JNK and ER stress signaling pathways mediate neuronal cell death in Experimental cerebral malaria: Role of SP600125 and Pentoxifylline as neuroprotective agents

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By

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DECLARATION

I, Santosh Anand Sripada, hereby declare that this thesis entitled "JNK and ER stress signaling pathways mediate neuronal cell death in Experimental cerebral malaria: Role of SP600125 and Pentoxifylline as neuroprotective agents" submitted by me under the guidance and supervision of Prof. P. Prakash Babu is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that this thesis entitled "JNK and ER stress signaling pathways mediate neuronal cell death in Experimental cerebral malaria: Role of SP600125 and Pentoxifylline as neuroprotective agents" is a record of bonafide work done by Mr. Santosh Anand Sripada, a research scholar for Ph.D. programme in the Department of Animal Sciences, University of Hyderabad under my guidance and supervision.

The thesis has not been submitted previously in part or full to this or any other University or Institution for the award of any degree or diploma.

(Signature of Supervisor)

(Head of the Department)

(Dean of the School)

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Abbreviations

Ab	Antibody
BBB	Blood brain barrier
BW	Body Weight
CD	Cluster differentiation
CM	Cerebral malaria
CNS	Central nervous system
COX-2	Cyclooxygenase-2
DNA	Deoxyribonucleic acid
EC	Endothelial cells
ECM	Experimental cerebral malaria
GFAP	Glial fibrillary acidic protein
GPI	Glycosylphosphatidylinositol
H & E	Hematoxylin and Eosin
ICAM-1	Intercellular adhesion molecule 1
IL	Interleukin
IFN	Interferon
IP	Intra peritoneal
iNOS	Inducible nitric oxide synthase
iRBC/PRBC	Infected/Parasitized red blood cell
kDa	Kilo Dalton
mRNA	Messenger ribonucleic acid
MSP 1	Merozoite specific protein 1
NK-cells	Natural killer cells
PbA	*Plasmodium berghei* ANKA
PfEMP-1	*P. falciparum* erythrocyte membrane protein-1
PMSF	phenylmethanesulfonylfluoride
PI	Post inoculation
PTX	Pentoxifylline
RBC	Red blood cell
ROS	Reactive oxygen species
RT-PCR	Real Time Polymerase chain reaction
TNF	Tumor necrosis factor
VCAM-1	Vascular endothelial cell adhesion molecule 1
WHO	World Health Organization

Contents

INTRODUCTION

Burden of Malaria disease

A recent report by the World Health Organisation (WHO, 1996) has recognized malaria as a major cause of morbidity and mortality in tropical and sub-tropical regions of the world. Nearly all of these deaths are caused by *Plasmodium falciparum*, one of the four species of malaria parasites infecting humans. This high burden of mortality falls heavily on sub-Saharan Africa, where over 90% of these deaths are thought to occur, and 5% of children die before the age of 5 years (Fig. 1) (Snow *et al.*, 2005). The death toll from malaria is still growing, with malaria specific mortality in young African children estimated to have doubled during the last 20 years. This increase has been associated with drug resistance of the parasite, spread of insecticide-resistant mosquitoes, poverty, social and political upheaval, and lack of effective vaccines.

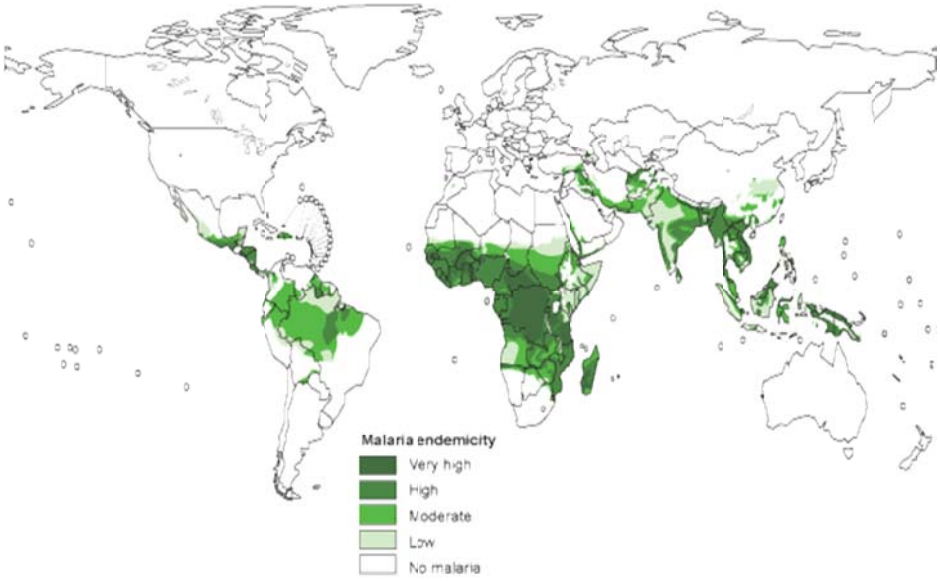


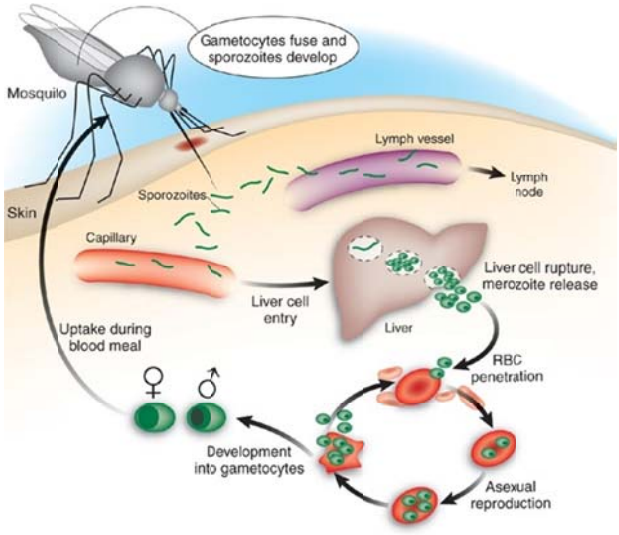
Fig 1. This disease is geographically restricted and is prevalent in a broad band around equator, in areas of Americas, many parts of Africa, Indian subcontinent, and also in South East Asia. Globally, the annual disease burden in over 90 endemic countries is estimated at 250–500 million clinical cases with the greatest impact in sub-Saharan Africa, where it claims the lives of 1.5–2 million children every year. **Source:** World Malaria Report 2006 from WHO and UNICEF

Failure of the host to control a malaria infection is in part related to the complexity of the parasite and the interaction with the immune system of the host. Although there is now considerably more investment in malaria research, and there are encouraging signs in vaccine development, we are a long way from understanding the nature and control of protective immunity or the pathological consequences of the host's response to *Plasmodium*. With the

major advances in knowledge in basic immunology, inflammation, and the genomic information on the host, vector and parasite, we are now in a position to elucidate the key unknowns in the immune response to malaria.

The parasite, *Plasmodium falciparum*

Malaria is caused by a protozoan parasite from the genus *Plasmodium*, transmitted through the bite of the female *Anopheles* mosquito. Four species of *Plasmodium* are known to cause disease in humans, *P. vivax*, *P. ovale*, *P. malariae* and *P. falciparum*, the latter being the most virulent and the major cause of mortality. Cerebral Malaria is the most severe neurological complication of infection caused by *Plasmodium falciparum* and is characterized by fever, malaise, headache, joint and body aches, seizures, coma and finally leading to death. *Plasmodium* infection begins with the inoculation of sporozoites into the dermis of a vertebrate host during the blood feeding of an infected female Anopheles mosquito. From the dermis, the sporozoites make their way into the nearby blood vessel and travel all the way to the liver sinusoid where they interact with sulfated heparan sulfate proteoglycans of hepatocytes. This results in the switching of sporozoites from migratory mode to invasive mode, wherein the parasites actively transmigrate through several hepatocytes before invading a final one and establish a productive infection. Once inside a parasitophorous vacuole (PV) formed during invasion, the parasite manipulates the host cell for its own benefit and divides by schizogony to produce at the end thousands of merozoites.



Merozoites once released into the bloodstream invade erythrocytes and undergo multiple intraerythrocytic cycles. This phase elicits the clinical manifestation of the disease. In the erythrocytes they become trophozoites which, in case of *P. falciparum*, develop into new merozoites within 48 hrs. After a variable number of erythrocytic schizogony cycles, some of the merozoites differentiate into gametocytes (sexual blood stages), which are taken up by the mosquito during a blood meal. Gametocytes differentiate into gametes, and sexual reproduction results in the formation of an ookinete, which traverses the mosquito midgut epithelium and develops into oocysts, leading to the release of sporozoites that migrate to the salivary glands.

Invasion of RBCs by *Plasmodium*

The erythrocyte invasion by the parasite is a crucial step and is vital to our understanding of the pathogenesis of malaria (Chitnis and Blackman, 2000). Invasion is a highly specific, ordered and sequential process lasting less than a minute (Gilson and Crabb, 2009). Numerous receptor–ligand interactions, and a number of merozoite proteins were implicated in invasion of the RBC by the parasite (Cowman and Crabb, 2006). It is well recognized that the components of initial attachment include antigens on the surface of the merozoite, such as merozoite surface protein 1 (MSP1) which is a GPI-anchored molecule (Gilson *et al.*, 2006; Goel *et al.*, 2003). Further, it is believed that MSP1 is the most abundant of the proteins found in that location (Goel *et al.*, 2003; O'Donnell *et al.*, 2001) and its expression is restricted to late blood stages and liver stages. At the time of invasion, MSP1 undergoes proteolytic cleavage to produce a 42 kDa C-terminal fragment, which is further processed to yield fragments of 33 and 19 kDa. The 19kDa fragment of the MSP1 remains on the surface of a merozoite that is invading an erythrocyte (Blackman *et al.*, 1990). After attaching to the susceptible red cell the merozoite undergoes apical re-orientation, which involves the integral membrane protein called the apical membrane antigen (AMA1) (Fig. 3) (Mitchell *et al.*, 2004). The parasite then uses actin-based motility to enter erythrocyte (Baum *et al.*, 2008) where in it slowly moves into a localized invagination of the red cell which subsequently envelops it as the parasitophorous vacuolar membrane (PVM). Identification of the molecules on the erythrocyte membrane to which merozoites bind has been the subject of intensive research. Pasvol *et al.*, first identified the red cell sialoglycoproteins or 'glycophorins' (GPs) especially GPA and GPB as the major receptors that mediate binding and subsequent invasion of the merozoites (Pasvol *et al.*, 1982). Of the sites involved on GP, the O-

linked tetrasaccharides, rather than the much larger N-linked sugars on these molecules, were found to be important. The resistance of the Tn red cell phenotype to invasion (Tn cells lack a glycosyl transferase which results in cells deficient in sialic acid), and the sensitivity of invasion to neuraminidase/sialidase, led to the identification of sialic acid on GP as a major recognition site on these O-linked sugars (Pasvol *et al.*, 1982). Further, a sialic acid- independent pathway has been identified for the erythrocyte invasion by *Plasmodium falciparum* (Dolan *et al.*, 1990; Goel *et al.*, 2003; Mitchell *et al.*, 1986). Attachment and orientation of the parasite to the RBC is followed by the internalization, accompanied by deformation of the red cell membrane. However, the number and diversity of molecules involved in this part of the process and their individual roles have not as yet been ascertained (Cowman *et al.*, 2000). Moreover, alterations in the physicochemical properties of the red cell membrane also play an important role in regulating invasion (Clough *et al.*, 1995). The ability to invade red blood cells might be a crucial factor in the pathogenesis of falciparum malaria since it has been shown to relate to disease severity.

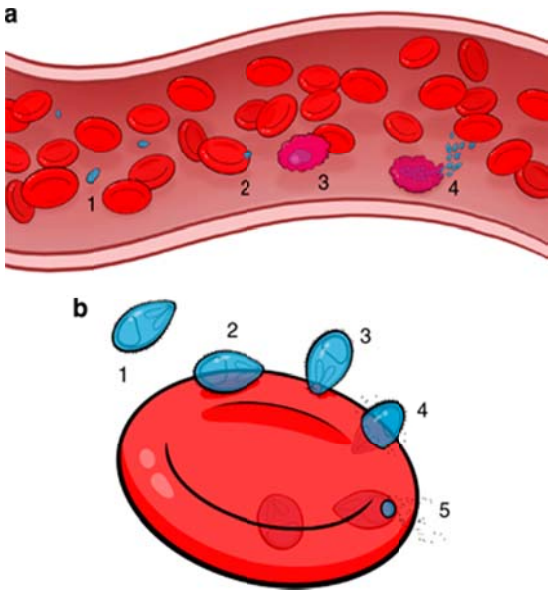


Fig. 3. The blood-stage lifecycle of *Plasmodium* (**a**) Merozoites are an extracellular form of *Plasmodium* (1) that attach and invade erythrocytes (2). The parasite then matures and divides through asexual replication inside the erythrocyte (3). The expression of parasite proteins on the surface of the infected erythrocyte enables interactions with receptors on the endothelial surface, facilitating sequestration of parasite-infected erythrocytes in various organs. After asexual replication, the schizont form ruptures and releases new merozoites into the circulation (4). (**b**) Merozoite invasion of erythrocytes. Merozoites are initially free in the intravascular space after schizont rupture (1), and the process of erythrocyte invasion commences when a merozoite binds to the surface of an erythrocyte (2). The merozoite then reorients its apical end to come into contact with the erythrocyte surface (3). A series of irreversible high-affinity ligand–receptor interactions then occur between the parasite and the erythrocyte, enabling tight-junction formation (4) and entry of the erythrocyte through an actin–myosin motor. As it does so, the parasite

invaginates the erythrocyte membrane to form the parasitophorous vacuole. Outer surface proteins are partially cleaved as the merozoite enters the erythrocyte (5). **Source**: Immunology and Cell Biology, 2009, 377–390

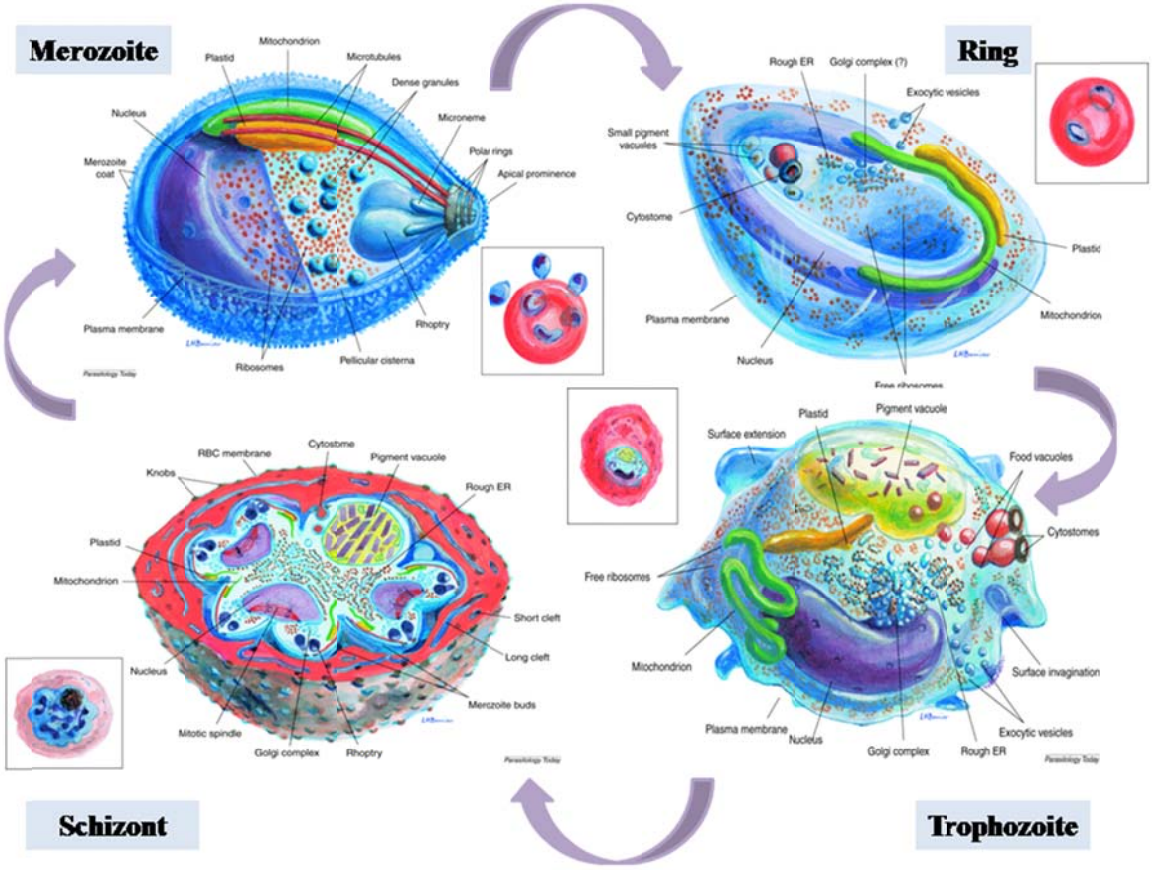


Fig 4: 3-D organization of a *P. falciparum* merozoite, early ring, mid-trophozoite and schizont with the pellicle partly cut away to show the internal structure. Inset: relative sizes of various blood stages and the red blood cell (RBC). **Source**: *Parasitology Today, 2000,427-433.*

Rosetting

The spontaneous binding of nonparasitized erythrocytes to erythrocytes containing trophozoites and schizonts of *Plasmodium* is termed as rosetting (Fig 5) (David *et al.*, 1988; Fernandez and Wahlgren, 2002; Handunnetti *et al.*, 1989; Udomsangpetch *et al.*, 1989b). Comparison of rosetting capacity of parasites from cases of cerebral malaria with those of uncomplicated malaria revealed that rosetting is highly prominent in cerebral malaria (Carlson *et al.*, 1990). Moreover, serum from patients with mild disease could more often disrupt preformed rosettes

than serum from patients with cerebral malaria suggesting that serum from cases with severe malaria usually contains a low titer of anti-rosette specific antibodies. In contrast, the presence of high titer of anti-rosette antibodies in patients with uncomplicated malaria indicate that rosetting is a phenotype associated with the severity of malarial disease and that the molecular mechanisms underlying this phenomenon exhibit a high degree of complexity and are likely based on multiple receptor-ligand interactions (Heddini *et al.*, 2001). Further, the anti-rosette immunity is considered as an important factor in disease outcome (Chen *et al.*, 2000b).

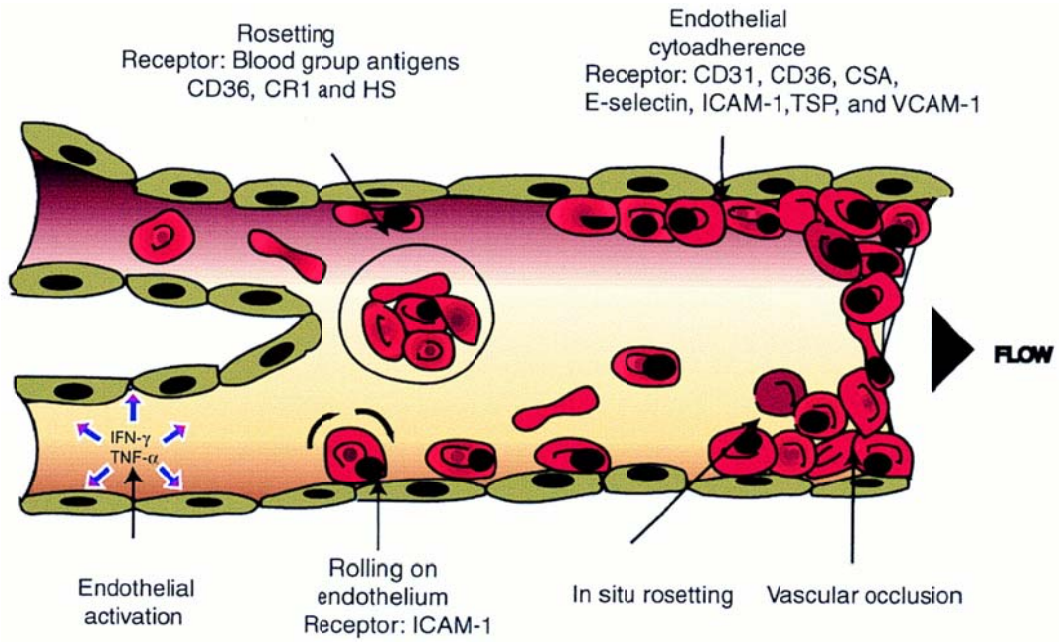


Fig. 5: Cytoadherence and rosetting in postcapillary vasculature. *P. falciparum*-infected RBCs bind to the postcapillary endothelial lines and to noninfected RBCs. Both phenomena are thought to contribute to the occlusion of blood flow and consequent severe disease. Parasite antigens could stimulate IFN-g and TNF-a release, which upregulates receptor expression (such as ICAM-1) and redistribution (such as CD31) on the endothelium. ICAM-1 is suggested to mediate pRBC rolling on endothelium, while CD36, CD31, and other receptors are responsible for more stable binding. Sequestration could be augmented by the ability of spontaneous rosetting (in situ rosetting) and cytoadherence of the parasite. **Source:** Clinical Microbiology reviews, July 2000, p. 439–450

Till date, 5 rosetting receptors have been identified on RBCs, *viz.,* blood group antigens A and B, CD36, complement receptor 1 (CR1), and HS-like GAGs. Handunetti *et al.*, suggested CD36 as a rosetting receptor (Handunnetti *et al.*, 1992). However the contribution of CD36 receptor in the rosetting phenomenon is limited to immature RBCs since the copy number of CD36 molecules on mature erythrocytes is low. In contrast, blood group antigens A and B function as rosetting receptors has been systematically investigated since they are widely

distributed on the surface of non-O RBCs (Carlson and Wahlgren, 1992). The high sensitivity of *P. falciparum* rosettes in type O blood to heparin and N-sulfated glycans suggest that malaria parasites use a GAG on the surface of RBCs as a rosetting receptor (Carlson *et al.*, 1992; Carlson and Wahlgren, 1992). CR1 seems to be an important rosetting receptor, as it is widely distributed on the RBC surface and some parasites cultured in CR1-deficient RBCs lose their capacity to form rosettes (Rowe *et al.*, 1997). Further, some serum proteins including IgM (non-immune) fibrinogen and albumin are also involved in rosette formation (Treutiger *et al.*, 1998; Treutiger *et al.*, 1999). The mechanisms by which rosetting leads to disease remain obscure till date. Rosette formation has not convincingly been observed *in vivo*. Rigorous experiments with a rosetting and non-rosetting parasite line, under static conditions or with red cells maintained under conditions of flow (i.e. in suspension), demonstrated that rosetting neither increases invasion nor targets merozoites within a rosette into adjacent uninfected cells (Clough *et al.*, 1998). Rosetting may perhaps reflect the phenotypic expression of some other parasite property such as adherence to a specific cell type, which in turn relates to pathogenesis.

Cytoadherence

Cytoadherence is a survival strategy adopted by the parasite to protect itself from the host immunity and to avoid splenic clearance. In this process the mature PRBCs specifically bind to endothelial cells in post-capillary venules (Fig 6). The process results in microcirculatory obstruction and subsequent hypoxia, metabolic disturbances, and multiorgan failure, which are detrimental to the host. Understanding the molecular events involved in these adhesive interactions is therefore critical both in terms of pathogenesis and implications for therapeutic intervention. SEM studies show a number of regular, symmetrically arranged 'knobs' on the surface of the infected cell as the parasite of *P. falciparum* matures (Howard, 1988; MacPherson *et al.*, 1985) . It is through these knobs the parasitised red cell mediate binding to endothelial cells in the deep tissues, although parasites without knobs are capable of cytoadherence *in vitro* (Biggs *et al.*, 1990; Udomsangpetch *et al.*, 1989a). As the parasite matures inside, the red cell becomes spherical and less deformable. Parasites sequester in various organs including heart, lung, brain, liver, kidney, subcutaneous tissues, and placenta. The various endothelial cells in these organs and syncytiotrophoblasts in placenta express different and variable amounts of host receptors. To successfully adhere to these cells, the parasite can bind to a large number of

receptors. The adhesion phenotype is not homogenous, and different parasites can attach to variable numbers and combinations of host receptors (Beeson *et al.*, 1999; Newbold *et al.*, 1997). This variability is believed to affect the tissue distribution and pathogenesis of parasites. Till date five parasite-derived proteins are known to be associated with the cell membrane of an infected erythrocyte at various stages of the developmental cycle (Howard, 1988). Of these five parasite derived proteins, three are associated with knobs [*P. falciparum* erythrocyte membrane proteins 1 and 2 (PfEMP1 and PfEMP2) and PfHRP1 or KAHRP]. Of these three, PfEMP1 is the only protein that extends beyond the cell surface to mediate cytoadherence (Magowan *et al.*, 1988), whereas PfEMP2 and PfHRP1 or KAHRP remain on the internal face of the erythrocyte membrane in association with electron-dense material. The proteins appear to be exported from the intracellular parasite through the erythrocyte cytoplasm to the surface membrane via a complex system of vesicle trafficking pathways (Pouvelle *et al.*, 1994). PfEMP-1 (molecular weight 200-350 kDa) appears to be the most important molecule expressed on the PRBC and mediates its binding to various receptors (Chen *et al.*, 2000b; Newbold *et al.*, 1999). PfEMP1 is encoded by the large and diverse *var* gene family that is involved in clonal antigenic variation and plays a central role in *P. falciparum* pathogenesis (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). The extracellular region of PfEMP1 has multiple adhesion domains like DBL (Duffy binding like) and CIDR (1-2 cystine-rich interdomain regions), that can simultaneously recognize several host receptors including platelet-endothelial cell adhesion molecule (PECAM), the blood group A antigen, non-immune IgM, a heparan sulphate-like glycosaminoglycan and CD36 (Chen *et al.*, 2000a). The binding domains for several host receptors were recently mapped to various DBL and CIDR present on the extracellular region of PfEMP1 (Smith *et al.*, 2001). Recently Pouvelle et al, detected two previously unknown parasite proteins on the surface of ring-stage PRBC, the ring surface proteins (RSP 1 and 2) (Pouvelle *et al.*, 2000). These proteins disappear shortly after the start of PfEMP1-mediated adhesion. It is thus possible that not only is the degree to which PRBCs cytoadhere important in pathogenesis, but also the stage in the erythrocyte life cycle when cytoadherence commences.

Sequestration

The histopathological hallmark of cerebral malaria is the sequestration of RBCs containing mature forms of parasite (trophozoites and meronts) to the cerebral microvasculature (MacPherson *et al.*, 1985). This event is thought to cause the major complications of falciparum malaria, particularly cerebral malaria (White and Ho, 1992). The sequestration of PRBCs in the relatively hypoxic venous beds allows optimal parasite development and prevents the PRBCs from splenic clearance (Marsh *et al.*, 1988). Parasite binding to the endothelial cells is mediated by a group of variant surface antigens expressed at the PRBC surface during development. The best described is *P. falciparum* erythrocyte membrane protein-1 (PfEMP1), a polypeptide of 200-350kDa, which is encoded by a family of about 50-150 variant genes associated with different binding phenotypes (Baruch *et al.*, 1995; Chen *et al.*, 1998; Su *et al.*, 1995). Each parasite expresses the transcript of only one variant gene at any one time but can switch to express a different variant gene which is about 2% per generation *in vitro* in the absence of any immunological selection mechanisms acting at the level of PRBC (Roberts *et al.*, 1992). However if all the parasites in a typical human infection, were to switch at this rate the variant repertoire would be rapidly exhausted and therefore it was hypothesized that *in vivo* only a limited number of antigenic types are expressed, and homologous anti-variant antibody acts as a signal for switching (Sherman *et al.*, 2003). Nevertheless it is not known how such switching would influence adhesion patterns.

PfEMP1 binds to many host receptors on endothelial cells, among which PfEMP1 interaction with CD36 and the intercellular adhesion molecule 1 (ICAM1) are well studied (Craig and Scherf, 2001; Newbold *et al.*, 1997). PfEMP1 binding to host endothelium does not always lead to pathogenesis, as most infections result in malaria that is devoid of any cerebral complications (Snow and Marsh, 1998). However it is not clear at present as to what factors contribute to the transition from uncomplicated malaria to cerebral malaria. One possibility is that expression of particular binding properties will lead to distinct patterns of sequestration and to pathogenic consequences. For example, sequestration of PRBC within the placenta causes premature delivery, low birth weight, and increased mortality in the newborn and anemia in the mother. Unlike parasites collected from non-pregnant individuals, PRBC isolated from placentas bind to CSA but not to CD36, the critical host receptor for sequestration in microvasculature (Beeson *et*

al., 1999; Fried and Duffy, 1996). One possible explanation to this observation is that PfEMP1 adhesion to these receptors was selected to allow the parasite to sequester not to endothelium but in placenta, perhaps a site of reduced immunity. Moreover, CSA-binding parasites express PfEMP1 with a DBLγ domain that binds CSA and a non-CD36-binding CIDR1 (Buffet *et al.*, 1999; Gamain *et al.*, 2001). In contrast, CD36-adherent parasites express a PfEMP1 with a CD36-binding CIDR1 (Gamain *et al.*, 2001). The glycosaminoglycan chondroitin-4-sulfate (CSA), is expressed throughout the microvasculature along with various proteoglycans such as thrombomodulin, has been shown to mediate the cytoadherence of IRBC selected on Chinese hamster ovary cells (Rogerson *et al.*, 1995). CSA is the principal molecule mediating cytoadherence in the human placenta, where it is expressed on syncytiotrophoblasts (Fried and Duffy, 1996). The glycoprotein ICAM-1 acts as a ligand for the leukocyte integrin lymphocyte function-associated antigen- 1 (LFA-1) and plays a central role in the generation of an immune response (Rothlein *et al.*, 1986). ICAM-1 is also the receptor for human rhinoviruses (Staunton *et al.*, 1989). Further, proinflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interferon-γ (IFN-γ) induces expression of ICAM-1 on endothelial cells during inflammation (Pober *et al.*, 1987). Molecular studies have shown that PRBC binds to the first immunoglobulin domain of ICAM-1 at a site that is different from the binding sites of both LFA-1 and rhinovirus (Berendt *et al.*, 1992; Ockenhouse *et al.*, 1992a). Further it was shown that PRBC bind to three other endothelial receptors viz., VCAM, E-Selectin and NCAM (Amodu *et al.*, 2005; Ockenhouse *et al.*, 1992b; Pouvelle *et al.*, 2007).

