was used as the housekeeping gene. Histopathological studies of brain samples from infected and uninfected mice were conducted.
Results: CM mice had highly up-regulated of all chemokines except IFN γ and RANTES than MM mice (all P<0.0001)); IFN γ (P=0.5133) and RANTES (P=0.9292) were found to be decreased in CM as compared to MM wherein IP-10 (P=0.1419), MIP1-α (P=0.1664), MIP1-β (P=0.6294) and MCP (P=0.8262) mRNA were significantly up regulated at peak parasitemia and remains high in the CM of experimental mouse model. Histopathology results revealed tissue section in severely parasitized mice exhibited massive degeneration of the parenchyma, consistent with marked inflammation.
Conclusion: It might be concluded from the findings of the present study that up regulation of IP-10, MIP1α, MIP 1β and MCP-1 and low expression of IFN γ and RANTES in the brains of CM mice are associated with mortality of *P.yoelii 17XL* infected CM mice as compared to mice with MM.
Keywords: Chemokines, *P. yoelii 17XL,* Pathogenesis, Murine Cerebral Malaria, Murine Mild Malaria

1. Introduction

Malaria, caused by the intracellular parasite, *Plasmodium*, is the major public health problem affecting about 80% of cases in Africa and 13% in South East Asia region [1-2]. One of the most life threatening hurdle of *Plasmodium falciparum* severe malaria is the Cerebral Malaria (CM) responsible for the mortality in children [3]. The process of sequestration results in the obstruction in the blood flow and is postulated to result in hemorrhages and hypoxia because of cytogenic edema, which are the predominant parameters in CM [3-4]. However, along with sequestration, loss of Blood Brain Barrier (BBB) integrity, significant accumulation of platelets and activation of inflammatory responses most likely contribute in the pathogenesis of human CM [5-6].

However, the difficulty in examining the human CM pathological cases, the immuno- pathology of CM is not fully understood [7]. Therefore animal studies using the rodent malaria parasite *Plasmodium berghei ANKA* and *Plasmodium yoelii* 17XL on susceptible mouse strain serves as a model to clarify the immunological mechanism involve in the CM [8-10]. Although no animal model fully enumerates the human disease, the *Plasmodium yoelii* 17XL strain has an advantage including parasitized RBCs (pRBCs) sequestration in the brain [8]. Cytoadherence of pRBCs to endothelial cells via ligands present on the erythrocyte membrane knots is thought to be responsible for the cause of CM in *P.yoelii 17XL* infected mice [11]. The endothelial cells involved in the inflammatory chemokines up-regulate the adhesion molecules in CM [12-13]. Other previous studies support the hypothesis that recruitment of leukocyte in the brain by chemokine and chemokine receptor interactions play a pivotal role in the pathogenesis of murine malaria [14-16].

Chemokines, a superfamily of small, structurally related proteins, plays important role in the generation of inflammatory immune responses [17]. Although IFN γ and IP-10 (IFN-induced protein of 10 kDa) have been implicated in infectious neurological diseases [17-18]; IP 10 in human malaria has been reportedly associated with CM mortality in Ghanaian Children [19]. Other chemokines of the C-C or α-subfamily- including Regulated upon activation, normal T cell expressed and secreted (RANTES), Microphage Inflammatory Protein (MIP)-1α, MIP 1β, Monocyte Chemoattractant Protein1 (MCP-1) are potent chemotactic cytokines. Low levels of RANTES are associated with disease severity and mortality in sepsis individuals [20] and meningococcal infections [21]. Decreased level of RANTES and increase in its corresponding receptors have been seen in the children infected by severe malaria [22] but in another study, researchers showed that RANTES level in the brain increased in some individuals who died by CM [23]. During the inflammation process, MCP-1 is responsible for increased leukocyte infiltration into the CNS through permeability of BBB [24-25].

