Role of lymphotoxin-α in neuronal cell death during fatal murine cerebral malaria

DOCTOR OF PHILOSOPHY

By

Prabhakar Eeka

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
Hyderabad-500 046, India

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for the award of a Ph.D. degree in Animal Sciences

By

Prabhakar Eeka

Department of Animal Sciences
School of Life Sciences
University of Hyderabad
PO Central University, Gachibowli
Hyderabad 500 046
Andhra Pradesh, India

Enrollment No: 07LAPH06

October 2012

In the loving memory of my parents

University of Hyderabad
(Central University established in 1974 by Act of Parliament)
School of Life Sciences
Department of Animal Sciences
Hyderabad-500 046, INDIA

DECLARATION

I, Prabhakar Eeka, hereby declare that this thesis entitled "Role of lymphotoxin-α in neuronal cell death during fatal murine cerebral malaria" 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.

Date:

Name: Prabhakar Eeka

Signature:

Regd. No.: 07LAPH06

University of Hyderabad
(Central University established in 1974 by Act of Parliament)
School of Life Sciences
Department of Animal Sciences
Hyderabad-500 046, INDIA

CERTIFICATE

This is to certify that this thesis entitled "Role of lymphotoxin-α in neuronal cell death fatal murine cerebral malaria" is a record of bonafide work done by Mr. Prabhakar Eeka, 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

PbA	*Plasmodium berghei* ANKA
RBC	Red blood cell
pRBC	parasitized RBC
iRBC	infected RBC
BBB	Blood brain barrier
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
IAEC	Institutional Animal Ethical committee
p.i.	Post infection
CM	Cerebral malaria
SE	Experimental Serum (Serum from CM mice)
SA	Severe Anemia
LT-α	Lymphotoxin-α
LT-α(T)	Recombinant Lymphotoxin-α treatment
Ab(T)	LT-α Antibody treated/ LT-α inhibited
TNF-α	Tumor necrosis factor alpha
Cas-3	Caspase-3
Cal1	Calpain1
Gra-b	Granzyme-b
iNOS	Inducible nitric oxide synthase
NFk-B	Nuclear kappa B
COX-2	Cyclooxygenase-2
NSE	Neuronal specific enolase
NeuN	Neuronal nuclear antigen

MAP-2	Microtubule associated protein
CTLs	Cytotoxic T lymphocytes
FACS	Fluorescent activated/assisted cell sorter
SEM	Standard error mean
TEM	Transmission electron microscope
CXCR3	C-X-C chemokine receptor type 3
CXCR4	C-X-C chemokine receptor type 4
CCR5	C-C Chemokine receptor type 5
CCR4	C-C Chemokine receptor type 4
ICAM-1	Intra cellular cell adhesion molecule 1
VCAM-1	Vascular cell adhesion molecule 1
LFA-1	Lymphocyte function associated antigen 1
VLA-4	Very late antigen/integrin alpha4beta1
GAPDH	Glyceraldehyde phosphate dehydrogenase
BCIP	5-Bromo-4-chloro-3-indoyl phosphate
NBT	Nitro blue tetrazolium chloride

Contents

INTRODUCTION

Plasmodium life cycle

Malaria is a parasitic disease caused by the *Plasmodium species* belong to *Protist* family of Eukaryota kingdom. The parasite that causes malaria has complex life cycle in two different hosts, primary host being humans and secondary being female Anopheles *mosquitoes*. Four types of Plasmodium species, namely *Plasmodium falciparum, Plasmodium vivax (P.vivax), Plasmodium ovale (P.ovale) and Plasmodium malariae (P.malariae))* have been reported to cause malaria in human beings. Among these *Plasmodium falciparum (P.falciparum)* is the most common and major cause of deaths around the world. Malaria is a chronic disease and symptoms are, in general, due to anaemia, regular lysis of RBCs and toxicity of the hemozoin (Hunt and Stocker, 2007), a secondary metabolite of the parasite heme metabolism which causes fever associated with chills. The lifecycle of Plasmodium *involves* sexual (mosquito) and asexual stages (humans or primates), sexual stage starts when a mosquito bites an infected person during its blood meal. Gametocytes (Male & Female) from the infected person along with blood enters the mosquito gut fuse to form zygote which transverse in to gut wall, matures to form sporozoites. These sporozoites travel to salivary glands of mosquito and get injected in to person's blood stream during mosquitos subsequent feeding on humans. Mature sporozoites in the blood stream reach to the liver (liver) through blood circulation and enter the hepatocytes where they divide and form merozoites; this asexual stage of lifecycle is called **exoerythrocytic lifecycle** or **liver stage** of Plasmodium (capital) in humans. Erythrocytic cycle starts when the mature merozoites ruptures out from the hepatocytes and invade RBCs, where they transform into different stages like early and late ring stages, trophozoites and merozoites by consuming haemoglobin of the RBCs. Matured trophozoites and merozoites ruptures out of iRBCs and invade fresh RBCs. Thus Erythrocytic cycle starts, the late RBCs forms into mature gametocytes which are taken by the mosquito when it takes blood meal from humans and the sexual cycle starts again in mosquito. During the liver stage, parasites undergo dormant stage and do not show their presence in the blood but they relapse and cause disease after dormancy called Hypnozoite. The lifecycle of Plasmodium is shown below (Fig.1).

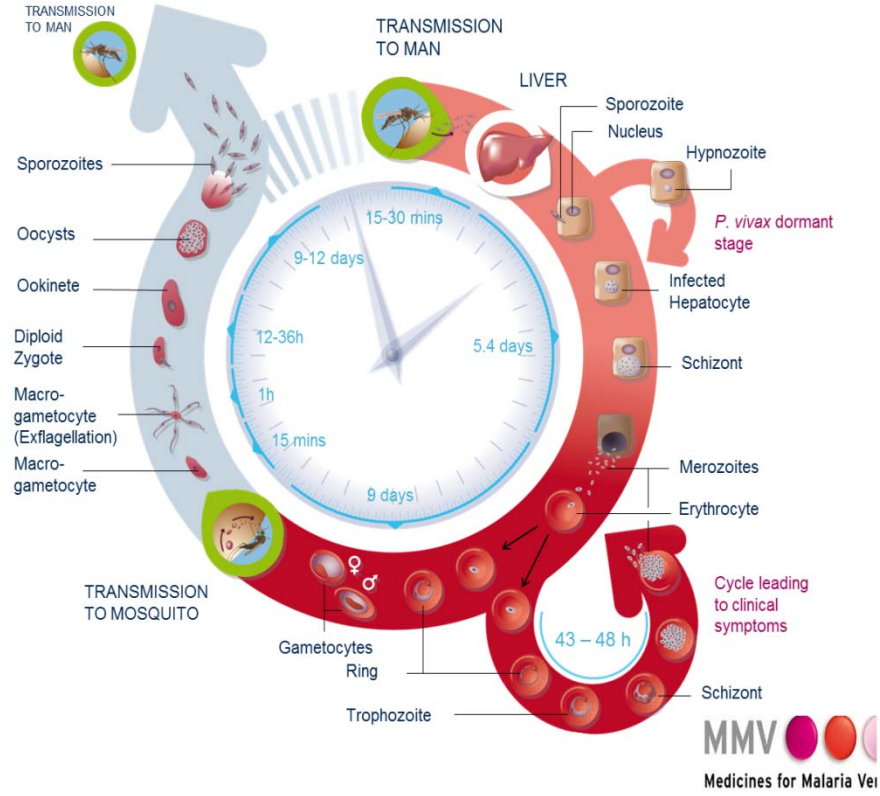


Fig. 1. Showing the nutshell diagram of complete *Plasmodium* life cycle with different stages with respect to average time for each of respective stages. Courtesy: Medicines for malaria venture (http://www.mmv.org/).

Distribution and severity

According to the WHO World Malaria Report 2011, malaria is prevalent in 108 countries of the tropical and semitropical world, with 35 countries in central Africa bearing the highest burden of cases and deaths. Of the 35 countries that account globally for ~98% of malaria deaths, 30 are located in sub-Saharan Africa, accounting for 98.5% of the deaths in Africa, with four countries alone accounting for ~50% of deaths on the continent (Nigeria, Democratic Republic of Congo, Uganda and Ethiopia). In sub-Saharan Africa, approximately 365 million cases occurred in 2002 and 963 thousand deaths in 2000, equating to 71% of worldwide cases and 85.7% of worldwide deaths. Almost 1 out of 5 deaths of children under 5 yrs. of age in Africa are due to malaria. With increased efforts in controlling malaria in Africa in the recent years, it is reported that a total of 11 countries and one area in the African region showed a reduction of more than 50% in either confirmed malaria cases or malaria admissions and deaths (Algeria, Botswana, Cape Verde, Eritrea, Madagascar, Namibia,

Rwanda, Sao Tome and Principe, South Africa, Swaziland, Zambia, and Zanzibar, United Republic of Tanzania), whereas there was evidence of an increase in malaria cases in 3 countries in 2009 (Rwanda, Sao Tome and Principe, and Zambia) [1-4]. However, these claims of improved malaria situation in Africa, as presented by the WHO, have been challenged [5-6].

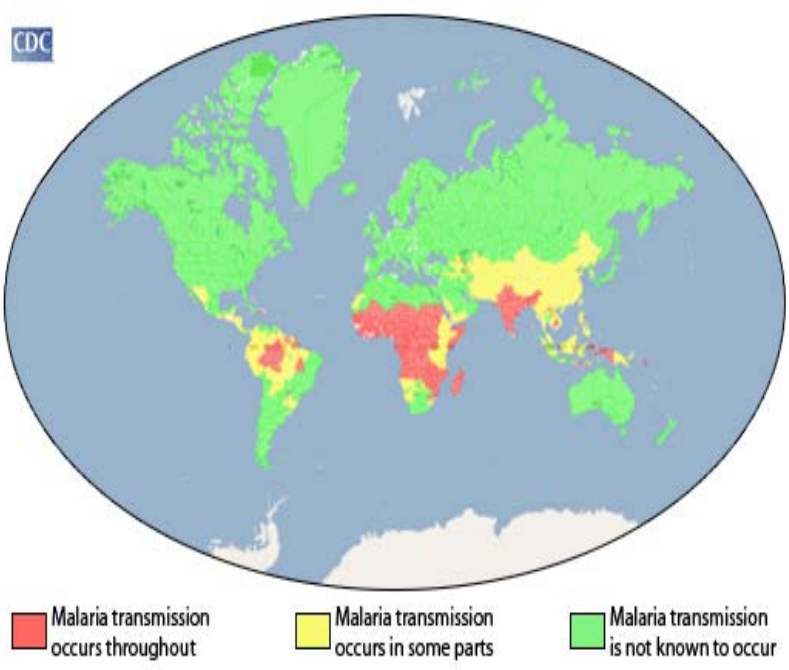


Fig.2. World map showing the global malaria transmission and is indicated by only in certain regions of North America, Africa and Asia. CDC (Courtesy: centres for disease control and prevention; http://www.cdc.gov/)

Cerebral malaria (inclusion of clear distinction of HCM and ECM)

Cerebral malaria (CM) is an acute pathological syndrome arises due to the occlusion of parasites associated with increased levels of cell adhesion and chemokine receptors overexpression on the brain endothelium and blood brain barrier (BBB) disruption, hyper activation of immune cells especially CD4+ and CD8+ T cells. The severe complication of cerebral malaria includes dysfunction of all the vital organs of the body which leads to coma and death. The symptoms include impaired consciousness with non-specific fever, generalized convulsions and neurological abnormalities, and coma that lasts for 24-72 hours, initially rousable and then unarousable. If not treated in time, it can lead to complications like jaundice, hemoglobinuria, a tender and enlarged spleen (splenomegaly), acute renal failure, and uremia, and is fatal in about 20% of patients. Further, it will manifest with signs of

increased intracranial pressure, hemiplegia (total paralysis of the arm, leg and trunk on the same side of the body), encephalopathy, delirium (sudden severe confusion and rapid changes in brain function that occur with physical or mental illness), seizures and coma.

Pathophysiology of cerebral malaria

The pathology of cerebral malaria is complex and involves many factors that range from parasite triggered cell death mechanisms (Barcinski and DosReis, 1999; Bienvenu et al., 2010), toxicity of secondary metabolites (Jaramillo et al., 2004) (Dostert et al., 2009), parasite derived microparticles (Carcaboso et al., 2004; Combes et al., 2004; Couper et al., 2010; Faille et al., 2009; Nantakomol et al., 2011), host immune factors (Claser et al., 2011; Finley et al., 1983; Haque et al., 2010; Haque et al., 2011b; Nie et al., 2007; Vigario et al., 2007; Wu et al., 2010) and host parasite interactions (Olszewski et al., 2009). All these factors can be put into two main hypotheses to explain the pathogenesis of cerebral malaria. First being the **hypoxic** theory that arises due to the regular lyses of RBCs or the blockade of blood vessels by the pRBCs as a result of acquired properties of cytoadherence (Eda and Sherman, 2002), rosetting (Carlson et al., 1990; Carlson et al., 1994; Ghumra et al., 2011) and sequestration (Amante et al., 2010; Ponsford et al., 2012). The second being the **cytokine** theory which arise as a result of cytokine release by activated immune cells (Tregs) which activate the immune cells as loopback mechanism (Campanella et al., 2008; John et al., 2008) and also helps in expression of chemokine receptors (Van den Steen et al., 2008) and adhesion molecules on the vascular endothelium of blood vessels in the brain (Armah et al., 2005; Ockenhouse et al., 1992). The activated immune cells also sequester in to the brain and kill the cells through perforin (Nitcheu et al., 2003; Potter et al., 2006) and granzyme-b (Haque et al., 2011b) mediated pathway or by activating microglial cells locally.

RBCs membrane modifications

During the asexual stage of lifecycle in humans, parasites once leaves the liver and infect RBCs, they undergo repeated cycles of erythrocytic life cycle and they escape immune cells by RBC membrane modifications. RBCs modifications not only help the parasite to evade host immune system but also help in the pathogenesis because they acquire the property of sluggishness which blocks blood vessels. A special feature acquired by the parasitized RBCs has a prominent role in pathogenesis besides other factors of host, parasite and their interactions. The special features of RBCs are: 1) **rosetting** is property of the

parasitized RBCs to bind to uninfected RBCs to form rosette like structure. This makes the RBCs to blocks the blood vessels (Kaul et al., 1991) thus leading to hypoxia (Dondorp et al., 2004; Doumbo et al., 2009; Penet et al., 2005) in brain and BBB disruption (Lackner et al., 2006; Pino et al., 2005; Treeratanapiboon et al., 2005), an important feature of CM and also dysfunction of different vital organs in the periphery (As shown by Department of Tropical Pathology, Department of Tropical medicine- google-tm.mahidol.ac.th/eng/tmpt/tmpt_malaria_lec.pdf). This property of the RBCs is due to the expression of the parasite proteins on the membrane of parasitized RBCs which not only helps in the adhesion of pRBCs with the uninfected RBCs but also helps in attaching to the cell adhesion molecules on endothelium of the brain leading to hypoxia and ultimately BBB breakdown. During the infection of pRBCs with the parasites, RBCs develop special structures (protrusions) on their surface called "**knobs**". Knobs are formed by a group of parasite expressed proteins, among PfEMP1 encoded by *var* genes (Albrecht et al., 2011; Flick and Chen, 2004), rosettin/rif (Helmby et al., 1993) proteins encoded by rosetting/rif multigene family. All these proteins are synthesised by the intracellular parasite and are inserted in to RBC and protrude out from the membrane (Glenister et al., 2002; Maier et al., 2009) which bind to cell receptors helps in sequestration also (Joergensen et al., 2010). During infection parasites are well protected themselves from the host immune system. however some parasites also undergo death by apoptosis to survive by natural selection (Pollitt et al., 2010) and the antigens and secondary metabolites are presented (Langhorne et al., 2004; Lundie, 2011) by the antigen presenting cells to NK (Hansen et al., 2007) cells and T (Renia et al., 2006; Yanez et al., 1999) cells for which kills the cells through direct cytotoxicity or by releasing the cytokines which have array of functions to clear the infected RBCs. Cytokines released by activated immune cells have other functions which helps in the expression of chemokine receptors and cell adhesion molecules on the endothelium and thus infiltration of peripheral immune cells into brain (Haque et al., 2001; Sarfo et al., 2011). This property of infected RBCs to adhere to the blood vessels in different organs to keep away or escape from the immune system is called **Sequestration.** Sequestration is associated with the binding of parasite expressed GPI anchored proteins on the RBCs membrane.

Immune system/cytokine theory

Immune system is the host defence system against the foreign molecule or the pathogen which enters the host. This surveillance protects the host from the attack or encounter of different pathogens which may cause diseases. However the role of immune system has been implicated in many diseases like autoimmune diseases (Bachmann and Kopf, 2001; Gregersen and Behrens, 2006; Teichmann et al., 2010), arthritis (Dhaouadi et al., 2007; Wang et al., 2006), cancer (Davidson-Moncada et al., 2010; de Visser et al., 2006), encephalitis (Bauer et al., 2012; Deckert-Schluter et al., 1994), many pathogen diseases like tuberculosis and cerebral malaria. During cerebral malaria the role of monocytes (Srivastava et al., 2010; Ward et al., 1984), neutrophils (Chen et al., 2000; Chen and Sendo, 2001; Porcherie et al., 2011) and platelets (Barbier et al., 2011; Combes et al., 2006; Cox and McConkey, 2010) have been reported to play a role in pathogenesis, but the role of T (Belnoue et al., 2002; Claser et al., 2011; Renia et al., 2006) cells have been considered to be very critical in the CM pathogenesis. Role of T cells have been studied at different stages of malaria life cycle and have been shown that CD4+ T (Nie et al., 2007) cells are important for the survival of parasites and CD8+ T (Haque et al., 2011a) cells have been shown to involve in pathogenesis by their cytotoxic effect. Both knockout and inhibition studies have been shown their importance in pathogenesis, but the mechanisms by which they induce CM are not clearly understood. Recent progress in understanding the disease throws light on the critical role of cytokines in immunopathogenesis (Hunt and Grau, 2003), among many cytokines that are associated with CM outcome , LT-α (Engwerda et al., 2002) is proven to be critical for ECM pathogenesis. Lymphotoxin-a, a cytokine which has many functions in normal conditions ranging from bone marrow neogenesis, T cell maturation and maintenance, lymphoid organogenesis, secondary lymph node formation and immunity against pathogens and cell signalling. During CM this molecule is reported to be up regulated in the brain and the knockdown of this molecule has been shown to protect the mice from CM by bring down the expression levels of cell adhesion molecules (ICAM1 and VCAM1) and chemokine receptors (CXCR4). The cytokine theory explains the role of cytokines which induces the cell adhesion molecules and chemokine receptors thus helps in the adhesion of pRBCs (sequestration) and also helps the immune cells to infiltrate in to brain where they may be cytotoxic to neuronal cells or by activating the resident immune cells that causes the damage.