Cerebral malaria has mortality between 25 and 50% and, without treatment, is fatal within 24-72 h. Although the underlying causes of the pathogenesis of cerebral malaria remain unclear, the most plausible explanation is provided by the mechanical/sequestration hypothesis. According to this hypothesis, disease severity in *P. falciparum* malaria is related more to the number of parasites sequestered than to the number circulating parasites. Further, the neuropathologic effects that is frequent in cases of cerebral malaria result from the occlusion of microvessels in the brain by clumps of sequestered parasitized red blood cells (Berendt *et al.*, 1994).

Cytokines in the pathogenesis of CM

The importance of cytokines, especially TNF-α and IFN-γ, and their contribution to severe malaria have been extensively surveyed (Grau *et al.*, 1987; Hommel, 1996; Miller *et al.*, 1994). High circulating levels of TNF-α and IFN-γ are more often found in patients with severe malaria than in uncomplicated cases (Kwiatkowski *et al.*, 1990). Extensive deposition of TNF-α, IFN-γ, IL-1, IL-6 in organs with massive sequestration (especially in brain) is more frequently seen in patients who died of CM. TNF is also raised in placental malaria and is associated with low birth weight (Fried *et al.*, 1998; Moormann *et al.*, 1999). Grau and colleagues hypothesized that excessive TNF-α production plays a decisive role in the pathogenesis of murine CM (Grau *et al.*, 1987). This fact is backed by the subsequent findings: (i) elevated levels of serum TNF-α is found only at the time of the neurological complications; (ii) a single injection of anti-TNF-α antibody on day 4 or 7 fully protected infected mice from cerebral malaria without modifying the parasitemia and (iii) injection of recombinant TNF-α to a CM-resistant strain of mouse makes it susceptible to CM. In addition, de Kossodo and Grau found decreased levels of IL-4 (which antagonizes the effects of TNF-α) along with upregulation of TNF-α mRNA in the brains of CM-susceptible mice suggesting a role for TNF-α in the pathology of CM (de Kossodo and Grau, 1993). Further it was found that in tumor necrosis factor receptor 2 (TNFR2) deficient mice are resistant to CM (Lucas *et al.*, 1997). However in another study, infected mice were not protected even after administration of TNF-α neutralizing antibody (Hermsen *et al.*, 1997b). In a study involving 178 Gambian children, Kwaitowski et al, found that plasma TNF-α level in fatal cases involving CM were at least 10 fold higher than their normal counterparts (Kwiatkowski *et al.*, 1990). This suggests that excessive TNF-α production during the pathology of CM makes humans susceptible to neurological manifestations and its fatal outcome. Paradoxically, in another study involving 600 Gambian children it was found that neurological sequelae increased after the administration of TNF-α antibody suggesting that the antibody may act to retain TNF within the circulation and thereby prolong its effect (van Hensbroek *et al.*, 1996). TNF-α produced by monocytes or glia enhances the release of cytokines, ROS, nitric oxide, superoxide production and potentiates glutamate receptor induced neurotoxicity. All these factors have been implicated in the pathogenesis of cerebral malaria. However, recent studies strongly suggest that

murine CM requires Lymphotoxin-α (LT-α) rather than TNF-α as mice deficient in LT-α were resistant to CM, whereas those deficient in TNF-α remained susceptible (Engwerda *et al.*, 2002).

The proinflammatory cytokine IFN-γ has been shown to reduce hepatocyte invasion by malaria sporozoites and conferred protection against both hepatic and asexual blood stages of malaria (Ferreira *et al.*, 1986; Maheshwari *et al.*, 1986; Ockenhouse and Shear, 1984). Moreover, *invitro* and *invivo* anti parasitic effects of IFN-γ have been demonstrated conclusively (Bienzle *et al.*, 1988; Clark *et al.*, 1987; Ockenhouse *et al.*, 1984; Rockett *et al.*, 1991). In human cases serum levels of IFN-γ in immunity and pathology of CM were often conflicting, where in there is a prominent rise in IFN-γ levels in serum of patients infected with *P. falciparum* (Kwiatkowski *et al.*, 1990; Ringwald *et al.*, 1991), but such an increase was not reported in other studies (Butcher *et al.*, 1990; Kremsner *et al.*, 1989). Apart from its anti-parasitic effect, IFN-γ could also be deleterious to the host by virtue of its pro-inflammatory actions (Ho *et al.*, 1995). Injections of neutralizing monoclonal antibody against recombinant murine IFN-γ prevent cerebral complications in CM susceptible mice (Grau *et al.*, 1989). Northern blot analysis and semi-quantitative PCR analysis showed that there is significant accumulation of IFN-γ mRNA along with TNF-α, and a decreased expression of IL-4 and TGF-β genes in the brains of CM-susceptible mice compared with CM-resistant and uninfected mice(Grau and de Kossodo, 1994). Increased expression of IFN-γ in the brain of CM susceptible mice results in the activation of endothelial cells, macrophages and glial cells. Moreover, IFN-γ may upregulate ICAM-1 and TNF receptors on cerebral vascular endothelial cells and could also prime macrophages to release more TNF-α rendering them more susceptible to TNF-α (Grau and de Kossodo, 1994).

Low doses of IL-1 prevent the development of cerebral complications in C57BL/6 mice infected with PbA. Further IL-1 given intraperitoneally at a dose of 80ng for 6 consecutive days starting from day 1 of infection suppressed the parasitemia (Curfs *et al.*, 1990). However it was not clear as to how the IL-1 treatment suppresses parasitemia and prevents CM. Kern *et al.*, suggested that TNF-α and IL-6 could serve as markers of severe malaria in human *P. falciparum* infections (Kern *et al.*, 1989). Although elevated levels of serum IL-6 were found in both CM- susceptible and CM –resistant mice infected with *P. berghei* ANKA, it was suggested that IL-6 does not play a major role in the pathogenesis of ECM, since passive immunization against IL-6 did not prevent ECM (Grau *et al.*, 1990). IL-10 plays a protective role against experimental cerebral

malaria by inhibiting PbA antigen-specific interferon-gamma (IFN-gamma) production in vitro but not tumour necrosis factor (TNF) serum levels in vivo (Kossodo *et al.*, 1997).

Central nervous system is the major site of complications during malaria infection

According to immunopathogenesis hypothesis host toxic mediators, directed against the intraerythrocytic form of the parasite, could cause non-specific tissue damage due to their untargeted mechanism of action (Clark *et al.*, 1981). Due to this non-specific mode of action the CNS vascular endothelium is damaged resulting in cerebral oedema and haemorrhage ultimately leading to coma and death. Indeed cerebral microvascular injury is a common phenomenon of both murine CM and human CM (Brown *et al.*, 1999; Chang-Ling *et al.*, 1992; Neill and Hunt, 1992; Thumwood *et al.*, 1988). Supporting evidence put forward by Medana *et al.*, suggests that astrocytes and microglia are actively involved in the development of the cerebral complications associated with malaria infection. Accordingly BBB disruption during the pathogenesis of CM results in the release of cytokines, malarial toxins and immune cells into the brain parenchyma thereby locally altering the immune and supportive functions of astrocytes and microglia. Activated astroglia and microglia in turn produce proinflammatory mediators and toxins which are detrimental to the neurons leading to CNS dysfunction (Medana *et al.*, 2001).

Astrocytes, collectively known as astroglia are a subtype of glial cells found in the CNS. They perform many functions including maintaining acid-base, electrolyte and neurotransmitter balance, maintaining BBB properties in the vascular endothelium and regulating the concentration of neurotransmitters, such as glutamate, in the extracellular fluid (Sykova *et al.*, 1992; Walz, 1992). Alteration in any of these astrocyte functions has profound effects on the normal neuronal function. Astrocytes express many inflammatory mediators and their receptors in the injured or infected brain. When stimulated *in vitro* by a variety of agents such as viruses, IL-1, TNF-α, IFN-γ, LPS and calcium ionophore, primary rodent astrocytes produce IL-1, IL-3, IL-6, TNF-α, IFN-α and IFN-β (Benveniste *et al.*, 1990; Frei *et al.*, 1985; Frei *et al.*, 1989; Lieberman *et al.*, 1989; Sawada *et al.*, 1989). A role for astrocytes in immune-mediated neurological disease has been demonstrated in EAE where in, production of TNF-α by the astrocytes has been correlated with the severity of the disease (Chung *et al.*, 1991). Furthermore,

astrocytes in HIV-1 infected brains express TGF-β (Wahl *et al.*, 1991). Astrocytes also contribute to the inflammation within the CNS by producing chemokines such as RANTES and MCP-1 which are thought to be vital for leucocyte migration and activation (Persidsky *et al.*, 1999). Using retinal wholemount model Medana *et al.*, have shown astrocytes play a critical role in the pathogenesis of experimental cerebral malaria (ECM). Redistribution of astrocytes is an early event following malaria inoculation in mice, and it progresses to astrogliosis and eventually to loss of astrocytes in some locations at the time when mice are exhibiting the symptoms of cerebral malaria (Medana *et al.*, 1996). Further S-100B an astrocyte marker was found to be elevated in cerebrospinal fluid (CSF) of patients with neurological symptoms and was linked to neurological outcome, disability and death (Medana *et al.*, 2007b).

Microglial cells are the resident macrophages of the CNS and comprise 5–20% of the total glial population in the brain. Three types of microglia are known to exist in the CNS viz., amoeboid, ramified, and reactive microglia, although these are currently viewed as different forms of a single cell type. Amoeboid microglia are active macrophages during development and are precursors of resting or ramified cells, which can, in response to a variety of insults such as infection, traumatic injury, or ischemia, reactivate in the postnatal brain, assume an amoeboid shape, and move to the site of injury (Thomas, 1992). Microglia secretes IL-1, IL-6, TNF-α like all other tissue macrophages when stimulated by LPS and IFN-γ (Frei *et al.*, 1989; Frei *et al.*, 1987; Giulian *et al.*, 1986; Giulian and Lachman, 1985; Lavi *et al.*, 1988; Sawada *et al.*, 1989). Microglia are the major antigen-presenting cell of the CNS and they have been shown to express major histocompatibility class I and II molecules as well as costimulatory molecules such as B7, ICAM-I and the αXβ2 integrin, which may activate T cells in the presence of specific antigen (Raivich *et al.*, 1999). In HIV-1 encephalitis, monocyte migration into the brain parenchyma is enhanced by the activated microglia through the production of chemokines (Persidsky *et al.*, 1999). Furthermore, proinflammatory cytokines such as IFN-γ induces major histocompatibility complex class II molecules on the microglia (Vass and Lassmann, 1990; Wong *et al.*, 1984). Reactive microglia expressing major histocompatibility complex class II have been found in affected areas in post-mortem brains of patients with senile dementia of the Alzheimer's type, Parkinson's disease, Huntington's chorea and multiple sclerosis (Hayes *et al.*, 1987; Itagaki *et al.*, 1989; Mattiace *et al.*, 1990; McGeer *et al.*, 1988; McGeer *et al.*, 1987). Major histocompatibility complex class II expressing cells concentrated at the BBB could modulate the

immune response by presenting antigen to CD4+ T cells. Antigen presentation occurring locally, resulting in T-cell activation, could result in a sustained or amplified immune response that could contribute to cerebral complications. In human CM there has been a report of cuffs of lymphocytes and microglia around veins (Janota and Doshi, 1979). Using a retinal whole-mount technique Medana *et al.*, reported that in the early stages of ECM, microglia were found to have distinct morphologic changes which includes a decrease in process length, an increase in soma size, an increasingly amoeboid appearance, and vacuolation. Redistribution of the microglia to the venous side of the vascular endothelium, with compromised barrier properties, was also found (Medana *et al.*, 1997a). TNF-α production by microglia, astrocytes, peripheral blood monocytes adherent to the meningeal vessels, and cerebrovascular endothelial cells prior to onset of cerebral symptoms was also detected (Medana *et al.*, 1997b).

The pathophysiological events leading to neurological damage and sequelae in CM are not known. Earlier Medana et al proposed Axonal injury as a key event leading to CNS dysfunction in CM infected individuals (Medana *et al.*, 2002). Earlier we have reported the activation of intrinsic cell death cascade in the brain during the pathology of murine cerebral malaria (Kumar and Babu, 2002). PbA infection in mice also leads to disturbances in calcium homeostasis leading to the activation of calpains, calpastatin and cleavage of spectrin (a substrate for active caspase-3) in the brain during fatal murine cerebral malaria (Shukla *et al.*, 2006). The role of calpains in mediating neuronal cell death in CM infected patients is well documented (Medana *et al.*, 2007a). A recent study in experimental cerebral malaria by Lackner *et al.*, have proposed a role for active caspase-3 in mediating neuronal cell death during the late stage of the disease (Lackner *et al.*, 2007). This fact is further supported by observations where in significant increase in Metallothionein I + II (MT-I + II) expression in reactive astrocytes, macrophages/microglia and vascular endothelium is accompanied by a localized CM-induced neuronal apoptosis (detected by TUNEL) indicating severe and irreversible pathology (Wiese *et al.*, 2006). Eeka *et al.*, in a recent paper linked the activation of multiple suicidal proteases (Caspase-3, Calpain-1 and Cathepsin B) to breakdown of neuronal cytoskeletal proteins leading to neuronal demise in experimental cerebral malaria (Eeka *et al.*, 2011). Seemingly many more pathways are involved as revealed by our latest findings wherein we have proposed the activation of c-Jun N-terminal kinases playing a critical role in neuronal cell death in experimental cerebral malaria (Anand and Babu, 2011).

Scope of the present study

The following specific questions were addressed to delineate the mechanisms of host parasite interactions set *in vivo* during the course of cerebral malaria pathology:

1) To study whether JNK signaling pathway is activated in ECM.
2) To study whether administration of SP600125 (a specific JNK inhibitor) is neuroprotective in ECM.
3) To study whether ER stress signaling pathway is activated in experimental cerebral malaria (ECM).
4) To study whether administration of Pentoxifylline (TNF-α inhibitor and immunomodulatory agent) is neuroprotective in ECM.

CHAPTER 1

c-Jun N-Terminal kinases are activated in the brain during the pathology of Experimental Cerebral Malaria

c-Jun N-Terminal kinases are activated in the brain during the pathology of experimental cerebral malaria

Cerebral malaria (CM) is a life threatening complication of *Plasmodium falciparum* characterized by the sequestration of parasitized red blood cells (PRBC), extensive endothelial apoptosis, blood brain barrier disruption followed by T-cell infiltration, secretion of proinflammatory cytokines, microglial activation and neuronal cell death (Idro *et al.*, 2005; Lackner *et al.*, 2007; Medana *et al.*, 1997a; Pongponratn *et al.*, 2003; Potter *et al.*, 2006; Wiese *et al.*, 2006). Histopathological and electron microscopic analysis of post mortem brain tissues obtained from CM infected individuals reveal adherence of PRBC and inflammatory cells to the brain microvasculature, petechial hemorrhages in the brain parenchyma, neurological lesions and perivascular oedema (Pongponratn *et al.*, 2003). Because of its resemblance to the human disease, *Plasmodium berghei ANKA* (*PbA*) infection in mice has been the widely used model to unravel cellular and molecular mechanisms involved in the pathogenesis of cerebral malaria (de Souza and Riley, 2002). Even though, some apoptotic and necrotic pathways which are activated in the brain during cerebral malaria had been reported from our lab (Kumar and Babu, 2002; Shukla *et al.*, 2006) and elsewhere (Lackner *et al.*, 2007; Wiese *et al.*, 2006) several signaling pathways are not yet completely studied.

c-Jun N-terminal kinases (JNK) belong to the family of mitogen activated kinases called MAP kinases, which are activated in response to inflammatory cytokines and environmental stress conditions. There are three isoforms of JNK: JNK 1, 2 and 3, of which JNK 1 and 2 are ubiquitously expressed while the expression of JNK3 is restricted to the neuronal and cardiac tissues (Davis, 2000). JNK, for its complete activation needs to be phosphorylated at Thr 183 and Tyr 185 and this is mediated by either JNKK1/MKK4/SEK1 or JNKK2/MKK7. These JNKKs in turn have differential preferences for the phosphorylation sites, with JNKK1 preferring Tyr 185 and JNKK2 preferring Thr 183 residue. Further, the activity of JNK is also regulated by protein phosphatases and scaffold proteins like JIP, NFκB, β-arrestin and JSAP1. Once activated, JNK translocates into the nucleus and phosphorylates c-Jun, its major downstream substrate, inducing transcription-dependent apoptotic signaling pathway. Moreover, it was reported that JNK also promotes apoptosis in cardiac myocytes by interacting with mitochondrial apoptotic machinery (Aoki *et al.*, 2002). The role of JNK in neuronal cell death has been well documented in several

neurodegenerative diseases like, Alzheimer's and Parkinson's disease (Colombo *et al.*, 2009; Pan *et al.*, 2009), cerebral ischemia (Kuan *et al.*, 2003), excitotoxicity- induced apoptosis (Yang *et al.*, 1997) and in axotomy-induced cell death (Keramaris *et al.*, 2005). In the current study we addressed the possible role of JNK in the induction of neuronal cell death during ECM. Our results show for the first time that JNK is involved in the neuronal cell death.

Materials and Methods

Pathogen-free C57BL/6J mice 6-8 weeks old, weighing 18–24 g, were obtained form NCLAS (National Center for Laboratory Animal Sciences), Hyderabad. All the protocols followed for the use of animal experimentation were strictly in accordance with the institutional and national ethical committee guidelines. C57BL/6J mice of either sex were inoculated intraperitoneally with 10^6 parasitized red blood cells, suspended in 200µl of PBS (pH 7.4). Uninfected mice of same age and sex were used as controls. Parasitemia was assessed from Geimsa-stained thin smears of tail blood prepared every day post inoculation (PI). On day 6-9 PI mice displayed clinical signs typical of CM such as ataxia, hemiplagia, seizures, paralysis and coma followed by death, with parasitemias not exceeding 15%. Brains were dissected out from control and *PbA* infected animals at 3rd, 5th and 7th day PI, snap frozen in liquid nitrogen and stored in -80^{0}C until further use.

Western Blotting: Western immunoblotting was performed according to the procedure published earlier [32]. Briefly, 50µg of tissue lysates were resolved on 10% SDS-PAGE and transferred onto nitrocellulose membrane and probed with rabbit polyclonal antibodies raised against p-MKK4, JNK, p-JNK, p-c-Jun and β-tubulin (Cell Signaling Technology; USA). Then, blots were probed with the HRP-conjugated anti- rabbit secondary antibodies and developed with ECL reagent (Pierce). Immunoreactivity was analyzed quantitatively using ImageJ software (NIH).

JNK activity assay: JNK activity was performed using a nonradioactive kinase assay kit according to the manufacturer's instructions (Cell Signaling Technology). Briefly, tissues were homogenized in lysis buffer, sonicated on ice, and spun at 14000 x *g* for 15 min. Protein content in the supernatant was determined by the method of Bradford (Biorad). Endogenous JNK was immunoprecipitated from 200µg of cell lysate with c-Jun fusion protein linked to glutathione agarose beads overnight at 4^{0}C. Precipitates were washed twice with lysis buffer and twice with

kinase buffer provided along with the kit. The kinase reaction was initiated by suspending the pellet in kinase buffer supplemented with cold ATP for 30 min at 30^0C. The reaction was terminated by the addition of 3X sample buffer and samples were resolved by 10% SDS-PAGE followed by western blot analysis. Membranes were probed with p-c-Jun antibody provided along with the kit.

Immunohistochemistry: Immunohistochemistry was performed according to an earlier published protocol (Bhaskara *et al.*, 2006) on coronal sections through the straitum of control and infected mouse brain cortices and probed with p-JNK antibody (1:100 dilution). For immunoflouroscent analysis, the same procedure was followed except that the sections were not pre-treated with 1% hydrogen peroxide. Briefly, after deparaffinization, sections were blocked for 1 hr, and probed with p-JNK antibody (1:100) overnight at 4^0 C. After washing with PBS, sections were incubated for 1 hr with Alexaflour 594 coupled goat anti-rabbit antibodies (1:500, Invitrogen) followed by counterstaining with DAPI (Invitrogen), mounted using Vectashield, coverslipped, and visualized under Leica confocal microscope. For double labelling analysis the above protocol was followed except that the sections were first incubated in p-JNK (1:100) overnight at 4^0C followed by incubation for 1 hr with a cocktail of Alexaflour 594 coupled goat anti-rabbit antibody and FITC-tagged mouse monoclonal antibody raised against MAP2 (1:200).

Flouro-Jade B staining: For colocalisation studies of p-JNK with Flouro-Jade B, deparaffinized sections were washed once with PBS for 5 min, microwaved in citrate buffer for 15 min, blocked with 5% goat serum and probed with p-JNK antibody for 1 hr, followed by three PBS washes. Sections were then incubated in secondary antibody, washed thrice with PBS and subjected to graded alcohol series. Sections were pretreated for 2 min with 0.06% potassium permanganate, rinsed in double distilled water for 3 min and immersed in Flouro-Jade B solution (0.0004% concentration) for 30 min at RT. After this step, sections were washed thrice with PBS for 5 min each, cleared with xylene and air dried on a slide warmer at 50^0C, mounted with DPX and coverslipped. Later, sections were analysed under confocal microscope. For Flouro-Jade B staining of the sections the same protocol was followed except that sections were not microwaved, primary antibody step is deleted and sections were immersed in potassium permanganate solution for 10 min.

Results

To investigate whether *PbA* infection leads to the activation of JNK pathway in the mice, we performed western blot analysis using antibodies against phosphorylated forms of MKK4, JNK, c-Jun and nonphosphorylated form of JNK, during the course of infection (Fig 1A). Distinct increases in immunoreactivity for p-MKK4 and p-c-Jun were seen at 3rd day PI. Densitometric analysis revealed that relative immunoreactivities of both p-MKK4 and p-c-Jun were significant on 7th day PI (Fig 1B, 1D). Although the levels of p-JNK at 3rd day PI were slightly higher than the control, a significant increase in expression was observed on 7th day PI (Fig 1C). Hence a strong and temporally regulated increase in MKK4/JNK/c-Jun pathway was produced in the murine brain upon *PbA* infection. Since the upregulation of p-JNK and p-c-Jun is not always adequate to prove the activity of JNK, we have performed a non radioactive capture JNK activity assay. As shown in the Fig 1E, JNK activity was dramatically increased on 3rd day PI and remained significantly elevated on 5th and 7th day PI; thus correlating with the results we obtained in our western blot analysis. In addition, immunohistochemistry was performed on brain sections (striatum) of *PbA* infected animals or uninfected controls, to confirm the induction of p-JNK that was so evident in our western blot analysis. In control mouse brain striatal sections (Fig 2A, C, D), p-JNK immunoreactivity was poorly distributed. However, in infected mouse brain sections (Fig 2B, E, F), signals for p-JNK increased in intensity and showed nuclear accumulation in some cells. Furthermore, double immunoflouroscent analysis of p-JNK with MAP2, a neuronal marker (Fig 2J-L); revealed neuronal induction of p-JNK in infected mouse brain sections, implying that neurons are the principal cell types in which p-JNK is activated in murine cerebral malaria. Since p-JNK is implicated in neuronal cell death in many neurodegenerative disorders (Colombo *et al.*, 2009; Kuan *et al.*, 2003; Pan *et al.*, 2009) we next investigated the role of p-JNK on the viability of neurons using Flouro-Jade B a novel fluorescent stain that specifically binds to dying neurons (Schmued and Hopkins, 2000). To this end, we have first confirmed neuronal viability by staining the sections with Flouro-Jade B. As shown in the Fig 3A, control mouse brain sections stained very little for Flouro-Jade B, where as in infected mouse brain sections (Fig 3B) a robust increase in Flouro-Jade B staining cells was observed. This sort of staining pattern clearly indicates that neuronal cell death is wide spread in infected mouse brain sections as compared to controls. Moreover, colocalisation studies of p-

JNK with Fluoro-Jade B on infected mouse brain sections (Fig 3C-E) clearly established that p-JNK is up regulated in degenerating neurons following *PbA* infection. Together, these results proved unequivocally that p-JNK is involved in neuronal cell death in murine cerebral malaria.

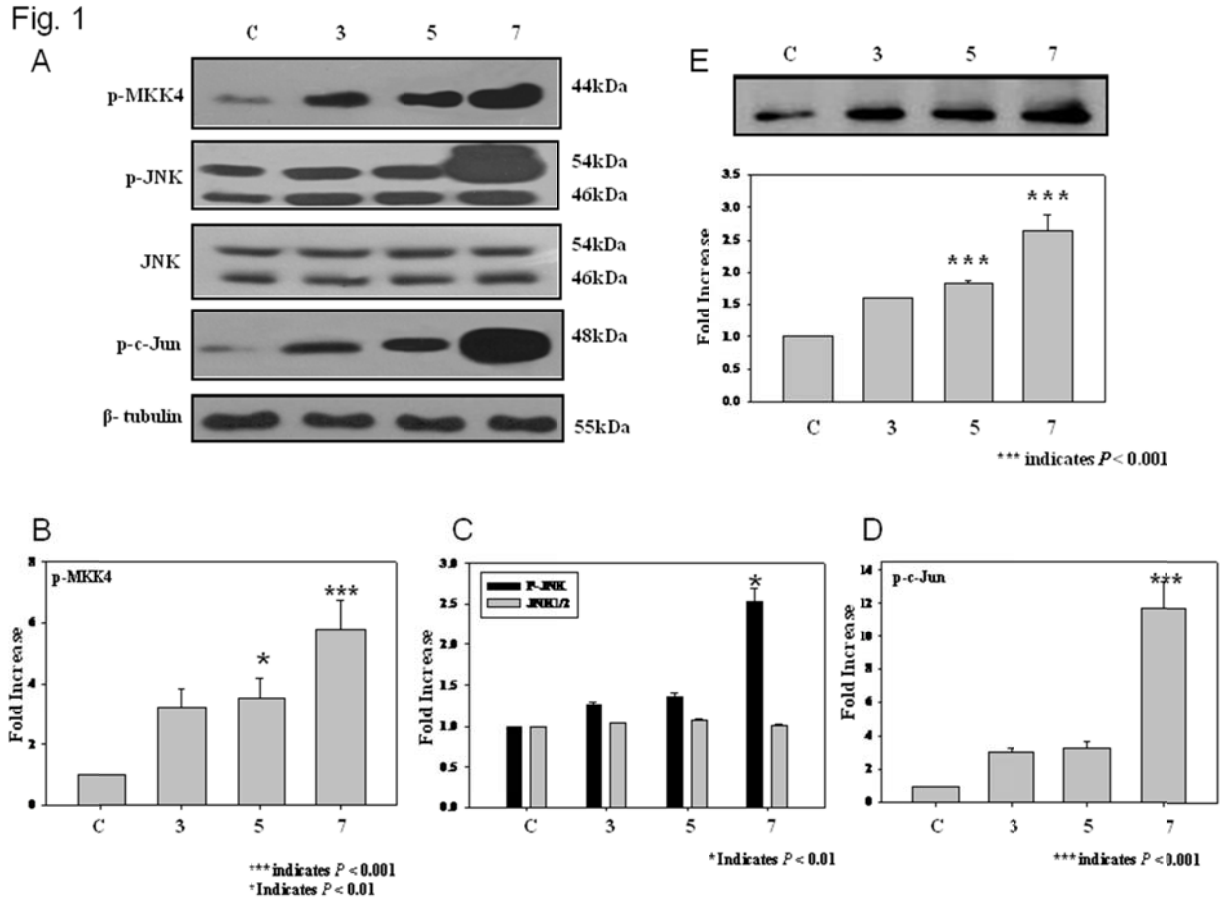


Fig 1 (A) Immunoblot analysis of p-MKK4, p-JNK, JNK, p-c-Jun, β-Tubulin in the control and infected mice brains. Equal amount of protein was electrophoresed by SDS-PAGE and transferred to nitrocellulose membrane and probed with primary antibody to p-MKK4, p-JNK, JNK, p-c-Jun and β-Tubulin. The result of β-Tubulin was shown in the lower panel as an internal control for equal protein loading. The result is representative of four independent experiments with similar results. The lanes: C, 3, 5, 7 indicates uninfected control mouse brain and *PbA* infected mice brains sacrificed on 3rd, 5th, and 7th day PI respectively. **(B-D)** Bands corresponding to p-MKK4, p-JNK, JNK and p-c-Jun were scanned and intensities were represented as folds vs. uninfected control. Data were expressed as mean ± SD (n = 4). *P < 0.01 vs. respective control, ***P < 0.001 vs. respective control. **(E)** Total JNK activity was measured using the capture JNK assay using GST-c-Jun beads as the substrate and detected using p-c-Jun antibody (upper panel). The graph show semiquantitative measurements of p-c-Jun immunoreactivity on C, 3rd, 5th, and 7th day PI and were represented as folds vs. uninfected control. Data were expressed as mean ± SD (n = 3), ***P < 0.001 vs. control (lower panel).