However, data till date seems to be contradictory to each other in the differential level of CC chemokines, we therefore, in this study investigated the alternation in gene expression of chemokines in the Brain samples of *P. yoelii 17XL* infected mice with Cerebral Malaria (CM) as well as Mild Malaria (MM).

2. Materials and methods

2.1 Mice

6-8 weeks old, male *Swiss albino* mice were purchased from Advanced Centre for Treatment, Research and Education in Cancer (ACTREC). All mice were maintained in specific pathogen-free environment and kept at 65-75ºF and 40-60% relative humidity with 10-12 hr light-dark cycle. They were given autoclaved pelleted, non contaminated and nutritionally adequate feed and fresh potable drinking water as per the guidelines. All mice experiments were approved by the Institutional Animal Ethics Committee (IAEC) of Haffkine Institute and all procedure were followed as per the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) guidelines. (HITRT/IAEC/026/2011)

2.2 Infection with *P.yoelii 17XL* parasite

Mice were injected Intraperitoneally (i.p.) with $2x10^6$ *P.yoelii 17XL* parasitized RBCs, kindly provided by National Institute of Malaria Research (NIMR), New Delhi, India. For the i.p. injection, mice were sedated through anesthetic chamber for the induction of successful anesthesia. Daily parasitemia were monitored and mice comprising of six mice in each group were sacrificed by inhalation of CO_2 by Euthanasia method. Brains of infected and control mice were stored at -80º C for RNA isolation.

2.3 Brain Histological analysis

Whole brains of infected and uninfected mice at mild and peak parasitemia were fixed in formal saline and were processed for routine histological analysis with Haematoxylin and Eosin staining (H and E staining). Brains sections were noted for the changes in parenchymal cells and erythrocyte sequestration.

2.4 RNA isolation

Harvested mice brains were homogenized and messenger RNA (mRNA) was isolated using NucleoSpin® RNA II (Macherey-Nagel) kit according to the manufacturer's instruction. The extraction procedure was carried out at different time points after symptoms of MM and CM. Extracted mRNA was stored at -80º C until further processing. Five different aliquots of each mRNA samples were made to avoid repeated freeze and thaw conditions.

2.5 Gene expression analysis using Real-Time SYBR Green PCR

Each aliquot of total RNA extracted was used for the gene expression analysis using TaKaRa One Step SYBR® Ex Taq™ qRT-PCR kit as per manufacturer's instructions. Glyceraldehyde-3 Phosphate Dehydrogenase (GAPDH) was used as a housekeeping gene. Primers were used at a concentration of 0.8µM for each gene. (Table 1) Amplification, data acquisition and expression analysis were carried out by using ABI StepOne™ instrument (Applied Biosystem, CA). Fold change was calculated using ΔΔCt determination using mean ΔCt value [26-27].

2.6 Statistical analysis

Data were statistically analyzed using GRAPHPAD PRISM™ 5 software. (GraphPad software, Inc. La Jolla, CA). The results obtained in this work were performed in triplicate by identical methods. Results were expressed as Mean ± Standard Deviation (SD) for each group of mice. Group differences were assessed using unpaired two-tailed t test. In all cases $P<0.0001$ was considered as significant.

Table 1: Primers sequence of target genes and housekeeping gene with annealing temperature