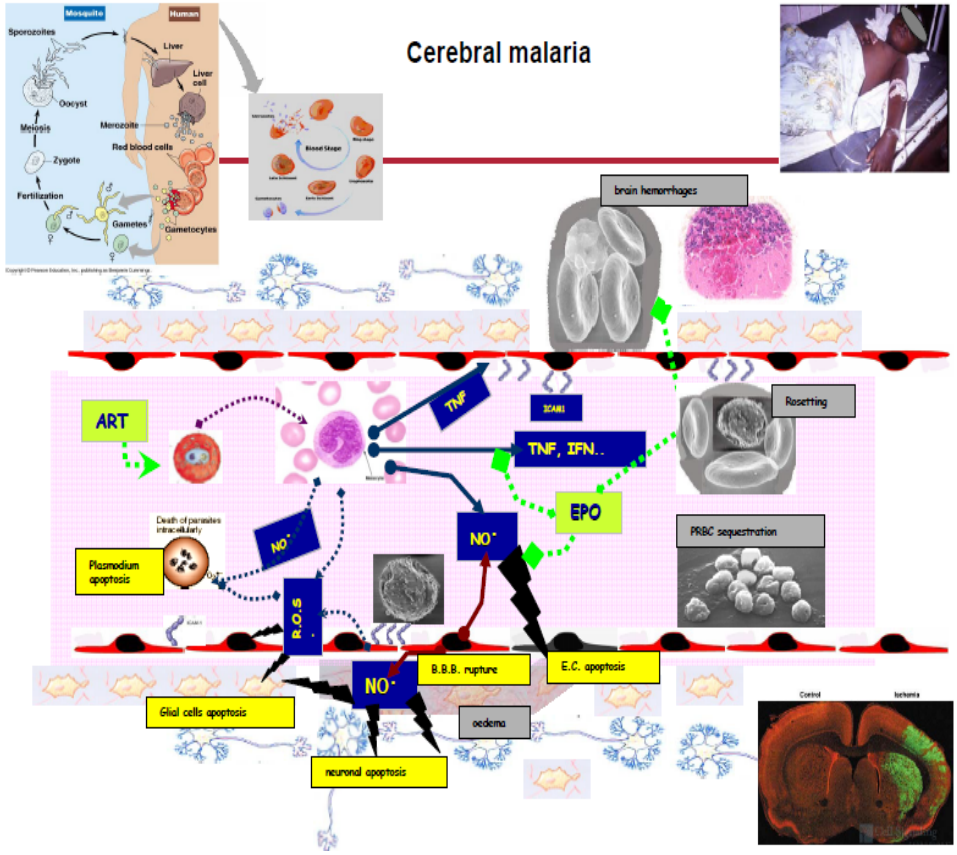


Fig. schematic representation of the events that occur during *Plasmodium* infection, above figure showing the life cycle of malaria parasite in mosquito and human host, blood stage infection and regular RBC lyses which leads to CM. Below figure showing the events that arises during *Plasmodium* infection from RBCs modification, cytokine releases and their effect on endothelium and brain cells cumulatively explained in two theories of pathogenesis: a) Hypoxia theory and b) Cytokine theory which explains the CM pathogenesis.

Pathophysiology of cerebral malaria

Cerebral malaria is a complex syndrome and the pathology can be explained broadly in two hypothesis, one being arises as a result of anoxic/hypoxic conditions that arises a result of regular lyses of RBCs as a part of erythrocytic cycle that occurs at regular time period or the blockade of blood vessels by the pRBCs as a property of cytoadherence and sequestration. This lead to the insufficient supply of blood to the brain resulting in the oxidative burst, generation of reactive oxygen species (ROS) and nitric oxide which causes the endothelial, glial cell and neuronal apoptosis (Rae et al., 2004). This also leads to metabolic disturbances in the brain resulting the oedema formation and cell death by necrosis. Second being the cytokine theory which explains the role of cytokine in the CM pathogenesis. During the Plasmodium (capital) infection the parasites in the RBCs undergo death by apoptosis (Pollitt et al., 2010) to keep check on their overgrowth for the survival,

these dead parasites proteins and/or malarial pigment are presented to the T helper cells by the antigen presenting cells. The pulsed antigen presented cells get activate and release cytokine which helps in the activation of T cells and NK cells which kills the infected cells by direct cytotoxicity. But of the few cytokines released has diversified effects, may activate the cells in a paracrine or autocrine mode and thus activate the cells. These cytokine also induces the expression of chemokine and cell adhesion molecules on the endothelium of blood vessel which helps in the anchorage of pRBCs and T cells and get sequestered or infiltrated in to the brain of CM patients / or animal models. The blockage of blood vessels by the pRBCs also leads to the breakdown of blood brain barrier also aggravate to the pathology.

All the events lead to the neurodegeneration and the events that occur at the molecular levels are not fully explored. Reports suggest that there are elevated levels of cell death proteases, caspases, calpains and cathepsin-b indicating the mode of cell death. Further and it is reported that these proteases induce the cell death in the brain through cytoskeletal breakdown (Eeka et al., 2011a). Inhibition studies have also proved the role of these cell death proteases (Helmers et al., 2008a) in CM pathogenesis that prevented mice from CM. But this is also well proved with the role of immune cells (CTLs) in the pathogenesis where it has been shown that T cells (CD8+) lacking Gra-b and perforin (Potter et al., 2006) protected mice from CM. This is also same case with the lymphotoxin-a, an immunomodulator, shown to have protected mice from CM lacking lymphotoxin-a by decreasing the cell adhesion molecules and chemokine receptors in the brain. Indicating the inter convergence of cell death and cytotoxic proteases in function in the CM pathogenesis and this lymphotoxin-α is playing a role in coordinating these molecules during CM.

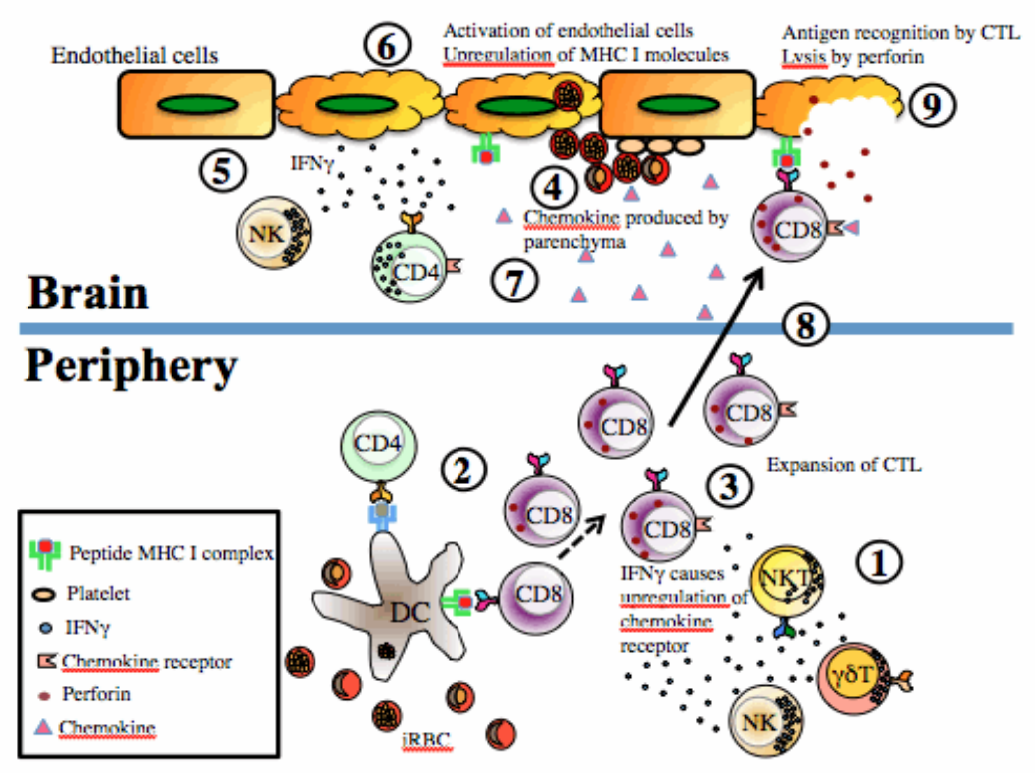


Fig.3. A proposed mechanism of ECM pathogenesis showing different event which lead to activation of CTLs and endothelial cytolysis which may occur as result of direct cytotoxic effect of CTLs or by the hypoxic effects that arises due to sequestration and cytoadherence. **Source:** Lei Shong Lau and Daniel Fernández Ruiz, Department of microbiology and immunology, Melbourne University Home page.

Neuronal cell death is the important hallmark of cerebral malaria and understanding mechanism of neuronal cell death is of critical importance. Neurons are delicate cells and important in brain function and behaviour of the organism as a whole, due to their non-regenerative nature or if regenerated, their proper original intricate synapse or contact formation is almost impossible. Thus neuronal cell death forms a permanent cognitive impairment. Hence understanding the basis of neural cell death helps us to know the ways of finding the solution to the problem. With this background we framed following objectives to study

1. Role and mechanism of action of cell death proteases (caspase-3, calpain1 and cathepsin-b) and CTLs in neuronal cell death during CM.

2. Status of lymphotoxin-α and role in neuronal cell death; whether it has any effect on caspase-3 and calpain1 mediated cell death.

3. *In vivo* inhibition of lymphotoxin-α and caspase-3 and calpain1.

CHAPTER I (a)

Role and mechanism of action of cell death proteases (caspase-3, calpain1 and cathepsin-b) in neuronal cell death during CM.

Introduction

Cerebral malaria is a clinical manifestation of mental status and coma leading to death of patient within the hours of onset (Looareesuwan, 1992). Cerebral malaria often results in as high as 30% mortality and about 10% of survivors suffer with long and short term neurological problems. The pathophysiology of this disease involves the adherence of the parasitized RBCs (pRBCs) to the activated/inflamed endothelium of the blood vessels in the (Hatabu et al., 2003) brain initiating cell death signaling cascades (Pino et al., 2003b). Increased adherence of pRBC to the vascular wall leads to blockade of blood vessels resulting in diminished oxygen supply leading to hypoxia and disruption of blood brain barrier (Pino et al., 2003a, 2005). Increased BBB permeability facilitates extravasation of peripheral immune cells into brain (Belnoue et al., 2002; Bisser et al., 2006; Patnaik et al., 1994). These immune events in the brain promote hyperactivation of resident microglia either directly or through the release of cytokines and toxic metabolites (Deininger et al., 2002). though a well-defined pathogenesis of CM remains to be understood, recent reports have shown that involvement of various mechanisms including vascular occlusion in the presence of sequestered pRBCs, leukocytes, inflammation, apoptosis/necrosis, platelet activation and angiogenic failure may contribute to the pathogenesis and exacerbation of cerebral malaria (Hunt et al., 2006; Idro et al., 2005, 2006; Jain et al., 2008; Nacher, 2008). Cellular degeneration is one of the leading components in exacerbation of the etiology of cerebral malaria. However, the role of apoptotic and necrotic suicidal proteases, their cross talks and action on various cellular substrates like cytoskeletal proteins remain elusive. Apoptosis and necrosis are tightly regulated processes and can be induced by alterations in several biochemical intermediates, including alterations in high-energy phosphates, intracellular calcium accumulation and reactive oxygen species (Berger and Garnier, 1999). Caspases, calpains and cathepsins are some of the crucial proteases involved in apoptotic and/or necrotic cell death (Marks and Berg, 1999; Yakovlev et al., 2001). Calpains and cathepsins can mediate apoptotic or necrotic cell deaths independent of caspases (Liu et al., 2004; Liu et al., 2006; Yamashima, 2000), whereas caspases were specifically involved in executing apoptosis (Riedl and Shi, 2004). However, calpains, cathepsins and caspases share similar substrate specificity and cleave several target proteins like poly-(ADP-ribose) polymerase-1 (PARP-1), post-synaptic density protein-95 (PSD- 95), spectrin, neurofilament (NF) and fodrin (Wang, 2000a). Neuronal cytoskeleton breakdown by calpains and caspase-3 has also

been shown in many neurological diseases (Stys and Jiang, 2002; Takamure et al., 2005). Axonal injury or neuronal cell death is implicated in many of the neurological diseases including virus induced encephalitis (Strachan et al., 2005), Alzheimer's disease (Higuchi et al., 2005; Marcilhac et al., 2006; Raynaud and Marcilhac, 2006), amylotrophic sclerosis (Locatelli et al., 2007) and cerebral ischemia (Chaitanya et al., 2010a, b; Chaitanya and Babu, 2008; 2009). In the present study, we have investigated the status of these proteases, cross talks between these proteases, proteolysis of cytoskeleton and the ultrastructure of cell morphology during cerebral malaria in the brains of mice infected with *Plasmodium berghei ANKA*.

MATERIALS AND METHODS

Antibodies

For western blotting and co-immunoprecipitation Neurofilament light (NF (L) is purchased from sigma immunochemically (Cat No. N5139), Caspase-3 from cell signaling and technology Cat.No 9662, calpain1 from chemicon international, Termicula, USA (MAB3164), Granzyme-b (Cat.No AM52) from Calbiochem and B-actin from epitomics Cat.No.1879-1. For FACS and immunofluorescence FITC-CD4 (Cat.No11-003-81) and PE-CD8 (Cat.No 12-0083-81) antibodies from eBiosciences, anti-NSE (AB951) from Chemicon international, Termicula, USA and Cy3- anti mouse secondary is a kind gift from Dr. Shiva Kumar (UoH, India). FACS sheet fluid was purchased from BD Biosciences, and Plasmodium parasites (PbA) was procured from NIMR (National Institute of Malaria Research) parasite bank, New Delhi, India.

Mice

C57 BL/6J mice were purchased from the NIN Hyderabad and maintained under strict hygienic conditions. Experiments are conducted as per the guidelines of the institutional animal ethical committee (IAEC), surgical procedures operated are approved by the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). Plasmodium berghi ANKA (*pbA*) parasites procured from parasite bank, National Institute of Malaria Research (NIMR), New Delhi.

Induction of cerebral malaria

C57BL/6J mice are intraperitonially injected with 10^6 *pbA* parasites and the control mice was injected with the same amount of PBS. Infection was monitored by taking blood smears through caudal puncture stained with giemsa and JSB-I and JSB-II and the cerebral symptoms through behavioural symptoms. The percentage of cerebral symptoms is around 85-90% and the mice were found to die in between 7-15 days. Mice showed convultions and partially in coma stages in between 6-9 days of post infection are considered to cerebral malaria mice. Mice that died at later stages was due to severe anaemia (percentage of anaemia was higher than those died at 6-7days). Based on the behavioural symptoms, mice were sacrificed at 6-9 days of post infection were taken for further experiments. Mice were sacrificed at 6^{th} or 7th day by transcardial perfusion with 4% paraformaldehyde in PBS or snap frozen in liquid nitrogen. Brains obtained from sacrificed mice were made into two

groups. One group consists of samples stored at -80^{o}C which are later homogenized in RIPA Buffer for western blotting. The second group of samples which are used for histochemical studies were fixed in the same perfusion solution for 12 hrs. and then processed in series of graded alcohol followed by chloroform. The processed samples were wax embedded and 3µm sections were cut using microtome. The tissue sections were taken on to the poly-L-Lysine coated slides which are used for immunohistochemical and immunofluorescence studies.

Western blots

For western blot analysis, control and infected mice were sacrificed with an overdose of pentobarbital. Brains were dissected out (n=5) and immediately snap frozen in liquid nitrogen and were homogenized in the modified radio immuno precipitation assay buffer containing protease inhibitor 1 mM PMSF and phosphatase inhibitors 10 mM β-glycerophosphate, 10 mM NaF and 0.3 mM Na_3VO_4. The lysates were sonicated, centrifuged and 50µgms of supernatant was separated on SDS-PAGE, transferred onto nitro-cellulose membrane and immunobloted Granzyme-b and NF (L).

H&E and Cresyl violet staining

Tissue sections stained with haematoxylin and eosin following standard protocol, air dried, mounted in DPX and observed under microscope. For cresyl violet staining tissue section slides were processed and stained with 0.1% cresyl violet for 5 min, mounted with DPX and observed under microscope.

Real Time PCR and PCR

For Real Time PCR analysis, total RNA by TRIZOL reagent method (Invitrogen) and synthesized the cDNA by using first strand cDNA synthesis kit (Blueprint-TAKARA). Specific forward and reverse primers for cell adhesion molecules (ICAM1 and VCAM1) and chemokine receptor (CXCR4) by taking the sequence from NCBI nucleotide database by using online OligoCalC software. Forward and reverse primers for CXCR4,5’GGAACCGATCAGTGTGAGTAT3’; ’CACCAATCCATTGCCGACTAT3’, for ICAM1 and VCAM1,GAGATCACATTCACGGTGCTG3’, 5’AGCTGGAAGATCGAAAGTCCG3’ and 5’ GACATCTACTCTTTCCCCAAGG 3’, 5’TGTTCATGAGCTGGTCACCCT3’ respectively. T cells presence in the brain of control

and infected mice brain samples are detected by the normal PCR using specific forward5' GGGGGTTTGTTCTCTATCTCTTCC3' and reverse 5' TATCCACAGGAGATGATGGTGCAC 3' primers for TCR-a segment of TCR gene. Forward 5'ATCTTCTTGTGCAGTGCCAGC 3' and reverse 5' TTGAGGTCAATGAAGGGGTCG 3' GAPDH primers are used as control gene for Real Time and normal PCR.

Immunohistochemistry

For immunohistochemical and immunofluorescence analysis**,** 3µ thick paraffin sections of CM and control mice brains were processed, antigen retrieved in citrate buffer (pH-6.0) and permeabilized in 0.1% Triton-X 100 for 15 min. Endogenous peroxidase was inhibited by incubating sections in 3% H_2O_2, washed and blocked using 10% normal goat serum for 60 minutes. The sections were incubated in primary antibody (CD4 and CD8) overnight at 4^oC, followed by washes in PBS and incubation with goat anti mouse and rabbit HRP conjugated secondary for 90 min and developed using Vectastain Elite ABC kit (Vector Laboratories).

Triple immunofluorescence

Triple immunofluorescence was done by incubating the sections first with mouse anti Granzyme-b primary overnight at 4^0 C after processing them in alcohol series and permeabalising with 0.1% triton-x 100 in PBS. Washed in PBS, followed by blocking with 10% goat serum and incubated CD4 and CD8 primary antibody cocktail conjugated with FITC and PE flourochromes for 90 min. washed in PBS, stained with and mounted in 90% glycerol and analysis was done using Leica laser scanning confocal microscopy.

Double immunofluorescence

Double immunofluorescence was performed by following the same procedure as cited above, blocked with 10% serum and incubated with Gra-b primary antibody at 4^0 C overnight. Washed and incubated with antimouse CY3 secondary antibody for 90 min. at room temperature and stained with Fluoro Jade B for 30 min, washed and mounted in 90% glycerol and observed under Leica confocal microscopy.

TEM (Transmission electron microscopy)

Mice with cerebral symptoms were sacrificed and decapitated; brains were removed, washed extensively in PBS and transferred into 2.5% glutaraldehyde in 0.1M cacodylate buffer and post fixed in 2% osmium tetroxide for 2hrs. Cortical regions were taken and dehydrated in series of graded alcohol. The slices were embedded in epoxy resin, 0.5 micron sections were made using ultra microtome, examined by light microscope for overall view. Ultrathin sections were then cut, taken on to grid and observed under electron microscope.