Fig. 2

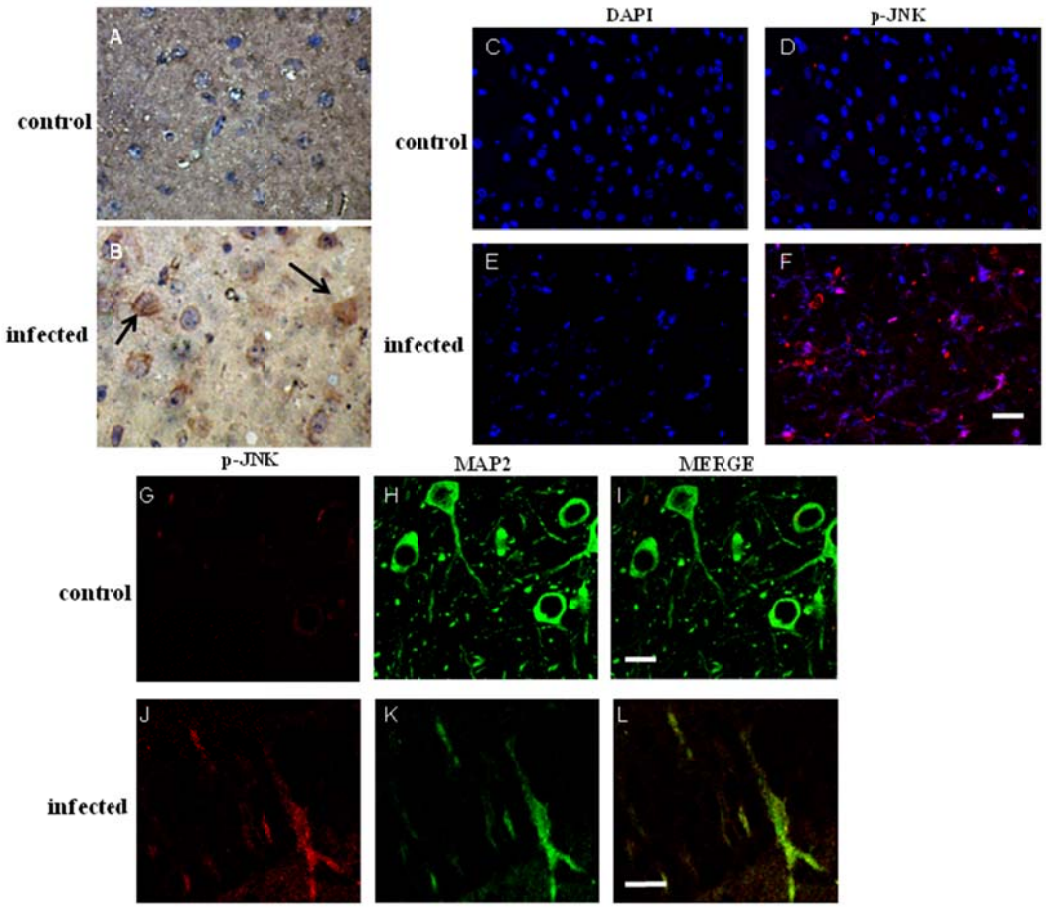


Fig 2 In control mouse brain sections p-JNK immunoreactivity was barely detectable (Fig 2A, D). However, in infected mouse brain sections there is a marked increase in p-JNK immunoreactivity with nuclear localization (Fig 2B, F). Figs 2A, B were taken at 40X magnification Black arrows in Fig 2B indicate p-JNK immunoreactivity. DAPI was used as a nuclear counterstain (Fig 2C, E). Note several pycnotic nuclei indicating wide spread cell death in infected brain sections (Fig 2E) compared to controls (Fig 2C). Double immunostaining of p-JNK (Fig 2G, J) and MAP2 (Fig 2H, K) showed neuronal induction of p-JNK in infected brain sections (overlay, panel L), whereas very little or no staining was observed in control brain sections (overlay, panel I). Panels G-I indicates control brain sections; panels J-L indicates infected mouse brain sections. Photomicrographs shown here in this figure is a representative of three individual animals from each group. Bar: F = 50 µm, I = 10 µm, L = 15 µm.

Fig. 3

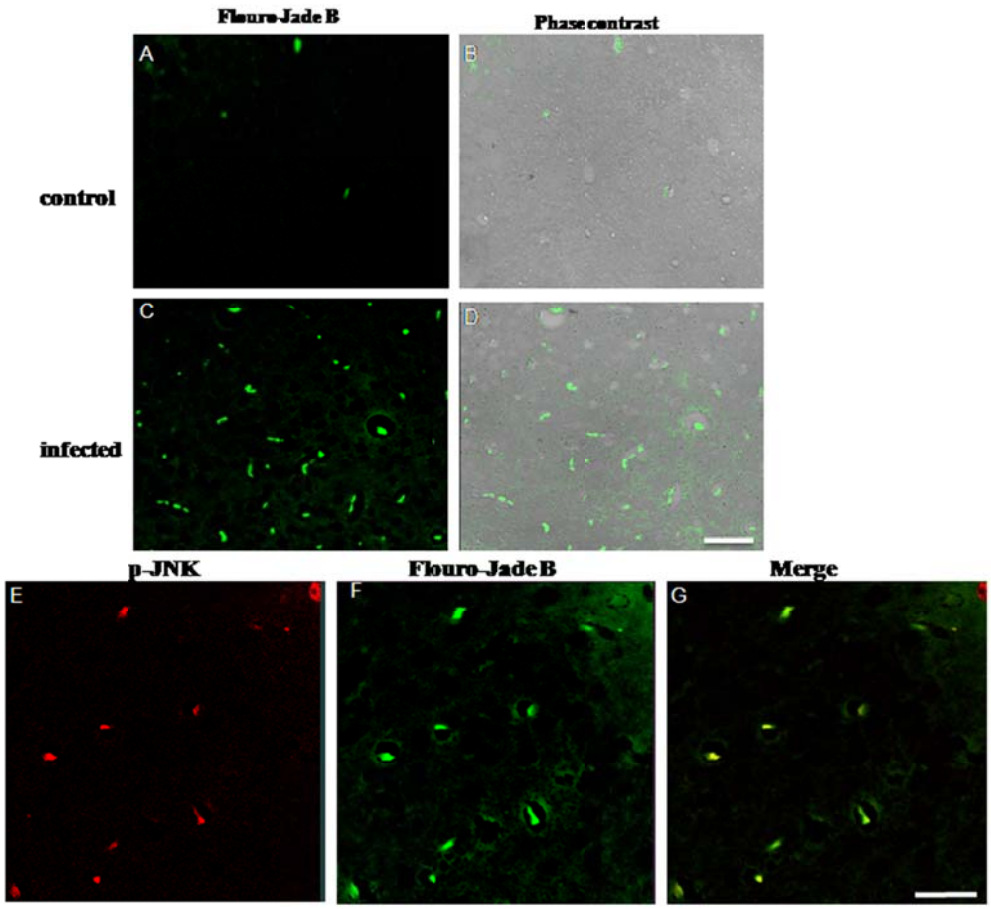


Fig 3 Paraffin embedded sections of both control (Fig 3 A, B) and *PbA* infected (Fig 3 C, D) mouse cortical sections were stained for Flouro-Jade B. While control sections have very few Flouro-Jade B positive cells, infected sections have significantly higher number of Flouro-Jade B positive cells. Double label staining of p-JNK (Fig 3 E) and Flouro-Jade B (Fig 3 F) showed that p-JNK positive cells were also positive for Flouro-Jade B (Fig. 3G). Photomicrographs shown here in this figure is a representative of three individual animals from each group. Bar: D = 30µm, G= 20 µm

Discussion

In this study, we have investigated the activation of JNK pathway in the mouse brain with evidence of JNK mediated neuronal cell death in experimental cerebral malaria. Though the pathophysiological mechanisms implicated in neuronal apoptosis were still under investigation, an overwhelming evidence indicate cerebral complications as a consequence of acute inflammatory and immune responses involving the infiltration of Cytotoxic T Cells, macrophages and neutrophils leading to the production of proinflammtory cytokines like IFN-γ and TNF-α (Belnoue *et al.*, 2002; Deininger *et al.*, 2002; Hermsen *et al.*, 1997a; Nitcheu *et al.*, 2003; Renia *et al.*, 2006; Yanez *et al.*, 1999). In fact, a central role for TNF-α as an important

mediator in this disease was proposed by Grau G *et al*, (Grau *et al.*, 1987) and JNK play a critical role in mediating TNF-α induced apoptosis (Deng *et al.*, 2003). Based on this information we sought to examine the role of JNK signal transduction pathway during ECM and further define the cell types that activate this pathway following proinflammatory assaults of TNF-α.

We observed that in infected brains, p-MKK4, p-JNK, and p-c-Jun were significantly upregulated compared to uninfected controls. Since terminally ill mice (7^{th} day PI mice) showed the highest levels of these proteins compared to controls, we have chosen this time point for immunoflouroscent analysis. Consistent with this, immunostaining of control and terminally ill mouse brain straital sections with p-JNK, revealed robust increase of p-JNK positive cells only in infected brains. The nuclear localization of p-JNK hence can be envisioned as a consequence of its activation leading to its translocation into the nucleus.

Recently, it was shown that *Plasmodium falciparum* GPI stimulates macrophages to secrete TNF-α in a JNK2 dependent manner that could explain the higher survival rates of *PbA* infected JNK2 deficient mice but not JNK1 deficient mice, as compared to wild type controls (Lu *et al.*, 2006). In another study targeted deletion of *Jnk3* protected the mice from neuronal injury after cerebral ischemia (Kuan *et al.*, 2003). Moreover Brecht *et al* studied the specific roles of JNK isoforms in four different neurodegenerative processes and concluded that the protective effects of JNK 1 and destructive actions of JNK 3 depend on a pathophysiological context (Brecht *et al.*, 2005). These studies confirm the role of various JNK isoforms in diverse models of inflammation and pathology. However, a direct proof of evidence linking TNF-α activity to JNK activation is lacking in ECM model, even though the role of TNF-α in pathological manifestation of the disease is proven beyond doubt. Our studies not only provide a direct evidence for activation of JNK as evinced by increased expression of p-MKK4, p-JNK, and p-c-Jun, but most importantly prove that such pathways are activated in neuronal cell types. The extensive modulation of neuromotor functions seen during ECM may then be implicated to these observations where straital cell types seem to be vulnerable targets for TNF-α mediated effects. To further confirm the role of p-JNK in neuronal cell death, we have used Flouro-Jade B, to localize degenerating neurons. Double labeling experiments revealed that Flouro-Jade B positive cells are also immunoreactive for p-JNK, implying that p-JNK plays a crucial role in mediating neuronal cell death. Considering the primary role of JNKs as an activator of c-Jun, its

translocation into the nucleus may have several implications in immunomodulating the host responses during ECM. Although the role of JNK in neuronal apoptosis was proven beyond doubt, recent studies also confirm that JNKs are activated in microglia and control the release of cytokines (Hidding *et al.*, 2002). Hence there is a possibility that the JNK mediated release of proinflammatory cytokines from microglia could also be responsible for neuronal apoptosis. Mechanisms of JNK mediated neuronal apoptosis in our model is far from clear since it is not known at present whether it is due to the release of these microglial cytokines or the direct neuronal activation of JNK3 isoform itself. Also it will be interesting to unravel whether JNK mediated neuronal apoptosis follows caspase dependent or caspase independent pathways in *PbA* infected mice.

Apart from c-Jun phosphorylation, JNK also is shown to affect the activity of multiple proteins like ATF2, p53, Elk1, c-Myc and Bcl-2 family of proteins involved in apoptosis (Liu and Lin, 2005), and thereby implicating its role in cell death. Numerous evidences reveal that constitutive activation of JNK by overexpressing the catalytically active form of MEKK1 promotes apoptosis (Minden *et al.*, 1994), while the hippocampal neurons obtained from *jnk3* null mice resist kainite induced apoptosis (Yang *et al.*, 1997). Similarly, mice deficient in *jnk1* and *2* showed reduced apoptosis in response to stressors such as UV irradiation, MMS and anisomycin, by preventing the release of cytochrome c (Liu and Lin, 2005). The pro-apoptotic function of JNK is possibly mediated by c-Jun which inhibits p53 mediated cell cycle arrest and therefore promotes apoptosis (Shaulian and Karin, 2002); as blocking c-Jun by specific antibodies or expression of a mutant and truncated form of c-Jun reduced apoptosis in response to NGF withdrawal (Ham *et al.*, 1995). However, multiple proteins like BIM, a pro-apoptotic member of the Bcl-2 family and cytochrome c (Lei and Davis, 2003) could also influence the pro-apoptotic function of JNK. A more detailed understanding of the biochemical events underlying JNK activation in experimental cerebral malaria may prove useful, since selective JNK inhibitors can be developed and screened for neuroprotective efficiency thus mitigating the disease.

CHAPTER 2

The specific, reversible JNK inhibitor SP600125, improves survivality and attenuates neuronal cell death in Experimental Cerebral Malaria (ECM)

SP600125, a small molecule inhibitor of JNK

c-Jun N-terminal kinases (JNK) are serine/threonine protein kinases that phosphorylate serine 63 and 73 at the N-terminal domain of c-Jun thus activating the transcriptional activity of AP-1 (Davis, 2000; Karin *et al.*, 1997). JNK signalling is activated by various stress stimuli such as UV and ionizing radiation, heat shock, inflammatory cytokines, metabolic inhibitors, and osmotic or redox shock (Davis, 2000). There are three different isoforms of JNK with 10 different splice variants. Of these JNK 1 and 2 are ubiquitously expressed and have a critical role in neural development, while JNK 3 is restricted to neural and cardiac tissues and is more closely involved in stress-induced neuronal apoptosis. JNK is activated by dual phosphorylation of Thr 183 and Tyr 185 situated in the activation loop by the upstream JNKK1/MKK4/SEK1 and JNKK2/MKK7. Once activated JNK phosphorylates a variety of transcription factors like c-Jun, ATF2, Elk, p53 and c-Myc and also members of Bcl-2 family (Bim, Bcl-2, Bcl-xL and BAD) (Liu and Lin, 2005). Phosphorylated c-Jun forms homo or hetero dimers forming AP-1 transcription factor which then regulates the expression of genes in response to a variety of stimuli such as cytokines, growth factors, stress, and bacterial and viral infections. In addition, phosphorylation of c-Jun and subsequent AP-1 mediated gene expression is a key event that mediates neuronal apoptotic processes since blockade of c-Jun activity by antisense oligonucleotides attenuated neuronal cell death in primary rat hippocampal cultures (Schlingensiepen *et al.*, 1994).

"During the past decade, pharmaceutical research on these diseases has shifted focus from symptomatic benefit to developing novel disease modifying agents. A key driver of this focus is the enhancement in fundamental knowledge of the mechanisms governing neuronal survival and death. JNK plays an integral role in neuronal death and this pathway might be operative in various central nervous system (CNS) disease states" (Manning and Davis, 2003). The small molecule chemical inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2*H*)-one), has been reported to be a potent and selective inhibitor of JNK1, -2, and -3 with more than > 20 fold selectivity over other related MAP kinases (Bennett *et al.*, 2001). Administration of SP600125 has prevented neuronal apoptosis following ischemia or ischemia/reperfusion of the brain. Apart from its inhibition of JNK activity, SP600125 was also shown to inhibit a number of pro-apoptotic events such as the activation of pro-apoptotic Bcl2 family members, the release of mitochondrial

cytochrome c into the cell cytosol, or the activation of pro-apoptotic caspase family of proteases (Guan *et al.*, 2006). SP600125 administered intraperitoneally 1 h before and 6 h in model of early brain injury after subarachnoid hemorrhage demonstrated benefits such as the suppression of caspase activation and concomitant neuronal injury, improved blood–brain barrier preservation, reduced brain swelling, and improved neurological function (Yatsushige *et al.*, 2007). SP600125 also prevented apoptosis of dopaminergic neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's Disease (Wang *et al.*, 2004) as well as neurons in the acute injury accompanying spinal cord trauma (Yin *et al.*, 2005). Taken together, these results support the further development of JNK inhibitors as neuroprotective agents and their use in a variety of brain insults. We recently reported that JNK group of MAP kinases are activated and mediates neuronal cell death in ECM (Anand and Babu, 2011). Here we report for the first time that SP600125, a specific, reversible, ATP competitive inhibitor of JNK (Bennett *et al.*, 2001), attenuates neuronal cell death and improves survivality in mice infected with PbA.

Materials and Methods

Study Groups: All the protocols followed for the use of animal experimentation were approved by the institutional as well as national ethical committee guidelines. Three study groups (n=15 per group) of animals were used in order to evaluate the effect of SP600125 in ECM. In the first group, six to eight weeks old C57BL/6J mice of either sex (~20g body weight) were inoculated intraperitoneally with 10^6 parasitized red blood cells, suspended in 200µl of phosphate buffered saline (pH7.4). The second group consists of uninfected mice of same age and sex and was used as negative controls. The animals infected with PbA strain showed behavioral changes around day 5 after inoculation followed by cerebral symptoms like paralysis, hemiplegia, convulsions and coma eventually succumbing to death by day 12. The third group consists of mice infected with PbA and were subsequently treated with SP600125 (Sigma) at 30mg/kg bodyweight dissolved in DMSO and given intraperitoneally starting from day 1 post infection (PI). This treatment regimen was followed for the next 9 days after which treated mice as well as control and PbA infected mice were sacrificed and brains were separated and stored either in -80^0 C or the mice were transcardially perfused with saline and later with 4% paraformaldehyde and the tissue was processed for histopathological studies. To evaluate the effect of SP600125 on the survivality of mice infected with PbA, a comparative survival curve of both infected and treated

groups was performed using MedCalc software, and the course of infection was followed daily by monitoring parasitemia on periodic blood smears prepared from day1 PI and was typically between 10-15% at the time of sacrifice.

Western Blot analyses: Western blot analysis was performed as described earlier (Anand and Babu, 2011). Briefly, mouse brain tissues were homogenized in RIPA (radioimmunoprecipitaiton assay) buffer and lysates were sonicated and centrifuged at 14,000 g for 15 min at 4^0C. Cellular proteins were subjected to electrophoresis on 10% SDS-polyacrylamide gels and resolved proteins were transferred onto nitrocellulose membranes. Then, membranes were blocked in non-fat dry milk (5%) in tris buffered saline (TBS) (10 mM Tris (pH 7.5), 150 mM NaCl) for 1 h at room temperature followed by incubation with primary antibodies for overnight at 4^0C. Then membranes were incubated with respective secondary antibodies conjugated to alkaline phosphatase (ALP) for 1-2 h at room temperature. Before and after incubation with secondary antibodies, membranes were washed with TBS and TBST (TBS containing 0.1% Tween-20). Blots were developed by incubating the membranes with BCIP-NBT solution. The primary antibodies used for Western Blot analysis are rabbit polyclonal antibodies raised against cleaved caspase-3, p-c-Jun, β-Actin (Cell Signaling Technology), TNF-α (Sigma-Aldrich) and mouse monoclonal antibodies raised against COX-2 (Cayman chemical).

Immunohistochemistry: Immunohistochemical analysis was performed as described previously (Anand and Babu, 2011). Briefly, tissue sections were deparaffinized in xylene and passed through graded alcohols and further rehydrated in phosphate buffered saline (PBS). Antigen unmasking was carried out by micro waving the sections for 10–14 min in 10 mM citrate buffer (pH 6.0). Then, endogenous peroxide activity was inhibited by treating the sections with 3% H_2O_2 for 10 min followed by blocking with serum for 1 h at room temperature in a humid chamber. Sections were then incubated with primary antibodies against active-caspase-3 for 1 h at room temperature followed by incubation with peroxidase conjugated secondary antibody for 1 h at room temperature. After washing in TBS, diaminobenzidine (DAB) in buffer was applied till sections develop colour. Then, sections were washed with distilled water followed by dehydration in graded alcohols, xylene and mounted with DPX (kit obtained from Biogenex Pvt Limited, India). For NeuN immunostaining, the same protocol was followed except that inhibition of endogenous peroxidise activity step was skipped, the primary antibody used was

mouse anti-NeuN monoclonal antibody (Chemicon), the mouse secondary antibody used was tagged with AlexaFlour 594 (Invitrogen), sections were mounted with Vectashield (Vector Labs) and visualized under Leica confocal microscope. The deparaffinized sections were also stained for Haematoxylin and Eosin (H & E), cleared in xylene and mounted using DPX mounting medium and were visualized in light microscope to identify neurological lesions and degenerative cells.

Flouro-Jade B staining: Paraffin embedded brain sections from all the three groups were deparaffinized in xylene, rehydrated in alcohol series and pretreated for 5-10min with 0.06% potassium permanganate, rinsed in double distilled water for 3min and immersed in Flouro-Jade B (Chemicon) solution (0.0004% concentration) for 30 min at RT. After this step, sections were washed thrice with PBS for 5min each, cleared with xylene and air dried on a slide warmer at 50^0 C, mounted with DPX and coverslipped. Later, sections were analyzed under Leica confocal microscope.

Results

The effect of SP600125 on the survival of PbA-infected C57BL/6J mice was assessed in SP600125 treated and PbA infected animals. Results showed that all untreated PbA infected mice died by day 12 PI with a median survival of 8 days, while animals which received SP600125 have a median survival of 27 days and died by day 30 (Fig. 1 a). Thus the survival time of PbA infected mice treated with SP600125 was significantly prolonged in comparison to untreated group ($p = 0.0001$). We further sought to check whether administration of SP600125 in PbA infected mice has any effect on parasitemia. As shown in the Fig. 1 b, there is no significant difference in parasitemia between infected and treated groups. H&E staining of brain sections from control mice showed healthy parenchyma, with intact blood vessels with no hemorrhages. However, sections taken from infected mice showed distinct neurological lesions, with parenchymal microhemorrhages, disruption of vessel walls and adherence of leucocytes to the vessel walls. In contrast, SP600125 treated mice have a healthy parenchyma, clear vessels with little or no hemorrhage (Fig. 1 c).

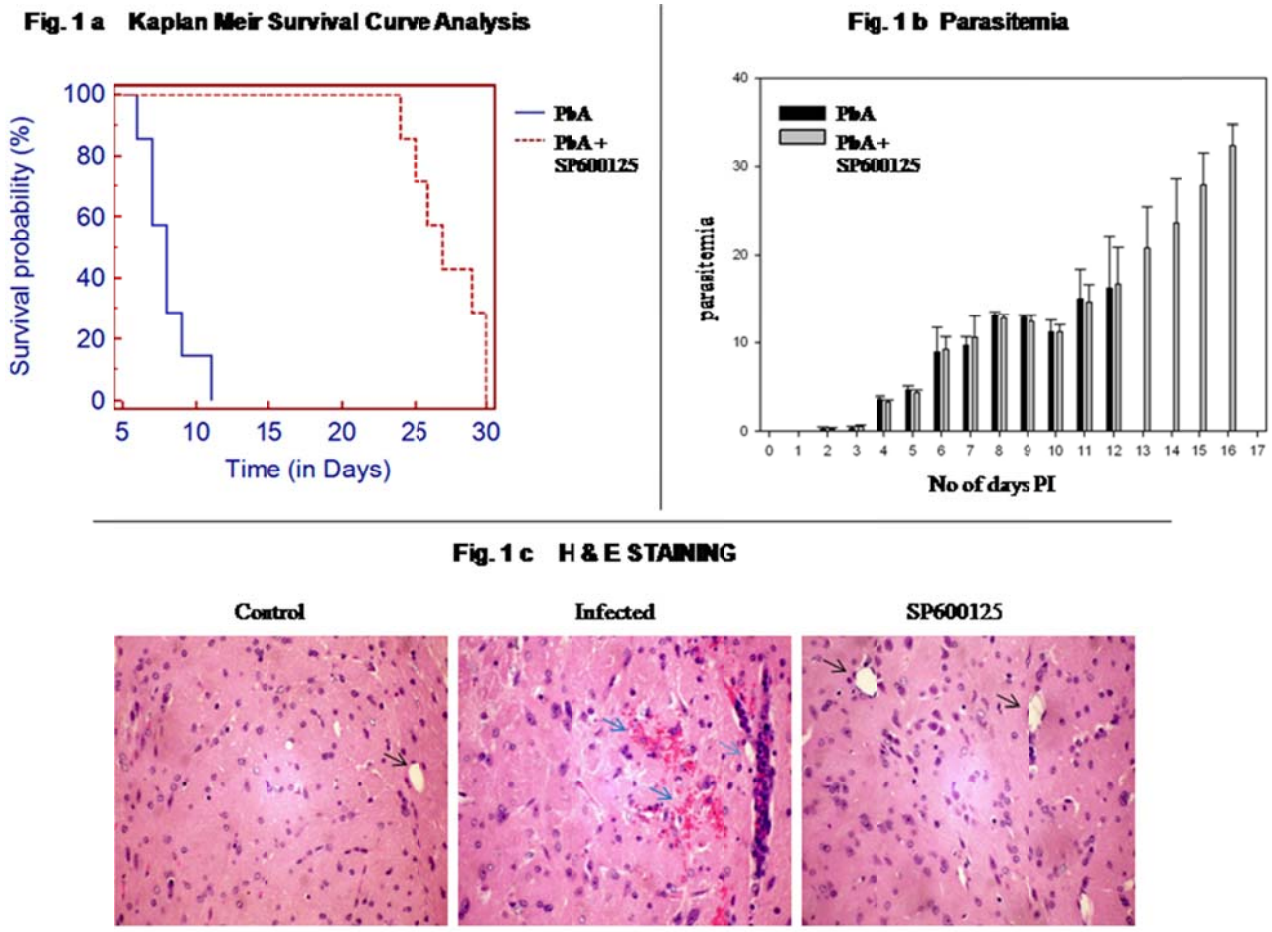


Figure 1 a Survival of mice infected with PbA was significantly increased (p = 0.0001) in group that received SP600125 from day 1 post-infection (30 mg/kg body weight). **(b)** Parasitemia levels in PbA infected mice and PbA infected mice treated with SP600125 (B). All infected mice developed ECM and died by day 12 PI with parasitemia not exceeding 15%. Treated mice did not show cerebral complications and there is no significant difference in parasitemia levels compared to infected mice. However all SP600125 treated mice succumbed to death by day 30 with high parasitemia. (**c**) H & E staining of the brain tissue sections taken from control, infected and SP600125 treated groups. Petechial cerebral hemorrhages (blue arrows), disruption of vessel walls and adherence of leucocytes to the vessel walls (blue arrows) with distinct neurological lesions were prominent in infected brain tissue sections. Healthy brain parenchyma and clear vessels (black arrows) were prominent in control and treated brain sections. Magnification = 40X.

We next wanted to investigate the status of key apoptotic proteins that are thought to be involved in JNK mediated cell death, in control, infected and treated mice. Therefore, we performed immunoblot analysis of p-c-Jun, active caspase-3, COX-2 and TNF-α. We found robust increase in the expression pattern of p-c-Jun, active caspase-3, COX-2 and TNF-α in infected mice brains compared to controls. However, administration of SP600125 in PbA infected mice dramatically reduced their levels indicating that SP600125 treatment attenuates inflammation and apoptotic cell death in ECM (Fig. 2).

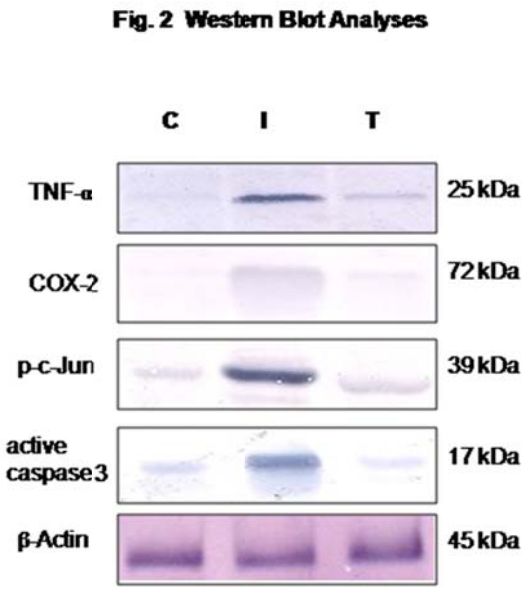


Figure 2 Immunoblot analysis of TNF-α, COX-2, p-c-Jun, cleaved caspase-3 and β-actin in the control, infected and treated mice brains. Equal amount of protein was electrophoresed by SDS-PAGE and transferred to nitrocellulose membrane and probed with primary antibody to TNF-α, COX-2, p-c-Jun, cleaved caspase-3 and β-actin. The result of β-actin was shown in the lower panel as an internal control for equal protein loading. The result is representative of four independent experiments with similar results. The lanes: C, I and T indicates uninfected control, PbA infected and PbA infected mice treated with SP600125 respectively.

We further performed immunohistochemical analysis to prove our findings. Active caspase-3 immunostaining (Fig. 3 a, b, c) of sections taken from control, infected and treated animals revealed caspase 3 positive cells in infected brain sections but not in control and treated groups indicating the protective effect of SP600125 in ECM. Since active caspase-3 is implicated in neuronal cell death we next investigated the viability of neurons by staining the sections taken from all the 3 groups with Flouro-Jade B, a novel fluorescent stain that specifically binds to dying neurons (Schmued and Hopkins, 2000). As shown in the figure (Fig. 3), control and treated mouse brain sections (Fig. 3 d, f) stained very little for Flouro-Jade B, where as in infected mouse brain sections a robust increase in Flouro-Jade B staining was observed (Fig. 3 e). This sort of staining pattern clearly indicates that neuronal cell death is wide spread in infected mouse brain sections as compared to control and SP600125 treated animals. To further validate this finding we did immunoflouroscent analysis on infected mouse brain sections with NeuN, a pan-neuronal marker. As shown in the figure (Fig. 3 g, h, i) NeuN expression was significantly attenuated in infected brains but not in control or treated brains indicating that neuronal cell death is wide spread in ECM and treatment with SP600125 effectively rescued the

neurons from demise. Together, these results proved unequivocally that SP600125 treatment inhibits neuronal cell death and improves survivality of mice infected with PbA.