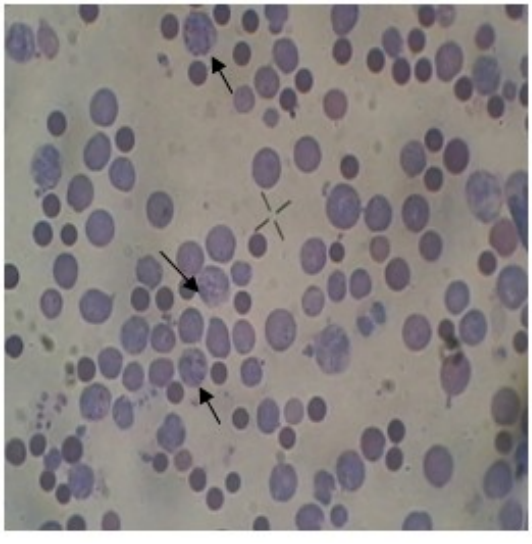
Gene	Direction (5'-3')	Sequence	Annealing temperature used (°C)	Amplified product length
IFN-γ	Forward Reverse	CTCAAGTGGCATAGATGTGGAAGA GAGATAATCTGGCTCTGCAGGATT	56.4	415
RANTES	Forward Reverse	CCCTCACCATCATCCTCACT CCTTCGAGTGACAAACACGA	52.2	185
IP-10	Forward Reverse	CGTCATTTTCTGCCTCATCCT GGTCTTAGATTCCGGATTCAG	52.8	227
MIP1-α	Forward Reverse	ATGAAGGTCTCCACCACTG GCATTCAGTTCCAGGTCA	51.6	275
MIP1-β	Forward Reverse	CCCACTTCCTGCTGTTTCTCTTAC AGCAGAGAAACAGCAATGGTGG	61.4	427
MCP1	Forward Reverse	GTCACCTGCTGCTACTCATTC GCTTGAGGTGGTTGTGGAAAA	53.5	318
GAPDH	Forward Reverse	GGAGAAGCTGCCAATGGATA GTGGTCTTCACGTTCGCATT	54	218

3. Results

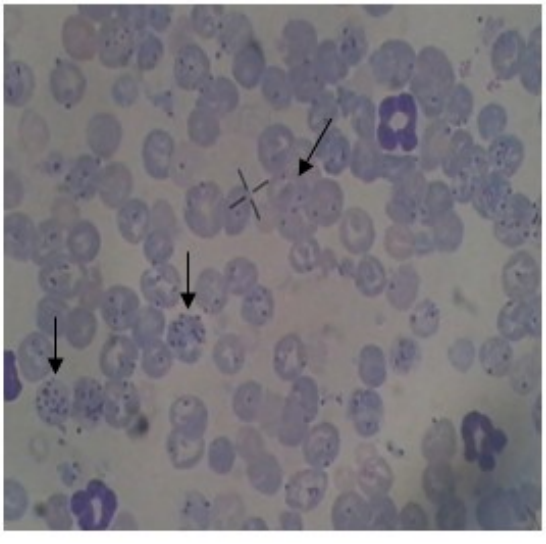
3.1 Parasitemia and clinical signs of *P.yoelii 17XL* murine malaria

Symptoms of malaria including appearance of ruffled fur, retinal whitening, and change in gait and shivering at MM and CM were noticed in all mice infected with *P.yoelii 17XL* parasite (Figure 1 and Table 2). After dissecting mice, examination of liver and spleen showed hepato- and spleno-megaly respectively at peak parasitemia (data not shown). None of the control or uninfected mice showed any sign and symptoms as were seen in infected mice. At up to 90% parasitemia mice developed clinical symptoms like hind limb paralysis but did not show convulsion and coma, as seen in *Plasmodium berghei ANKA* strain in murine CM [9].

Fig.-1: Level of parasitemia in *P.yoelii 17XL* infected *Swiss albino* mice. Parasitemia were monitored from the tail vein blood and counting at least 500 RBCs, stained with Giemsa, under immersion oil (100x). 15-20% parasitemia was considered as MM and 70-90% parasitemia was considered as CM. Black arrow indicates the parasitized RBCs with MM and CM.