Isolation of brain sequestered leukocytes

Mice are anaesthetized and sacrificed after perfusing them with ice cold PBS to remove circulating blood and non-adherent lymphocytes. Brains were dissected out and minced into small pieces with teasers loosen the tissue by passing through pasture pipettes with different bore sizes from normal to very small. The resultant cells were treated with DNase 2U/ml for 30 min. at room temperature followed by passing the extract through sterile 18 gauze needle and centrifuged for 5 min at 4000 rpm. The resultant supernatant was deposited on 30% percoll gradient and centrifuged at 700xg for 11 minutes, the pellet is collected resuspended in FACS sheet fluid (eBiosciences), labelled the cells with FITC conjugated CD4 and PE conjugated CD8 antibodies (following the dilutions recommended by the provider) and counted by using BD Biosciences cell sorter.

Co-immunoprecipitation

Mice brain samples were homogenized in Nonidet P-40 lysis buffer (50mM Tris, pH 8.0; 150mM Nacl; 1% Nonidet P-40; complete protease inhibitor) and centrifuged at 15000xg for 10 min at 4^0 C. Supernatants collected were used for Co-immunoprecipitation. Briefly, 250 µg of protein from control and CM mice brain samples were incubated with caspase-3 and calpain1 and NF (L) overnight at 4^0 C followed by 20 µl of protein-A-sepharose-A beads for 90 min at RT. Beads were washed and resolved on SDS- PAGE, transferred on to nitrocellulose and probed with NF (L) and Gra-b respectively.

Statistics

Densitometry analysis for the western blots was performed by using the NIH ImageJ software and graphs were plotted by using sigmaplot 3.5. The p values less than or equal to 0.05 were considered as significant. Survival analysis graphs were plotted by using MedCalC Kaplan-Meiyer survival analysis curve. Real Time data analysis was done by using Δ^{-ct} method.

Results

CM induces neuronal degeneration in Plasmodium berghei ANKA infected mice brains

Neuronal death in the CM brains was observed by staining brain sections with hematoxylin, eosin and cresyl violet. We found an extensive disruption of neuropil and altered cellular architecture in the brain parenchyma. Increased cellular and tissue vacuolation, crenulated cells observed in the cortices of infected mice brain samples over the controls indicating apoptotic mode of cell death and decreased H&E staining and enlarged cells indicate the edema and cell death by hypoxic conditions. Moreover, we also observed heavily condensed cells with intense hematoxylin staining suggesting pyknotic cell death (Fig-4a). Cresyl violet staining clearly showed an increased neuronal degeneration as indicated by condensed cell shape, crenulated appearance, pyknosis, intense cresyl violet accumulation, vacuolation and detachment from the brain parenchyma (anoikosis). Extensive tissue disruption in the present study suggests of necrosis in all the infected brain samples (Fig- 4b).

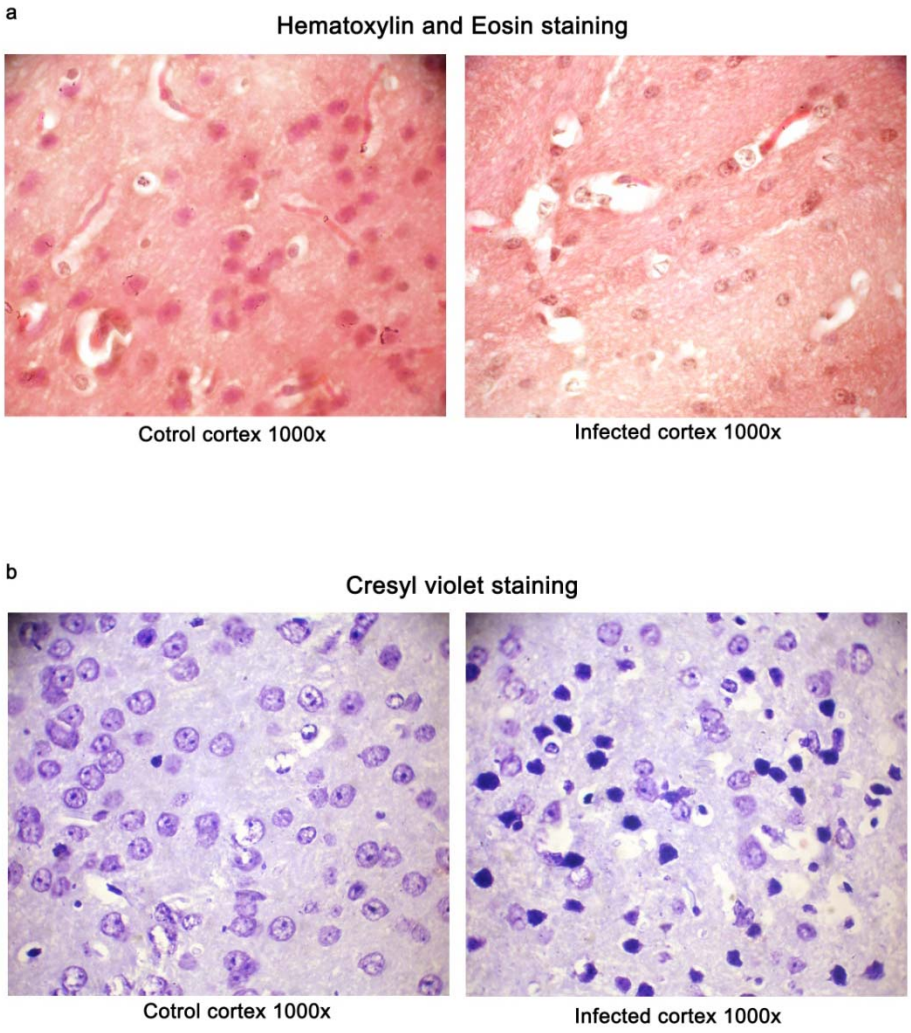


Fig. 4 – Histology of brain tissue sections stained with H&E and cresyl violet. a) Increased cell death in the cortical region of infected mice brain evident by their crenulated appearance, cell shrinkage, vacuolation, detachment from the brain parenchyma, and tissue disruption over the control mice can be observed. Images were obtained at 1000× magnification with Olympus fluorescent microscope. b) Cresyl violet staining was used to identify the status of neurons in the infected mice cortices over the control. Increased neuronal death was observed in the infected mice brain over the control evident by their shrunken, crenulated shape and increased vacuolation and tissue disruption and decreased intensity to stain with cresyl violet. Images were obtained at 1000× magnification with Olympus fluorescent microscope

Cerebral malaria results in elevated interactions between activated caspase-3, calpain-1 and cathepsin-b and a mixed apo-necrotic form of neuronal death.

Western blot analysis of caspase-3, calpain-1 and cathepsin-b clearly indicated a significant increase in their protein levels in the cortical samples of infected mice over the controls (Fig-5a, Fig-5b, Fig-5c). Immunohistochemistry of active caspase-3 showed a predominant elevation in the infected mice brain cortices over the controls. Both full form and catalytic fragments of calpain-1 levels were significantly increased in the infected mice brains over the control mice brain cortices. Furthermore, lysosomal protease cathepsin-b levels were also elevated along with active caspase-3 and calpain in the infected mice brain samples. Immunohistochemistry was performed to observe the localization pattern of these proteases in the infected and control mice brain cortical regions. A significant increase in the levels of active caspase-3 cells were observed in the cortical region of infected mice brain over the controls indicating an increase in the apoptotic cell death in the infected mice brain cortices (Fig-6a). Immunohistochemical analysis of calpain-1 showed a significant increase in the number of calpain-1 positive cells in the infected mice brains over the controls. The elevation of calpain suggests either apoptotic or necrotic modes of cell death chosen by the cell at later stages (Fig-6b). Further, immunohistochemistry of cathespsin-b also showed a significant increase in the levels of cathespsin-b immunoreactivity in the infected mice brains over the control along with active caspase-3 and calpain-1 (Fig-6c). This increase correlating with elevated calpain-1 levels indicates that the cell might choose either apoptotic or necrotic modes of cell death. Co-localization studies using triple immunofluorescence was used to study the interactions between these proteases (Fig-7). We observed some cells in the infected mice brain cortices stained positive for all these three proteases suggesting a tendency for that specific cell to undergo apo-necrotic cell death continuum. Hence, in order to check whether any of the cells in the infected mice brains shows apo-necrotic mode of cell death ultrathin brain sections from infected mice brain were obtained and observed under transmission electron microscope. Ultrastructural analysis showed a mixed type (apo-necrotic) of cell death with morphological features containing both apoptosis and necrosis. We observed marginalization of neuronal nuclei, membrane blebbing, and proapoptotic body formation indicating classical features of apoptosis and mitochondrial swelling, endoplasmic reticulum breakdown, vacuolation and disintegration of nuclear membrane indicating necrotic mode of cell death in a single degenerating neuron (Fig-8a, 8b).

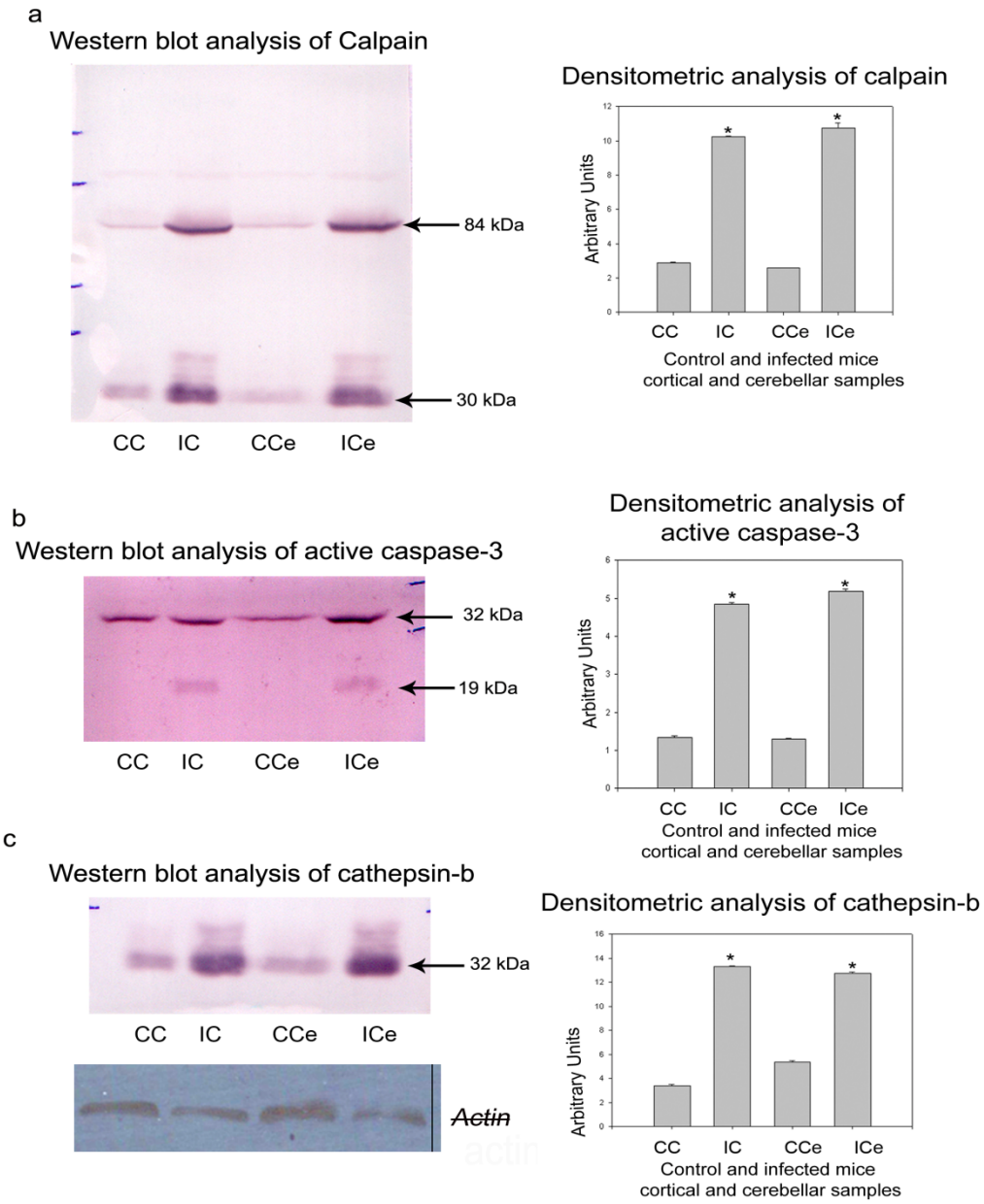


Fig. 5 – Western blots of calpain-1, caspase-3 and cathepsin-b. a) Western blot of calpain-1 showed a significant increase in the calpain-1 levels in the infected mice brain cortical regions and an internal control cerebellum samples. Both full length and catalytic fragments (30 kDa) were found to be increased in the cytosolic fraction of infected mice brain samples over the controls. $p<0.05$ is considered to be statistically significant. b) Western blot of caspase-3 showed no difference in the levels of pro-caspase-3 in the cytosolic fractions of infected mice brain samples over the controls. However significant increase in the active caspase-3 was observed in the infected over the controls. $p<0.05$ is considered to be statistically significant. c) Western blot of lysosomal protease cathepsin-b was found to be increased in the infected mice brain samples over the control. $p<0.05$ is considered to be statistically significant. n=5; each blot was repeated 3 times. Bars represent standard error.

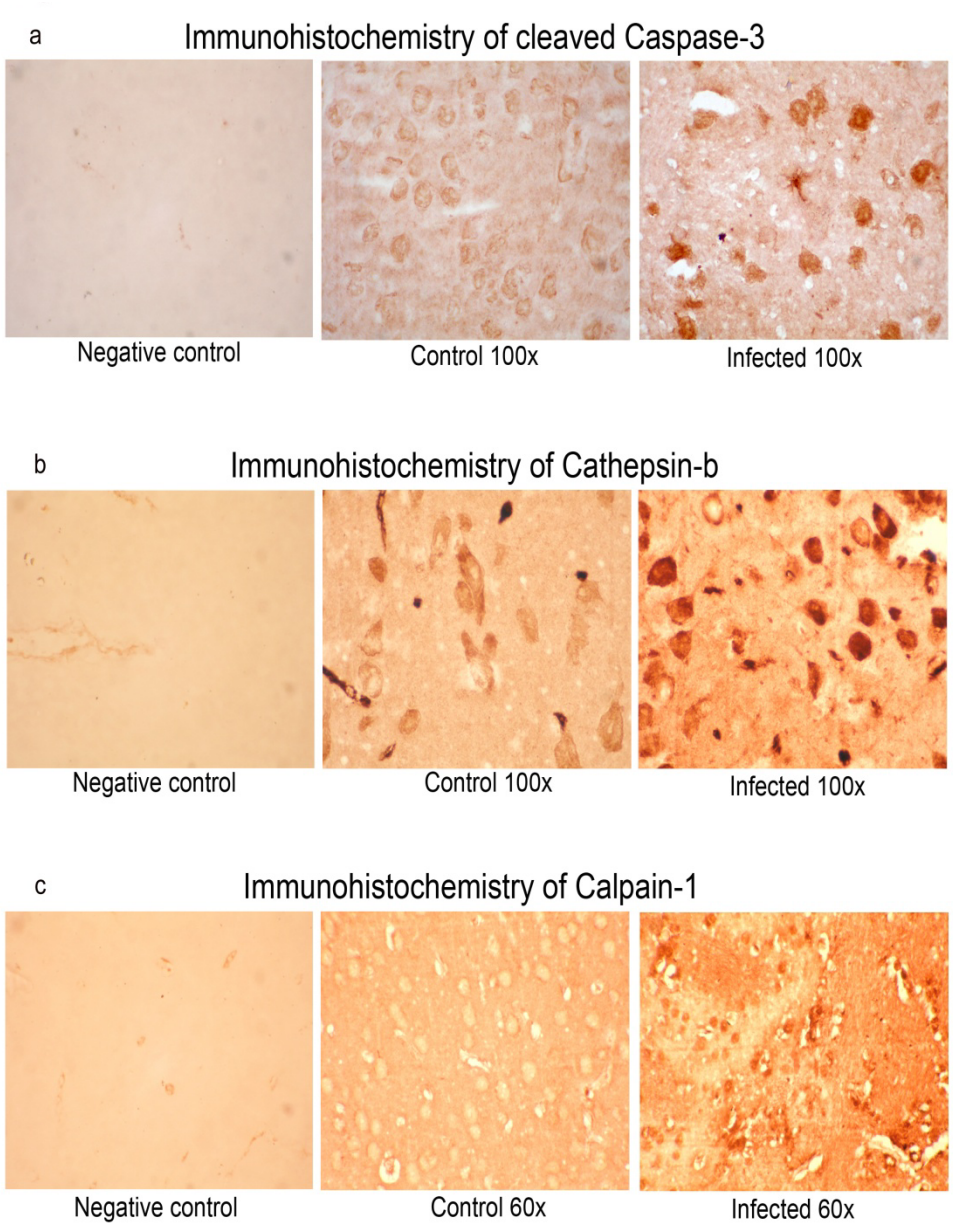


Fig. 6 – Immunohistochemical analysis of cell death proteases, a) Immunohistochemistry of active caspase-3 (antibody specific for active caspase-3 was used) indicated an increase in active caspase-3 in the brains of infected mice over the controls. Magnification 600×. b) Immunohistochemistry of calpain-1 showed a dramatic increase in the intensity of calpain1 in the infected mice brain cortices over the controls correlating with our previously published results. Magnification 600×. c) Immunohistochemistry of cathepsin-b. Lysosomal protease cathepsin-b was found to be elevated in the infected mice brain cortices over the controls. In addition to its localization in the cells, increased cathepsin-b was also found in the brain parenchyma or brain matrix. Magnification 400×. No immunoreactivity was observed in negative controls in which primary antibody addition was omitted. n=3; 3 parallel sections from each mice brain were used

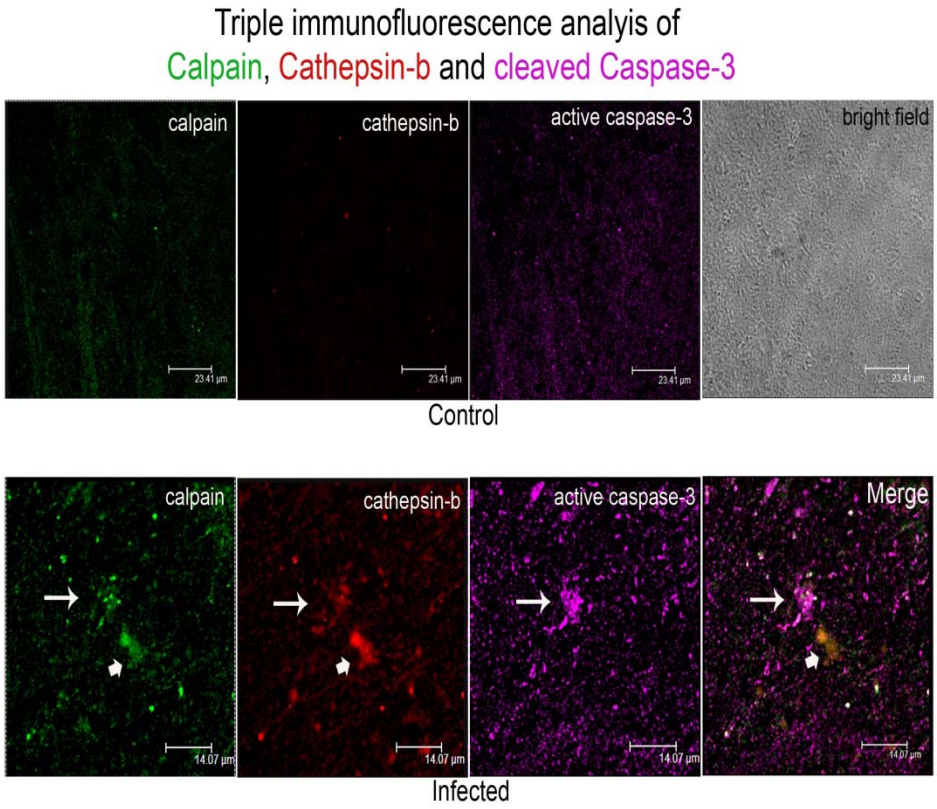


Fig. 7. Triple immunofluorescence analysis of active caspase-3, calpain-1 and cathepsin-b. Triple immunofluorescence was used to observe the cross-talks between these proteases. Arrow points towards the degenerating cell positive for active caspase-3, calpain-1 and cathepsin-b indicating cross talks among them. Moreover due to the presence of active caspase-3 the resulting cell death might be apoptotic. Cell pointed with arrow head shows the absence of active-caspase-3 and increased presence of calpain-1 and cathepsin-b indicating that the resultant cell death might be necrotic.