Fig. 3 Immunohistochemistry and Flouro-Jade B staining

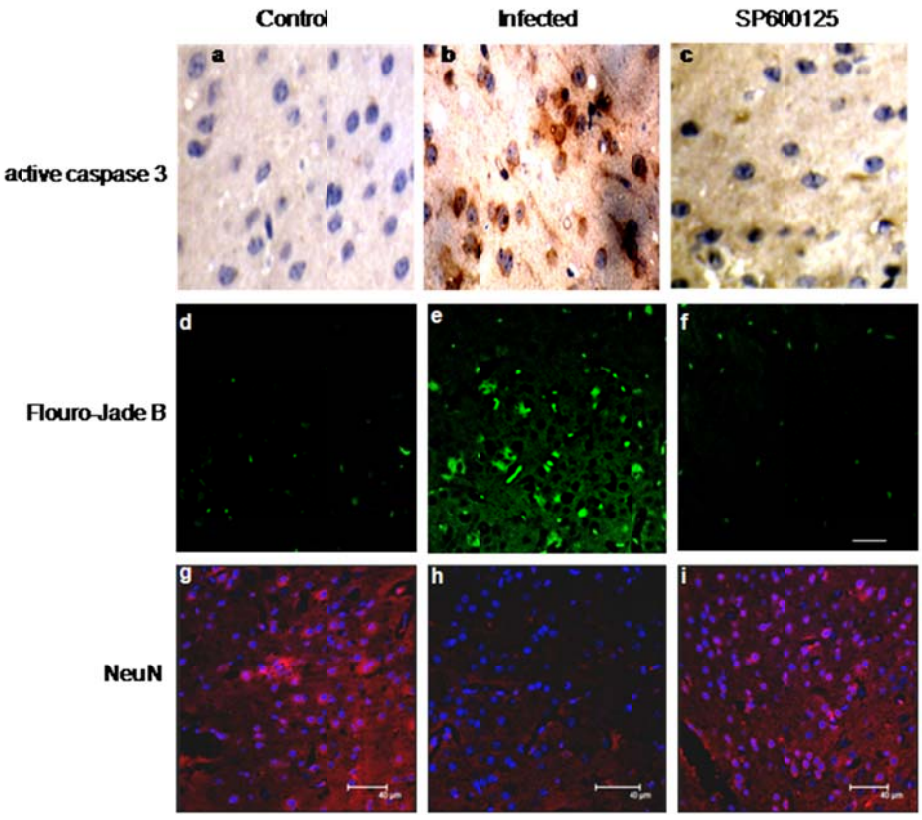


Figure 3 In control and treated mouse brain sections cleaved caspase-3 immunoreactivity was barely detectable (panels a and c) while NeuN immunoreactivity was prominent (panel g and i). However, in infected mouse brain sections there is a marked increase in cleaved caspase-3 immunoreactivity (panel b) with a corresponding decrease in NeuN immunoreactivity (panel h). Paraffin embedded sections of both control (paned d), PbA infected (panel e) and treated (panel f) mouse cortical sections were stained for Flouro-Jade B. While control and treated sections (panels d and f) have very few Flouro-Jade B positive cells, infected sections (panel e) have significantly higher number of Flouro-Jade B positive cells indicating neuronal cell death. The result is representative of four independent experiments with similar results. f = 40µm.

Discussion

The results of our study show that administration of SP600125 increases survivality and attenuates neuronal cell death in an experimental model of CM. The decision to use C57BL/6J mice in our research was made in order to enable a more precise manifestation of human CM susceptibility and the possible effect of treatment. C57BL/6J mice infected with PbA is the most widely used model and its resemblance to human CM has been previously demonstrated (de

Souza and Riley, 2002). Further, the decision to use SP600125 at a dose of 30 mg/kg bodyweight per mouse was taken based on earlier reports that confirm the inhibitory effects of SP600125 in the brain after IP injection (Wang *et al.*, 2004; Yatsushige *et al.*, 2007). There is every possibility that changing the dosage/frequency/route of SP600125 administration may improve our findings and so certainly a follow-up study will be required to determine the optimum dosing schedule.

The major finding in this study is that SP600125 treatment increases the survivality in mice infected with PbA, although all treated mice invariably succumbed to death with high parasitemia and severe anemia. But the significant prolongation in survivality may open a considerable time window for adequate treatment in future. Further SP600125 treatment has no effect on parasite since the parasitemia levels in infected and treated mice are almost similar. One possible explanation for this observation is that the genome of *Plasmodium* lacks typical three-component (MEKK-MEK-MAPK) modules which are the hallmark of the ERK1/2, p38 and JNK pathways (Ward *et al.*, 2004). The other reason could be the specificity of SP600125, which has been shown to exhibit a selectivity of >20 fold relative to other closely related kinases (Bennett *et al.*, 2001).

Apart from its inhibition of JNK activity and subsequent apoptotic cell death, SP600125 is also known to inhibit the expression of inflammatory genes and prevent the activation and differentiation of primary human CD4 cell cultures (Bennett *et al.*, 2001). Hence the significant survivality that we observed in treated mice can be attributed to the immunomodulatory effects of SP600125 and this fact is confirmed by our immunoblot analysis where in we found marked reduction in the levels of proinflammatory mediators, TNF-α and COX-2 upon treatment with SP600125. These results are in consistence with the earlier *in vitro* reports where in SP600125 effectively inhibited the expression of TNF-α and COX-2 (Bennett *et al.*, 2001; Nakahara *et al.*, 2004; Nieminen *et al.*, 2006). In fact, TNF-α and COX-2 have been implicated in the pathogenesis of cerebral malaria (Ball *et al.*, 2004; Grau *et al.*, 1987). Moreover, JNK up regulates both COX-2 and TNF-α under inflammatory conditions via c-Jun/AP-1 mediated transcription (Das *et al.*, 2009; Hunot *et al.*, 2004; Rhoades *et al.*, 1992) and both COX-2 and TNF-α are implicated in neuronal cell death (Hunot *et al.*, 2004; Venters *et al.*, 2000).

Another related finding in our study is that SP600125 significantly inhibited the levels of active caspase-3 and p-c-Jun in infected mice. JNK interacts with both pro-apoptotic proteins,

such as c-Jun, p53, Bim and Bax enhancing cell death (Gillardon *et al.*, 1999; Lei *et al.*, 2002; Tsuruta *et al.*, 2004) and phosphorylates anti-apoptotic proteins like Bcl-2 and Bcl-xL thereby inhibiting their anti-apoptotic function (Fan *et al.*, 2000; Maundrell *et al.*, 1997; Yamamoto *et al.*, 1999). These interactions lead to the activation of caspase-3 in the brain through the intrinsic apoptotic pathway and this fact is supported by our findings (Fig. 2) wherein we observed inhibition of active caspase-3 in infected mice treated with SP600125. Phosphorylated c-Jun on the other hand increases the AP-1 transcription activity there by influencing the expression of key cell death promoting genes and controls neuronal cell death and survival in the mammalian brain (Herdegen *et al.*, 1997). Earlier Ma *et al.,* have found increased expression of c-fos protein with nuclear accumulation in the brains of terminally ill mice infected with PbA (Ma *et al.*, 1997). c-fos, like c-Jun belongs to Jun family and is an important constituent of AP-1 transcription factor, and has been suggested to be a sign of neuronal degeneration and cell death in the brain (Smeyne *et al.*, 1993). Further it is the concentration of phospho c-Jun and not c-Jun *per se* that is critical to the neuronal cell death (Rossler *et al.*, 2002). We further confirmed neuronal cell death by staining the sections taken from all the three groups with Flouro-Jade B, a marker for neuronal degeneration and NeuN a pan neuronal marker. The staining pattern supports our hypothesis that SP600125 is neuroprotective and rescues the neurons from cell death that is widespread in cerebral malaria. Our findings are also in agreement in animal models of ischemic and related neurodegenerative disorders, wherein treatement with SP600125 attenuated neuronal cell death (Gao *et al.*, 2005; Wang *et al.*, 2004). Thus, the SP600125 used in our study inhibits both transcription dependent cell death mediated by c-Jun, and transcription independent cell death mediated by caspase-3 possibly by interacting with mitochondrial cell death machinery.

The results presented here confirm that JNK is involved in neuronal cell death in murine cerebral malaria and also provide some new insights into the mechanism by which JNK triggers cell death execution pathways. In conclusion we hypothesize that SP600125 increases survivality in mice infected with PbA, reduces neurological lesions, attenuates neuronal cell death and has no effect on parasitemia.

CHAPTER 3

ER stress & Neurodegeneration in Experimental Cerebral Malaria

ER Stress and Neurodegeneration in experimental cerebral malaria

Cerebral malaria (CM) is the life-threatening complication of *Plasmodium falciparum* infection in humans, responsible for more than one million deaths annually (Miller *et al.*, 1994)**.** Cerebral dysfunction becomes evident through a variety of symptoms, including extreme lethargy and febrile convulsions and can progress to coma and death in approximately 20% cases (Idro *et al.*, 2005)**.** Although a number of studies have described the neurological complications of human CM, the pathogenesis remains controversial. Apart from post mortem brain studies, an experimental murine model, induced by the infection of susceptible mice with *Plasmodium berghei ANKA*, has been used to further understand the pathogenesis of CM (de Souza and Riley, 2002)**.** Studies with this model have suggested that ECM complications are multifactoral involving activation of platelets, upregulation of proinflammatory cytokines and endothelial cell adhesion molecules, disruption of blood brain barrier, infiltration of leucocytes together with the mechanical blockage of microvessels by the monocytes and infected erythrocytes (van der Heyde *et al.*, 2006). The pathologic features of fatal stage include PRBC and monocyte adhesion to cerebral vascular endothelial cells, edema, petechial heamorrhages, glial activation and neuronal cell death in the central nervous system (Lackner *et al.*, 2007; Medana *et al.*, 1997a; Pongponratn *et al.*, 2003; Potter *et al.*, 2006; Wiese *et al.*, 2006)**.** The mechanisms leading to cell death are complex and several pathways have been implicated, including mitochondrial dysfunction, calcium activated kinases, phosphatases and proteases, Caspases and c-Jun N-terminal Kinases (JNK) (Anand and Babu, 2011; Kumar and Babu, 2002; Kumar *et al.*, 2003; Lackner *et al.*, 2007; Shukla *et al.*, 2006). Though the events central to tumour necrosis factor (TNF) and interleukin are activated during FMCM (Medana *et al.*, 2001), to our understanding no reports exists on the role of ER stress proteins during cell death in the brain.

The Endoplasmic reticulum (ER) is a cell organelle which plays a pivotal role in the synthesis, folding, posttranslational modifications and trafficking of secretory and membrane proteins, calcium storage and release, lipid biogenesis and apoptosis. Perturbations in ER functioning may lead to accumulation of misfolded proteins in the ER lumen and ER in turn initiates an adaptive response known as the unfolded protein response (UPR) that protects the cell against the accumulation of misfolded proteins (Kaufman, 1999). However, if the ER stress is excessive and

prolonged, these adaptive responses fail to compensate and UPR leads to cell death by both caspase-dependent and caspase-independent pathways (Kim *et al.*, 2008; Rao *et al.*, 2004). UPR is initiated by the binding of ER chaperone BiP/GRP78 to the misfolded proteins. Under normal physiological conditions, BiP forms a complex and suppress the activity of three proximal ER-resident stress sensors: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Kim *et al.*, 2008). During ER stress, BiP binds to misfolded proteins thereby releasing PERK, IRE1 and ATF6 from the complex. Upon its release from BiP, PERK is activated by homodimerization and autophosphorylation, and subsequently the kinase domain of PERK phosphorylates eIF2α (peIF2α) thereby shutting down global protein synthesis (Harding *et al.*, 1999). However, phosphorylation of eIF2α can also lead to selective translation of activating transcription factor 4 (ATF4) which in turn activates the transcription of pro-survival genes such as GRP78 and GRP94; genes that are resistant to oxidative stress; and genes that are involved in amino-acid metabolism and transport (Harding *et al.*, 2003; Lu *et al.*, 2004). IRE1 is activated by dimerization and *trans*-autophosphorylation upon its release from BiP (Bertolotti *et al.*, 2000). Activated IRE1 splices *XBP1* mRNA, resulting in a translational frameshift and formation of potent transcriptional activator, inducing the transcription of ER stress responsive genes (Kim *et al.*, 2008). ATF6 is an ER resident protein which binds to BiP under normal physiological conditions. However during ER stress BiP binds to unfolded proteins releasing ATF6 which translocates to Golgi and is cleaved by site 1 and site 2 proteases to release transcription factors (Haze *et al.*, 1999). The released transcription factors migrate to the nucleus and induces the transcription of ER chaperone proteins such as GRP78, GRP94, protein disulphide isomerase, and the transcription factors CHOP and X box-binding protein 1 (XBP1) (Kim *et al.*, 2008; Szegezdi *et al.*, 2006). Further the role of ER stress pathways in mediating neuronal cell death has been well documented in several neurodegenerative diseases like Alzheimer's and Parkinson's disease, Amyotrophic Lateral Sclerosis, Transmissible Spongiform Encephalopathies and ischemia (Lindholm *et al.*, 2006).

The present study examines the role of ER stress proteins in modulating neuronal cell death in ECM, with particular emphasis laid on PERK-eIF2α axis. Our study for the first time shows that peIF2α mediates neuronal cell death in murine cerebral malaria.

Materials and Methods

Induction of cerebral malaria in mice: All the protocols followed for the use of animal experimentation were approved by the institutional as well as national ethical committee guidelines. Six to eight weeks old C57BL/6J mice of either sex (~20g body weight, n=4 per each group) were inoculated intraperitoneally with 10^6 parasitized red blood cells, suspended in 200µl of phosphate buffered saline (pH7.4). Uninfected mice of same age and sex were used as negative controls. The animals infected with *Plasmodium berghei* ANKA (PbA) strain showed behavioral changes around day 5 after inoculation followed by cerebral symptoms like paralysis, hemiplegia, convulsions and coma eventually leading to cell death. The parasitemia was monitored by preparing periodic blood smears from the day 1 of parasite inoculation and was typically between 15-20% at the time of sacrifice. The duration between parasite inoculation and sacrifice of terminally ill animals was approximately 6-12 days.

Preparation of tissue lysates: Mouse brain tissues were homogenized in 5 volumes of RIPA (radioimmunoprecipitaiton assay) buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.4% deoxy-cholate, 1% NP-40 containing protease inhibitors including 2µg/ml leupeptin, 2µg/ml aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF) and phosphatase inhibitors including 10 mM β-glycerophosphate, 10 mM NaF, 0.3 mM Na_3Vo_4 and. The lysate was sonicated for 2 min and centrifuged at 14,000 g for 15 min at 4^0C. The supernatant was collected as whole tissue lysate and frozen at -80^0C before use. Protein concentrations were determined by the method of Bradford (BioRad).

Western Immunoblotting: Western immunoblotting was performed according to the procedure published earlier (Anand and Babu, 2011). Briefly, 50µg of tissue lysates were separated by SDS-PAGE, and transferred onto nitrocellulose membrane and probed with primary antibodies for 1hr. Then membranes were incubated with secondary antibodies conjugated to alkaline phosphatase (ALP) (anti-rabbit and anti-mouse IgG conjugated to ALP obtained from Genei Pvt Ltd, Bangalore, India), and immunoreactivity was visualized by incubating the membranes with BCIP-NBT solution (Genei Pvt Ltd, Bangalore, India). Immunoreactivity was analyzed quantitatively using ImageJ software (NIH). The primary antibodies used in these experiments included rabbit polyclonal antibodies raised against phosphor-PERK (p-PERK), eIF2-α, GADD34, BiP, Calregulin, Calnexin (Santa Cruz Biotechnology); phospho-IRE1(p-IRE1),

ATF4 (Abcam); phospho-eIF2-α (Epitomics), Caspase-3, BCL-2, BAX, cleaved caspase-7, caspase-12 (Cell Signaling Technology); CHOP/GADD153 (Pierce) and mouse monoclonal antibodies raised against ATF-6 (Abcam).

Histopathology: Formalin fixed, paraffin embedded mouse brain sections were deparaffinized in xylene, rehydrated in alcohol series and incubated in 0.1% Cresyl violet solution for 3–5 min. The sections were then rinsed in distilled water and differentiated in 95% alcohol, followed by dehydration in 100% alcohol. The sections were then cleared in xylene and mounted using DPX mounting medium. Tissue sections were also stained with hematoxylin and eosin (H&E) for histological analysis following the above protocol.

Immunohistochemistry: For immunohistochemistry infected and control mice brains were perfused first with 0.9% saline solution followed by 4% paraformaldehyde in 0.1M PBS, pH 7.4 and were embedded in paraffin. Paraffin embedded mice brains were sectioned horizontally (5µm) by automated rotary microtome (Leica), deparaffinized in xylene, passed through graded alcohols and further rehydrated in phosphate buffered saline (PBS). Antigen retrieval was carried out by microwaving sections in 10mM citrate buffer, pH 6.0, for 10 min. Sections were then treated with 3% H2O2 for 10 min to inhibit endogenous peroxidase followed by incubation with serum for 1 h at room temperature (RT) in a humid chamber. Primary antibody (1:100 dilution of rabbit polyclonal antibodies against p-eIF2α and CHOP) was diluted in blocking solution and incubated overnight at 4^0C. Peroxidase conjugated secondary antibody was used for 1hr at RT followed by TBS washes (3x5 min each). DAB in buffer was used till sections developed color. Sections were then, counter stained with haematoxylin for 10s and washed with dH_2O followed by dehydration in graded ethanol and xylene and coverslipped with DPX mount.

Double Immunofluorescence analysis: For double immunoflouroscent analysis, the same procedure was followed except that the sections were not pre-treated with 1% hydrogen peroxide. Double immunofluorescence analysis was performed by incubating sections in a cocktail of primary antibodies (rabbit polyclonal p-eIF2α 1:100 dilution with mouse monoclonal Synaptophysin (Abcam) 1:100 dilution) and (rabbit polyclonal CHOP (1:100) dilution with mouse monoclonal Synaptophysin (Abcam) 1:100 dilution) overnight at 4^0C. The sections were washed in PBS and then incubated in fluorochrome-conjugated secondary antibodies 1:100 for 1 hour at room temperature and further incubated with DAPI and mounted using Vectashield (Vector Labs). The sections were visualized using a Leica confocal microscope.

Flouro-Jade B staining: For Flouro-Jade B staining, deparaffinized sections were subjected to 100% Ethanol for 10 min followed by Basic alcohol (1% sodium hydroxide in 80% alcohol) for 5 min. This was followed by immersing the sections in 70% alcohol for 2 min and double distilled water for 2 min. Sections were then immersed in potassium permanganate solution for 10 min, washed once with PBS for 2 min, and immersed in Flouro-Jade B solution (0.0004% concentration) for 30 min at room temperature. After staining, the sections were washed three times with double distilled water, cleared with xylene, air dried and coverslipped using DPX (Fluka). For colocalisation studies of CHOP with Flouro-Jade B, deparaffinized sections were washed once with PBS for 5 min, microwaved in citrate buffer for 15 min, blocked with 5% goat serum and probed with CHOP antibody for 1 hr, followed by three PBS washes. Sections were then incubated in secondary antibody, washed thrice with PBS and subjected to graded alcohol series. Sections were pretreated for 2 min with 0.06% potassium permanganate, rinsed in double distilled water for 3 min and immersed in Flouro-Jade B solution (0.0004% concentration) for 30 min at RT. After this step, sections were washed thrice with PBS for 5 min each, cleared with xylene and air dried on a slide warmer at 50^0C, mounted with DPX and coverslipped. Later, sections were analysed under confocal microscope.

Data Analysis: Data are reported as means +/- SEM of n experiments. All parameters were compared using a two-tailed Student's-t-test between infected and control animals. A level of $*p < 0.05$ was considered statistically significant and was determined using Sigma plot 2000 for windows version 6.00, SPSS Inc., Illinois, USA.

Results

As shown in Fig 1 a, all the infected mice succumbed to the disease and died by day 12 PI with a median survival of 7.5 days ($p < 0.0001$) often with parasitemia not exceeding 15% (data not shown), whereas all the uninfected control mice have 100% survivality. H&E staining of brain sections taken from PbA infected animals (Fig 1 c, e and g) showed distinct neurological lesions, with parenchymal microhemorrhages, cells with irreversible damage, shrinkage of the nucleus, vacuolization of the cytoplasm with distinct apoptotic morphology, disruption of vessel walls and adherence of leucocytes to the vessel walls. In contrast, brain sections taken from uninfected controls (Fig 1 b, d and f) have a healthy parenchyma, clear vessels with no hemorrhage.

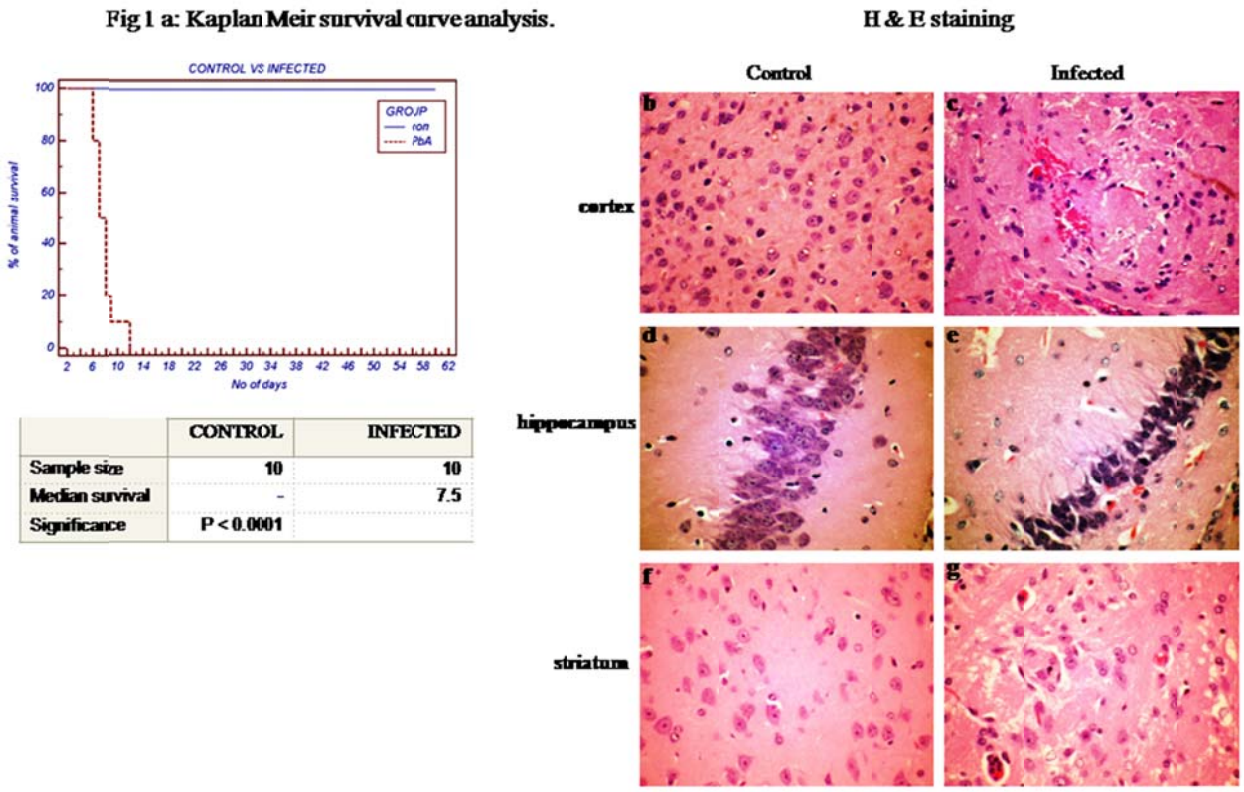


Fig 1: Kaplan Meir survival curve analysis (a) showing C57BL/6J mice (n=10) inoculated intraperitoneally with 10^6 parasitized red blood cells died by day 12 PI with a median survival of 7.5 days ($p<0.0001$), while all uninfected controls (n=10) has 100% survivality. Observation of animal survival experiments was done in a masked manner so as to avoid bias toward any group of animals. H&E staining of brain sections encompassing cortex (c), hippocampus (e) and striatum (g), taken from PbA infected animals showing widespread neurological lesions, cells with apoptotic morphology, peticheal hemorrhages and blood vessels clogged with leucocytes and PRBCs. Sections taken from control animals (b, d and f) have healthy parenchyma, with no vascular clogging or hemorrhages. Figures were taken at 60X magnification.

To investigate whether PbA infection leads to the activation of ER stress sensors, we performed Western blot analyses using antibodies against the phosphorylated forms of PERK and IRE1. Significant increases in immunoreactivity for p-PERK ($p = 0.004$) and p-IRE1 ($p = 0.03$) was seen in PbA infected mice brains as compared to controls. We further assessed the cleavage status of ATF6 in both PbA infected and control mice brains using an antibody which detects spliced and unspliced versions of ATF6. As shown in the Figure 2, ATF6p50 (the activated cytosolic form of ATF6) protein levels were significantly increased in PbA infected mice brains as compared to uninfected controls ($p < 0.001$). Taken together our results indicate the activation of all the three major arms of ER stress signaling pathway.

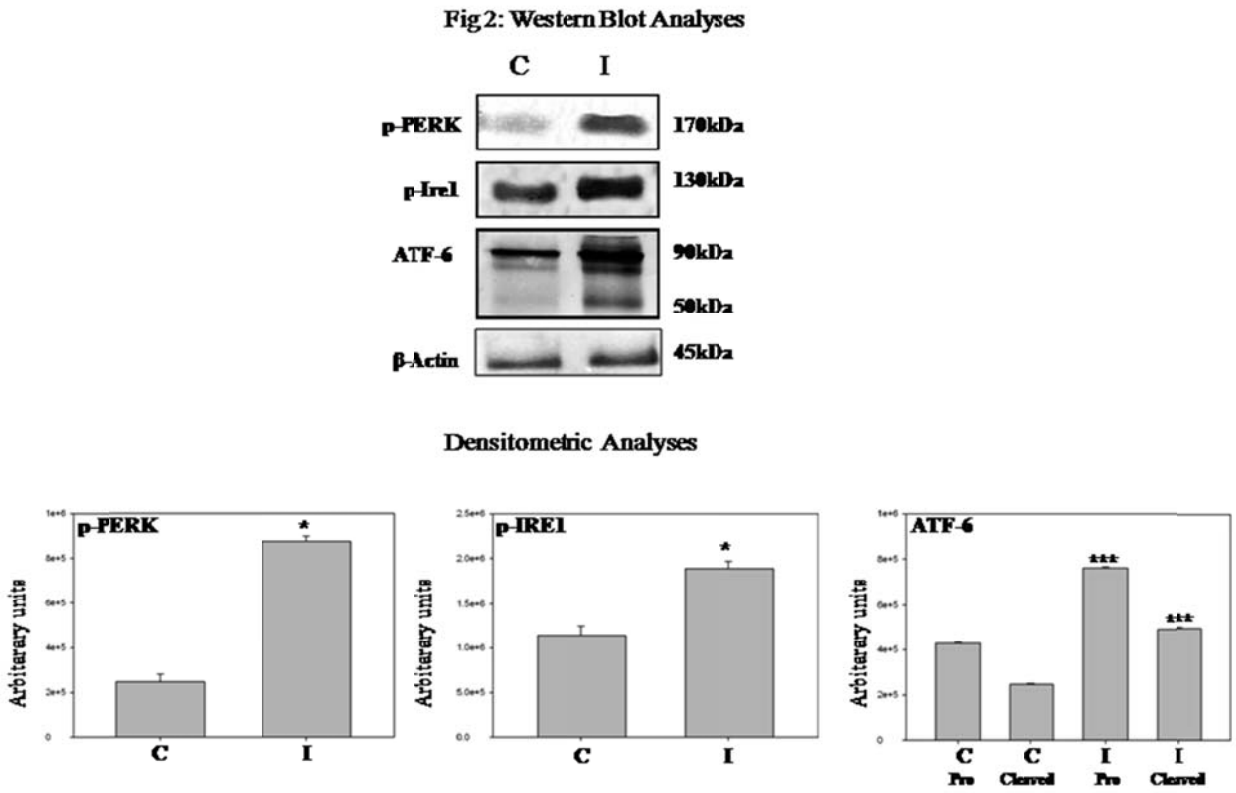


Fig 2: Immunoblot analysis of p-PERK, p-IRE1, ATF6 and β-Actin in the control and infected mice brains. Equal amount of protein was electrophoresed by SDS-PAGE and transferred to nitrocellulose membrane and probed with primary antibody to p-PERK, p-IRE1, ATF6 and β-Actin. β-Actin was used as loading control. The result is representative of four independent experiments with similar results. The lanes C and I indicates uninfected control and PbA infected mice brains sacrificed on day 7 PI respectively. Densitometric analysis (Lower three panels) showing significant increases in the levels of p-PERK, p-IRE1 and ATF6 in infected samples as compared to their respective controls. (*$p < 0.05$ and ***$p<0.001$ indicate significant difference relative to the corresponding control).

Since activation of PERK induces the phosphorylation of eIF2α, we next checked the status of eIF2α phosphorylation. As shown in Fig 3 a, there is significant increase in the p-eIF2α levels in the infected brain compared to controls ($p = <0.001$), while unphosphorylated eIF2α levels remained the same. In addition, we performed immunohistochemistry on brain sections encompassing cortex, hippocampus & striatum of PbA infected animals or uninfected controls, to confirm the induction of p-eIF2α. In control mouse brain sections (Fig. 3 b, d and f), p-eIF2α immunoreactivity was poorly distributed. However, in infected mouse brain sections (Fig. 3 c, e and g), signals for p-eIF2α increased in intensity thus correlating with our Western blot results. Furthermore, double immunoflouroscent analysis of p-eIF2α with synaptophysin, a neuronal marker (Fig. 3 h-k); revealed neuronal induction of p-eIF2α in infected mouse brain sections,

implying that neurons are the principal cell types in which p-eIF2α is activated in murine cerebral malaria.