Mild Malaria (MM)



Cerebral Malaria (CM)

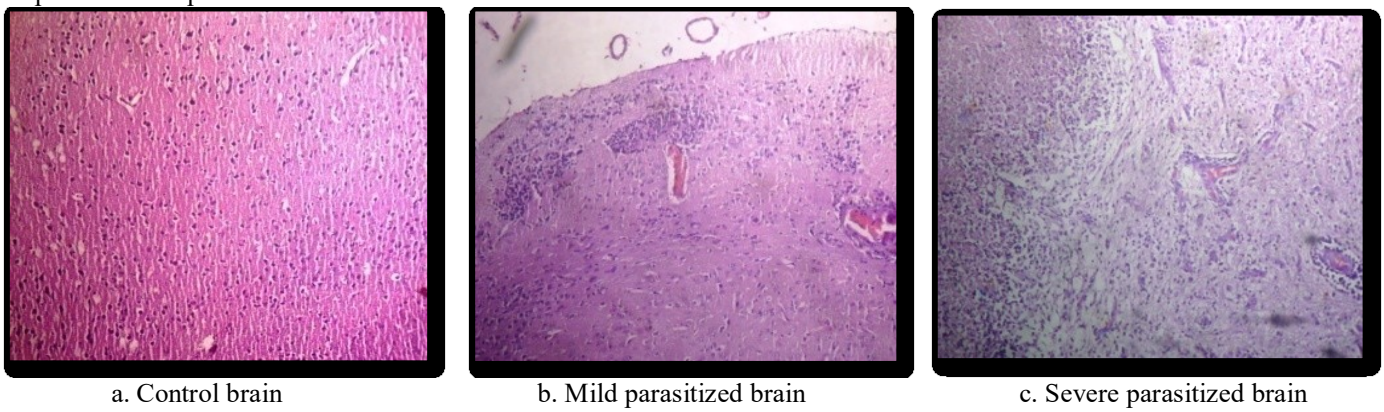
Table-2: Comparison between Clinical manifestations in mice of Cerebral Malaria (CM) & Mild Malaria (MM)

	Mice with CM (n= 6)	Mice with MM (n= 6)
Ruffled fur & Shivering	05	05
Retinal whitening	05	05
Change in gait	04	02
Splenomegaly	03	01
Hepatomegaly	04	01
Hind Limb paralysis	03	01
Death	01	00

3.2 Histopathology of mouse brain

Sagittal section from the midline region of the brain taken from CM, MM and control uninfected mice were stained and examined. Sections were carefully scanned over the entire area including the parenchymal cells. Histological analysis revealed mild infiltration of polymorphs in the cerebral parenchyma. (Fig.2). Tissue section in severely parasitized mice exhibited massive degeneration of the parenchyma, consistent with marked inflammation of all the infected mice, but none of the uninfected mice.

Fig.-2: Histological brain section of a. control brain, b. mild parasitized brain, c. severe parasitized brain; *P.yoelii 17XL* infected mice induces mild infiltration of polymorphs in the cerebral parenchyma of severely parasitized brain of mice as compared to mild parasitized brain.