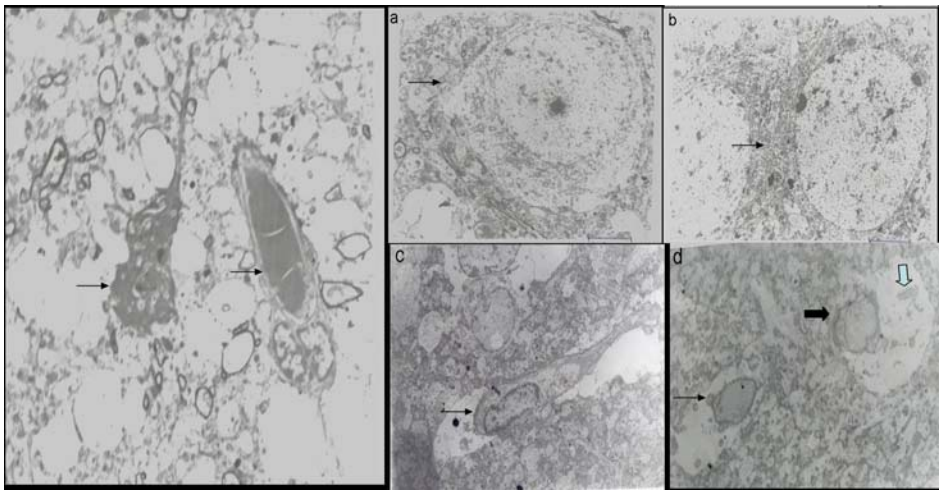


Fig. 8 – TEM photographs of the control brain cortical region showing the intact neuronal cell with intact neuropil and connections with parenchyma tissue. Adjacent right panel showing the oval shaped normal nucleus with intact chromatin. Intact nuclear membrane with normal nuclear pores and intact inter nuclear tight junctions are shown by arrows (panels a and b). c) Electron micrographs of a neuron that lost its contact with brain parenchyma and degenerating dendrites (arrow). Ultrastructural changes of the neuronal cell of cerebral malaria mice brain cortical region with crenellated neuronal nucleus and apoptotic blebbing formation (thin arrow), endoplasmic reticulum breakage (blue arrow), and swollen edematous nucleus and outer nuclear membrane disintegration and vacuolation (thick arrow).We can also observe the internuclear tight junction disintegration in the infected samples(d), suggesting apo-necrotic continuum . Overall magnifications of electron micrographs are 3580×.

Status of PSD-95, Vimentin, NF-L and synaptophysin

Effects of the increased cell death protease levels on cellular substrates like vimentin, PSD-95 and synpatophysin of infected mice brains were analysed by western blot analysis. Western blot analysis showed a significant increase in the breakdown products of vimentin (Fig-6a), NF-L (Fig-6b) and decreased levels of PSD-95 and synaptophysin (Fig-6c, 6d). Correlating with increased neuronal death, NF-L was extensively proteolysed to 57 and 53 kDa fragments. Moreover, we observed vimentin breakdown products of 50 and 44 kDa known to be mediated by calpain. We also observed decreased synaptophysin levels in infected over control mice brain cortical samples (Fig-6d). This might be associated with neuronal degeneration in the infected mice brain cortices over the controls (Fig-6e). In order to identify the interaction of caspase-3 and calpain with vimentin, PSD-95 and NF-L, we performed co-immunoprecipitation experiments by immunoprecipitating the protein complexes in the cytosol with calpain1 and caspase-3 antibodies and western blotting with vimentin, PSD-95 and NF-L antibodies. Significant increase in the binding of vimentin (Fig-7a), PSD-95 (Fig-7b) and NF-L (Fig-7c) with caspase- 3 and calpain were observed in the infected mice brain samples over the control mice brains. Negative controls showed no binding of these protein complexes. The increased binding of vimentin, PSD-95 and 7 NF-L correlated with their breakdown and subsequent decrease in the infected samples over the control mice brain samples. The breakdown of these cytoskeletal proteins also correlated with significantly increased cell death in the infected mice brain samples over the controls. Importantly, the decrease in the PSD-95 levels and breakdown of NF-L correlated with the increased neuronal death observed in the cresyl violet staining and neurological deficit of the infected mice.

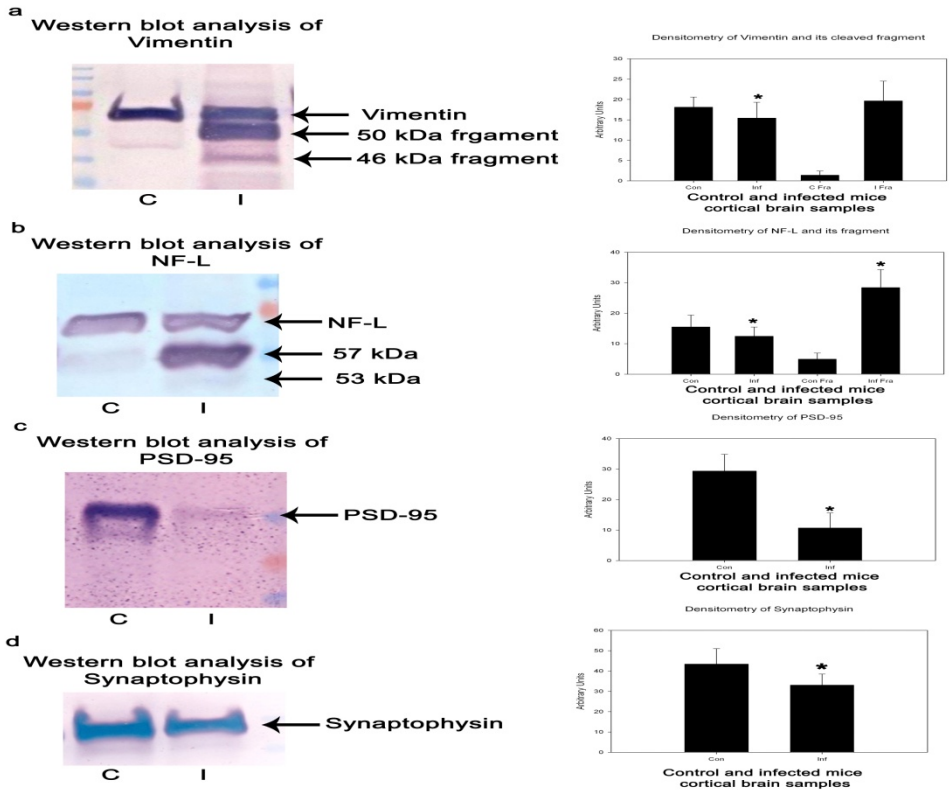


Fig. 9 –Western blots of cytoskeletal proteins. a)Western blot of vimentin. Significantly increased breakdown products of vimentin were observed in the cortical samples of infected mice brains over the controls. The identified breakdown products were having the molecular weight of 50 and 46 kDa. b) Western blot of NF-L. Increased breakdown of neuronal filament was observed in the infected mice brain over the control. The observed molecular weight of NF-L fragments was 57 and 53 (less distinct) kDa. c) Western blot of PSD-95. Significant decrease in the PSD-95 levels was observed in the infected mice brain samples over the controls in the cortical brain samples. Decrease in the PSD-95 levels correlated with the breakdown of NF-L in the infected mice brain samples. d) Western blot of synaptophysin. Decrease in the synaptophysin levels was observed in infected mice cortices over the control mice brain samples. n=5; each blot was repeated 3 times. $p<0.05$ was considered to be statistically significant. Bars represent standard error.

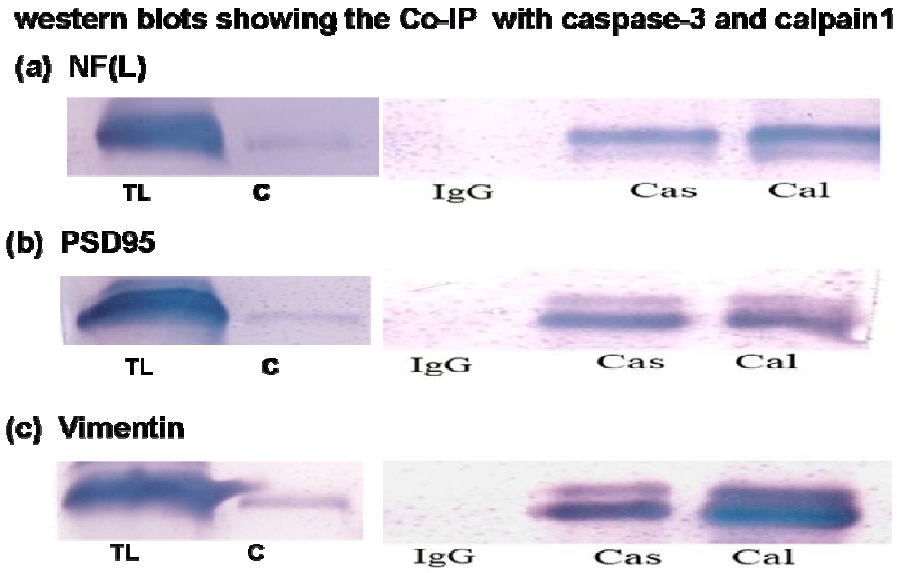


Fig. 10 – Co-immunoprecipitation of vimentin, PSD-95 and NF-L. a) Co-immunoprecipitation of vimentin with caspase-3 and calpain antibodies revealed a significant increase in its binding with the suicidal proteases in the infected mice brain samples correlating with its subsequent breakdown. b) Coimmunoprecipitation of PSD-95 revealed an increase in the binding of calpain and caspase with PSD-95 in the infected mice brain cortical samples correlating with its decreased presence in the Western blots. c) Co17 immunoprecipitation of NF-L indicated an increase in the binding of NF-L with caspase-3 and calpain in the infected mice brain samples. n=5; each blot was repeated 3 times. No binding was observed in the primary antibody omitted negative controls.

Discussion

Pathophysiological features of cerebral malaria in humans and mouse models include sequestration of pRBCs in the brain vasculature, blood brain barrier (BBB) breakdown (due to the ischemic conditions, toxic metabolites or both) (Hatabu et al., 2003; Hunt et al., 2006; Johnson et al., 1993; Medana and Turner, 2006; Pino et al., 2005), elevated immune cell infiltration into the brain (resulting in dampened peripheral immune responses) (Patnaik et al., 1994), cerebral hemorrhages (White and Silamut, 2005), brain edema (Sanni, 2001), degeneration of vascular and non-vascular cells of the brain (Medana and Turner, 2007; Pino et al., 2003; Wiese et al., 2006), increased oxidative load (Becker et al., 2004) and activation of resident immune cells in the brain (glial cells) culminating in increased mortality or persistent neurological deficits. Several hypotheses indicate that cerebral malaria (CM) might be a result of increased cytoadherence of pRBCs in the brain vasculature leading to focal hypoxic conditions and BBB dysregulation leading to the pathogenesis of the disease (van der Heyde et al., 2006). However, it is becoming well accepted that the pathogenesis is multifactorial.

In the present study we have observed some important pathological features in CM including elevated suicidal proteases which were involved in apoptotic and necrotic cell deaths. Moreover, in accordance with the previous reports in other models like cerebral ischemia and brain tumor, these proteases were found to interact with each other. Cross talks between suicidal proteases and the ability of these proteases to act independently or in synergy with each other are some of the most troubling phenomena that greatly attenuate therapeutic benefits (Neumar et al., 2003; Rami, 2003). For example, the ability of caspases to cleave calpastatin an endogenous substrate for calpain helps in the activation of calpain (apart from other stimulus like elevated intracellular calcium levels) (Kato et al., 2000). Calpains were known to interact with lysosomes (storage houses of lysosomal proteases) and help in the spillage of cathepsins into the cytosol (apart from acidification of the cell which helps in the activation and spillage cathepsins) (Yamashima et al., 2003). Increased cathepsin levels and activity were shown to be involved in activation of calpains. Once activated calpains and or cathepsins can mediate either apoptotic or necrotic cell deaths and caspases can mediate apoptotic cell death depending on the intensity and nature of the insult.

More importantly, these proteases share similar substrates specificity (with varied intensities) cleaving several cellular substrates (Liu et al., 2006). PARP-1 is another best known molecule which is cleaved by caspases, calpains, cathepsins and granzymes leading to the production of specific signature fragments like 89 and 21 kDa (caspase specific

fragments), 72 and 64 kDa (granzyme-specific fragments), 50 (necrotic fragment produced by cathepsin-b), and 45 (calpain specific fragment) (Froelich et al., 1996; Gobeil et al., 2001; Wang, 2000; Zhu et al., 2009). However, the conditions and the molecular mechanisms that were involved in a cell choosing the protease required for the specific execution of cell death form still remains to be understood. Hence, it becomes difficult to choose a beneficial therapeutic approach by inhibiting a single suicidal protease. Caspases, calpains and cathepsins are some of the crucial proteases in the cell death machinery. Though they are well known to interact and activate each other, their activation and localization profiles in a temporal, spatial specific manner are still lacking in cerebral malaria. Moreover, their role in mediating either apoptotic or necrotic cell deaths independent or dependent of each other makes it very difficult to understand their downstream signaling events that participate in executing specific cell death. A recent report that also describes the failure of decreasing CM pathology despite inhibiting caspase-3 and calpain-1 is a very good example of highly complex pathology of cerebral malaria (Toure et al., 2008); Helmers et al., 2008). Previous reports from our lab showed increased levels of caspase-3, calpain-1 and cathepsin-b in cerebral malaria mice (Shukla et al., 2006) and in rat model of focal cerebral ischemia (Chaitanya and Babu, 2008). In general, caspases mediate the cell death through apoptosis by cleaving the cytoskeletal proteins which leads to the shrinkage of cell and eventual death (Fan et al., 2005), whereas calpains execute the cells through necrosis and help in the spillage of cathepsins from lysosomes (storage houses of lysosomal proteases) into the cytosol (apart from acidification of the cell which helps in the activation and spillage cathepsins) (Yamashima et al., 2003) which in turn kills the cells that aggravate the effect. Moreover, the colocalization of these proteases indicates their cross talk during the pathology. The major two types of cell death mechanisms (apoptosis and necrosis) are clearly distinct by their anatomical changes and hence the mode of cell death. Our TEM study showed degenerating neurons exhibiting the features of both necrosis and apoptosis indicating an intermediate stage/type of cell death "apo-necrotic" continuum, which correlated with the elevated levels of these cell death proteases and their cross talk within the same cell undergoing death.

Cell death in fatal murine cerebral malaria is heterogeneous; in the present study we have observed cells undergoing apoptotic and necrotic cell deaths apart from previously published apo-necrotic continuum. Neuronal apoptosis has been shown to be a main cause for the pathology of cerebral malaria (Wiese et al., 2006). Here we show that neuronal cell death during cerebral malaria involves extensive cleavage of the neuronal cytoskeletal proteins by

the cell death proteases belonging to apoptotic and necrotic cell deaths. Cytoskeleton, the filamentous protein and tubules present in the cytosol of the cell are important in maintaining the cell integrity, transport of materials, cell signaling and also for the cell survival (Dillon and Goda, 2005). Through immunofluorescence studies we found the co-localization of the active caspase-3, cathepsin-b and calpain-1 in a single cell undergoing cell death. This indicates the cross talks between these cell death proteases, their synergy in aggravating the cell death and contributing to increased neuronal deficits and or mortality. Our results show that the elevated levels of cell death proteases during cerebral malaria and their action on cytoskeletal proteins might result in increased neuronal death. The decreased intensity of PSD-95, a synaptic density protein, clearly indicates a severe compromise in the neuronal synaptic function which correlates with neuronal filament breakdown and observed neurological deficits in the infected mice over the controls. Moreover, we observed increased interactions of suicidal proteases involved in apoptotic and necrotic cell deaths which correlated with the ultra-structural features of cell undergoing apoptosis, necrosis and apo-necrotic forms of cell death. The inhibition of these proteases during the earlier stages of cerebral symptoms may seem to increase the longevity of the infected, which otherwise leads to sudden onset of un-arousable coma and death. Molecular mechanisms involved in the regulation of these proteases' activation, their involvement in execution of apoptotic or necrotic or apo-necrotic forms of cell death in a temporal and spatial manner during this pathology need to be addressed. Moreover, these need to be traced out to identify the role of infiltrating immune cells, resident immune cells, hypoxia and the toxins released by the sequestered parasites during the pathology and their link with the activation of suicidal signaling cascades in this pathology.