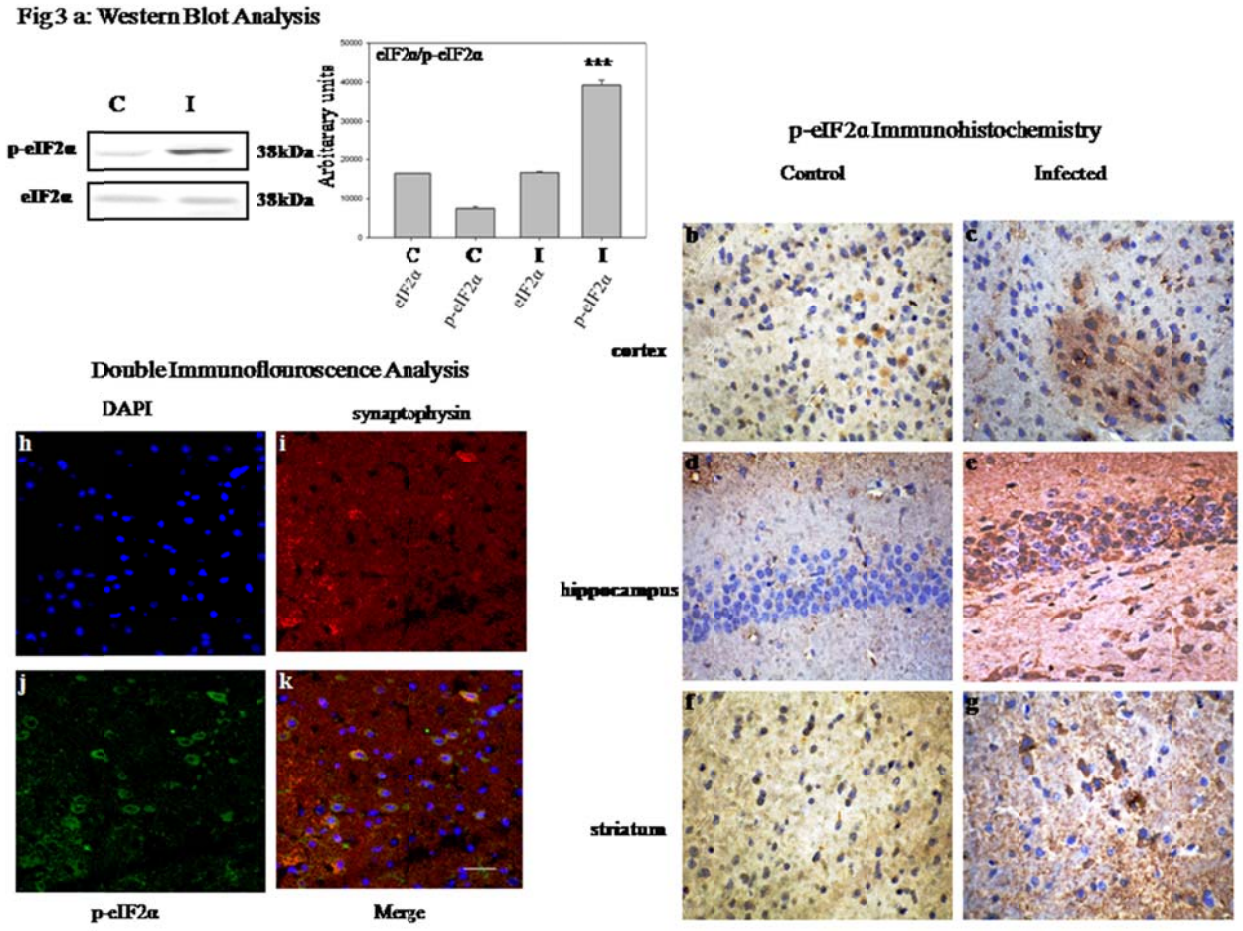


Fig 3: Immunoblot analysis of p-eIF2α and eIF2α in the control and infected mice brains (a). The result is representative of four independent experiments with similar results. The lanes C and I indicates uninfected control and PbA infected mice brains sacrificed on day 7 PI respectively. Densitometric analysis (vertical bar graph) showing significant increases in the levels of p-eIF2α in infected samples as compared to controls while the levels of eIF2α remained the same in both the groups. (***$p<0.001$ indicate significant difference relative to the corresponding control). In control mouse brain sections p-eIF2α immunoreactivity was barely detectable (b, d and f). However, in infected mouse brain sections there is a robust increase in p-eIF2α immunoreactivity in all the three brain regions (c, e and g). Pictures were taken at 60X magnification. Double immunostaining of p-eIF2α (j) and synaptophysin (i) showed neuronal induction of p-eIF2α in infected brain sections (overlay, panel k). DAPI was used as nuclear counterstain (h). Photomicrographs shown here in this figure are a representative of three individual animals from each group. Bar: k=40μm.

To further gain insight into the downstream effectors of PERK-eIF2α branch of UPR during PbA infection, we asked if factors which are normally activated by high levels of phosphorylated eIF2α, such as ATF4 and GADD34, are expressed under these conditions. In our immunoblot

analyses (Fig 4) we found significant increases in the expression of both ATF4 and GADD34 ($p = 0.002$ and 0.005 respectively) in infected mice brain as compared to controls indicating activation of PERK-eIF2α branch of UPR during PbA infection.

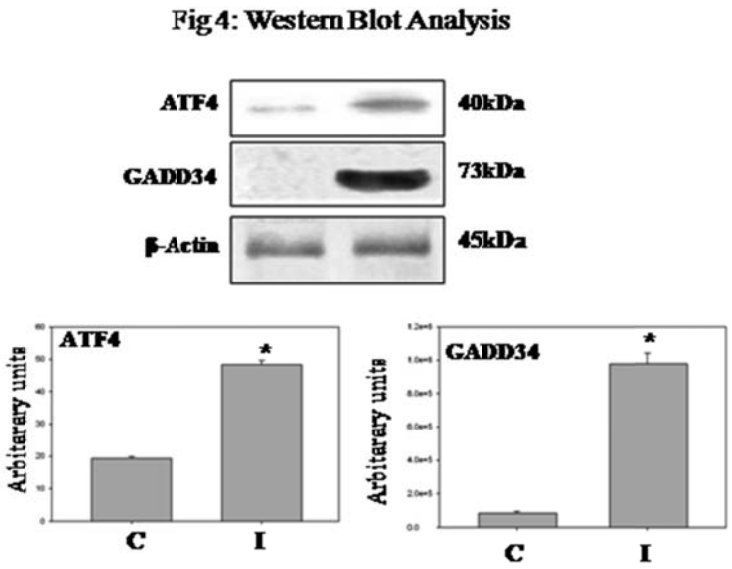


Fig 4: Immunoblot analysis of ATF4 and GADD34 in the control and infected mice brains. β-Actin was used as loading control. The result is representative of four independent experiments with similar results. The lanes C and I indicates uninfected control and PbA infected mice brains sacrificed on day 7 PI respectively. Densitometric analysis (Lower two panels) showing significant increases in the levels of ATF4 and GADD34 in infected samples as compared to their respective controls. (*$p < 0.05$ indicate significant difference relative to the corresponding control).

We next investigated the status of ER chaperones such as, BiP, Calregulin and Calnexin which are prosurvival and are expressed during UPR and ER stress (Fig 5). Western blot analysis revealed a significant down regulation of Calregulin ($p = 0.02$) and Calnexin ($p = 0.002$) in infected mice when compared to controls. On the other hand although we observed the down regulation of BiP levels in infected mice compared to controls, the decrease is not that significant ($p = 0.073$).

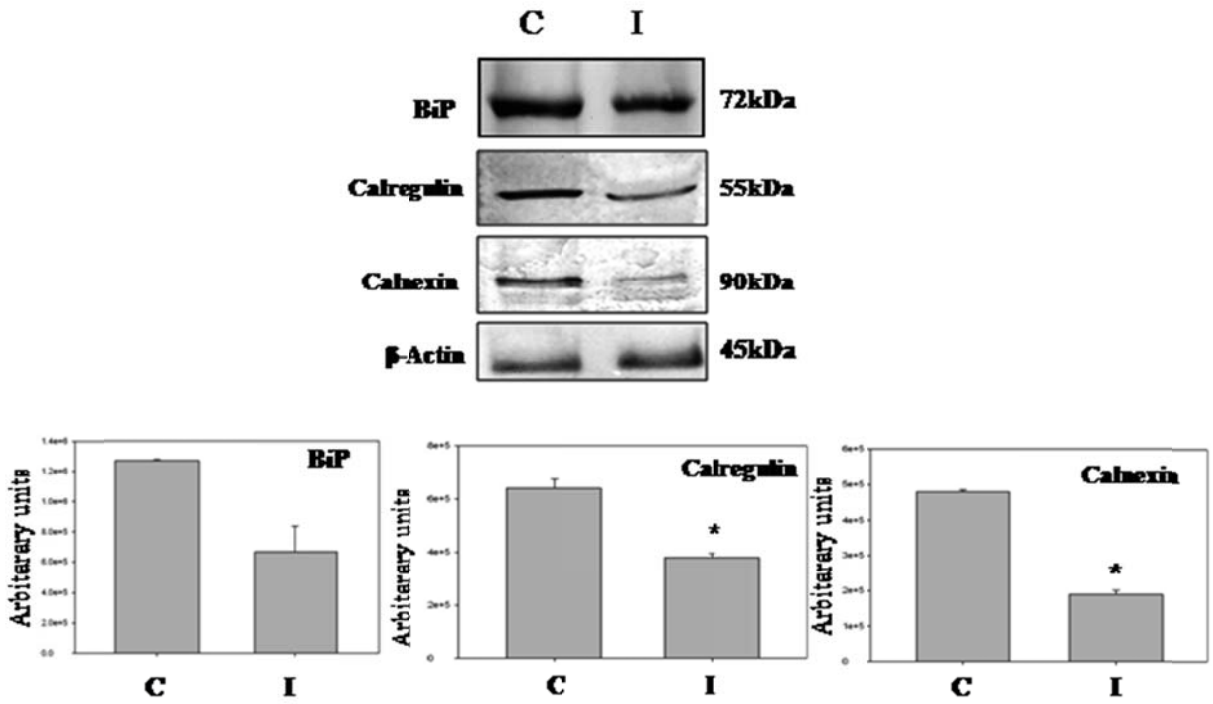


Fig 5: Immunoblot analysis of BiP, Calregulin and Calnexin in the control and infected mice brains. β-Actin was used as loading control. The result is representative of four independent experiments with similar results. The lanes C and I indicates uninfected control and PbA infected mice brains sacrificed on day 7 PI respectively. Densitometric analysis (Lower three panels) showing significant decreases in the levels of Calregulin and Calnexin in infected samples as compared to their respective controls. Changes in BiP levels in the infected samples as compared to controls were not found to be significant. (*$p < 0.05$ indicate significant difference relative to the corresponding control).

All the three major arms viz., PERK, IRE1 and ATF6 of UPR and ER stress signaling pathway, converge on the elements in CHOP promoter region to induce CHOP expression. To test this, we first performed Western blot analysis and later the bands are quantiated by densitometry (Fig 6 a). Our results show a prominent increase in the levels of CHOP protein in infected samples ($p = 0.009$) but in control samples it is barely detectable. Immunohistochemistry of brain sections using CHOP antibody revealed robust increase in immunoreactivity with distinct nuclear localization in all the three brain regions in infected sections (Fig 6 c, e and g) but not in control brain sections (Fig 6 b, d and f). Further double immunoflouroscence labeling on infected brain sections revealed colocalization of CHOP (Fig. 6 j) with synaptophysin (Fig 6 i), a neuronal marker suggesting the neuronal expression of CHOP in the infected brain.

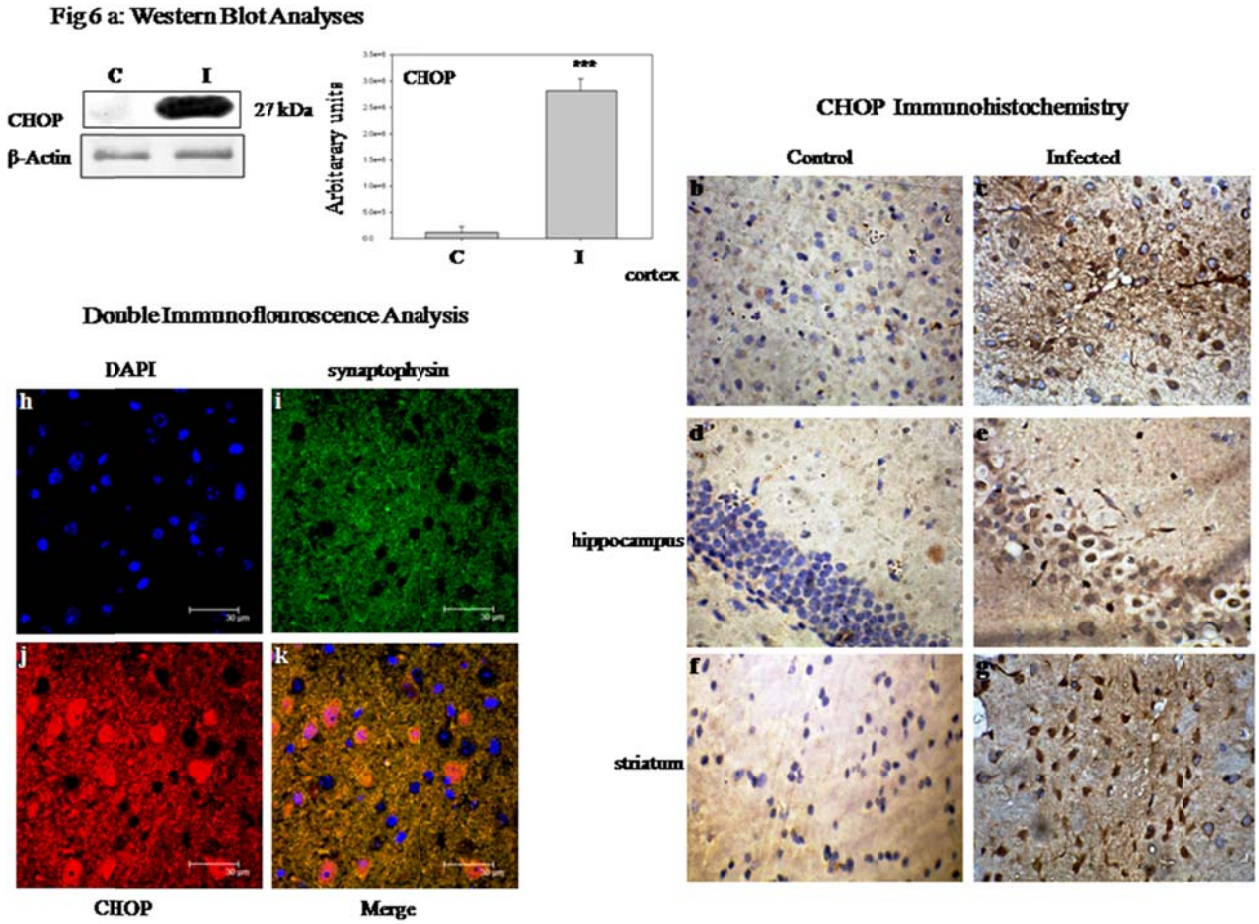


Fig 6: Immunoblot analysis of CHOP in the control and infected mice brains (a). The result is representative of four independent experiments with similar results. The lanes C and I indicates uninfected control and PbA infected mice brains sacrificed on day 7 PI respectively. Densitometric analysis (vertical bar graph) showing significant increases in the levels of CHOP in infected samples as compared to controls. β-Actin was used as loading control. (***$p<0.001$ indicate significant difference relative to the corresponding control). In control mouse brain sections CHOP immunoreactivity was barely detectable (b, d and f). However, in infected mouse brain sections there is a marked increase in CHOP immunoreactivity in all the three brain regions with nuclear localization (c, e and g). Pictures were taken at 60X magnification. Double immunostaining of CHOP (j) and synaptophysin (i) showed neuronal induction of CHOP in infected brain sections (overlay, panel k). DAPI was used as nuclear counterstain (h). Note nuclear localization of CHOP in the overlay panel k. Photomicrographs shown here in this figure is a representative of three individual animals from each group.

Moreover, several studies have shown CHOP playing as a proapoptotic molecule induced by ER stress (Kim *et al.*, 2008; Rao *et al.*, 2004; Szegezdi *et al.*, 2006) and earlier studies from our lab have shown that the pathology of ECM manifests neuronal apoptosis (Anand and Babu, 2011; Kumar and Babu, 2002; Kumar *et al.*, 2003; Shukla *et al.*, 2006). To substantiate our results we checked the levels of pro-apoptotic proteins like BAX, caspase-3, active caspase-7, caspase 12 and anti-apoptotic BCL-2. Immunoblotting with caspase-3 antibody revealed significant increases in the levels of cleaved caspase-3 ($p < 0.001$) in the infected brain (Fig 7 a). Further,

PbA infection resulted in the elevated protein levels of BAX ($p = 0.002$), active caspase-7 ($p < 0.001$) and active caspase-12 ($p < 0.001$) in the infected brain (Fig 7 a). On the other hand, anti-apoptotic BCL-2 levels in infected brain were barely detectable indicating predominance of apoptosis in the infected samples. Moreover, cresyl violet staining revealed neurons with distinct apoptotic morphology indicating irreversible neuronal injury in all the three regions taken from infected brain sections (Fig. 7 c, e and g) but not in control brain sections which showed prominent nucleolus within the cell nucleus and healthy parenchyma (Fig 7 b, d and f).

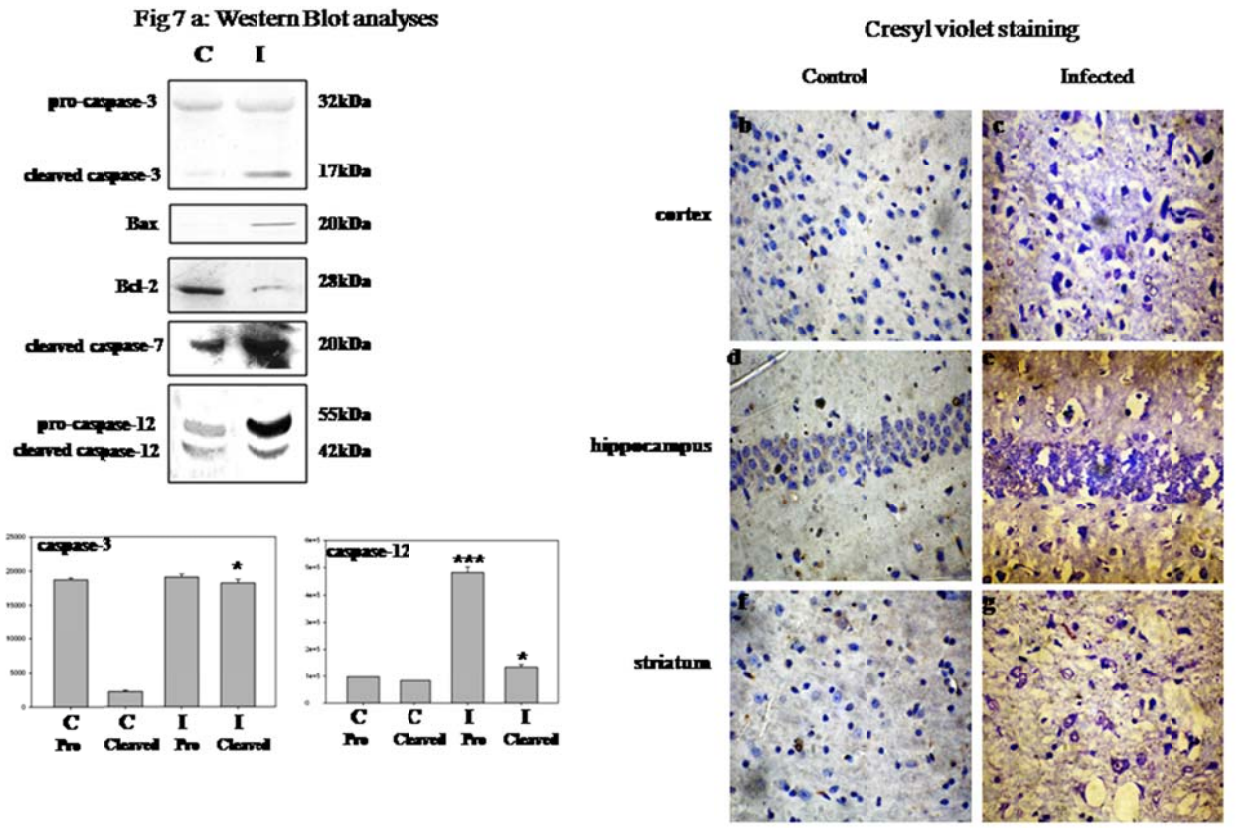


Fig 7: Immunoblot analysis of caspase-3, BCL-2, BAX, active caspase-7 and caspase-12 in the control and infected mice brains (a). β-Actin was used as loading control. The result is representative of four independent experiments with similar results. The lanes C and I indicates uninfected control and PbA infected mice brains sacrificed on day 7 PI respectively. Densitometric analysis (Lower two panels) showing significant increases in the levels of cleaved caspase-3 and significant increases in the levels of total and cleaved versions of caspase-12 in infected samples as compared to their respective controls. (*$p < 0.05$ and ***$p<0.001$ indicate significant difference relative to the corresponding control). Cresyl violet staining of brain sections encompassing cortex (c), hippocampus (e) and striatum (g), taken from PbA infected animals revealed pyknotic cells and apoptotic neurons (deep-blue neurons) indicating irreversible neuron injury. (b, d and f) indicates normal neurons with clear nucleus and nucleolus taken from control animals. Photomicrographs shown here in this figure is a representative of three individual animals from each group taken at 60X magnification.

To confirm neuronal cell death in PbA infected mice we stained the sections with Flouro-Jade B, a novel fluorescent marker which binds to dying neurons (Schmued and Hopkins, 2000). Our results revealed several Flouro-Jade B positive cells in all the three brain regions in infected samples (Fig 8 c, g and k) but not in control brain sections (Fig 8 a, e and i) indicating widespread neuronal cell death in PbA infected murine brain. Moreover, colocalization studies of CHOP with Flouro-Jade B on infected mouse brain sections (Fig. 8 m, n and o) clearly showed that CHOP is up regulated in degenerating neurons following PbA infection. Together, these results proved unambiguously that CHOP is involved in neuronal cell death in murine cerebral malaria.

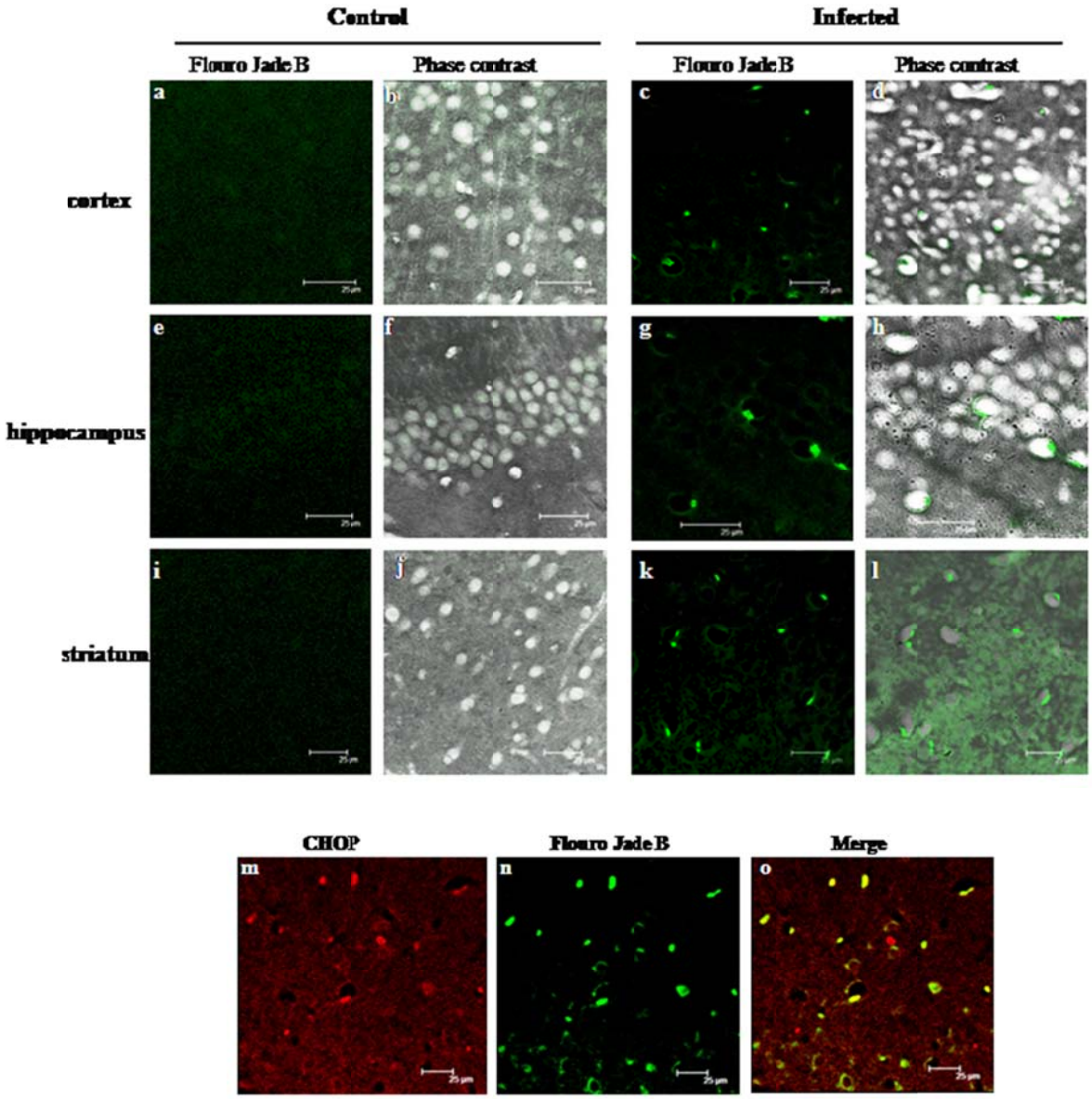


Fig 8: Paraffin embedded sections of both control (a, e and i) and PbA infected mouse brain sections (c, g and k) were stained for Flouro-Jade B. While control sections stained very little for Flouro-Jade B, infected sections have significantly higher number of Flouro-Jade B positive cells in all the three regions. Panels (b, f and j) and (d, h and l) were phase-contrast images of control (a, e and i) and infected (c, g and k) panels respectively. Double label staining of CHOP (m) and Flouro-Jade B (n) showed that CHOP positive cells were also positive for Flouro-Jade B (o). Photomicrographs shown here in this figure are a representative of three individual animals from each group.

Discussion

Murine CM model has provided important insights into the pathogenesis of CM and enabled potential targets for modulation to be identified for study in humans. C57BL/6J mice infected with *PbA* develop neurological signs and symptoms typical of human CM and die within 6-12 days PI (de Souza and Riley, 2002). Studies with this model have identified the upregulation of several pro-apoptotic mediators as major cause of neuronal cell death during the course of infection (Anand and Babu, 2011; Kumar and Babu, 2002; Kumar *et al.*, 2003; Lackner *et al.*, 2007; Shukla *et al.*, 2006; Wiese *et al.*, 2006). An overwhelming evidence points out the activation of inflammatory pathways, upregulation of cytokines such as TNF-α, IFN-γ, IL-1, IL-6 and IL-8 and pRBC sequestration during the pathophysiology of CM (van der Heyde *et al.*, 2006). Further, sequestration results in reduced blood flow leading to tissue hypoxia and hypoglycemia (Marsh *et al.*, 1995), which in turn causes protein misfolding and ER stress. Although there is growing evidence underlying the role of the UPR and ER stress in several neurodegenerative disorders (Lindholm *et al.*, 2006), there is no data currently available as to which of the three possible branches of UPR are activated, or the kinetics of activation or to the extent of their involvement in neuronal cell death in ECM.

To this end, we performed Western blot analyses and our results for the first time showed the induction of p-PERK and p-IRE1 and cleavage of ATF6 in infected mice brains but not in uninfected controls indicating the activation all the three branches of UPR. Our results are in agreement with earlier studies of brain ischemia/reperfusion and other neurodegenerative disorders which implicate the activation of multiple pathways of UPR following insult (Lindholm *et al.*, 2006). However we emphasized the role of PERK-eIF2α axis in this study mainly because our aim here was to investigate a well-established pathway of translation arrest and to provide deeper insight into the mechanisms by which PERK-eIF2α axis mediates neuronal cell death in ECM.

Along with hypoglycemia which is known to occur in cerebral malaria (Marsh *et al.*, 1995), depletion of ER calcium stores is known to activate PERK (Brostrom and Brostrom, 1990; Prostko *et al.*, 1992). Further, a direct proof of evidence linking disturbances in neuronal calcium homeostasis to PERK activation is lacking in ECM model. However, supporting evidence in the form of calpain activation owing to altered calcium homeostasis in pathological manifestation of the disease is well established (Shukla *et al.*, 2006). Activation of PERK leads to eIF2α phosphorylation, thereby shutting off mRNA translation and reducing the protein load on the ER. In this study, we evaluated PbA -induced alterations in both eIF2α phosphorylation and eIF2α protein levels by western blotting using the respective antibodies. While there is no change in the levels of eIF2α protein, p-eIF2α levels increased significantly in infected mice as compared to controls. Further we performed Immunohistochemistry to determine whether there was any regional difference in the expression of p-eIF2α. We selected cortex, hippocampus and striatum, the three different neuroanatomical regions that control movement because, CM effects movement and coordination in terminally ill mice with symptoms ranging from but not limited to stupor, hemiplegia, dysplegia, paraplegia coma and finally death. Robust increases in immunoreactivity for p-eIF2α was observed in cortex, hippocampus and striatum indicating that all the three regions are equally vulnerable to ER stress and infact this regional vulnerability coincides with neuronal cell death as observed in our Cresyl violet and Flouro-Jade B staining. We further performed p-eIF2α/synaptophysin colocalisation experiments in infected mice brain sections to confirm the neuronal induction of p-eIF2α. Our results show that p-eIF2α was significantly upregulated in neurons and taking into account the pattern of Flouro-Jade B staining we conclude that neurons are particularly vulnerable to ER stress mediated cell death.

eIF2α phosphorylation was followed by the induction of the transcription factor ATF4 and GADD34, both genes being specific targets of the PERK/eIF2α pathway. GADD34 is a regulatory subunit of protein phosphatase 1(PP1), which is required for eIF2α dephosphorylation and recovery from a shutoff of total protein synthesis in response to ER stress (Novoa *et al.*, 2001). In our results we found distinct increases in both ATF4 and GADD34 emphasizing the point that PERK/eIF2α pathway is activated in ECM. Increased levels of p-eIF2α and GADD34 observed in infected mice throws light on the dynamic stress response involving regulation of eIF2α kinases and phosphatases. Increased GADD34 levels in infected animals also support the

notion that the increase in the levels of p-eIF2α is due to elevated activity of the upstream kinase and not due to decreased activity of phosphatase.