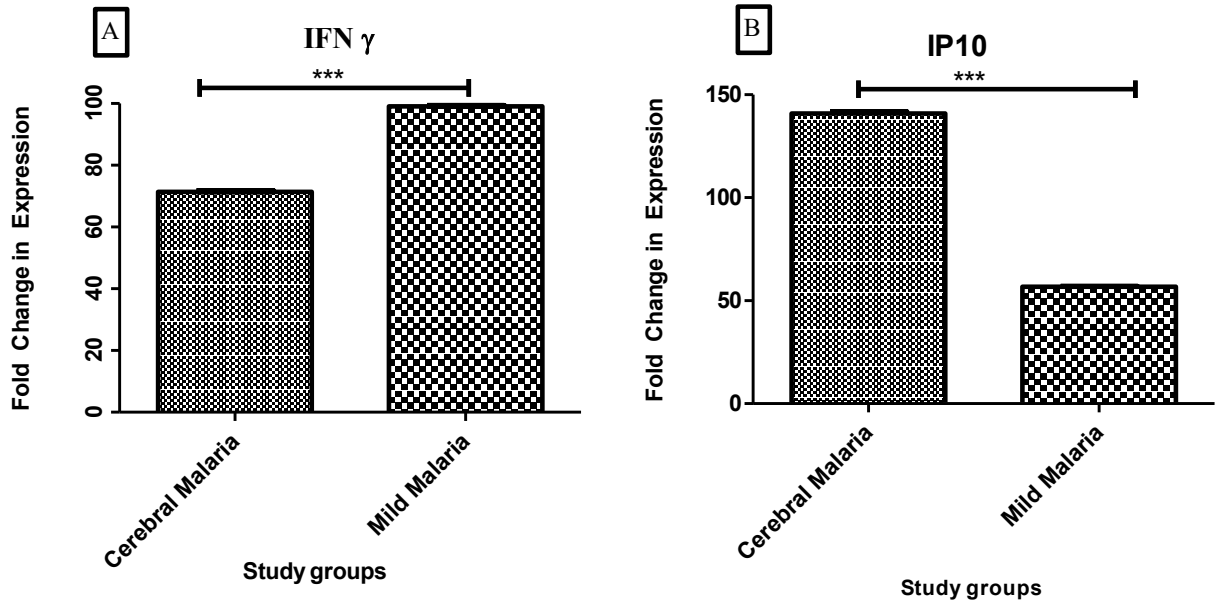


a. Control brain b. Mild parasitized brain c. Severe parasitized brain

3.3 CXCL10 chemokine is highly Up-regulated in the Brains of mice with CM

We examined the expression of IFN γ and IP-10 in the brain during murine MM and CM. Nine to ten days after infection, when mice displayed signs of CM, IP10 was the most highly expressed of the tested chemokines in the brains in terms of total mRNA level. IP10 was the initial chemokine to be induced in the brain 6 days after infection (56-fold) in MM and increasing further by day 9 (140-fold increase) in CM. (Fig-3). Significantly higher level of IP10 was observed in mice with CM compared with those with MM (Table 3). However, in case of IFN γ, on day 9, low parasitized mice produced significantly higher amount of IFN γ compared to severe malaria mice. There was 99-fold increased in IFN γ total mRNA level in MM as compared to 71-fold in CM. (Table 3).