CHAPTER I (b)

Role of CTLs in neuronal cell death during fatal murine cerebral malaria

Introduction

Cerebral malaria is a complex, pathological syndrome that is broadly caused by hypoxic conditions, immune system effects, the pathogenic infiltration of effector T cells (Boubou et al., 1999; Nie et al., 2009) and toxins that are released by the parasites (Bate and Kwiatkowski, 1994; Jakobsen et al., 1995; Parroche et al., 2007). Recently, the role of the immune system in central nervous system (CNS) pathology has drawn much attention (Bartholomaus et al., 2009). In particular, focus has been on activated T cells entering into the CNS and causing damage. The role of T cells in *Plasmodium berghei* ANKA induced (*PbA*) pathology has been studied at the blood (Miyakoda et al., 2008; Shibui et al., 2009) and liver stages (Chakravarty et al., 2007) of malaria. The functional behaviour of T cells differs at varying stages of pathology. CD8+ T cells are reported to be protective during the blood and liver stages but are pathogenic in the brain (Ocana-Morgner et al., 2003; Reyes-Sandoval et al., 2011). In contrast, CD4+ T cells that are pathogenic during the liver and blood stages (Haque et al., 2010; Weiss et al., 1993) are protective in the brain (Haque et al., 2011b). Indicating the function of these cells depends on their location or in which tissue they are present. Much evidence is being accumulated on the infiltration of peripheral immune cells into the brain (Coban et al., 2007; Nie et al., 2009). During experimental cerebral malaria (ECM) pathology, a series of events occurs that leads to unarousable coma and death. The series of events occurs due to occlusion of parasitized red blood cells (pRBCs) in blood vessels, leading to breakdown of the blood brain barrier (Hearn et al., 2000), an insufficient supply of blood, oxidative stress and oedema (Penet et al., 2005; Schluesener et al., 2001; Thumwood et al., 1988) and the infiltration of activated immune cells into the brain parenchyma, which may directly kill the resident cells, or to activation of the resident immune cells (Potter et al., 1999; Potter et al., 2006) .

The cell death that occurs during cerebral malaria has been shown to be associated with elevated levels of cell death proteases such as cathepsin b, calpain1 and active caspase-3, indicating apo-necrotic cell death. We have recently shown that activation of these cell death proteases affects the breakdown of the neuronal cytoskeleton (Eeka et al., 2011b), but this appears to be stress induced rather than the result of a pathogen-related signalling cascade. This is also evident from studies that showed no role of TLRs in the pathology of CM (Togbe et al., 2007).

The infiltration of immune cells, particularly CTLs (activated CD4+ and CD8+ T cells), into the brain in human cerebral malaria (observed in post-mortem samples (Dorovini-Zis et al., 2011; Hunt and Grau, 2003; Taylor et al., 2004) and in mouse models has been shown (Belnoue et al., 2002; Belnoue et al., 2008), and the pathogenicity of the infiltrated CTLs has been demonstrated in other neurodegenerative diseases, such as cerebral ischemia (Chaitanya et al., 2010). CTLs have been shown to kill the resident cells by direct cytotoxicity that is caused by Granzyme-b/perforin-mediated cell death. There is no evidence indicating whether CTLs directly kill neuronal cells in the brain. Exploring the role of each component involved in the complex pathology helps in understanding the mechanism of cell death and may help in pathological intervention. Hence, we studied the role of infiltrated CTLs in the pathology of experimental cerebral malaria.

Cytotoxic T lymphocytes (CTLs) are activated immune cells belong to subset of mature CD8+ T cells and are very critical for CM pathogenesis. They kill the infected cells by direct cytotoxicity through Gra-b and perforin pathway. Role of these cells in CM pathogenesis is of interesting as there is no infection to the cells of brain during CM and how they are involved in neuronal cell death if at all. We have earlier reported the Gra-b mediated neuronal cell death in human ischemic post-mortem samples and others during *pbA* infection mice with CD8+ T cells lacking perforin and Gra-b are protected from CM.

Results

Overexpression of CXCR4, ICAM-1, VCAM-1 and CTLs infiltration

Up regulation of cell adhesion molecules (ICAM-1 and VCAM-1) and chemokine receptor (CXCR3) have been reported during CM conditions. Hence we investigated we studied the expression levels of cell adhesion molecules and chemokine receptors and found CXCR4, ICAM and VCAM-1 expression is significantly in CM compared to control (Fig.11a). This is consistent with the presence of increased levels of TCR-a receptor gene in the infected compared to control (Fig.11b). Infiltration of immune cells into the brain was studied by immunohistochemical analysis and found infiltration of CD4+ and CD8+ T cells into the infected brain, but no infiltration in the PBS injected control mice, indicating the infiltration of CTLs during the pathology of cerebral malaria (Fig.12d-g). We also studied the expression levels of other chemokine receptors and found that those molecules are also expressed in others conditions like severe malaria and are not critical for the CM, because protection of mice from CM also brought down the CXCR4, ICAM-1 and VCAM-1 expression levels significantly but not the other molecules

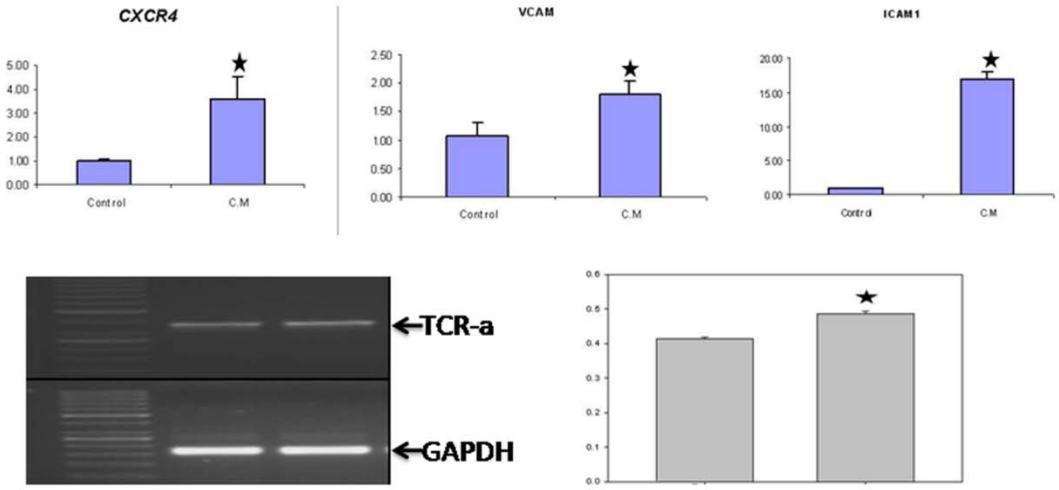


Fig.11.Showing the graphs of Real Time analysis of CXCR4, ICAM1 and VCAM1 indicating the increased significant levels of these molecules compared to control and the b) PCR analysis showing the increased levels of TCR-α gene in the infected mice ECM mice brain samples compared to control mice. Graph showing the densitometry analysis of the TCR-α gene, indicating the infiltration of CTLs through upregulation of chemokine receptor and cell adhesion molecules. Astrick above the bar indicates the significance (P< .05).

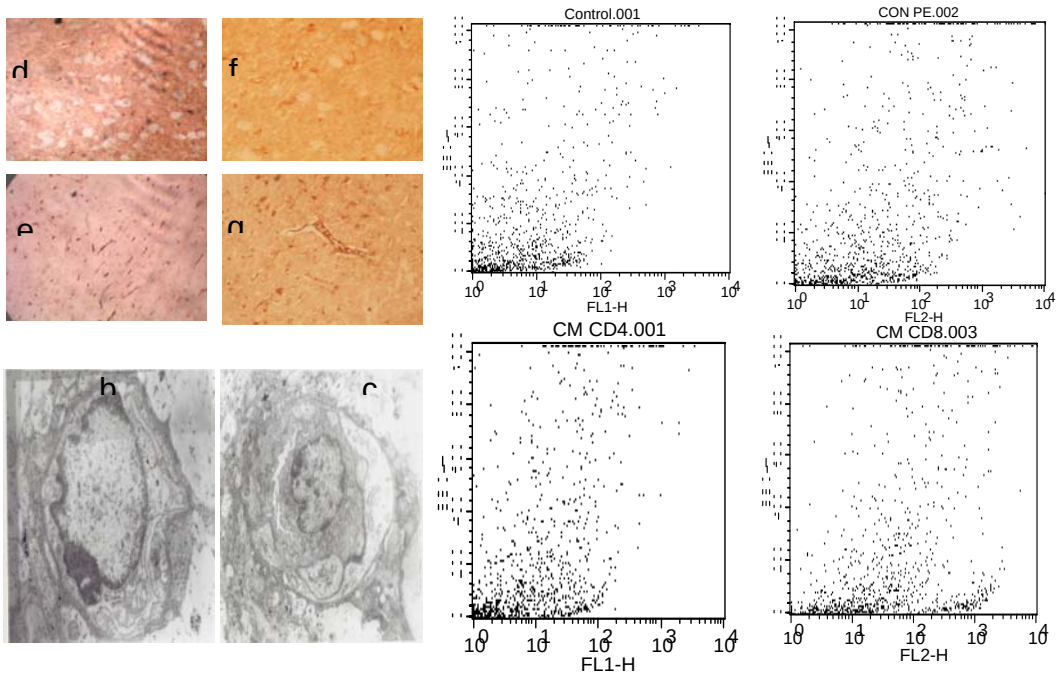


Fig. 12 Showing A, C) Immunohistochemisrty of control mice brain sections for CD4+ and CD8+ T Cells and B, D) are CM mice brain sections for CD4+ and CD8+ T Cells showing the increased T cells infiltration when compared to control respectively. E and F) are TEM photographs of cerebral malaria mice brain sections showing the infiltrated lymphocytes with large nucleus and pseudopod like structures indicates their infiltrating nature. G, I) FACS analysis of the CD4+ (FL-2(FITC) and CD8+(FL-1(PE) T Cells isolated from the spleen of normal mice and H, J) showing the FACS analysis of CD4+ and CD8+ T Cells isolated from the brain of CM mice. The FACS analysis showing the presence of T Cells in the CM mice brain samples indicating T cells infiltration during this pathology.

Interaction of infiltrated T cells with the neuronal cells

To study whether these CTLs are interacting with neuronal cells, we performed triple immunofluorescence analysis with CD4+ and CD8+ T cell markers and NeuN (Fig.13). We found the close apposition of these CTLs with neuronal cells, indicating the interaction of CTLs with neuronal cells. This is also evident from the co-immunoprecipitation (Co-IP) results showing the interaction Gra-b with NF (L).

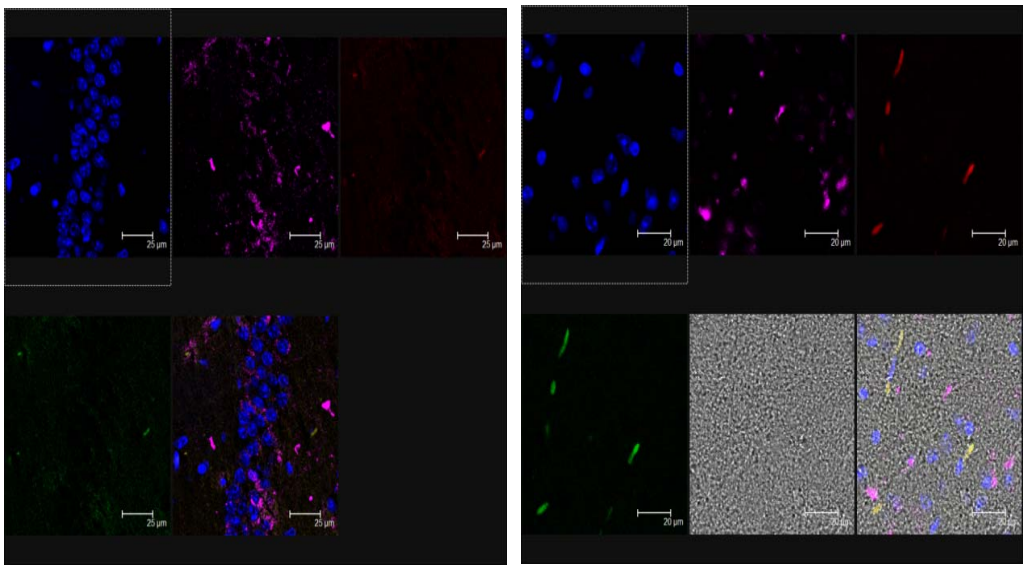


Fig.13 Triple immunofluorescence analysis of control and CM mice brain sections showing the infiltration of CD4+ T (FITC-green) Cells and CD8+ T (PE-red) Cells into brain of CM mice (A) compared to control (B) and their close apposition with the neuronal cells (neuronal marker, NSE-magneta indicated by arrows) indicating the direct interaction of T Cells with the neurons during this pathology. This is also evident from the Western blot showing co-immunoprecipitation of Gra-b with NF(L) (C). Graph showing the densitometry analysis of significant interaction of Gra-b with NF(L) in ECM compared to control (D).

Granzyme-b secreted by the CTLs kills the neuronal cells

CTLs kills the neuronal cells through Gra-b/perforin mediated pathway, therefore we studied the protein levels of Gra-b through western blotting and immunofluorescence analysis (Fig.6). Further, we studied whether the neuronal cell death during CM is Gra-b mediated, hence performed triple immunofluorescence analysis of CTLs with Gra-b. Immunofluorescence analysis showed colocalisation of Gra-b with the degenerating neurons (FluoroJade-b B positive cells), implying the Gra-b is indeed responsible for neuronal cell death (Fig.14). This is evident from the Co-IP showing the interaction of Gra-b with NF (L) and detection of NF (L) into its signature fragments in the CM compared to control (Fig.14 western blots). We have earlier reported the neurodegeneration in CM is caused by the cleavage of neuronal cytoskeleton by the elevated levels of capase-3 and calpain1 during CM (Eeka et al., 2011a). Gra-b interaction with caspase-3 have been shown in cerebral ischemia rat model (Chaitanya et al., 2010), hence we performed the Co-IP experiments to check if Gra-b here also playing a role in activating caspase-3 and calpain1 (Fig.15). We found Gra-b is interacting with caspase-3 and calpain1 and activating them in CM mice compared to

control. We also found that Hsp70 is getting cleaved during CM, which is reported to exist in complex with caspase-3 and prevents it to become active.

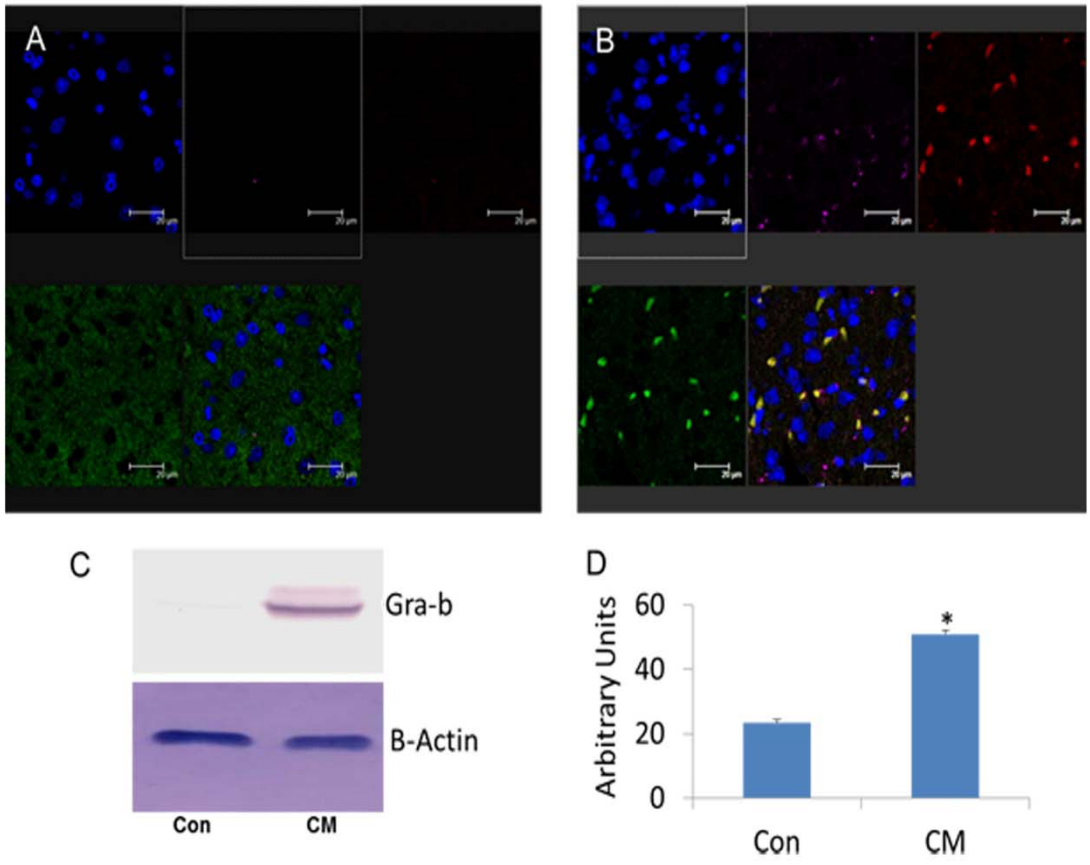


Fig. 14. Triple immunofluorescence analysis showing the co localization of Gra-b with the infiltrated CD4+ and CD8+ T Cells in the CM mice brain (Fig.6 (B) when compared with control mice brain samples where is no infiltration of CTLs and the Gra-b (Fig. 6(A), indicating release of Gra-b by the infiltrated cells which is also shown by the western blot analysis (Fig.6(C) that Gra-b present only in the CM mice brain samples. Graph showing the densitometry analysis of the Gra-b showing the significant increase of Grab during ECM compared to control mice brain samples.

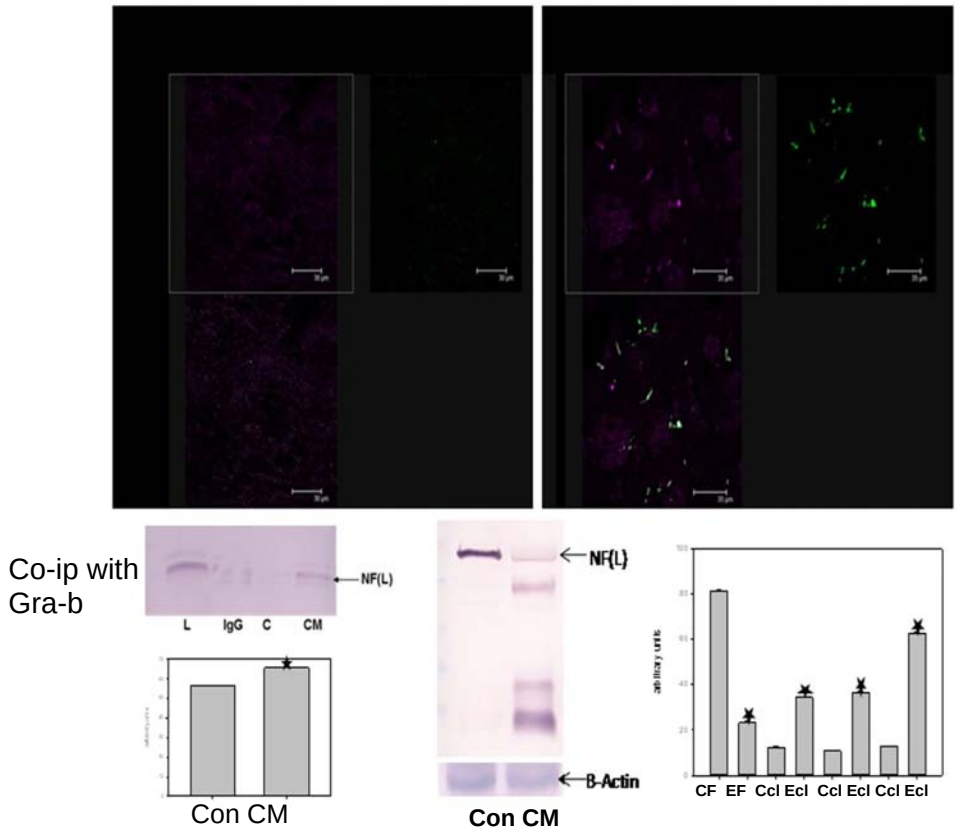


Fig. 15 Double immunofluorescence analysis showing the colocalization of Fluorojadeb (specific marker for degenerating neurons) dying neurons with Gra-b in the CM mice brain sections (A) indicating the neurons are dying by Gra-b mediating killing where as there is no Gra-b and neuronal cell death in control (B). This also evident from Western blot showing the breakdown of NF(L)(C) and the graph showing the densitometry analysis of NF(L) breakdown evident from decrease in NF(L) full form in ECM compared to control indicated by CF for full form of control, EF is full form of CM sample, Ccl and Ecl indicate the cleaved products of NF(L) form control and CM mice brain samples respectively.