While ATF4 on one hand induces the transcription of pro-survival genes such as GRP78, GRP94 and genes that are involved in amino-acid metabolism and transport, on the other hand it also leads to the transcription of CHOP/GADD153 which is a pro-apoptotic ER stress marker. Infact, PERK-eIF2α signaling pathway directly regulates the transcriptional arm of the UPR, affecting both the pro- and anti-apoptotic components (Luo *et al.*, 2003). In this connection we performed Western Blot analyses of BiP and CHOP and our results show a decrease in BiP levels and a corresponding increase in CHOP levels suggesting that the pro-apoptotic component of PERK-eIF2α signaling pathway is dominant over its anti-apoptotic counterpart in the PbA infected mice. This fact is further strengthened by our results wherein we also found the downregulation of Calreticulin and Calnexin, the two ER resident pro-survival chaperones (Liu *et al.*, 1997; Rosenbaum *et al.*, 2006) in infected mice as compared to controls. Our results are in agreement with earlier studies where in, BiP expression was reduced while CHOP expression was increased in the late phase of permanent middle cerebral artery occlusion (MCAO) in mice (Morimoto *et al.*, 2007). Thus, in light of these observations we conclude that in ECM, ER stress accompanied by failure of adaptive response, may eventually results in apoptotic cell death.

CHOP is a transcription factor that translocates to nucleus after its activation. Infact all the three axes of ER stress pathway converge on CHOP induction (Oyadomari and Mori, 2004) and it is one of highest inducible genes during ER stress as revealed by microarray analysis (Okada *et al.*, 2002). Numerous evidences reveal that overexpression of CHOP or microinjection of CHOP in cells promotes apoptosis (Matsumoto *et al.*, 1996; Maytin *et al.*, 2001; Oyadomari *et al.*, 2001), while overexpression of BiP attenuates CHOP induced apoptosis (Wang *et al.*, 1996). Similarly, mice deficient in CHOP showed reduced apoptosis in response to ER stress (Oyadomari *et al.*, 2001; Zinszner *et al.*, 1998). Our results from CHOP immunohistochemistry show that CHOP immunoreactivity is robust in all the three brain regions of the infected mouse brain as compared to controls. Further CHOP is localized exclusively to nucleus and CHOP/synaptophysin colocalisation experiments revealed that CHOP is expressed in neurons in infected mice. Moreover, CHOP/Flouro-Jade B dual staining confirm that CHOP is involved in neuronal cell

death in PbA infected mice. Taken together, our results highlight the importance of CHOP mediated neuronal cell death in ECM.

Apart from its intrinsic ribonuclease activity, IRE1, also has a Ser/Thr kinase domain which once activated is involved in ER stress mediated cell death (Kim *et al.*, 2008). In fact modulation of UPR signaling via IRE1α is also dependent on its association with pro-apoptotic members of BCL-2 family BCl-2-associated X protein (BAX) and BCl-2 antagonist/killer (BAK) (Hetz *et al.*, 2006). Our results, in conjunction with this previous study, show the activation of IRE1, upregulation of BAX, down regulation of BCL-2 and cleavage of caspase-3 indicating ER stress mediated apoptotic pathway in PbA infected mice brains. Activated IRE1α also mediates apoptosis via its interaction with the cytoplasmic domain of TRAF2 and activation of JNK via IRE1α–TRAF2-ASK1–JNK signaling pathway (Kim *et al.*, 2008; Urano *et al.*, 2000). In fact Liu *et al* recently demonstrated IRE1α- ASK1–JNK mediated pro-apoptotic pathway in the hippocampus of patients with chronic epilepsy (Liu *et al.*, 2011). Earlier, we have demonstrated the activation and involvement of JNK in mediating neuronal cell death in ECM model (Anand and Babu, 2011). Taken together these data implicate a role of IRE1α in neuronal apoptosis in PbA infected mice.

Unlike activated IRE1 which plays a major role in apoptosis in ER stressed cells, activation of ATF6 is prosurvival aimed to counteract ER stress (Szegezdi *et al.*, 2006). Moreover, in a recent study it was reported that ischemia activates ATF6 and induces ER stress responsive genes (Doroudgar *et al.*, 2009). Furthermore, sequestration of PRBC's, attachment of monocytes to cerebral endothelia, cerebral oedema and increase in brain lactate result in ischemic conditions (Sanni *et al.*, 2001) which may provide a plausible explanation for the activation of ATF6 in PbA infected murine brain.

Caspase-12 is present on the cytoplasmic side of the ER and mediates cell death associated with ER stress. Caspase 12 is activated either by calpain owing to Ca^{2+} imbalances (Nakagawa and Yuan, 2000) or it is activated by classical TRAF2-IRE1-JNK pathway (Yoneda *et al.*, 2001) or it is activated by caspase-7 (Rao *et al.*, 2001). Our results show the activation of caspase-12 in infected animals, but at this point of time we cannot exactly delineate as to which of these pathways are involved in caspase-12 activation. Infact, we have reported earlier the activation of

calpains and JNK (Anand and Babu, 2011; Shukla *et al.*, 2006) in murine malaria. Further we have also checked the levels of active caspase-7 in this study which suggests that activation of caspase-12 in ECM could be synergistic involving calpains, TRAF2-IRE1-JNK and also caspase-7.

In summary the results presented here show for the first time, activation of multiple apoptotic ER stress pathways which could shed new light on the mechanisms underlying the relationship between ER stress, the UPR and the cell death program in ECM.

CHAPTER 4

Pentoxifylline is neuroprotective in Experimental Cerebral Malaria

Pentoxifylline (PTX) is neuroprotective in experimental cerebral malaria

Pentoxifylline (PTX), 3,7-Dihydro-3,7-dimethyl-l-(5-oxohexyl)-lH-purine-2,6-dione, is the 1-(5-oxohexyl) analog of the methylxanthine theobromine with a molecular weight of 278.31. To begin with, PTX was characterized as a hemorrheologic agent for the treatment of peripheral vascular disease (Muller and Lehrach, 1981) and intermittent claudication (Porter *et al.*, 1982). This therapeutic benefit is mainly due to its effect on red blood cell deformability and therefore leading to increased red blood cell flexibility and reduced blood viscosity (Aviado and Dettelbach, 1984; Grigoleit and Jacobi, 1977). This leads to reduced red blood cell aggregation, reduced platelet aggregation, and fibrinogen levels resulting in improved microcirculation and tissue oxygenation (Jarret *et al.*, 1977; Leonhardt and Grigoleit, 1977; Weithmann, 1983). Further, PTX alters the biochemical and physical properties of platelets (Hammerschmidt *et al.*, 1988), endothelial cells **(Weithmann, 1980)**, polymorphonuclear leukocytes and macrophages (Hammerschmidt *et al.*, 1988; Ikossi *et al.*, 1986), and fibroblasts (Berman and Duncan, 1989). Additionally, PTX also known to be immunomodulatory and it exerts strong anti-cytokine activity, as it predominantly inhibits the proinflammatory actions of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) on neutrophil function and cytokine production by monocytic cells (Balazs and Kiss, 1994; Strieter *et al.*, 1988; Sullivan *et al.*, 1988; Tannenbaum and Hamilton, 1989).

PTX is known to inhibit phosphodiesterases, thereby increasing cytoplasmic cyclic adenosine monophosphate (cAMP) levels (Sinha *et al.*, 1995). High levels of cAMP suppress TNF-α gene transcription (Strieter *et al.*, 1988). In addition to inhibiting cytokines, PTX is also known to reduce the TNF-α induced upregulation of ICAM and E-selectin in endothelial cells which inhibits the adhesion of neutrophils (Bahra *et al.*, 2001; Zhang *et al.*, 2010). It also inhibits adhesiveness, degranulation, and superoxide production of activated neutrophils and the production of nitric oxide by macrophages (Bessler *et al.*, 1986).

Because of its ability to counteract multiple proinflammatory mediators simultaneously PTX seems a useful agent to attenuate the generalized inflammatory response leading to the organ failure observed in Experimental cerebral malaria. To test this hypothesis, we wanted to check

whether administration of PTX improves survivality and attenuates neuronal cell death in mice infected with PbA.

Materials and methods

Experimental CM model: Pathogen-free C57BL/6J mice 6-8 weeks old, weighing 18–24 g, were obtained from NCLAS (National Center for Laboratory Animal Sciences), Hyderabad. All the protocols followed for the use of animal experimentation were strictly in accordance with the institutional and national ethical committee guidelines. C57BL/6J mice of either sex were inoculated intraperitoneally with 10^6 parasitized red blood cells, suspended in 200µl of PBS (pH 7.4). Uninfected mice of same age and sex were used as controls. Parasitemia was assessed from Geimsa-stained thin smears of tail blood prepared every day post inoculation (PI). On day 6-9 PI mice displayed clinical signs typical of CM such as ataxia, hemiplagia, seizures, paralysis and coma followed by death, with parasitemia not exceeding 15%. Brains were dissected out from control, *PbA* infected animals and Pentoxifylline treated animals, snap frozen in liquid nitrogen and stored in -80^0C until further use. To evaluate the effect of Pentoxifylline on the survivality of mice infected with PbA, a comparative survival curve of both infected and treated groups was performed using MedCalc software, and the course of infection was followed daily by monitoring parasitemia on periodic blood smears prepared from day1 PI and was typically between 10-15% at the time of sacrifice.

Administration of Pentoxifylline: Mice were grouped into 3 categories namely control mice, infected mice, infected mice receiving PTX daily at a dose of 60mg/kg BW/twice a day with a gap of 12 hr intervals. And this dosage regimen continued for the next 10 days starting from day 0 PI. After which, blood was collected and brains were harvested for further analyses.

Histology: For histological analysis, brains were quickly removed, fixed in 10% formalin, embedded in paraffin and cut into 5µm sections. The sections were stained with hematoxylin and eosin (H&E). Slides were examined under a light microscope for neurological lesions, infiltration of leucocytes, sequestration of monocytes and PRBC's and neuronal cell death.

Behavioral analyses: Clinical signs of cerebral malaria were evaluated and used for scoring disease severity as shown in the following table (Waknine-Grinberg *et al.*, 2010). Mice with a cumulative score of 5 or above are sacrificed, and death deemed to be on the following day.

Parameter	Clinical signs	Severity (score)
Appearance	**Normal**	**0**
	Coat ruffled	**1**
	Coat staring; panting	**2**
Behaviour (undisturbed)	**Normal**	**0**
	Hunched; wobbly gait	**1**
	Partial paralysis; immobile*	**2**
	Convulsions; coma*	**3**
Food intake	**Normal 0**	**0**
	Up to 10% loss in body weight	**1**
	10%-15% loss in body weight *	**2**
	More than 15% loss in body weight*	**3**
Body temperature	**Normal (36-37°C)**	**0**
	34-35°C	**1**
	32-33°C*	**2**
	Below 32°C*	**3**

Assessment of BBB- vascular leakage by Evans Blue dye extravasation method: The evaluation of vascular permeability was performed after 9 days in all the three experimental groups (control, infected and pentoxifylline treated). One hour before the sacrifice of mice, Evans blue dye (2 ml/kg of 2% dye in 0.9% NaCl) was injected intravenously. Immediately after sacrificing the animals by an overdose of pentobarbital, the blood was cleared from the circulation by transcardiac perfusion (150 ml 0.9% NaCl). The brains were rapidly removed and dissected and analyzed for dye leakage into the brain parenchyma.

Western Blot Analysis: Western blotting was performed using the standard method (Anand and Babu, 2011). The following primary antibodies were used: mouse monoclonal antibodies raised against COX2 (Cayman chemical) and TNF-α (Sigma), rabbit polyclonal antibodies raised against iNOS (Chemicon), p-JNK, p-p38, p-ERK, JNK, p-AKT, AKT, active caspase 3, PARP, Bcl-2, Bax and cytochrome-c (Cell Signalling Technology).

Immunohistochemistry and analysis of infiltrating lymphocytes: Immunohistochemical analysis was performed as described previously (Anand and Babu, 2011). Briefly, tissue sections were

deparaffinized in xylene and passed through graded alcohols and further rehydrated in phosphate buffered saline (PBS). Antigen unmasking was carried out by micro waving the sections for 10–14 min in 10 mM citrate buffer (pH 6.0). Then, endogenous peroxide activity was inhibited by treating the sections with 3% H_2O_2 for 10 min followed by blocking with serum for 1 h at room temperature in a humid chamber. Sections were then incubated with primary antibodies against TNF-α (1:100 dilution), active-caspase-3 (CST, 1:100 dilution), COX-2 (1:100) and iNOS (1:100) for 1 h at room temperature followed by incubation with peroxidase conjugated secondary antibody for 1 h at room temperature. After washing in TBS, diaminobenzidine (DAB) in buffer was applied till sections develop colour. Then, sections were washed with distilled water followed by dehydration in graded alcohols, xylene and mounted with DPX (kit obtained from Biogenex Pvt Limited, India). For NeuN immunostaining, the same protocol was followed except that inhibition of endogenous peroxidise activity step was skipped, the primary antibody used was mouse anti-NeuN monoclonal antibody (Chemicon), the mouse secondary antibody used was tagged with AlexaFlour 594 (Invitrogen), sections were mounted with Vectashield (Vector Labs) and visualized under Leica confocal microscope. For cd4 and cd8 lymphocyte infiltration into the brain parenchyma we stained the sections with FITC tagged mouse anti-cd4 and TRITC tagged mouse anti-cd8 antibodies (eBioscience) following the same protocol as described above.

Flouro-Jade B staining: Paraffin embedded brain sections from all the three groups were deparaffinized in xylene, rehydrated in alcohol series and pretreated for 5-10min with 0.06% potassium permanganate, rinsed in double distilled water for 3min and immersed in Flouro-Jade B (Chemicon) solution (0.0004% concentration) for 30 min at RT. After this step, sections were washed thrice with PBS for 5min each, cleared with xylene and air dried on a slide warmer at 50^0 C, mounted with DPX and coverslipped. Later, sections were analyzed under Leica confocal microscope. For colocalisation studies of active caspase-3 with Flouro-Jade B, deparaffinized sections were washed once with PBS for 5 min, microwaved in citrate buffer for 15 min, blocked with 5% goat serum and probed with caspase-3 antibody for 1 hr, followed by three PBS washes. Sections were then incubated in secondary antibody, washed thrice with PBS and subjected to graded alcohol series. Sections were pretreated for 2 min with 0.06% potassium permanganate, rinsed in double distilled water for 3 min and immersed in Flouro-Jade B solution (0.0004% concentration) for 30 min at RT. After this step, sections were washed thrice with PBS for 5 min

each, cleared with xylene and air dried on a slide warmer at 50^0C, mounted with DPX and coverslipped. Later, sections were analysed under confocal microscope.

Caspase 3 activity assays: Briefly, brain tissues were homogenized in caspase assay buffer containing 50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.1% CHAPS, 10% sucrose and 5 mM DTT. Aliquots of 50µg of crude tissue lysate were incubated with caspase-3 substrate Ac-DEVD-AFC (Pharmingen, San Diego, CA) at 37°C for 30 min. AFC hydrolysis was monitored by measuring flouroscence (excitation, 400nm emission, 500nm) in a Flouromax-3, Jobin Yvon Horiba spectrofluorometer.

Cytokine Assays: The RayBio Mouse Inflammation Array was purchased from RayBiotech (Norcross, GA) and used according to the manufacturer's instructions. Briefly, after blocking, membranes were incubated for 2 h with 250µg of brain tissue lysate taken from control, infected and PTX treated groups. The membranes were washed and then incubated with biotin-conjugated antibodies for 1 h. The membranes were washed again and incubated with horseradish peroxidase-conjugated streptavidin for 30 min, washed, and then developed. Densitometric analysis of each spot was done using ImageJ software (NIH).

Quantitative SYBR Green real-time PCR: Intron/exon spanning, gene-specific PCR primers specific for mouse *ICAM-1, VCAM-1, MSP-1* and *GAPDH* as a housekeeping gene control (Table 1) were designed for real-time PCR using Primer Express 1.0 software (Applied Biosystems). PCR standards for determining copy numbers of target transcripts were cloned into the pT257R/T vector (Promega). Linearized plasmids were quantitated by nanodrop method, and from 10^8copies, tenfold serial dilutions were performed for each plasmid. These dilutions resulted in generating a dynamic range from 10^8 to 10 copies/µl and served as standards for real time PCR. RNA from the brains of individual animals was used for real-time PCR assays. cDNA was synthesized using Taqman reverse transcription reagents (Applied Biosystems). Each 20µl amplification reaction mixture and Thermal cycling conditions were given in the table below (Table 2 & 3). A GAPDH fragment was amplified in triplicate reactions by real-time PCR on the same plate as the gene of interest. The mean concentration of GAPDH in each sample was used to control for integrity of input RNA and to normalize values of target gene expression to those of the housekeeping gene expression. The final results were expressed as the mean number of copies per 200 ng total RNA for ICAM, or VCAM relative to values obtained for GAPDH RNA.

The same strategy was employed to analyze the expression levels of MSP-1 in control, infected and PTX treated samples.

Table 1 (Primers used in this study)

PRIMERS	FORWARD	REVERSE
ICAM1	GCAAGTAGGCAAGGACCTCA	CAGCACCGTGAATGTGATCTC
VCAM1	TGGAGGAAATGGGCATAAAG	CTCTGCCTCTGT TTGGGTTCA
MSP1	GGATTAATGCACGCAATAAAT	ATTTTCTATTGGTTTTCTATAACC
GAPDH	CCTCAACTACAT CCTTTACT	GCTCCTGGAAGATGGTGATG

Table 2 (PCR composition-20µl)

10X PCR buffer	2µl
MgCl2	2µl
2.5mM dNTPs	2µl
Forward primer	0.2µl
Reverse primer	0.2µl
Genomic DNA	20ng
Taq polymerase	0.2µl
Water	11.4µl

Table 3 (Thermal cycling conditions)

94°C	2 min
94°C	30 sec
56°C	30 sec
72°C	1 min
74°C	10 min

30 cycles

Statistical analysis: The significance of observed differences among all the three groups was assessed by paired *t* test for cytokine assays or analysis of variance (ANOVA) for real-time PCR, Western immunoblot analysis, behavioral tests and caspase-3 activity assays. Analysis was

carried out using Sigma Plot 11.0 software. Values were considered to be significant when *P* values were <0.05.

Results

Treatment with PTX protects against severe *P. berghei* malaria: To determine whether treatment with PTX protects against severe *P. berghei* malaria, we infected two groups of mice with *P. berghei* and compared the survival of mice inoculated intraperitoneally on day 1 of infection with PTX to the survival of infected controls (Fig 1). The mice that received PTX at a dose of 60mg/kg body weight twice a day (*n=15*) exhibited significantly (*P*< 0.0001) increased survival after *P. berghei* infection compared with the survival infected controls (*n=15*). Infact the infected mice which received PTX at 60mg/kg body weight twice a day, have shown 70% survivality compared to infected controls which died by day 12 post infection (PI). To evaluate whether the protective effects of Pentoxifylline was dose dependent, we treated animals with half the fully protective Pentoxifylline dose (60 mg/kg i.p. once a day). This dose only delayed the disease onset and progression but did not offer complete protection as observed in mice treated with a dose of 80 mg/kg dose i.p. twice a day. To further evaluate the protective effect of PTX, we have administered the drug at a dose of 60mg/kg BW once a day starting from sixth day post-infection (after onset of cerebral symptoms). Even at this time point PTX was found to be protective since the median survivality of mice increased to 27.8 days. Further, we have taken up a scoring chart for the definition and severity of CM as per the clinical signs. According to this scoring chart we have tested 4 parameters with corresponding clinical sigs. Each clinical sign was given a severity score, ranging from normal = 0 to abnormal = 3. In our results infected mice received a better score indicating cerebral complications, whereas PTX treated mice have a low scoring pattern indicating normal ($p<0.01$).

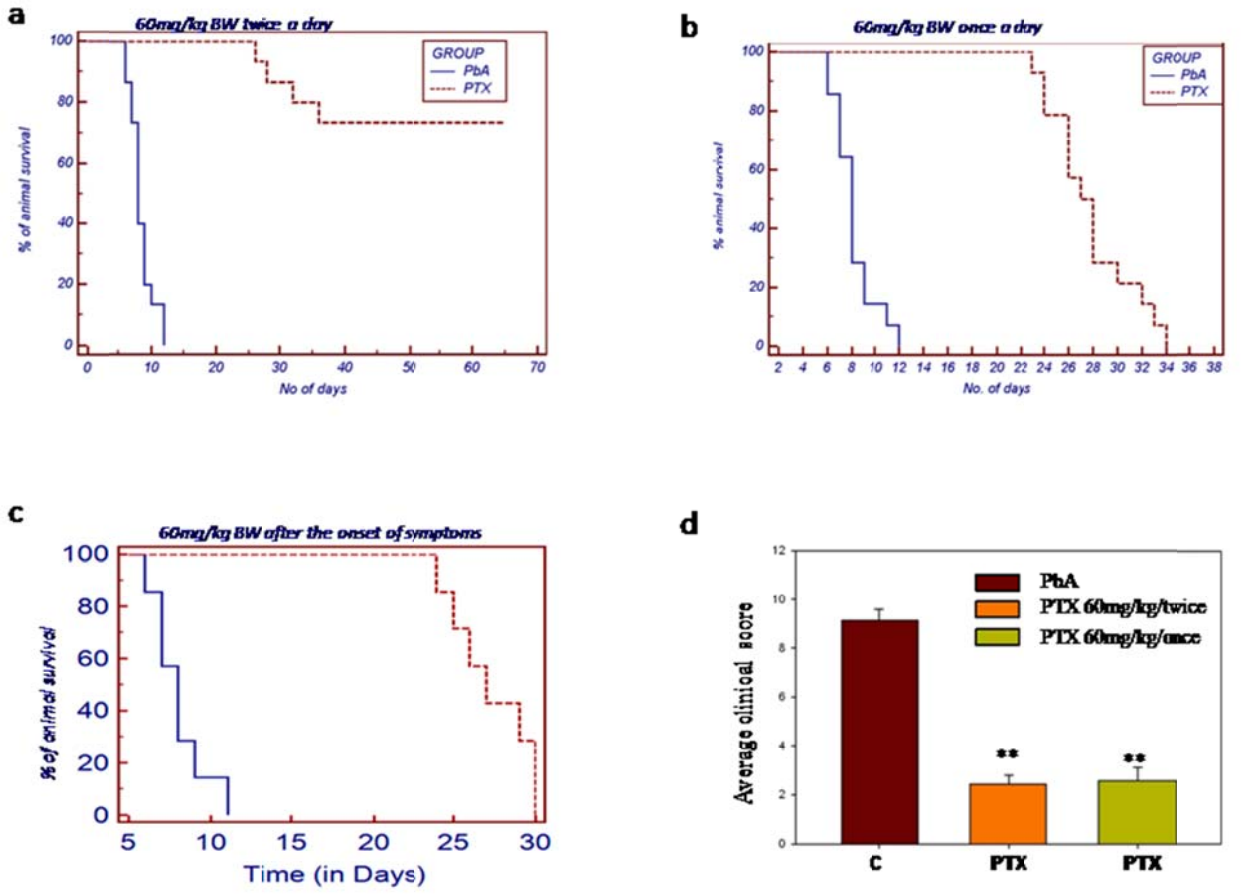


Fig 1: PbA infected mice treated with PTX twice a day conferred significant protection: Survival of ($p < 0.0001$) mice infected with 10^6 PRBC's of PbA was significantly improved in groups that received PTX (60 mg/kg body weights) treatment (n = 15 for each group) (a). PTX at a dose of 60 mg/kg body weight given once a day intra peritoneally delayed the mortality of animals (n=14) following PbA infection ($p < 0.0001$) (b). Survival of mice infected with 10^6 PRBC's of PbA was significantly improved ($p<0.0005$) in groups that received PTX from sixth day post-infection i.e after the onset of cerebral symptoms (60 mg/kg body weight). Mice receiving PTX showed significant reduction ($p<0.01$) in behavioral abnormalities when compared to infected controls.

Histopathological analyses: H&E staining of brain sections from control mice showed healthy parenchyma, with intact blood vessels with no hemorrhages. However, sections taken from infected mice showed distinct neurological lesions, with parenchymal microhemorrhages, disruption of vessel walls and adherence of leucocytes to the vessel walls. In contrast, SP600125 treated mice have a healthy parenchyma, clear vessels with little or no hemorrhage (Fig. 2).

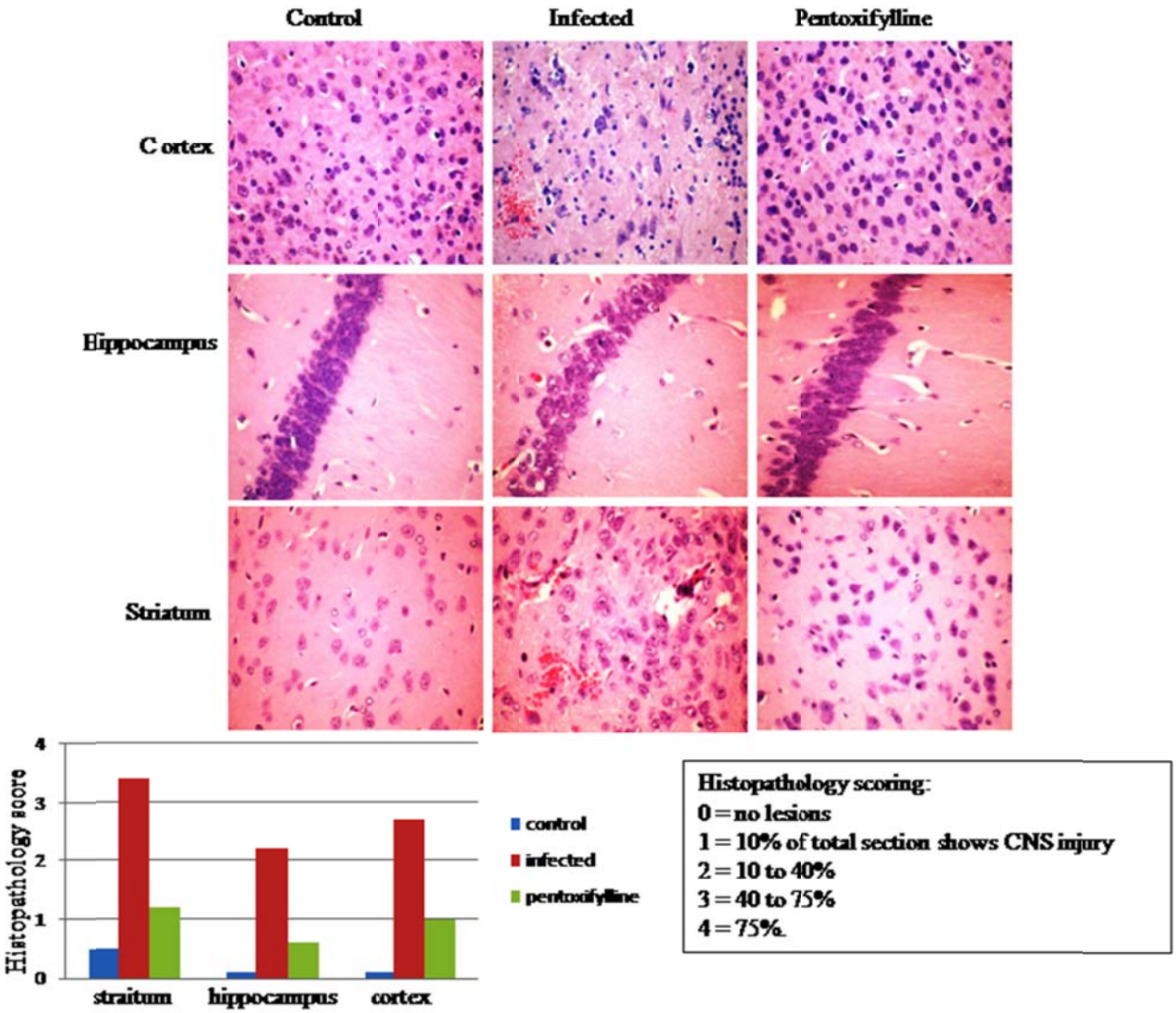


Fig 2 PTX treatment protects from PbA-induced CNS injury. Representative brain tissue samples were stained with hematoxylin and eosin in order to evaluate for evidence of histological damage. Samples represent four replicates of the experiment consisting of four mice per treatment group per replicate. Original magnification of images shown in panels 60X (B) Blind neuropathology scoring of histologic sections show significantly decreased injury in the PTX-treated mice compared to untreated, PbA-infected mice.

Pentoxifylline inhibits blood brain barrier disruption and inhibits lymphocyte infiltration: Brains taken from all the three experimental groups were assessed for the blood brain barrier integrity by Evan's Blue extravasation method. As shown in the figure 3 the brains of infected mice were stained intensely by Evans blue administered intravenously on day 7 after infection, indicating a widespread increase in vascular permeability and rupture of the BBB. In contrast, there was only little dye leakage in infected mice treated with PTX and the brain tissue looked similar to that of the uninfected control. Further as shown in Fig 3 (lower panels), cd4 and cd8 immunoreactivity

was robust in infected brain sections but not in control and PTX treated animals, indicating that cd4 and cd8 lymphocytes infiltrated the brain parenchyma in infected animals compared to uninfected controls. Moreover, this phenomenon is attenuated upon administration of PTX to the infected animals.