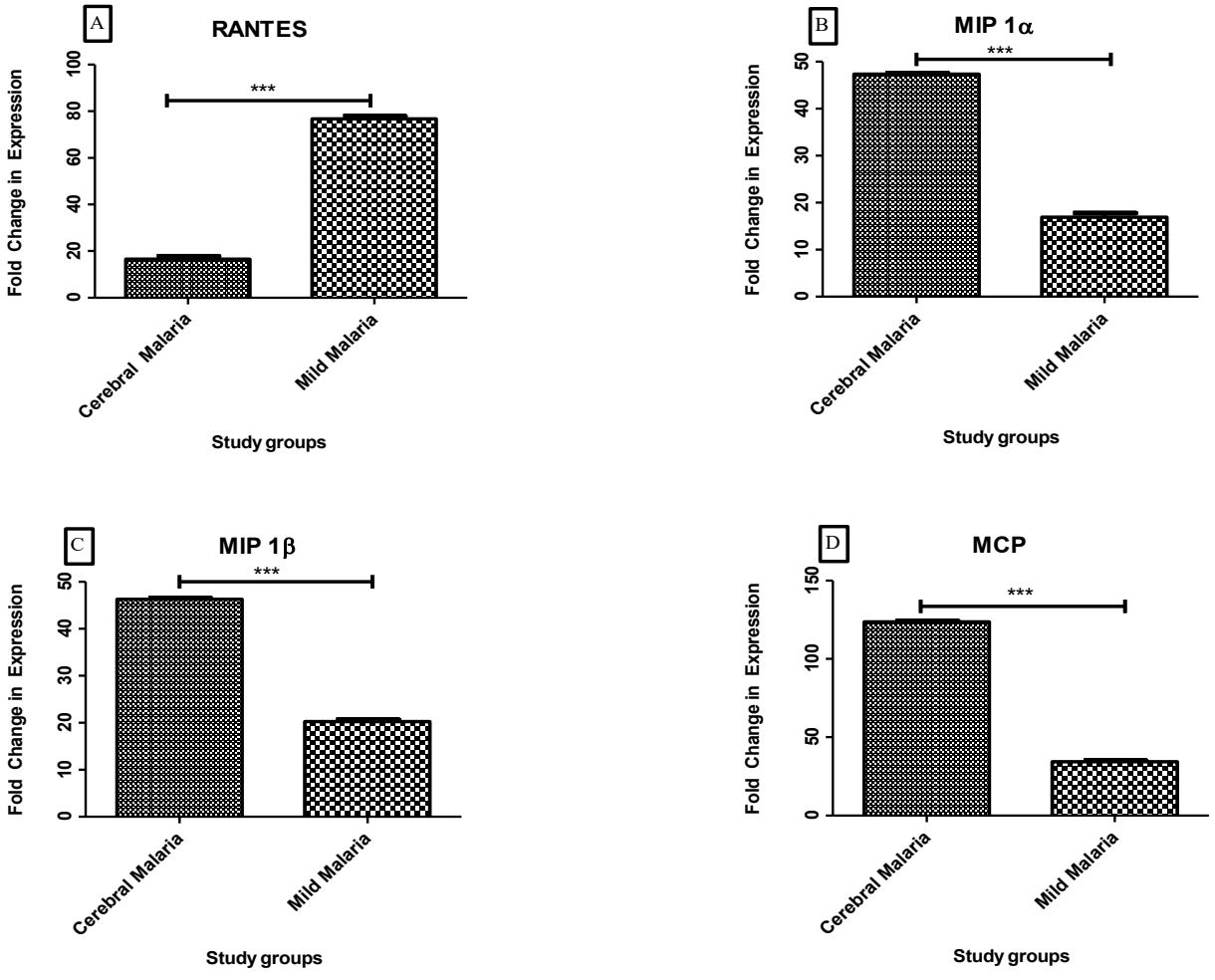
Fig.-3: Chemokine mRNA expression in brain during murine CM and MM. *Swiss albino* mice were i.p. injected with $2x10^6$ *P.yoelii 17XL* parasitized RBCs and brains were harvested at the indicated time point. IFN γ (A) and IP-10 (B) mRNA levels were analyzed. mRNA data was expressed as mean ±SD fold change in expression. Group differences were assessed using two-tailed unpaired t test. (P<0.0001 significant)



3.4 Association of C-C chemokines expression in *P. yoelii 17XL* induced Malaria

A strong positive correlation was seen in the mRNA level of RANTES and its corresponding receptors i.e. MIP1α and MIP 1β in CM as compared to MM. Level of MIP1α and MIP 1β were markedly higher in mice that showed severe symptoms because of CM (n=6) when compared with those who had MM (n=6). There was an up regulation of MIP1α and MIP 1β total mRNA in CM wherein it was lower in MM (Table 3) (Fig.-4). However, level of RANTES was significantly lower in mice with CM than in MM (Table 3). Level of RANTES, after 5 days of infection, was significantly up-regulated, 76-fold higher in MM than CM which was 16-fold up-regulation at the time of high parasitemia. (Fig.-4).

Fig-4: Association of C-C and CCL2 chemokines expression: *Swiss albino* mice were i.p. injected with parasitized RBCs and brains were harvested at the indicated time point. RANTES (A), MIP1α (B), MIP 1β (C) and MCP-1 (D) mRNA levels were analyzed. mRNA data were expressed as mean ±SD fold change in expression. Group differences were assessed using two-tailed unpaired t test. (P<0.0001 significant)



3.5 CCL2 chemokine level in *P. yoelii 17XL* induced Malaria

Brain samples from infected mice as well as uninfected control were assessed for MCP-1 chemokine expression. Systemic increase in MCP-1 expression began 6 days after *P. yoelii 17XL* infection in MM, further until peak parasitemia at day 9, it highly up-regulate with about 123-fold increased in infected brain samples of CM as compared with control. (Fig.-4; Table 3)

Table-3: Unpaired t test results for fold change in expression for the measured cytokine and chemokines.