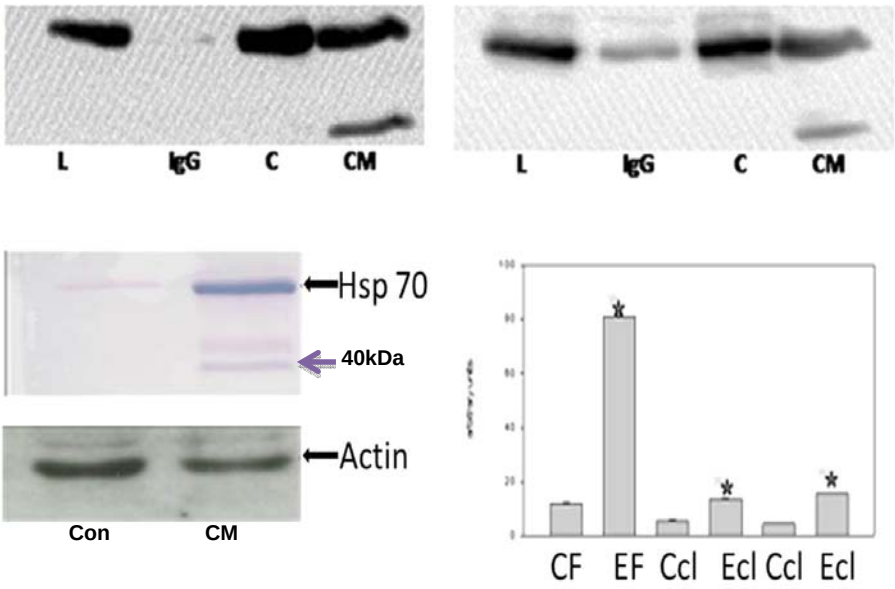


Fig. 16. Western blot analysis shows the co-immunoprecipitation of Gra-b with caspase-3 and calpain1 indicating the interaction. We can also see the interaction of Gra-b with caspas-3 and calpain1 in control also, but the interaction doesn't lead to the formation of active caspase-3 and calpain1 implying there might be some other protein complex that might be protecting or the Gra-b in the control samples is not sufficient to form the active product. B) Western blot of hsp70 showing its cleavage during ECM, also indicated by the increased cleavage products of hsp70 during ECM compared to controls. C) Densitometry analysis showing the increased levels of hsp70 and its increased breakdown during ECM, blue bar indicates hsp70 protein levels in control and red indicates the ECM samples. Numbers 1 indicates the full form, 2 and 3 indicates the cleavage products.

Discussion

Cerebral malaria is an acute, complex, pathological neurodegenerative disease that arises due to *Plasmodium* infection. Sequestration of parasitised RBCs (Franke-Fayard et al., 2005; MacPherson et al., 1985), infiltration of T cells (Hansen et al., 2007) and neuronal cell death (Anand and Babu, 2011; Stoltenburg-Didinger et al., 1993) are the most common features that are associated with this syndrome. Hence, the pathological outcome depends on many factors: activation of the cell death signalling pathways triggered by the pathogen (Amante et al., 2010; Coban et al., 2007), stress developed due to the hypoxic conditions (Hempel et al., 2011a), toxicity of secondary metabolites (Dostert et al., 2009; Jaramillo et al., 2004), toxins released by the parasites (Tachado et al., 1996) and adverse effects of the host immune system (Amante et al., 2010; Belnoue et al., 2003; Yanez et al., 1999). The pathology is the outcome of the neuronal sequalae that lead to the sudden onset of unarousable coma and death, which makes CM a deadlier disease than malaria. A clear understanding of the disease pathology is of critical importance to prevent the onset of cerebral pathology during *Plasmodium* infection. Currently, there are no available drugs that can be used to prevent the disease. Reports indicate the role of elevated levels of cell death proteases (Lackner et al., 2007; Medana et al., 2007), cytotoxic proteases (Nitcheu et al., 2003) and cytokines (Hanum et al., 2003) in the pathogenesis of CM. Elevated levels of cell death proteases, such as fodrin (Janicke et al., 1998) and spectrin (Shukla et al., 2006), are associated with the cytoskeleton. Previously, we have shown that cell death proteases can cleave NF (L) into its signature fragments (Eeka et al., 2011a). Further, *in vivo* inhibition of caspase-3 and calpain has been found to be neuroprotective, to prevent cerebral ischemia in a rat model (Cheng et al., 1998; Knoblach et al., 2004) and to increase the survival (up to 20 days post-infection in *z-VAD-fmk* and *ALLN* treated mice) of mice infected with PbA (data not shown). Lymphotoxin-α, an immunomodulator (Engwerda et al., 2002), and Granzyme-b (Haque et al., 2011b) have been shown to protect mice from CM. In view of these studies, we sought to investigate the synergistic activation and convergence of these molecules in the mechanisms of neuronal cell death in an ECM model.

Cell death during cerebral malaria follows an apo-necrotic continuum. Cells that are undergoing cell death in the brains of mice with cerebral malaria exhibit the anatomical and biochemical features of apoptosis and necrosis (Eeka et al., 2011a). Cresyl violet staining of the neurons shows shrunken, darkly stained neurons and lightly stained (diffuse) oedematous neuronal cells in the brains of mice with cerebral malaria (Fig. 1). These results are consistent

with the increased levels of cell death proteases, CTLs infiltration and the type of cell death reported in ischemia (Chaitanya et al., 2010). The results of our TEM studies (Fig. 3a) and increased Gra-b immunoreactivity (Fig.3 d-g) show that the infiltration of CTLs (Fig. 3h-k) into the brain parenchyma of infected mice may result in neuronal cell death. In view of CTLs infiltration and increased Granzyme-b levels, we questioned ourselves whether Gra B plays a direct role in neuronal cell death in ECM. To answer this question, we have performed colocalization experiments that reveal positive Gra-b staining colocalised with Fluoro Jade B staining (Fig. 6), indicating that Gra B released by effector T cells plays an important role in neuronal cell death in ECM. This is also evident from the Co-IP experiment showing an interaction of Gra b with NF (L), and the breakdown of NF (L) (Fig. 7). Our results are consistent with the Gra-b-mediated killing of neuronal cells in samples of human ischemic tissue (Chaitanya et al., 2011). This indicates the existence of a similar pathology in rat models and human patients during ischemia and ECM arising as a result of hypoxic conditions due to an insufficient supply of blood caused by blockage of blood vessels and regular RBC lysis, or by occlusion of blood vessels by parasitized RBCs pRBCs and CTL infiltration at the site of inflammation. We then studied whether Gra b activates cell death proteases. Our co-immunopreciptation results of Gra-b with caspase-3 and calpain1 showed an interaction between Gra-b and these proteases (Fig. 8). However, this interaction did not result in the formation of any active forms of caspase-3 and calpain1 in the controls, indicating that there might be some other protein complex formation that inhibits Gra-b mediated activation of these proteases or that the levels of Gra-b may be not sufficient to induce the activation of these proteases. Hsp70 is shown to prevent apoptosis by preventing caspase-3 activation (Nylandsted et al., 2004; Olsson et al., 2004). Therefore, we examined the status of hsp70 during CM and found that during CM, Hsp70 is cleaved (Fig. 8) and thus no longer prevents caspase-3 and calpain1 activation. The increased levels of Gra-b observed in CM may be sufficient to cleave Hsp70 and thus activate the cell death proteases and aggravate the pathology. This is inconsistent with results showing that the overexpression of Hsp70 is not neuroprotective in a mouse model of cerebral ischemia (Olsson et al., 2004). Increased levels may be the reason that Hsp70 aids in translocation of Granzyme from the membrane to the cytosol to exert its cytotoxic effects during ischemic conditions (Gross et al., 2003). Thus, our results provide insight into the complex syndromes of CM, where the cytotoxic protease, Gra-b, is secreted by the brain after CTL infiltration and plays a role in

killing neuronal cells directly by activating cell death proteases that, in turn, aggravate the pathology of CM.

CHAPTER II

Status of lymphotoxin-α and role in neuronal cell death; whether it has any effect on caspase-3 and calpain1 mediated cell death

Introduction

Cerebral malaria is complex neuropathological syndrome caused by the *Plasmodium falciparum*. It is major health problem and causes huge economic loss as it causes most of the child deaths. In humans four species of *Plasmodium* have been reported to cause malaria, among them *Plasmodium falciparum* is the most common and deadliest pathogen to cause the disease. Different mouse models are available to study the pathogenesis of CM, but C57BL/6 mice model is the widely acceptable and better model to study the immunopathogenesis of the disease and the pathology of this model is similar to human pathology.

Pathology of the disease is not clearly understood and this might arise as a result of Plasmodium life cycle which leads to regular lyses of RBCs. Thus there occurs dearth of oxygen supply to the brain (Hempel et al., 2011b; Lochhead et al., 2010), this is also in part with the blockage of blood vessels due to the special features acquired by the RBCs and pRBCs, rosetting (Carlson et al., 1990; Carlson, 1993) and sequestration (Franke-Fayard et al., 2005; Ponsford et al., 2012), which lead to hypoxia in the brain (Sanni, 2001), generation of reactive oxygen species(Postma et al., 1996), increased iNOS (Maneerat et al., 2000) levels are cytotoxic to cells or cells death by apoptosis (Pino et al., 2005). Binding of pRBCs to the endothelium may initiate cell death signaling, apoptosis in the brain. Studies on human post-mortem brain samples and murine models have shown the presence of infiltrated immune cells, especially T cells (Belnoue et al., 2002; Belnoue et al., 2008; Dorovini-Zis et al., 2011; Hunt and Grau, 2003; Taylor et al., 2004). Studies on mouse models have proved the critical role of Cytotoxic T cells in the pathogenesis (Lundie et al., 2008) and these cells might cause the pathology through direct cytotoxicity or by activating the resident immune cells which may be cytotoxic by releasing cytokines which initiate cell death. One of the important features cerebral malaria is the death of neurons (Wiese et al., 2006) and the mechanisms for neuronal cell death during CM is not clearly understood. During this pathology elevated levels of caspase-3 and calpain1 are reported to be associated with pathology (Shukla et al., 2006). This is also evident from the inhibition study of caspase-3 and overexpression of Bcl-2, anti-apoptotic protein protected mice from CM (Helmers et al., 2008b). Indicate the critical role of caspase-3 in the CM pathogenesis. It has been shown in animal models that T cells lacking perforin (Nitcheu et al., 2003; Potter et al., 2006) and Granzyme-b (Gra-b) (Haque et al., 2011b) have been shown to protect the mice from CM, indicating the role of T cells in the CM pathogenesis through Gra-b and perforin. But

knockdown studies of lymphotoxin-α, a cytokine and an immune modulator shown to protect the mice from CM by decreasing the cytokine receptors and cell adhesion molecules (Engwerda et al., 2002) in the mice infected with *PbA*, implying that this molecule might be a key regulator or to which all the factors involved in CM pathogenesis might be associated for them to show their effect. Recently it has been reported that T cell infiltration and parasite burden in the brain is essential for the disease outcome (Haque et al., 2010) , where it has been shown that blocking of T reg cells decreased the CTLA4 expression by Foxp3+ prevented the parasite sequestration and CM. So here we aimed at studying the mechanism by which this lymphotoxin-α and whether it has any role in regulating the cell death proteases (caspase-3 and calpain1) which we have already reported that increased levels of these proteases in the brain causes neurodegeneration and CM.

Lymphotoxin-α, cytokine belong to the TNF superfamily of proteins and shares the sequence homology and receptor binding with TNF-a, secreted by the activated immune cells. It is an immune modulator, cytotoxic and signalling molecule involved in bone marrow neogenesis, activation and maturation of T and B lymphocytes, lymph nodes and secondary lymphoid organs formation and maintenance and acts against pathogens. This molecule is implicated in many types of cancers and its knockdown protected mice from cerebral malaria. But the mechanism by which it causes the disease pathogenesis is not known and there is no evidence whether it has any role in capase-3 and calpain1 mediated neurodegeneration and CM paradigm. So here we aimed at studying the status of lymphotoxin-α during experimental CM.

There are three main ligands of TNF superfamily which are similar in function and the TNF ligands shares receptor binding with TNFRs (TNFRI, TNFRII and TNFR-β). Binding of these ligands to these ligands and their mode of signal depends on the stimulus and oligomerisation of these receptors decides the fate of the cell. And these TNF superfamily ligands and TNFRs are reported to translocate into the lipid rafts (Lotocki et al., 2004) during the cell signalling.

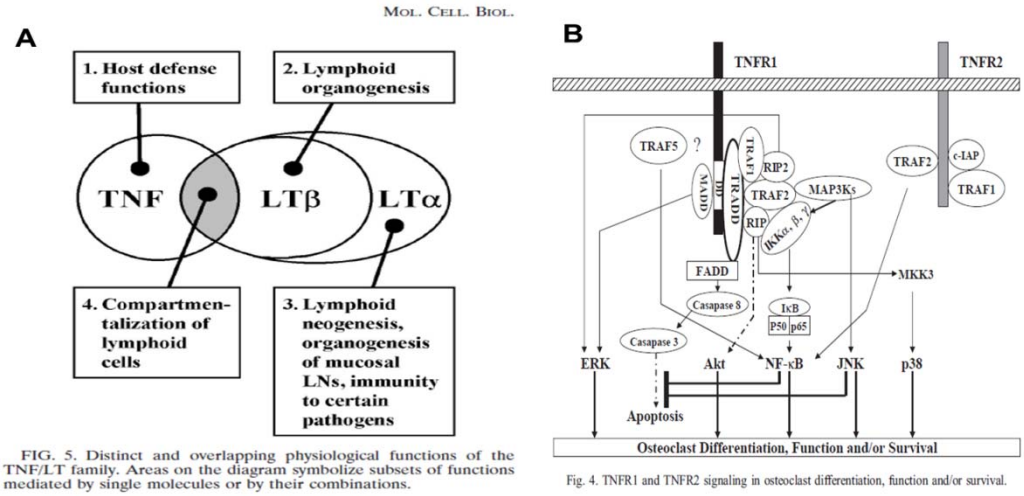


FIG. 5. Distinct and overlapping physiological functions of the TNF/LT family. Areas on the diagram symbolize subsets of functions mediated by single molecules or by their combinations.

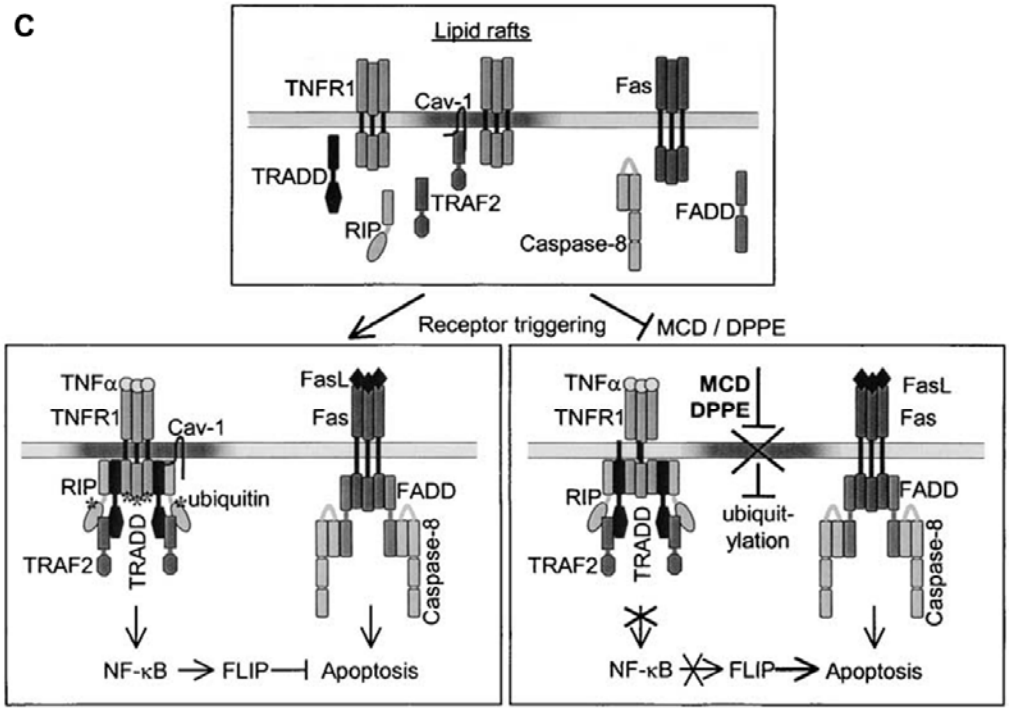
Fig. 4. TNFR1 and TNFR2 signaling in osteoclast differentiation, function and/or survival.

MOL. CELL. BIOL.

MOLECULAR AND CELLULAR BIOLOGY, Dec. 2002, p. 8626–8634
0270-7306/02/$04.00+0 DOI: 10.1128/MCB.22.24.8626–8634.2002

X. Feng / Gene 350 (2005) 1–13

Fig. 17. Schematic diagram showing the mechanism of TNFRs mediated regulation of apoptosis when it binds to their ligands by recruiting the complex to the lipid rafts.



Daniel F. Legler et al., Recruitment of TNF Receptor 1 to Lipid Rafts Is Essential for TNF-Mediated NF-B Activation. Immunity, Vol. 18, 655–664, May, 2003.

Serum collection

Blood from the control and the infected CM mice were collected by retro-orbital puncture and allowed the RBC to settle down by keeping the blood at 4^{o} C for 12 hrs., serum collected and kept at -80^{o} C.

Lipid rafts isolation from mice brain samples

Mice brains from the control and infected CM mice were dissected out and homogenised in ice cold RIPA buffer in a dounce homogeniser. Added 700µl of 1% Triton X-100 lysis buffer (modified HEPES buffer- 25mMHEPES-HCl, pH 6.5, 150mMNaCl, 1mMEDTA, 1mMPMSF, protease inhibitor cocktail. Prepare 2× and store at 4 °C (*see* Note 2).1% Triton X-100 lysis buffer: 1% (v/v) Triton X-100 in 10 ml modified HEPES Buffer) to 300µl of tissue sample and incubated at 4^{o} C for 30 min. After incubation, mix lysates with same volume (1 ml) of 80% sucrose cushion solution to yield a mixture at a final 40% sucrose gradient, and then transferred into a 12 ml polyallomer ultracentrifuge tube (for SW41 rotor, Beckman Instruments). At the top of the sample-sucrose mixture, overlay 6.5 ml of 30% and 3.5 ml of 5% sucrose cushion, respectively. Ultracentrifuged at 40000 rpm 20 hrs. , 4°C using an SW41 rotor. After centrifugation, the floating opaque band corresponding to the detergent resistant membrane fraction can be found at the interface between the 30% and 5% sucrose gradients.