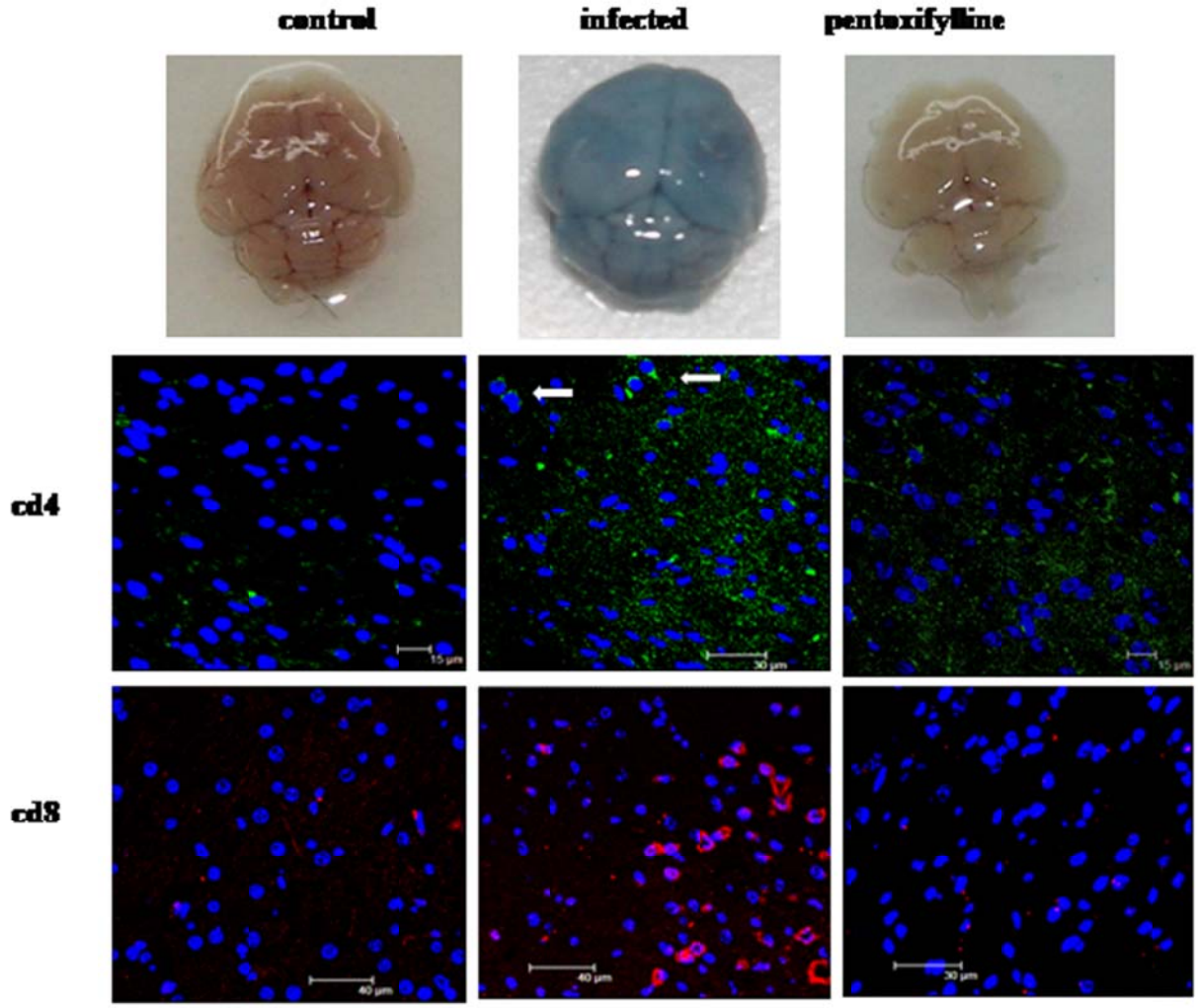


Fig 3: Blood–brain barrier damage of mice infected with PbA by Evan's blue dye exclusion test (Top 3 panels). Intravenous injection of Evan's blue results in the formation of a conjugate with serum albumin. Inclusion of the blue colored dye in the brain indicated the passage of the dye–protein conjugate into the brain that signifies increased permeability or damage of BBB. The picture showed here was selected from brain from different groups (control, infected and PTX treated) of three experiments. Representative Immunoflouroscent images of control, PbA infected and PTX treated brain sections incubated with cd4 (middle 3 panels) and cd8 (lower 3 panels). Note increased immunoreactivity in the brain sections taken from infected animals but not in control or PTX treated groups indicating cd4 (white arrows) and cd8 cell infiltration in infected mice was attenuated upon treatment with PTX.

Pentoxifylline inhibits TNF-α levels in the brain: Since PTX is a TNF-α inhibitor we first wanted to confirm the efficacy of PTX on TNF-α levels in our animal model. Brain tissue TNF-α levels in control, infected and from PTX treated animals were analyzed by Western Blotting. Infection of mice with *PbA* resulted in robust increase in TNF-α levels as compared to controls (Fig. 4). However administration of Pentoxifylline abolished the increased levels of TNF-α in infected animals implying that PTX inhibits TNF- α significantly. We further performed TNF-α immunohistochemistry on brain sections taken from control, infected and PTX treated animals. As shown in the figure 4, TNF-α positive cells were present significantly in *PbA* infected mouse brains but not in control or PTX treated animals. These results confirm that PTX administration in *PbA* infected animals attenuated the induction of proinflammatory cytokine TNF-α.

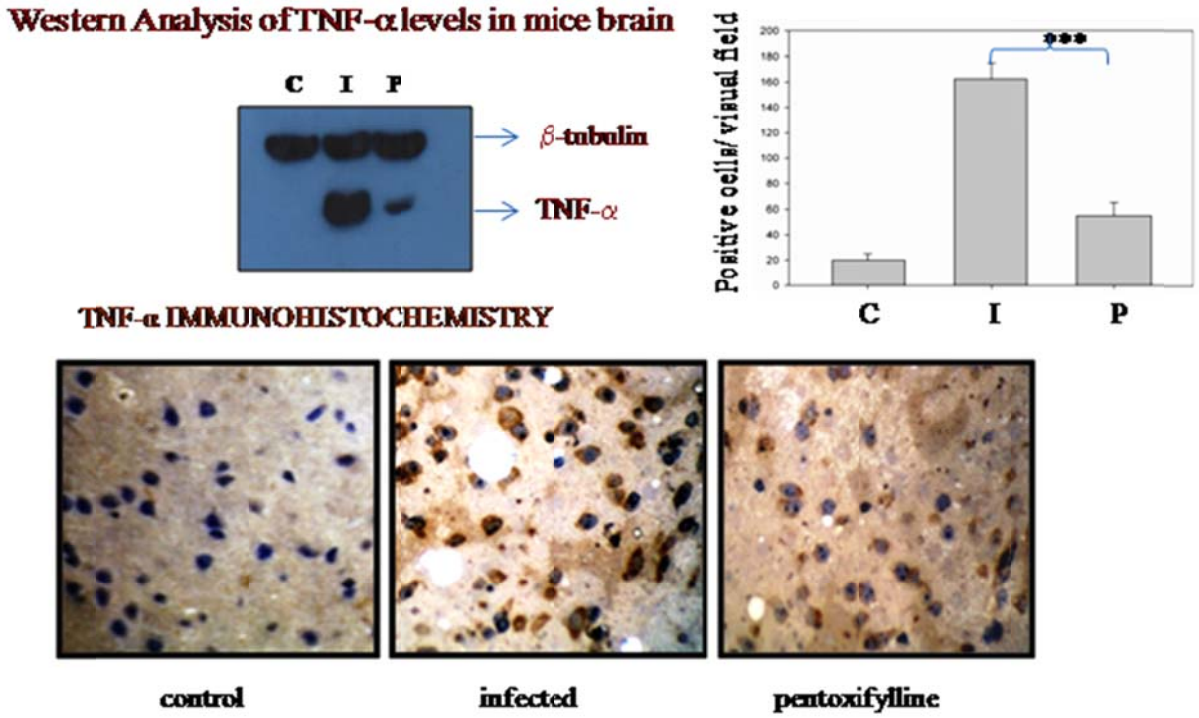


Figure 4: Immunoblot analysis (upper panel) of TNF-α in the whole brain lysates of control (C), infected (I) and PTX treated (P) samples. The result of β-tubulin was shown in the upper panel as an internal control for equal protein loading. The result is representative of four independent experiments with similar results. In control and PTX treated mouse brain sections TNF-α immunoreactivity was barely detectable (lower panels) while in infected brain sections there is a marked increase in TNF-immunoreactivity. The result is representative of four independent experiments with similar results. Magnification = 60X. Cell counting for TNF-α was performed in the striatum of control, infected and Pentoxifylline treated animals. Immunopositive cells were counted at 400 X magnification. Five visual fields within an area displaying TNF-α immunoreactivity were counted and expressed as average number (mean) per visual field (vertical bar graph).

Pentoxifylline has no effect on parasitemia, or tissue parasite load: We further wanted to check whether or not; the protective effect of PTX was conferred on the mice by inhibiting the growth of parasites. To this end we have checked the parasitemia levels of mice infected with *PbA* and PTX treated mice. Parasitemia was assessed from Giemsa-stained thin smears of tail blood prepared every day post inoculation (PI). Parasitemia levels of both Infected and PTX treated mice were found to be almost similar (Fig 5 a) indicating that PTX has no effect on parasitemia. To further assess whether or not PTX modulates the tissue parasite load we checked brain tissue parasite load by Real time PCR using primers directed against MSP1 (Merozoite Surface Protein 1) (Fig 5 b). Further we have checked the expression levels of ICAM and VCAM which mediate the binding of the parasite to the host endothelial cells lining the blood vessels (Fig 5 c).

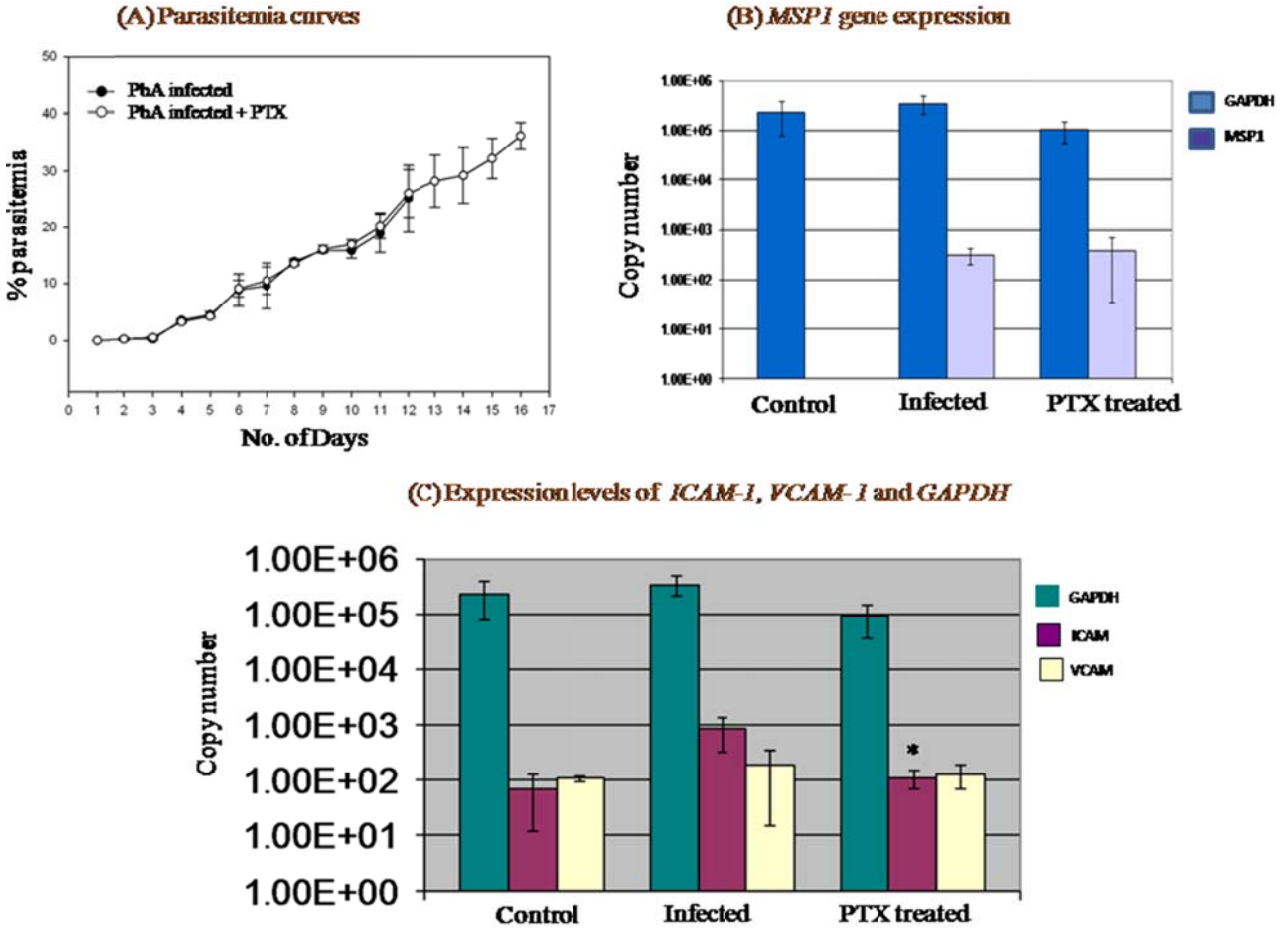
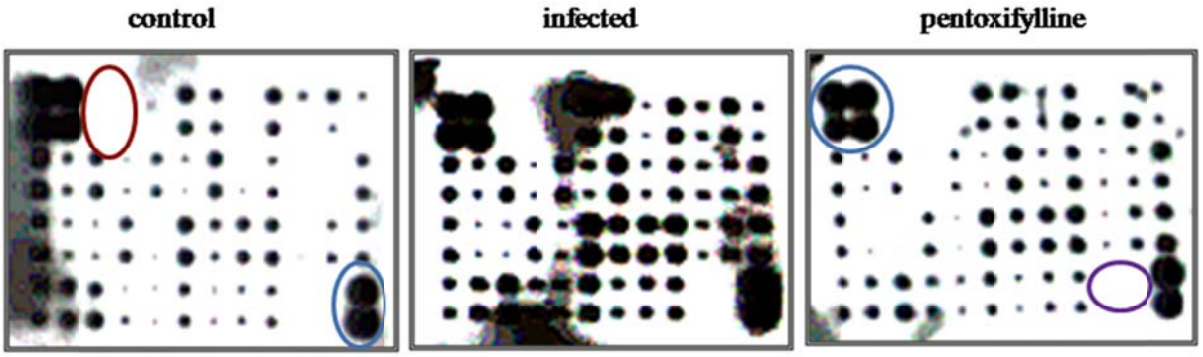


Fig 5 Parasitemia levels (A) of mice infected with *PbA* and PTX treated mice remained same as assessed by Geimsa-stained thin smears of tail blood prepared every day post inoculation. Further we observed no significant differences in tissue parasite load between infected and PTX treated groups (B). Further we observed an increase in ICAM1 transcript levels in infected mice, which was significantly attenuated ($p<0.05$) upon administration of PTX, However, we didn't find much difference in VCAM levels in all the three groups (C).

Pentoxifylline inhibited the up regulation of inflammatory cytokines: We next wanted to confirm whether the infiltration of leucocytes and gliosis leads to the production of proinflammatory cytokines and if so whether administration of PTX attenuates the release of these cytokines. To this end we checked the levels of various cytokines and chemokines by RayBio Mouse Inflammation membrane based antibody Array (Fig 6 top 3 panels) and quantified by densitometry using ImageJ software. We found that at least 21 different cytokines and chemokines (shown by asterisks in the lower panel) involved in inflammation, trafficking and homing of different cells including neutrophils, lymphocytes, macrophages and monocytes; are up regulated in the infected brain tissue and were significantly attenuated upon the administration of PTX ($p<0.05$).



	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	Blank	BLC	CD30 L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
2	POS	POS	NEG	NEG	Blank	BLC *	CD30 L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
3	GM-CSF	IFNγ	IL-1α *	IL-1 β *	IL-2 *	IL-3 *	IL-4 *	IL-6	IL-9	IL-10 *	IL-12p40p70 *	IL-12p70
4	GM-CSF	IFNγ	IL-1α *	IL-1 β *	IL-2	IL-3 *	IL-4 *	IL-6	IL-9	IL-10 *	IL-12p40p70 *	IL-12p70
5	IL-13	IL-17	I-TAC	KC	Leptin *	LIX *	Lymphotactin *	MCP-1 *	MCSF	MIG *	MIP-1α *	MIP-1γ
6	IL-13	IL-17	I-TAC	KC	Leptin *	LIX *	Lymphotactin *	MCP-1 *	MCSF	MIG *	MIP-1α *	MIP-1γ
7	RANTES	SDF-1	TCA-3 *	TECK *	TIMP-1 *	TIMP-2 *	TNFα *	sTNF RI *	sTNF R II *	Blank	Blank	POS
8	RANTES	SDF-1	TCA-3 *	TECK *	TIMP-1 *	TIMP-2 *	TNFα *	sTNF RI *	sTNF R II *	Blank	Blank	POS

Fig 6 Murine whole brain tissue lysates from control, infected and PTX treated samples were collected and assayed for cytokine/chemokine production using the Mouse inflammation Array I (RayBiotech, Norcross GA). Shown in this figure (top 3 panels) are scans of films developed from array filters following incubation with tissue lysates from control, infected and PTX treated samples. The pattern of cytokines, chemokines, growth factors, and inflammatory products printed on the array support was given below (lower panel): spots encircled by blue color indicate positive controls, red indicates negative color and purple indicates blank.

Pentoxifylline inhibits the proinflammatory mediators COX2 and iNOS: Protein lysates from the whole brain of control, infected, and PTX treated mice were used to perform western blots for COX2 and iNOS. We found significant increases in the expression pattern of COX2 and iNOS in infected mice brains only but not in control and treated groups indicating that PTX inhibits the overproduction of COX2 and iNOS. This was further confirmed by immunostaining the sections taken from all the three groups as shown in the Fig 7 (lower panels).

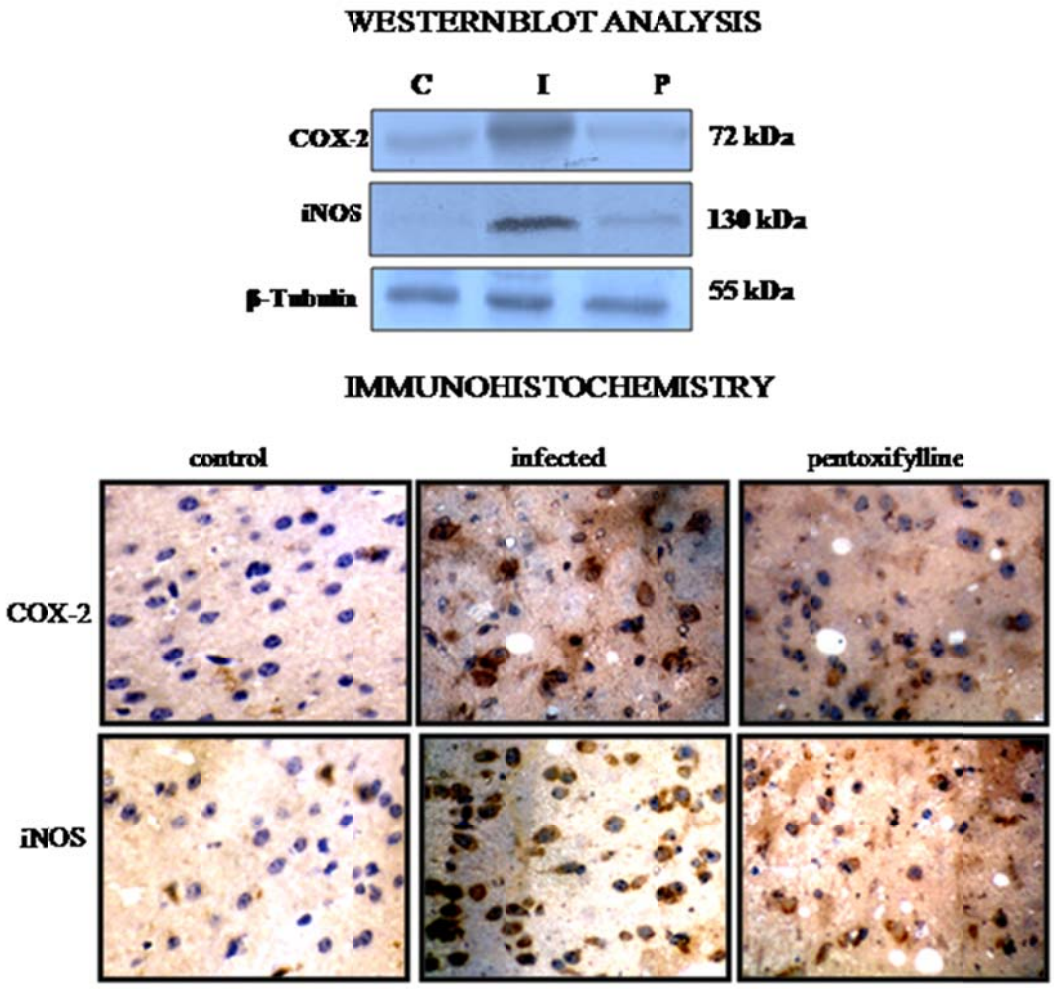


Fig 7 Western blot analysis of COX-2 and iNOS in the control, infected and PTX treated mice brains (upper panel). The result is representative of four independent experiments with similar results. The lanes C, I and P indicates uninfected control, PbA infected mice brains and PbA infected mice brains treated with PTX respectively sacrificed on day 10 PI respectively. In control mouse brain sections COX-2 and iNOS immunoreactivity was barely detectable. However, in infected mouse brain sections there is a robust increase in COX-2 and iNOS immunoreactivity which was attenuated upon administration with PTX (lower 6 panels). Pictures were taken at 60X magnification.

Pentoxifylline modulate the expression pattern of several key proteins associated with stress and apoptosis: Western blot analysis demonstrated a significant inhibition in the expression of different stress related proteins whose levels were elevated following PbA infection, upon PTX treatment (Fig. 8). There was a significant increase in the expression of phospho-p38 MAPK, phospho-Jun-N-terminal kinase, and phospho-ERK in PbA infected mice when compared with control. But Pentoxifylline treatment dramatically reduced their levels. Interestingly, the significant decrease in p-AKT expression following PbA infection was up-regulated in animals that received PTX treatment.

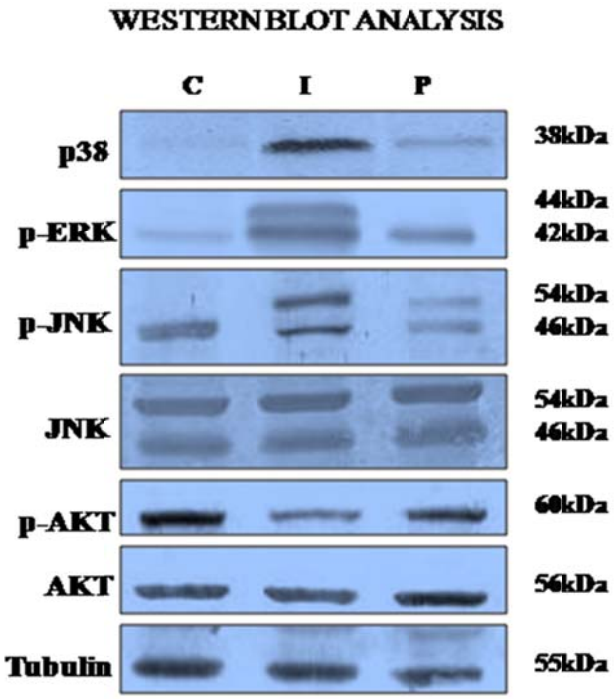


Fig 8: Western blot analysis of phospho-p38, phospho-ERK, phospho-JNK, JNK, phospho-AKT and AKT in the control, infected and PTX treated mice brains. β-Tubulin was probed as a loading marker for equal protein loading.The result is representative of four independent experiments with similar results. The lanes C, I and P indicates uninfected control, PbA infected mice brains and PbA infected mice brains treated with PTX respectively sacrificed on day 10 PI respectively

Further, protein lysates from the whole brain of control, infected, and PTX treated mice were used to perform western blots for active caspase 3, Bax, Bcl-2, and also assayed for caspase 3 activity as an index of apoptosis. There was a profound increase in active caspase 3 and Bax expression in PbA infected mice when compared with control and treated mice. The expression of Bcl-2 was reduced in infected brain compared with control and PTX treatment increased the expression of this antiapoptotic protein (Fig. 9 a). Active caspase 3 activity was increased within

the CNS after infection compared with control animals (Fig. 9 b & c). PTX treatment significantly abrogated the increase in caspase 3 activity ($p<0.001$). These experiments demonstrated that treatment with PTX could significantly rescue neuronal apoptosis and signaling events associated with cell death. Further caspase 3 immunostaining (Fig. 9 d, e & f) of sections taken from control, infected and PTX treated animals revealed caspase 3 positive cells in infected brain sections but not in control and PTX treated groups indicating the protective effect of PTX in cerebral malaria.

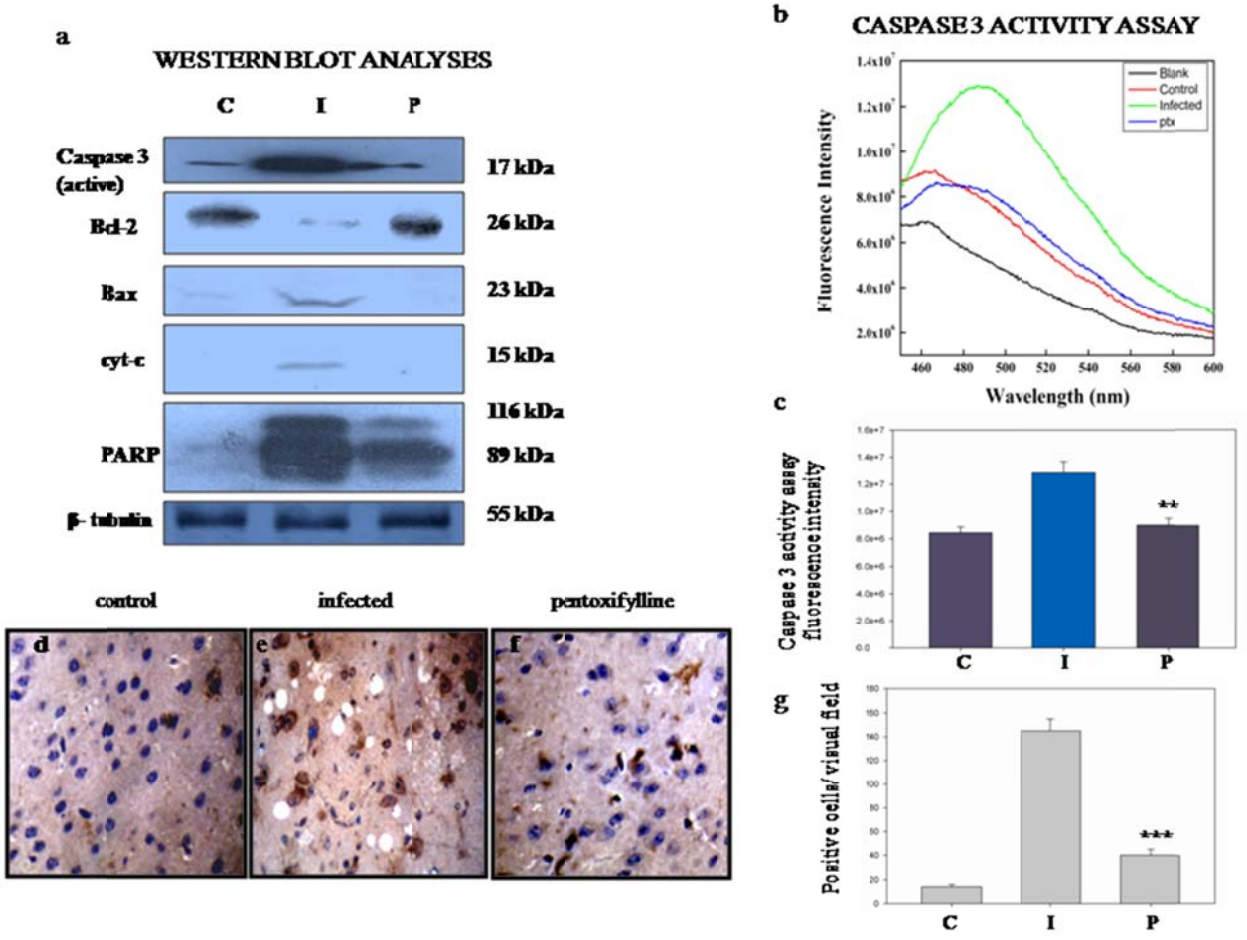


Fig 9 Western blot analysis (a) of caspase-3 (active), BCL-2, BAX, Cyt-c & PARP in the control, infected and PTX treated mice brains. β-Tubulin was probed as a loading marker for equal protein loading. Cleaved caspase 3 activity as measured by a fluorometric assay demonstrates significantly decreased caspase activity in PTX treated samples ($p<0.001$) when compared to PbA infected samples. In control and PTX treated mouse brain sections caspase-3 immunoreactivity was barely detectable (lower panels) while in infected brain sections there is a marked increase in caspase-3 immunoreactivity. The result is representative of four independent experiments with similar results. Magnification = 60X. Cell counting for caspase-3 was performed in the striatum of control, infected and Pentoxifylline treated animals. Immunopositive cells were counted at 400 X magnification. Five visual fields within an area displaying caspase-3 immunoreactivity were counted and expressed as average number (mean) per visual field (vertical bar graph).