Groups	Mean ± S.D Fold change in expression		P-value (P<0.0001)
	Cerebral Malaria	Mild malaria	
IFN-γ	71.38 ± 0.4256	99.02 ± 0.2501	0.5133
RANTES	16.50 ± 2.167	76.82 ± 2.018	0.9292
IP-10	140.9 ± 1.622	56.84 ± 0.4484	0.1419
MIP1-α	47.36 ± 0.4599	16.92 ± 1.527	0.1664
MIP1-β	46.27 ± 0.4273	20.26 ± 0.6305	0.6294
MCP1	123.6 ± 1.091	34.36 ± 1.300	0.8262

4. Discussion

In an attempt to study malaria immunology with respect to chemokine response, we infected *Swiss albino* mice by *P. yoelii 17XL* parasite for the development of malaria symptoms both in MM and CM. The *P.yoelii 17XL* model in *Swiss albino* mice showed similarity to human CM in terms of histopathology whereas *P. berghei ANKA* model showed similarity with human CM in terms of symptomatology [14]. Therefore we harvested the whole brains of infected and uninfected mice at mild and peak parasitemia and we noticed mild infiltration of polymorphs in the cerebral parenchyma of the infected brains (figure2). Tissue section in severely parasitized mice exhibited massive degeneration of the parenchyma, consistent with marked inflammation. These results support the observation led by Sarfo and colleagues that sequestered parasitized erythrocytes were observed in the brains' micro vessels of *P.yoelii 17XL* infected mice [14].

Further we studied chemokines, IFN γ, IP-10, RANTES, MIP 1α, MIP 1β and MCP, those play important roles in the pathogenesis of CM. The result of qRT-PCR analysis revealed that during the course of MM to CM infection, mRNA expression of IFN γ was significantly up-regulated in infected mice compared with control mice indicating that IFN γ is involved in the immuno pathogenesis in *P.yoelii 17XL*- infected mice. Different studies have reported that IFN γ was produced in large amounts during infection and plays an important role in the activation of vascular endothelium [12]. In addition to this, IFN γ and chemokines like ICAM-1, P-selectin and VCAM-1 were highly up-regulated on brain vascular endothelial cells during the development of experimental cerebral malaria (ECM) [3,28]. In our study, IFN γ was found to be increased more than 20-fold in MM as compared to CM in the infected brain samples.

The expression of chemokine IP10 has been shown to be highly up-regulated in CM than in MM. Different human studies have reported that IP10 and other CXCR3 ligand Mig, induced a greater fold increased in infected individuals than a normal individuals in CM [5,19,29], which is similar to what we found in our murine studies. Another group of researchers have reported the role of IP10 in HIV infection [17] as well as West Nile Virus infection [18] which showed that IP10 plays an important role in the pathogenesis of severity of the disease. In HIV infection, IP10 has a direct neurotoxin effect by correlating with HIV associated dementia in Cerebrospinal Fluid (CSF) level [17]. According to the Campanella *et al*. [5] IP10 and Mig recruit $CD8^+$ T cells in the brain by amplifying the induction of these cytokine and additional chemokines that leads to the development of CM through CXCR3 ligand. Our study supports the previous finding in the role of IP10 in the development of CM through direct neurotoxicity in addition to its role in $CD8^+$ T cell recruitment in the brain vessels.