MATERIALS AND METHODS

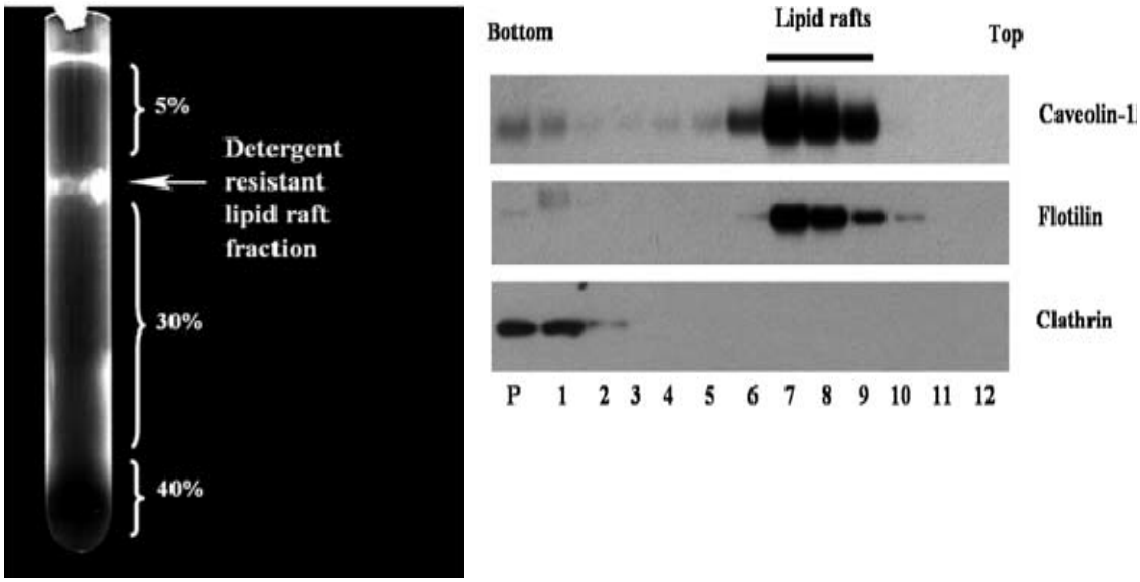


Fig.18. Showing the isolation and lipid rafts by sucrose density gradient separation and blots showing the fractions positive for the lipid rafts. Source: Ki-Bum Kim, Jae-Seon Lee, and Young-Gyu Ko. The Isolation of Detergent-Resistant Lipid Rafts for Two-Dimensional Electrophoresis. *Methods in Molecular Biology, vol. 424*

Injection of SE (serum from CM mice) to naïve mice

We found the increased levels of lymphotoxin-α in serum of CM mice compared to controls and its translocation in to lipid rafts, and evident from the reports from knockdown studies that lymphotoxin-α protects the mice from CM. We hypothesized that lymphotoxin-α might be having some role in regulating or mediating the cell death mediated or cytotoxic proteases mediated cell death that lead to neurodegeneration during ECM. Hence we injected the normal mice (n=10 for both control and experimental) with the serum from control and the CM mice intraperitonially and observed for survival.

Lymphotoxin-α injection (Dosage)

We also studied the effect of recombinant lymphotoxin-α by injecting the mice with 100-500ng (n=10 for each dose) of protein through caudal vein and observed the mice for survivality. Control mice were given same volume of PBS. Complete batches were left without collecting the samples to know the exact survival ratio and the next batch of mice were sacrificed when the mice get the cerebral symptoms.

Dot blot

Dot blot was done by allowing the equal concentration of protein samples to get dried on the nitrocellulose membrane at room temperature for 1 hr. after the sample got dried up, membrane was blocked with 10% milk solution and incubated with primary antibody at 4^{o} C overnight, washed with TBS and TBST. Incubated for 1 and half hr. with ALP secondary antibody and developed using BCIP/NBT substrate.

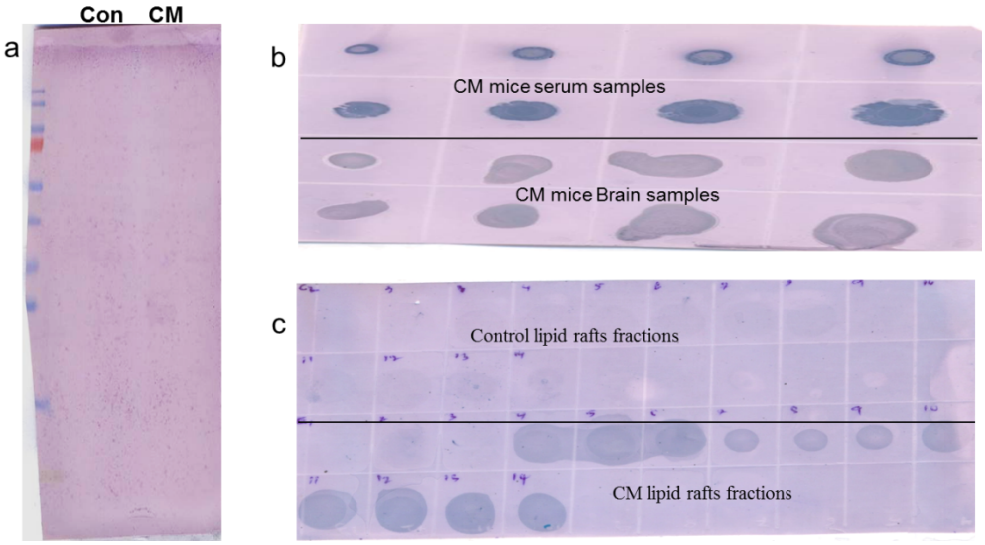
Survival analysis

Survival analysis was done using the Kaplan-Meiyer survival analysis (MedCalc) by giving the time mice survived in days with 0 and 1. “0” indicate the death of the mice and the “1” indicate the survival. For western blots (n=3 for each blot) densitometry was done using NIH ImageJ software and statistical analysis was done by ANOVA (sigmaplot 12) and graphs were by excel or sigma plot 12.

Results

Increased serum levels of lymphotoxin-α in infected CM mouse samples compared to control and translocation in to lipid rafts

We performed the Western blot analysis to check the status of lymphotoxin-α in the mice brain samples, serum and the lipid rafts fractions from control and the infected CM. Our results showed no presence of lymphotoxin-α in control and cerebral malaria mice brain samples. We found increased levels of serum lymphotoxin-α in CM mice compared to control. There is also increased levels of lymphotoxin-a in lipid rafts of CM mice brain samples compared to control indicates the translocation of increased levels of lymphotoxin-α in the serum is translocate to lipid rafts in the brains of CM mice compared to control. Our immunofluorescence and immunohistochemical analysis also indicate the same and increased levels of lymphotoxin-α predominantly along the blood vessels.



Immunohistochemical analysis showing the lymphotoxin-α infected mice brain

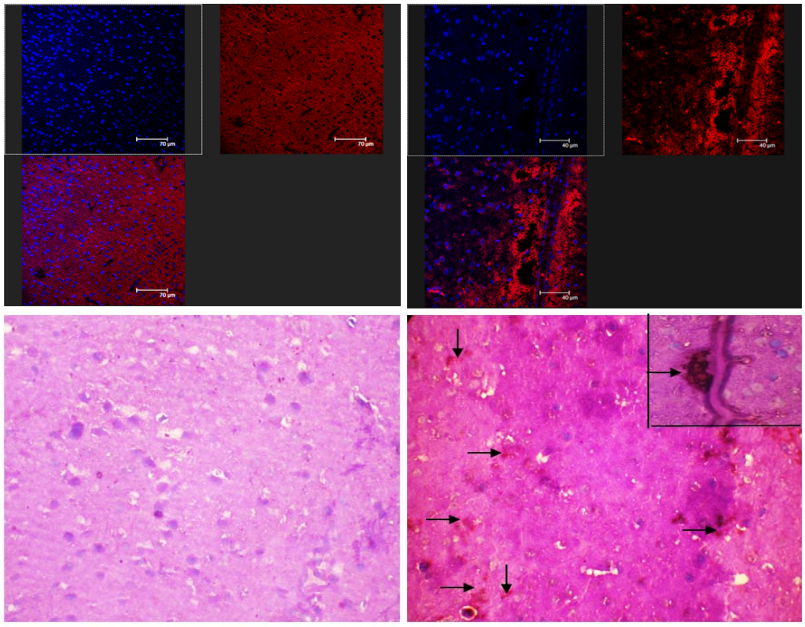
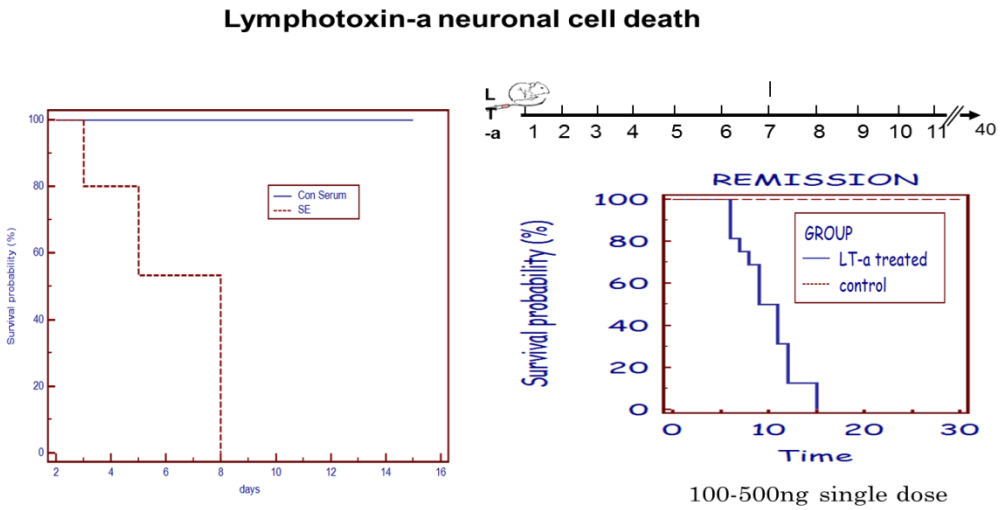


Fig. Representing the elevated levels of LT-a in cerebral malaria mice brain tissue sections

Fig. 19. Western blots showing the status of lymphotoxin-α during PBS treated control and the cerebral malaria mice conditions. There is no difference of lymphotoxin-α in control and cerebral malaria mice brain samples (a). Serum levels of lymphotoxin-α is increased during CM compared to control (b) and the lipid rafts fractions from CM mice brain samples showing the increased levels of lymphotoxin-α compared to control(c), indicating increased serum lymphotoxin-α during CM is translocated in to lipids of brain. Below immunofluorescence and immunohistochemical analysis showing the increased levels of lymphotoxin-α in the CM mice brain samples compared to control, especially along endothelial lining of the blood vessels in the brain indicated by arrows in the immunohistochemistry of brain sections.

SE (experimental serum) injection and lymphotoxin-α treatment is lethal to mice

Mice which received the serum from CM mice and lymphotoxin-α protein died with in 8 and 15 days respectively, indicating the increased levels of lymphotoxin-α is lethal to mice. We further studied the status of caspase-3, calpain1 and neuronal cell death in SE injected mice and lymphotoxin-α injected mice to see whether these has any effect on capase-3 and calpain1 and cytotoxic proteases mediated neuronal cell death and CM.



Kaplan-Meier survival curve

Survival time	Time
Endpoint	Remission
Factor codes	Group

	Factors			
	1		2	
Sample size	5		4	
Median survival	-		8.5	
Survival time	Survival Proportion	Standard Error	Survival Proportion	Standard Error
7	-	-	0.750	0.217
8	-	-	0.500	0.250
9	-	-	0.250	0.217
13	-	-	0.000	0.000
30	-	-	-	-
	Comparison of survival curves (Logrank test)			
Endpoint: Observed n	0.0		4.0	
Expected n	2.4		1.6	
Chi-square	6.0056			
DF	1			
Significance	P = 0.0143			

Kaplan-Meier survival curve

Survival time	time
Endpoint	remission
Factor codes	group

	Factors			
	1		2	
Sample size	5		5	
Median survival	-		8	
Survival time	Survival Proportion	Standard Error	Survival Proportion	Standard Error
3	-	-	0.800	0.179
4	-	-	-	-
5	-	-	0.533	0.248
8	-	-	0.000	0.000
15	-	-	-	-
	Comparison of survival curves (Logrank test)			
Endpoint: Observed n	0.0		4.0	
Expected n	2.0		2.0	
Chi-square	4.8000			
DF	1			
Significance	P = 0.0285			

Fig. 20. Graphs showing the survival probability of mice (n=8) which received the serum from CM mice (SE) and the control serum intraperitonially and lymphotoxin-α through caudal vein and the methodology is indicated

above the graph showing survival probability of LT-α injection. Mice which received the SE died by the 8th day of compared to the controls which were normal and the mice injected with lymphotoxin-α at a dosage of 100-500ng (n=8/group) died by the 15th day of post injection (p.i) compared to control mice injected with PBS survived till 30 days. Graphs showing the time in days and the remission (treated or the control) as group.

Effect of SE and lymphotoxin-α injection on caspase-3, calpain1 and cytotoxic proteases granzyme-b and perforin in normal mice

We studied the levels of caspase-3 and calpain1; our results showed the increased levels of caspase-3 and calpain1 in CM serum injected and severe anaemia mice brain samples which is consistent with the immunofluorescence analysis of SE mice brain sections showing the elevated levels of capase-3 and calpain1.

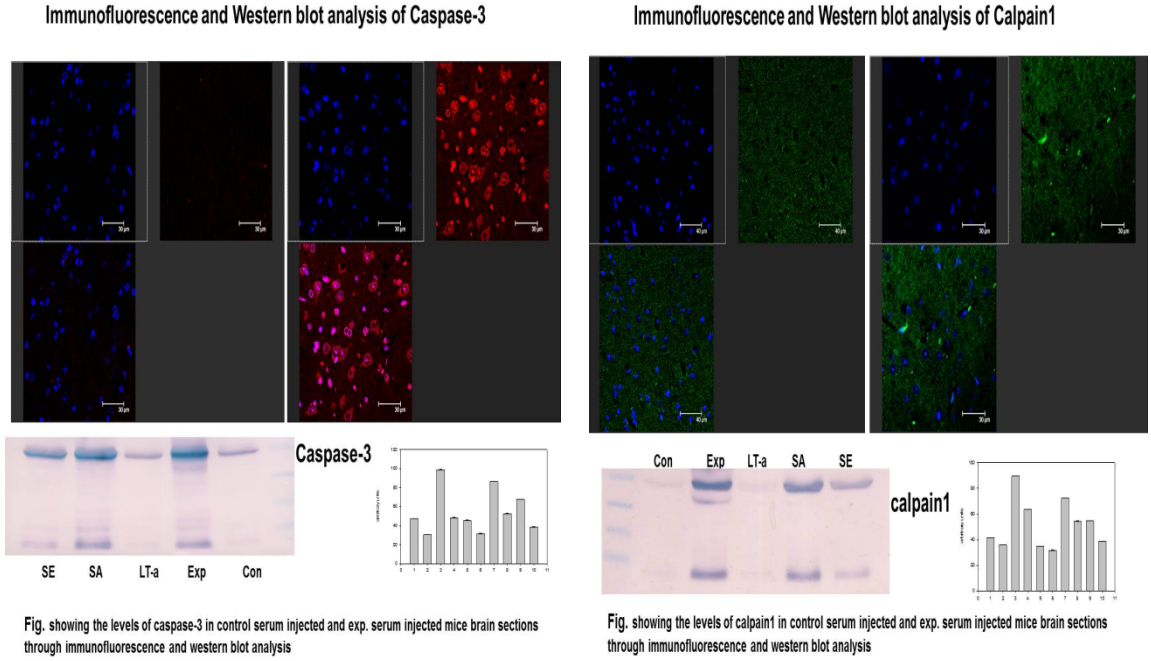


Fig. showing the levels of caspase-3 in control serum injected and exp. serum injected mice brain sections through immunofluorescence and western blot analysis

Fig. showing the levels of calpain1 in control serum injected and exp. serum injected mice brain sections through immunofluorescence and western blot analysis

Fig. 21. Immunofluorescence analysis showing the increased protein levels of caspase-3 and calpain1 in SE injected mice brain samples compared to control. Western blot analysis of caspase-3 and calpain1 showing increased active capsase-3 and calpain1 levels in CM, SE, SA mice brain samples, but the levels are normal in lymphotoxin-α (LT-a) treated mice brain samples. Graphs representing respective caspase-3 and calpain1 analyses in different experimental conditions.

Increased amount of apoptosis in LT-α, SE injected and severe anaemia (SA) mice brain samples

TUNEL experiments showed the increased amount of apoptosis in the brains of mice injected with lymphotoxin-α protein and serum from CM mice compared to control, we also found the increased amount of apoptosis in SA mice brain samples. Increased amount of apoptosis in LT-α mice brain samples without the elevated levels of caspase-3 and calpain1 indicates that LT-α might induce the cell death through different mechanism other than caspase-3 and calpain1.

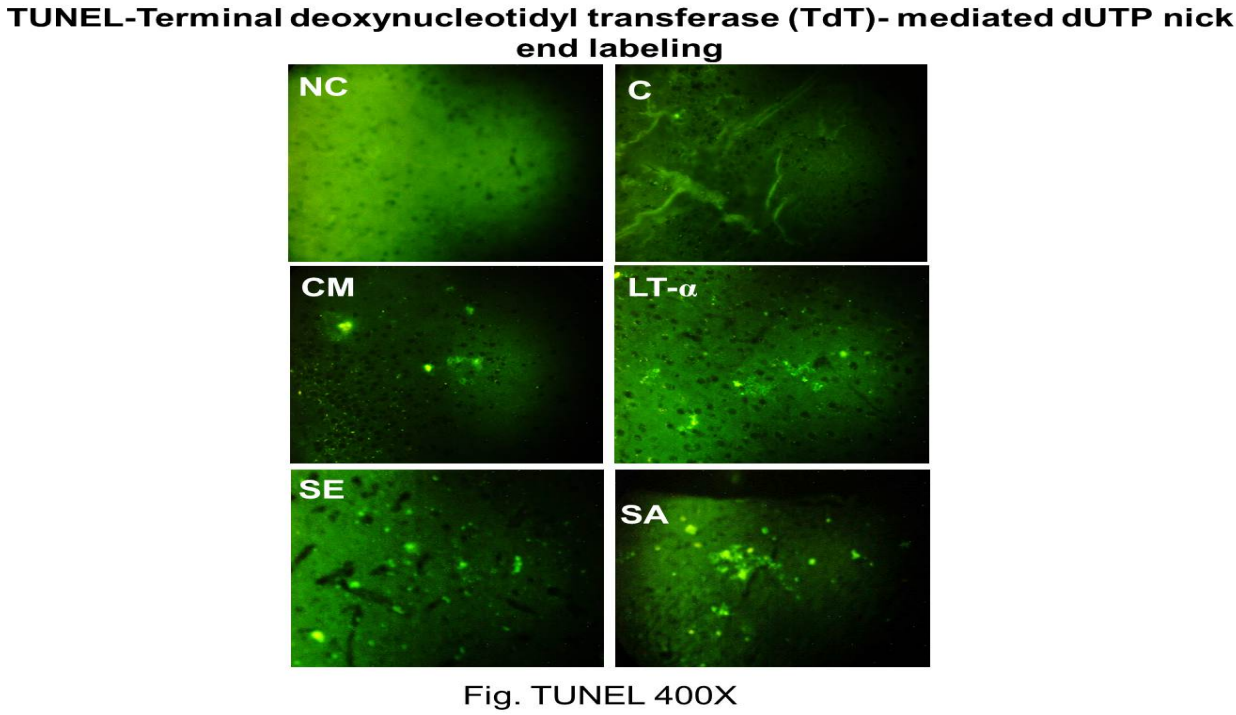


Fig. 22. Showing mice brain sections from control, CM, LT-α treated, SE and SA increased TUNEL positivity implying increased apoptosis. Increased TUNEL positivity in Lt-α treated pointing towards lymphotoxin-α induces cell death through other mechanism of programmed cell death, autophagy.