Pentoxifylline inhibits neuronal cell death: Since caspase-3 is implicated in neuronal cell death in many neurodegenerative disorders we next investigated the role of active caspase-3 on the viability of neurons using Flouro-Jade B a novel fluorescent stain that specifically binds to dying neurons. To this end, we have first confirmed neuronal viability by staining the sections with Flouro-Jade B. As shown in the Figure 10, control (a) and PTX treated (c) mouse brain sections stained very little for Flouro-Jade B, where as in infected mouse brain sections (b) a robust increase in Flouro-Jade B staining cells was observed. This sort of staining pattern clearly indicates that neuronal cell death is wide spread in infected mouse brain sections as compared to control and PTX treated animals. Moreover, colocalisation studies of caspase 3 (d) with Fluoro-Jade B (e) on infected mouse brain sections (Fig 20B) clearly established that caspase 3 is up regulated in degenerating neurons following *PbA* infection. Together, these results proved unequivocally that PTX inhibits neuronal cell death in murine cerebral malaria.

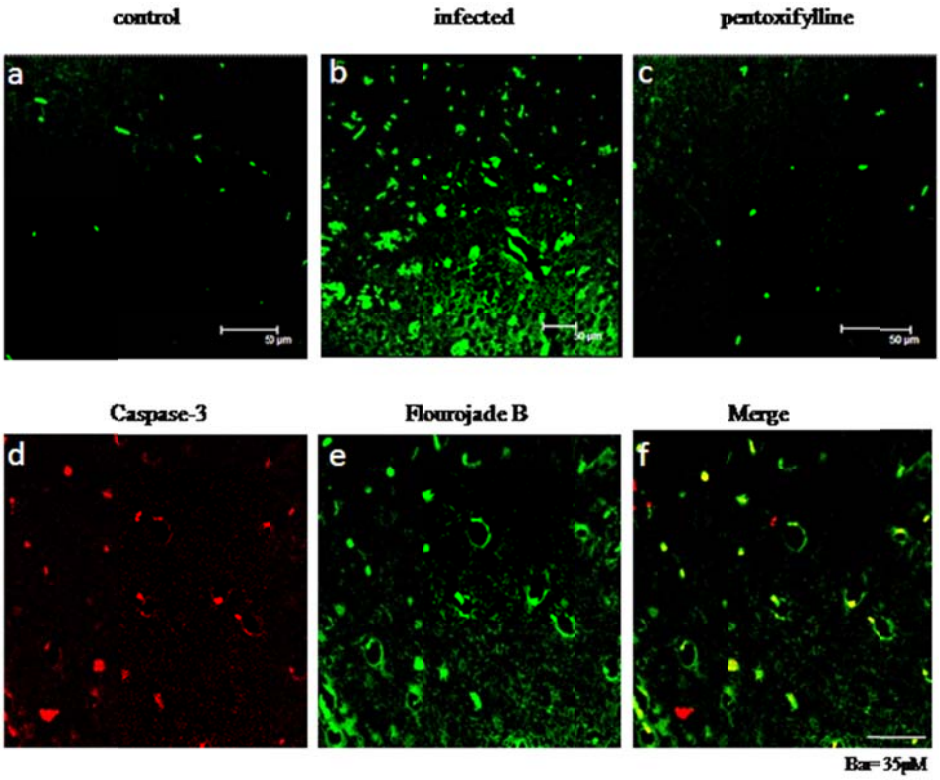


Fig 10: Paraffin embedded sections of both control (paned a), PbA infected (panel b) and treated (panel c) mouse cortical sections were stained for Flouro-Jade B. While control and treated sections (panels d and f) have very few Flouro-Jade B positive cells, infected sections (panel b) have significantly higher number of Flouro-Jade B positive cells indicating neuronal cell death. Double label staining of active caspase-3 (d) and Flouro-Jade B (e) showed that caspase-3 positive cells were also positive for Flouro-Jade B (f). The photomicrographs shown in this figure are representative of four independent experiments with similar results.

Discussion

The detailed pathophysiology of cerebral malaria remains far from completely resolved and contributes to high mortality rate especially among children. The pathological hall marks of CM include cerebral capillaries packed with parasitized erythrocytes, hemorrhages, global hypoxia-ischemia, increase in brain lactate levels and glial activation and proliferation. In addition, focal accumulation of proinflammatory cytokines in areas of brain with massive sequestration could be a major cause of neuronal cell death in cerebral malaria. In fact a central role for TNF-α in the pathological manifestation of the disease has been proposed. Moreover, extensive endothelial cell death, massive infiltration of lymphocytes into the brain parenchyma, raise in intracranial pressure due to cerebral oedema and activation of several stress and apoptotic pathways contribute to CM pathology. In this connection we were in search for drugs which could mitigate, if not all, atleast few of the above said processes that contribute to the disease. Pentoxifylline has been shown to have neuroprotective properties in diverse models of neurodegeneration and CNS injury and is of potential interest in the treatment of vascular diseases, in general, and of stroke, in particular, because of their vasodilating properties (Banfi *et al.*, 2004; Kruuse *et al.*, 2000; Vakili and Zahedi khorasani, 2007). The decision to use Pentoxifylline in our model is based on the fact that it is a potent TNF-α inhibitor. Further Pentoxifylline has been shown to reduce oxidative stress, downregulation of cell adhesion molecules on endothelial cells such as ICAM, VCAM and E-selectin. Moreover PTX also inhibits the activation and release of proinflammatory cytokines from microglia (Chao *et al.*, 1992). Further in earlier studies it was shown that Pentoxifylline was shown to protective in cerebral malaria and infact in atleast one study it was shown that administration of PTX attenuates hippocampal neuronal cell death in experimental cerebral malaria. However the mechanism by which PTX mitigates the disease is not known. So in order to gain mechanistic insights as to how PTX attenuates neuronal cell death we administered PTX in PbA infected C57BL/6J mice. The decision to use PTX at a dose of 60 mg/kg body weight was taken based on the earlier reports which confirm the inhibitory effects of PTX in mice (Kremsner *et al.*, 1991). Infact, our results were in consistent with earlier reports where in administration of PTX in PbA infected mice increased the survivality significantly (Kremsner *et al.*, 1991). However, in this study we decided to use PTX at a dose of 60 mg/kg body weight twice a day because of its short

half-life as revealed in a study after examining the detailed pharmacokinetics of PTX in mice (Wyska *et al.*, 2007). To assess whether PTX reduces neurological lesions and peticheal hemorrhages that are so frequent in PbA infected mice brain (Lackner *et al.*, 2006), we performed H & E staining of the brain sections taken from all the three groups, *viz*., control, infected and PTX treated mice. We selected the three different neuroanatomical regions *viz.*, cortex, striatum and hippocampus because they are involved in movement and coordination and infact PbA infected mice shows defects in movement and coordination with symptoms ranging from but not limited to stupor, hemiplegia, dysplegia, paraplegia coma and finally death. In our results we found more number of neurological lesions and irreversible neuronal damage in striatal regions and therefore we restricted our immunohistochemical studies mainly to striatum. Further treatment with PTX showed reduced hemorrhages, healthy parenchyma, very few to no neurological lesions and almost no cells indicating apoptotic morphology. Further infected mice which received PTX showed no behavioral abnormalities indicating that PTX is neuroprotective, with no hemorrhagic foci in the brain and increases survivality in mice.

Excessive synthesis of TNF-α during the pathology of cerebral malaria can be harmful to the host. In fact Grau *et al.*, has proposed that TNF-α is a central mediator in the pathological manifestation of the disease (Grau *et al.*, 1987). Although the exact mechanisms by which TNF-α mediates the neurologic changes that characterize CM remain obscure, it was shown that TNF-α up-regulates the expression of cell adhesion molecules on the endothelium lining the blood vessels in the brain. In this connection we first checked whether PTX attenuates the excessive production of TNF-α in the murine brain infected with PbA. Our results suggest that PTX effectively inhibited the excess production of TNF-α. Further, inhibition of TNF-α is also accompanied by the attenuation of ICAM1 expression which is the major docking site for the parasitized erythrocytes in the brain micro-capillaries. Since sequestration is complex process which involves upregulation of endothelial cell adhesion molecules and binding of parasitized erythrocytes to these molecules, we next wanted to check whether PTX has any effect on parasitemia and tissue specific parasite load. Further the protective effect of PTX that we observed in PbA infected mice could also be due to its inhibitory effect on the development of parasite. To rule out this scenario we have compared parasitemia of PTX treated mice with that of PbA infected animals. Our results suggest that PTX has no effect on the parasite as the parasitemia levels of both infected and treated groups were similar. Further we checked MSP1

expression levels (which is a marker for blood stage parasites), and our Real Time PCR results suggest that MSP1 expression levels in the brain taken from infected and treated animals are same suggesting that PTX does not affect sequestration.

One of the major findings in this study is that administration of PTX in a mouse model of cerebral malaria markedly reduces the influx of inflammatory cells into the region of injury. By this mechanism, PTX attenuates the production of proinflammatory cytokines, which in turn, results in a much smaller volume of injury. To this end we first checked the disruption of blood brain barrier (BBB) in mice infected with PbA. As shown in our results disruption of blood brain barrier is extensive in PbA infected mice brains whereas PTX administration inhibited the BBB disruption. Further in our model, disruption of BBB also leads to the infiltration of inflammatory cells specifically cd4 and cd8 T cells which are earlier implicated in the pathology of cerebral malaria. PTX administration, on the other hand, totally reversed the inflammatory cells infiltration into the brain parenchyma and associated proinflammatory cytokine production. In fact, results from our RayBio Tech inflammatory antibody array suggest that at least 21 different cytokines/chemokines which are up regulated in the brain during PbA infection are significantly attenuated upon administration of PTX in PbA infected mice suggesting that PTX is immunomodulatory. Our results are in agreement with earlier observations where in PTX was found to be immunomodulatory (Rieneck *et al.*, 1993).

Results from our lab and also elsewhere show that PbA infection is accompanied by profound neuronal apoptosis (Anand and Babu, 2011; Lackner *et al.*, 2007; Potter *et al.*, 2006; Shukla *et al.*, 2006; Wiese *et al.*, 2006). In addition to immunomodulatory role, PTX also has been shown to exert anti-apoptotic effects by inhibiting caspase-3, and inhibiting the release of cytochrome c from mitochondria (Jean Harry *et al.*, 2003; Okamoto, 1999). These actions likely contributed to our *in vivo* finding of reduced caspase-3 activity as well as in our immunohistochemistry findings (Flouro-Jade B staining) showing reduced apoptosis. Furthermore caspase-3 positive cells also stained positive for Flouro-Jade B, implicating that caspase-3 is involved in neuronal cell death in PbA infected mice brains. All these effects were reversed upon administration of PTX in PbA infected mice indicating that PTX interferes with caspase-3 induced neuronal cell death. Further we also observed the upregulation of stress activated protein kinases in PbA infected murine brain. Administration of PTX, however, resulted in attenuation of pro-apoptotic

stress activated protein kinases indicating that PTX also modulates the expression of these molecules. In short PTX was found to be neuroprotective in our model and could be an attractive candidate for clinical assessment because it might alter the course of the disease when given along with standard anti-malarials.

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Synopsis of the thesis entitled

JNK and ER stress signaling pathways mediates neuronal cell death in Experimental cerebral malaria: Role of SP600125 and Pentoxifylline as neuroprotective agents.

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Introduction

Malaria remains one of the world's greatest public health challenges and remains prevalent in tropical areas of Americas, many parts of Africa, Indian subcontinent, and also in South East Asia. According to WHO estimates, 90% of the global disease incidence was borne by Africa alone. Cerebral Malaria (CM) is a severe neurological complication of *Plasmodium falciparum* infection involving sequestration of parasitized red blood cells (PRBCs) in the cerebral microvasculature. The adhered PRBCs interact with the endothelial cells lining the vasculature leading to the dysfunction of blood brain barrier (BBB) resulting in infiltration of inflammatory cells into the brain parenchyma, release of proinflammatory cytokines, glial activation and neuronal cell death. The pathophysiological consequences of CM is poorly understood since most of the information regarding cerebral malaria is from post-mortem analyses, which cannot tell much about the sequence of events leading to the neuronal cell death. C57BL/6J mice infected with asexual stages of *Plasmodium berghei* ANKA (PbA) is the most widely accepted model for studying cerebral malaria and have been shown to develop similar neurological complications as that of humans. Further PbA infected mice develop neurological complications such as ataxia, hemiplagia, seizures, paralysis and coma by day 5 post infection (PI) and usually die by day 6-9 PI often with low parasitemias not exceeding 15%. The importance of cytokines, especially TNF-α and IFN-γ, and their contribution to severe malaria have been extensively surveyed (Grau et al 1987; Hommel 1996; Miller 1994). High circulating levels of TNF-α and IFN-γ are more often found in patients with severe malaria than in uncomplicated cases (Kwiatkowski et al. 1990). Extensive deposition of TNF-α, IFN-γ, IL-1, IL-6 in organs with massive sequestration

(especially in brain) is more frequently seen in patients who died of CM. TNF is also raised in placental malaria and is associated with low birth weight (Fried et al. 1998; Moorman et al. 1999). Grau and colleagues hypothesized that excessive TNF-α production plays a decisive role in the pathogenesis of murine CM (Grau et al., 1987). This fact is backed by subsequent findings: (i) elevated levels of serum TNF-α is found only at the time of the neurological complications; (ii) a single injection of anti-TNF-α antibody on day 4 or 7 fully protected infected mice from cerebral malaria without modifying the parasitemia and (iii) injection of recombinant TNF-α to a CM-resistant strain of mouse makes it susceptible to CM. In addition, de Kossodo and Grau (1993) found decreased levels of IL-4 (which antagonizes the effects of TNF-α) along with upregulation of TNF-α mRNA in the brains of CM-susceptible mice suggesting a role for TNF-α in the pathology of CM. Further it was found that in tumor necrosis factor receptor 2 (TNFR2) deficient mice are resistant to CM (Lucas et al. 1997). However in another study, infected mice were not protected even after administration of TNF-α neutralizing antibody (Hermsen et al., 1997). In a study involving 178 Gambian children, Kwaitowski et al (1990), found that plasma TNF-α level in fatal cases involving CM were at least 10 fold higher than their normal counterparts. This suggests that excessive TNF-α production during the pathology of CM makes humans susceptible to neurological manifestations and its fatal outcome. Paradoxically, in another study involving 600 Gambian children it was found that neurological sequelae increased after the administration of TNF-α antibody suggesting that the antibody may act to retain TNF within the circulation and thereby prolong its effect (van Hensbroek et al., 1996). TNF-α produced by monocytes or glia enhances the release of cytokines, ROS, nitric oxide,

superoxide production and potentiates glutamate receptor induced neurotoxicity. All these factors have been implicated in the pathogenesis of cerebral malaria. Further, the proinflammatory cytokine TNF- α, when produced in excess also upregulates the expression of cell adhesion molecules, ICAM and VCAM on the endothelial cells lining the brain microvasculature. This results in the congestion of blood vessels, as ICAM and VCAM mediate the binding of PRBC to the endothelial cells; leading to marked decrease in cerebral blood flow and neuronal damage at advanced stages. Therefore, the search is on for compounds which are cheap, easily available and with no tolerable side effects combined with a protective potential when administered several hours after infection.

Central nervous system is the major site of complications during malaria infection:

According to immunopathogenesis hypothesis host toxic mediators, directed against the intraerythrocytic form of the parasite, could cause non-specific tissue damage due to their untargeted mechanism of action (Clark et al., 1981). Due to this non-specific mode of action the CNS vascular endothelium is damaged resulting in cerebral oedema and haemorrhage ultimately leading to coma and death. Indeed cerebral microvascular injury is a common phenomenon of both murine CM and human CM (Brown et al., 1999; Chan-Ling et al., 1992; Thurnwood et al., 1988; Neill and Hunt 1992). Supporting evidence put forward by Medana et al., suggests that astrocytes and microglia are actively involved in the development of the cerebral complications associated with malaria infection. Accordingly BBB disruption during the pathogenesis of CM results in the release of cytokines, malarial toxins and immune cells into the brain parenchyma thereby locally altering the immune and supportive functions of astrocytes and microglia. Activated

astroglia and microglia in turn produce proinflammatory mediators and toxins which are detrimental to the neurons leading to CNS dysfunction (Medana et al., 2001). In the present study, we have addressed the possible role of ER stress signaling and JNK signaling in mediating neuronal cell death in an experimental model of cerebral malaria. We further tested the neuroprotective efficacy of SP600125 (a specific JNK inhibitor) and Pentoxifylline (TNF-α inhibitor and immunomodulatory agent) in a murine model of cerebral malaria.

The objectives of this work include:

1) To study whether ER stress signaling pathway is activated in experimental cerebral malaria (ECM).
2) To study whether JNK signaling pathway is activated in ECM.
3) To study whether administration of SP600125 (a specific JNK inhibitor) is neuroprotective in ECM.
4) To study whether administration of Pentoxifylline (TNF-α inhibitor and immunomodulatory agent) is neuroprotective in ECM.

ER stress and Neurodegeneration in cerebral malaria: PERK- p-eIF2α axis is activated and mediates neuronal cell death in cerebral malaria:

The Endoplasmic reticulum (ER) is a cell organelle which plays a pivotal role in the synthesis, folding, posttranslational modifications and trafficking of secretory and membrane proteins, calcium storage and release, lipid biogenesis and apoptosis. Perturbations in ER functioning may lead to accumulation of misfolded proteins in the ER lumen and ER in turn initiates an adaptive response known as the unfolded protein

response (UPR) that protects the cell against the accumulation of misfolded proteins. However, if the ER stress is excessive and prolonged, these adaptive responses fail to compensate and UPR leads to cell death by both caspase-dependent and caspase-independent pathways. UPR is initiated by the binding of ER chaperone BiP/GRP78 to the misfolded proteins. Under normal physiological conditions, BiP forms a complex and suppress the activity of three proximal ER-resident stress sensors: PKR-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). During ER stress, BiP binds to misfolded proteins thereby releasing PERK, IRE1 and ATF6 from the complex. Upon its release from BiP, PERK is activated by homodimerization and autophosphorylation, and subsequently the kinase domain of PERK phosphorylates eIF2α (peIF2α) thereby shutting down global protein synthesis. However, phosphorylation of eIF2α can also lead to selective translation of activating transcription factor 4 (ATF4) which in turn activates the transcription of pro-survival genes. IRE1 is activated by dimerization and *trans*-autophosphorylation upon its release from BiP. Activated IRE1 splices *XBP1* mRNA, resulting in a translational frameshift and formation of potent transcriptional activator, inducing the transcription of ER stress responsive genes. Following its release from BiP, ATF6 is transported to Golgi, where it is cleaved by site-1 and site-2 proteases, generating a 50kDa transcription factor (ATF6p50) that translocates to the nucleus thereby inducing the transcription of ER stress genes. Further the role of ER stress pathways in mediating neuronal cell death has been well documented in several neurodegenerative diseases like Alzheimer's and Parkinson's disease, Amyotrophic Lateral Sclerosis, Transmissible Spongiform Encephalopathies and ischemia (Lindholm et al., 2006). The present study examines the role of ER stress

proteins in modulating neuronal cell death in ECM, with particular emphasis laid on PERK-eIF2α axis.

Salient findings:

- ✓ Histopathological analysis of brain tissue sections taken from control and infected mice showed that apoptotic neuronal cell death is widespread in mice infected with PbA. This fact is further supported by induction of proapoptotic mediators like caspase-3, caspase-7, caspase 12, Bax and suppression of antiapoptotic Bcl-2 in CM infected brains.
- ✓ All the three branches of ER stress signaling pathway (PERK, IRE1 and ATF-6) is activated in the brains of mice infected with PbA.
- ✓ Western blot analysis and immunohistochemical analysis of brains taken from control and infected mice revealed that p-eIF2α is upregulated and is localized in neurons.
- ✓ Further, the downstream effectors of PERK-eIF2α axis *viz.* ATF4 and CHOP were upregulated and are localized in neurons in PbA infected brains.
- ✓ Flouro jade B (a fluorescent marker which detects dying neurons) staining of sections taken from both the groups revealed that Flouro jade B positive cells are present in only infected brain but not in control brains. Further p-eIF2α was found to be colocalized with Flouro Jade B positive cells indicating that p-eIF2α plays a crucial role in neuronal cell death in ECM.
- ✓ From this study we conclude that PERK-eIF2α axis of ER stress signaling pathway is activated and mediates neuronal cell death in murine cerebral malaria.

c-Jun N terminal kinases (JNK) are activated in the brain during the pathology of Experimental Cerebral Malaria.

c-Jun N-terminal kinases (JNK) are serine/threonine protein kinases that phosphorylate serine 63 and 73 at the N-terminal domain of c-Jun thus activating the transcriptional activity of AP-1 (Karin et al. 1997; Davis 2000). JNK signalling is activated by various stress stimuli such as UV and ionizing radiation, heat shock, inflammatory cytokines, metabolic inhibitors, and osmotic or redox shock (Davis, 2000). There are three different isoforms of JNK with 10 different splice variants. Of these JNK 1 and 2 are ubiquitously expressed and have a critical role in neural development, while JNK 3 is restricted to neural and cardiac tissues and is more closely involved in stress-induced neuronal apoptosis. JNK is activated by dual phosphorylation of Thr 183 and Tyr 185 situated in the activation loop by the upstream JNKK1/MKK4/SEK1 and JNKK2/MKK7. Once activated JNK phosphorylates a variety of transcription factors like c-Jun, ATF2, Elk, p53 and c-Myc and also members of Bcl-2 family (Bim, Bcl-2, Bcl-xL and BAD) (Liu and Lin 2005). Phosphorylated c-Jun forms homo or hetero dimers forming AP-1 transcription factor which then regulates the expression of genes in response to a variety of stimuli such as cytokines, growth factors, stress, and bacterial and viral infections. In addition, phosphorylation of c-Jun and subsequent AP-1 mediated gene expression is a key event that mediates neuronal apoptotic processes since blockade of c-Jun activity by antisense oligonucleotides attenuated neuronal cell death in primary rat hippocampal cultures (Schlingensiepen et al. 1994). While there is overwhelming evidence of involvement of JNK pathway in the neuronal cell death in several neurodegenerative diseases like Alzheimer's, Parkinsons's disease and stroke (Borsello and Forloni 2007),

little is known of its involvement in cerebral malaria. In the current study we addressed the possible role of JNK in the induction of neuronal cell death during ECM.

Salient findings:

- ✓ Western blot analysis using antibodies against phosphorylated forms of MKK4, JNK, c-Jun and nonphosphorylated form of JNK, during the course of infection revealed that JNK signaling pathway is activated in the brains of terminally infected mice.
- ✓ Immunohistochemistry of brain sections taken from *PbA* infected animals or uninfected controls, confirm the induction of p-JNK that was so evident in our western blot analysis.
- ✓ Double immunoflouroscent analysis of p-JNK with MAP2, a neuronal marker; revealed neuronal induction of p-JNK in infected mouse brain sections, implying that neurons are the principal cell types in which p-JNK is activated in murine cerebral malaria.
- ✓ Flouro jade B staining of sections taken from both the groups revealed that Flouro jade B positive cells are present in only infected brain but not in control brains. Further p-JNK was found to be colocalized with Flouro Jade B positive cells indicating that p-JNK plays a crucial role in neuronal cell death in ECM.
- ✓ From this study we conclude that p-JNK is activated and mediates neuronal cell death in murine cerebral malaria.

The specific, reversible JNK inhibitor SP600125, improves survivality and attenuates neuronal cell death in ECM.

"During the past decade, pharmaceutical research on these diseases has shifted focus from symptomatic benefit to developing novel disease modifying agents. A key driver of this focus is the enhancement in fundamental knowledge of the mechanisms governing neuronal survival and death. JNK plays an integral role in neuronal death and this

pathway might be operative in various central nervous system (CNS) disease states" (Manning and Davis, 2003). The small molecule chemical inhibitor SP600125 (anthra[1,9-cd]pyrazol-6(2*H*)-one), has been reported to be a potent and selective inhibitor of JNK1, -2, and -3 with more than > 20 fold selectivity over other related MAP kinases (Bennett et al., 2001). Administration of SP600125 has prevented neuronal apoptosis following ischemia or ischemia/reperfusion of the brain. Apart from its inhibition of JNK activity, SP600125 was also shown to inhibit a number of pro-apoptotic events such as the activation of pro-apoptotic Bcl2 family members, the release of mitochondrial cytochrome c into the cell cytosol, or the activation of pro-apoptotic caspase family of proteases (Guan et al., 2006). SP600125 administered intraperitoneally 1 h before and 6 h in model of early brain injury after subarachnoid hemorrhage demonstrated benefits such as the suppression of caspase activation and concomitant neuronal injury, improved blood–brain barrier preservation, reduced brain swelling, and improved neurological function (Yatsushige et al., 2007). SP600125 also prevented apoptosis of dopaminergic neurons in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of Parkinson's Disease (Wang et al., 2004) as well as neurons in the acute injury accompanying spinal cord trauma (Yin et al., 2005). Taken together, these results support the further development of JNK inhibitors as neuroprotective agents and their use in a variety of brain insults. Earlier we reported that JNK group of MAP kinases are activated and mediates neuronal cell death in ECM (Anand and Babu, 2011). Here we report for the first time that SP600125, a specific, reversible, ATP competitive inhibitor of JNK, attenuates neuronal cell death and improves survivality in PbA infected mice.

Salient Findings:

- ✓ Survival of mice infected with PbA was significantly increased ($p = 0.0001$) in group that received SP600125 from day 1 post-infection (30 mg/kg body weight).
- ✓ Administration of SP600125 in PbA infected mice has no effect on parasitemia indicating that the protective effect of SP600125 may be due to the modulation of host responses and not due to its inhibitory effect on parasitemia.
- ✓ Further, H&E staining of brain sections from infected mice showed distinct neurological lesions, with parenchymal microhemorrhages, disruption of vessel walls and adherence of leucocytes to the vessel walls when compared to uninfected controls. In contrast, SP600125 treated mice have a healthy parenchyma, clear vessels with little or no hemorrhage.
- ✓ Immunoblot analysis of p-c-Jun, active caspase-3, COX-2 and TNF-α revealed that administration of SP600125 in PbA infected mice dramatically reduced their levels indicating that SP600125 treatment attenuates inflammation and apoptotic cell death in ECM.
- ✓ Active caspase-3 immunostaining of sections taken from control, infected and treated animals revealed caspase 3 positive cells in infected brain sections but not in control and treated groups indicating the protective effect of SP600125 in ECM.
- ✓ Since active caspase-3 is implicated in neuronal cell death we next investigated the viability of neurons by staining the sections taken from all the 3 groups with Flouro-Jade B. We found Flouro-Jade B positive cells in infected group only but not in control or treated groups.

Pentoxifylline improves survivality and attenuates neuronal cell death in ECM.

Pentoxifylline (PTX) is a methylxanthine derivative that has been used for treatment of chronic occlusive arterial disease because of its rheological actions. PTX acts primarily by increasing red blood cell deformability, reducing blood viscosity, and decreasing the potential for platelet aggregation and thrombus formation. In addition, PTX has effects on the inflammatory response. It has been shown to improve survival in sepsis models by decreasing TNF-α, interleukin IL-1β and IL-6 levels and to inhibit the LPS-stimulated production of multiple cytokines in alveolar macrophages. PTX is known to inhibit phosphodiesterase, thereby increasing cytoplasmic cyclic adenosine monophosphate levels and resulting in serious inhibition of TNF-α gene transcription (Kremsner et al., 1991). In addition to inhibiting cytokines, PTX is also known to reduce the TNF-α induced upregulation of E-selectin, ICAM and VCAM in endothelial cells thereby inhibiting the adhesion of neutrophils. It also inhibits adhesiveness, degranulation, and superoxide production of activated neutrophils and the production of nitric oxide by macrophages. Because of its ability to counteract multiple proinflammatory mediators simultaneously PTX seems a useful agent to attenuate the generalized inflammatory response leading to the organ failure observed in cerebral malaria (Di Perri et al., 1995; Das et al., 2003; Looareesuwan et al., 1998).

Salient findings:

- ✓ Pentoxifylline (PTX) improves the survivality in *Plasmodium berghei ANKA* (PbA) infected mice.

- ✓ H & E staining of the brain sections taken from control, infected and PTX treated mice and we found that neurological lesions and neuronal cell death were widespread in infected mice when compared to control or PTX treated mice brain sections.
- ✓ Administration of PTX completely abrogated the induction of TNF-α.
- ✓ Administration of Pentoxifylline inhibits the disruption of blood –brain barrier that is so common in cerebral malaria.
- ✓ Administration of Pentoxifylline blocks the leukocyte infiltration and subsequently changes the cytokine profiles from Th1 to Th2.
- ✓ Pentoxifylline administration does not modulate the parasite adhesion and sequestration and Pentoxifylline has no effect on parasitemia.
- ✓ Pentoxifylline modulate the expression pattern of several key proteins associated with stress.
- ✓ Further caspase 3 immunostaining of sections taken from control, infected and PTX treated animals revealed caspase 3 positive cells in infected brain sections but not in PTX treated groups indicating the protective effect of PTX in cerebral malaria.
- ✓ Control and PTX treated mouse brain sections stained very little for Flouro-Jade B, where as in infected mouse brain sections a robust increase in Flouro-Jade B staining cells was observed. This sort of staining pattern clearly indicates that neuronal cell death is wide spread in infected mouse brain sections as compared to control and PTX treated animals.
- ✓ Our results suggest that Pentoxifylline inhibits neuronal cell death, improves neurological outcome and survivality in mice infected with PbA and Pentoxifylline can be used as an adjuvant along with standard anti-malarials.

Publications:

Published:

1. ***Anand S.S.****, Babu P.P.,* c-Jun N terminal kinases (JNK) are activated in the brain during the pathology of experimental cerebral malaria, Neurosci Lett. 2011, 488(2): 118-22.

Under Review:

2. ***Anand S.S.****, Babu P.P.,* The JNK specific inhibitor SP600125, is neuroprotective in experimental cerebral malaria (**manuscript under review**)

In Preparation:

3. ***Anand S.S.****, Babu P.P.,* ER stress and neurodegeneration in experimental cerebral malaria: PERK – peIF2α axis is activated and mediates neuronal cell death in murine cerebral malaria.
4. ***Anand S.S.,*** *Arun Kumar K., Babu P. P.,* Pentoxifylline is neuroprotective in experimental cerebral malaria (**manuscript under preparation**).

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