The importance of MIP1α, MIP 1β and RANTES have been demonstrated by many researchers in the mediation of Host Immune response to bacterial infection [20-21], viral infection [30] and human malaria by *P. falciparum* infection [22]. John and co-workers studied serum level of these C-C chemokines and their relation to pro- and anti-inflammatory cytokines in children with CM in Uganda [15]. They found serum levels of RANTES were low and β-chemokine levels were high in childrens with CM as compared to childrens with MM and it was associated indirectly with mortality with other expressed cytokines and chemokines. These findings are very similar to what we found in our murine model. In our study, we demonstrated increased level of MIP1α and MIP 1β and decrease in the RANTES expression in CM as compared to MM mice infected with *P.yoelii XL* strain. Addition to this, Ochiel et. al, demonstrated decreased level of RANTES but increased level of β-chemokines MIP1α and MIP 1β in Kenyan children with Severe malaria compared to mild malaria caused by *P.falciparum* [22]. They also suggested that in the context of malaria, reduced RANTES production may result in the ineffective immunological response. In contrast, a study with Ghanaian children with CM by Sarfo *et al*; showed increased mRNA expression of RANTES, CCR3, CCR5 in post mortem samples of cortex and cerebellum of children's brain but not in non malaria cases [23]. Same team of researchers also demonstrated that mRNA expression of RANTES, CCR1, CCR3 and CCR5 was up-regulated in the plasma of *P.yoelii* infected mice and induced ultra structural changes in the cerebellum in the brain [14]. Taken together, all the finding concluded that inability of RANTES production may be a factor in disease severity in mice with CM and may be an increased in expression in mild cases. However, as per Sarfo *et al*; up-regulation of RANTES in localized area of the brain is progressive towards CM [12], we do not exclude this possibility but the cells that produce RANTES may also be different in the brain (monocytes, microglia, astrocytes) [15] and the effect of RANTES may also different in these areas.

β chemokines, MIP1α and MIP 1β, plays a crucial role in the host parasite interactions. Increased expression of β chemokines has been shown in both murine as well as human cerebral malaria cases [22-23]. Previous results also showed that serum concentration of MIP1α and MIP 1β were elevated in children associated with cerebral *P. falciparum* malaria [22]. They indicated that elevated circulating concentration of MIP1α was the direct result of malaria infection prior to treatment with antimalarial drugs in children with acute falciparum malaria and not due to treatment intervention. Their investigation of MIP 1β revealed similar results in which MIP 1β gene expression in PBMCs was elevated in *P .falciparum* infected individuals [22]. This distinct profile of β chemokines expression appears to be due to Phagocytosis of Hemozoin, which is polymer of heme and produced by parasites during the digestion of host

heamoglobin within the Red blood cells [22,31]. In our study, gene expressions of MIP1α and MIP 1β were up-regulated in brains of CM. However, results obtained in our study failed to find the exact correlation between MIP1α, MIP 1β and brain endothelial cells. It is important to note that disease severity in the present study was defined according to only in dissected samples of brains. As such for disease severity, based on gene expression analysis from brains, its not be possible to fully elucidate the contribution of MIP1α and MIP 1β to the etiology of severity of malaria. MCP-1, also known as CCL2, is one of the best characterize β-chemokine and is strongly involved in the inflammatory processes [24]. At the site of inflammation, MCP-1 acts as a mediator for attraction of macrophages/monocytes [25]. In the brain, increased permeability of BBB and hence increased leukocyte infiltration into the CNS during the inflammation process is thought to be associated with MCP-1 chemokine expression [25,32]. In the present study, MCP-1 was highly up-regulated in the brains of CM mice (123 fold) as compared to MM malaria brain samples. Thus the statistical power of this gene was high enough to detect the association of MCP-1 with CM severity.

In conclusion, *P.yoelii 17XL* infection highly up-regulated IP-10, MIP1α, MIP 1β and MCP-1 in the brains of mice that developed CM and IFN γ and RANTES was up-regulated in MM as compared to mice with CM. The results and evidence of this study are in agreement to some extent with previous related research work. Though this work supports the role of chemokines in severity of experimental murine malaria in its least form, there may be more to discover before implicating this findings. More investigation with expression of cytokines and chemokines both at mRNA and protein levels should be carried out in similar study groups likewise here.

Conflict of Interest

The authors declare no competing interests.

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