As there is no difference of caspase-3 and calpain1 levels between control and the lymphotoxin-α treated mice brain samples and still we detected significant amount of apoptosis in lymphotoxin-α treated compared to control. Hence we studied the status of cytotoxic proteases (Gra-b and perforin) during lymphotoxin-α treatment, our western blot results showed the normal levels of Gra-b, significant increase in the levels of perforin when compared to control. Increased apoptosis in the lymphotoxin-α and increased levels of

perforin indicate cell death in lymphotoxin-α treated mice might be due to perforin or may be through different mechanism. Experimental serum (SE) injected mice showed the increased levels of caspase-3, calpain1 and perforin, suggesting that during *PbA* infection both the immune factors and parasite factors leads to lead to CM pathogenesis, but the increased levels of serum are as lethal as PbA infection which is also evident from the lymphotoxin-α treatment alone or the knockdown studies from earlier workers.

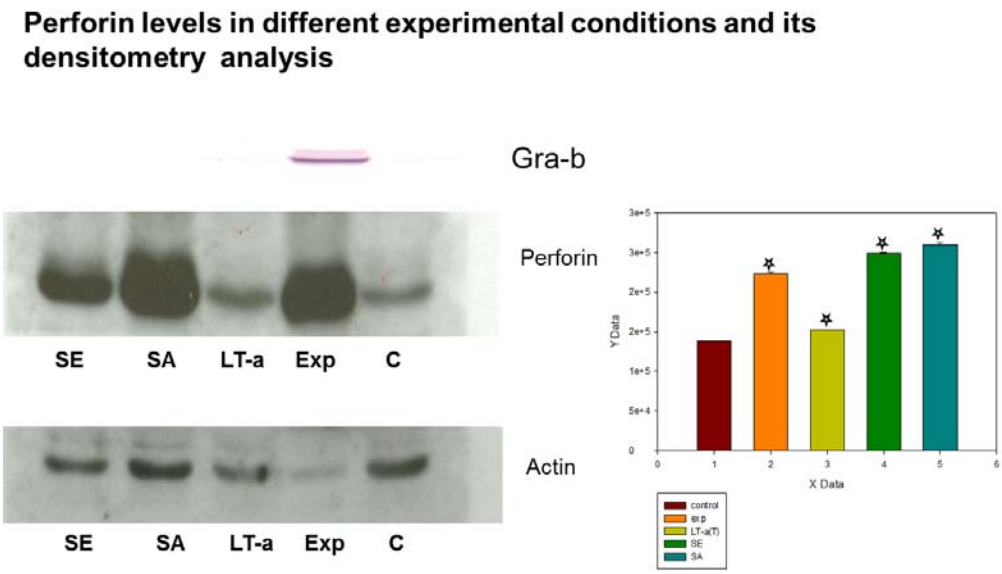


Fig. 23. Western blot analyses showing the status of cytotoxic proteases, Granzyme-b (Gra-b) and perforin in different experimental conditions. Increased levels of perforin in all the experimental conditions compared to control but not Gra-b. Gra-b is increased during CM only and increased perforin in the lymphotoxin-α treated mice brain samples indicates lymphotoxin-α indices the cell death through perforin. Graph showing the densitometry analysis of perforin. $p \leq .05$ is considered as significant indicated by * above the bars in the graph.

Increased levels of proinflammatory molecules in SE and SA mice brain samples, but not in lymphotoxin-α treatment

We studied the status of pro-inflammatory molecules NF-kB p50, NF-kB p65 and COX-2. Our Western blot results showed no difference in the levels of these pro-inflammatory in the lymphotoxin-α treated mice compared to control. We found increased levels of pro-inflammatory molecules in SE and SA mice brain samples. Suggest role of inflammation during SE and SA conditions, but not during lymphotoxin-α treatment.

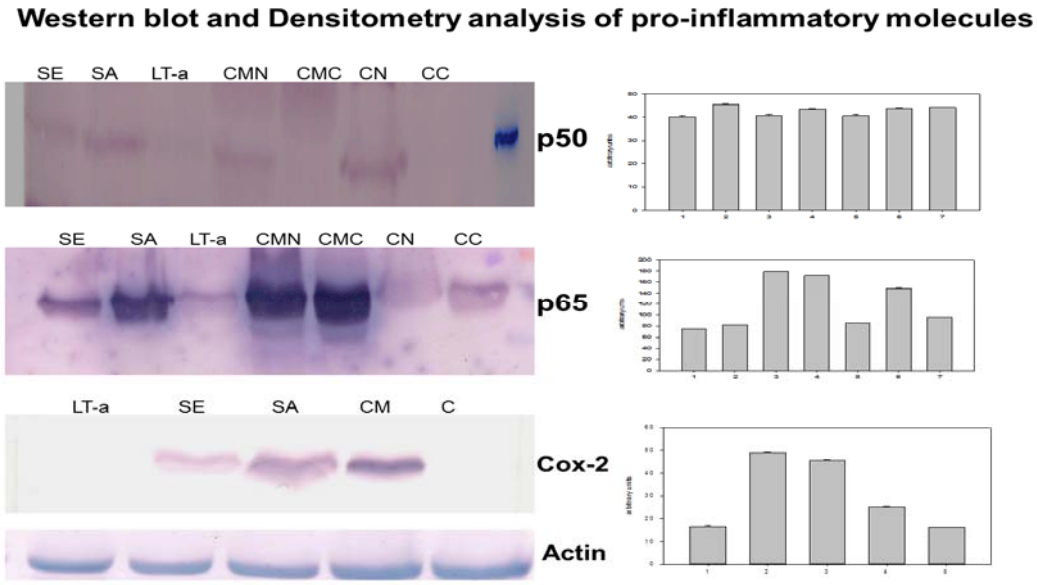


Fig. 24. Western blots showing the status of pro-inflammatory molecules, NF-kB p50, p65 and Cox-2 during different experimental conditions. All the three pro-inflammatory molecules are increased in different experimental conditions when compared to control, but not in LT-a treated mice brain samples. Graphs showing the densitometry analysis of respective pro-inflammatory in different experimental conditions.

Chapter III

In vivo inhibition of lymphotoxin-α and caspase-3 and calpain1

Introduction

As we observed injection of serum from CM mice (SE) and LT-α is lethal with increase in the cell death, pro-inflammatory molecules and cytotoxic proteases in SE mice brain samples and no difference of these molecules or proteases in LT-α treatment. We found only increased levels of perforin and increased apoptosis in LT-α treated mice, hence it is clear that lymphotoxin-α induces the cell death through perforin mediated cytotoxic cell death. But as mentioned earlier, CM is a complex disease involves many factors and these factors may be interdependent. Knockdown studies of lymphotoxin-α have shown to protect mice from CM, indicating this molecule might regulate the key factors for the disease outcome. So we aimed at inhibiting lymphotoxin-α by using specific antibody against lymphotoxin-α during infection and we also inhibited caspase-3 and calpain1 by using the specific inhibitors zVAD-fmk and Ac-DEVD-CHO (Calbiochem) respectively.

Methodology

In vivo inhibition of lymphotoxin-α and caspase-3 and calpain1 was done by given the mice with 5-10ng/mice (n=40; 8 mice for dose) dose of antibody against lymphotoxin-α and specific inhibitors zVAD-fmk and Ac-DEVD-CHO (Calbiochem) at a dose of 50-250µg/mice (n=40; 8 mice for dose) daily once till 7 day of post infection (p.i) respectively. First batches of the both experiments were used only for survival analysis by recording the no. of days survived. Same doses are given for the next batches and brain samples were collected when the mice are final stages of survival.

METHODS:

Blood Brain Barrier (BBB) disruption: Evan's blue extravagation assay

Mice were used to study the blood brain barrier disruption by injecting 2% Evan's blue dye intraperitonially and sacrificed the mice after 1 hr. Evan's blue extravagates into the brain only when there is blood brain barrier (BBB) disruption, so extravagation of Evan's blue into the brain indicates BBB disruption.

Brain water content

To study the edema role in neuronal cell death we measured the brain water content of PBS injected control and different experimental conditions mice brain. Brain water content was calculated by measuring the wet weight of the brain immediately after dissecting out the brain (n=3 for each group of experiment), these brains were allowed to dry at 110^0C for 24 hr and weighed the tissue again. This weight of the tissue is taken as the dry weight and the brain water content was expressed as the % water content increased as compared to control. The brain water content is the measure of the difference between wet and dry weight divided by 100.

Fluoro-Jade B Staining-Neurodegeneration

Fluoro-jade B specifically stains the degenerating neurons; we used this stain to study the degeneration of neurons during cerebral malaria and other experimental conditions from Ab(T) and Cas-3 & Cal1 inhibition mice brain samples. Briefly, 3µ paraffin sections were dewaxed in xylene and rehydrated in 100% alcohol followed by 70% for 5 and 2 min respectively. Slides were rinsed twice in ddH_2O for 1 min; sections were then incubated with freshly prepared 0.06% potassium permanganate for 17 min and rinsed for 1 min twice in ddH_2O. Then slides were incubated for 30 min in 0.001% Fluoro-Jade solution at room temperature in the dark and rinsed twice in ddH_2O 1 min. Slides were air dried for 10 min and mounted with permanent mounting media and stored the slides in dark, pictures were taken by observing under fluorescent microscope using fluorescein-5-isothiocyanate filter.

RNA isolation by TRIzol Reagent Method

Total RNA was isolated by homogenising 100mg of mice brain tissue in 1ml of Trizol reagent (Invitrogen) in porcelain motor and pestle. Samples were kept at room temperature for 10 min to permit complete dissociation of the nucleoprotein complexes. Then 0.2 ml of chloroform was added, vortexed the samples vigorously for 15 sec and kept at room temperature for 2 to 3 min. Samples were centrifuged at 12000xg for 15 min at 4^0C, RNA was collected from the upper aqueous phase and transferred into fresh eppendorff tubes. RNA from this aqueous phase was precipitated by adding 0.5 ml of isopropyl alcohol per ml of TRIZOL used for initial homogenisation. Incubated the tubes for 10 min at room temperature and centrifuged for 10 min, 12000xg at 4^0C. Supernatant was removed and the pellet washed

once with 1 ml 75% alcohol per ml of ml of TRIZOL used in initial step. Mixed the samples, vortexed and centrifuged at 7,500xg for 5 min at 4^0C, repeated the alcohol wash one more time and removed alcohol. Air dried the pellet for 10 min and dissolved the RNA pellet in 100µl of DEPC treated water by mixing gentle with the pipette tip. The quality and quantity of RNA was measure by Nano Drop 200 (Thermo Scientific).

Primer synthesis

Specific primers for cell adhesion molecules (ICAM-1 and VCAM-1), chemokine receptors (CXCR3, CXCR4, CCR4, CCR5, VLA-4 and LFA-1) and MHC molecules β_2-microglobulin for MHC-II and α chain of MHC-I were designed by taking the gene sequence from NCBI nucleotide database by using online oligo properties calculator software, OligoCalc and got the primers synthesised from eurofins. The list of the primers used is given below.

Gene	ACCESSION NUMBER	FORWARD	REVERSE	AMPLICON SIZE (bp)
CXCR4	NM009911.3	5'GGAACCGATCAGTGTGAGTAT 3'	5'CACCAATCCATTGCCGACTAT3'	188
CXCR3	NM_009910.2	5'GCCATGTACCTTGAGGTTAGTG3'	5'GGTTCTGTCAAAGTTCAGGCTG3'	178
CCR4	NM_009916.2	5'CAGAAGAGCAAGGCAGCTCAA3'	5'CAAGGCTTTGGCATGCTTTCGT3'	175
CCR5	NM_009917.5	5'AGACTCTGGCTCTTGCAGGAT3'	5'TGAATACCAGGGAGTAGAGTGG3'	187
ICAM1	NM_010493.2	5'GAGATCACATTCACGGTGCTG3'	5'AGCTGGAAGATCGAAAGTCCG3'	147
VCAM1	NM_011693.3	5'GACATCTACTCTTTCCCCAAGG3'	5'TGTTCATGAGCTGGTCACCCT3'	151
Itga4(VLA4)	NM_010576.3	5'TGGCTCTATCGTGACTTGTGG3'	5'TCTTTGTAACACGGGGCCATC3'	144
Itgb2(LFA1)	NM_008404.4	5'TTCTTCCTGGGATCTGCTGTG3'	5'ATCATCGGCTGGACAACCCTT3'	193
GAPDH	NM_008084.2	5'ATCTTCTTGTGCAGTGCCAGC3'	5'TTGAGGTCAATGAAGGGGTCG3'	154
TCR	BC147852	5'GGGGGTTTGTTCTCTATCTCTTCC3'	5'TATCCACAGGAGATGATGGTGCAC3'	347
MSP-1	XM_673413.1	5'GCTGCCCCTGTTACTACCGAAG3'	5'GCTCCTGTATTAGTGACGCCTGC3'	141

Real time reverse transcriptase (RT) PCR

cDNA was synthesised from 1µg of total RNA by using random hexamers with first strand cDNA synthesis kit (BluePrint^{TM} 1st Strand cDNA Synthesis Kit # 6115A), TaKaRa. Expression levels of cell adhesion cell adhesion molecules and chemokine receptors *Power* SYBR® Green PCR Master Mix (Applied Biosystems # 4367659). Quantitative PCR (qPCR) was performed in Applied Biosysyems 7900HT Fast-Real Time System (Applied Biosystems) in triplicates. The relative expression of target genes in control, CM, SE, SA, Ab (T) and Cas-3&Cal1 inhibition samples were compared by analysing the data by $2^{-\Delta\Delta C}{}_{T}$ Method.

Normal PCR (Sequestration of pRBCs and infiltration of T cells)

Normal PCR was done to study the pRBCs sequestration and T cell infiltration in the brain by designing the primers specific to MSP-1 (merozoite surface protein of *PbA*) and α-segment of the TCR (T Cell Receptor) gene, taking the nucleotide sequence from NCBI nucleotide database. PCR was run using thermo cycler with Fermentos Dream Taq PCR ready master mix.

Estimation of parasitemia

Parasitemia of the Giemsa stained caudal blood smears were counted manually by taking the photographs under light microscope (Nikon) at different positions of the smears (no. of fields, n=10/slide) from 3rd day of p.i till mice death.

Statistical analysis

Densitometry was done to quantify amount of MSP-1, TCR-α and expression levels of MHC-I and MHC-II by using NIH Image J software (n=3 individual experiments). Statistical significance was done using sigmaplot 11 statistical analysis (ANOVA) and .05 is considered as significant, the plots were plotted using Microsoft excel 2010. For parasitemia (n=10 fields/slide) was counted manually, statistical analysis, ANOVA was performed and the mean and respective standard error mean (SEM) was taken and graphs were plotted against the days survived by the mice on x-axis and the % parasitemia on y-axis using Microsoft excel 2010.

Results

LT-α inhibition and cas-3 & cal1 inhibition protected the mice from CM- increased survivality of mice to 30 and 20 days respectively

Our results of lymphotoxin-α and caspase-3 and calpian1 inhibition has increased the survivality of mice after *PbA* and thus protected the mice from CM indicating increased levels of lymphotoxin-α leads to CM pathogenesis and also this molecule as regulating the cell death mediated CM pathogenesis. But this could be either effecting parasite life cycle or effecting the mechanisms by which parasite may cause the pathogenesis, so further studied extended our study to dissect out the each of the component by which this molecule might be contributing to the CM outcome. Hence we studied the parasitemia levels also during different experimental conditions.

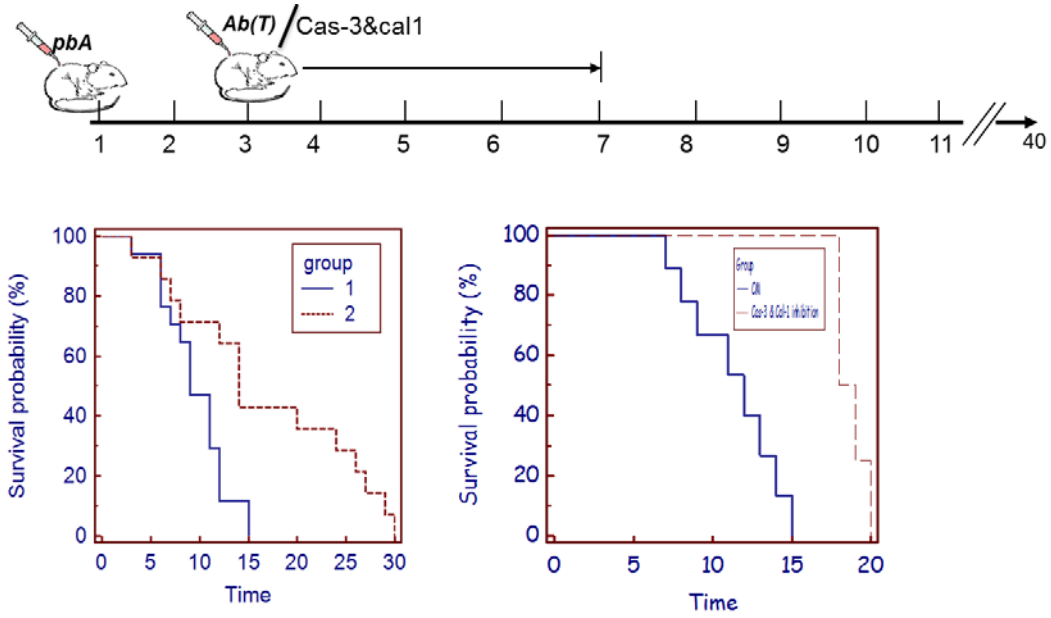


Fig.25. Showing the protocol followed for the *in vivo* inhibition of lymphotoxin-α and Cas-3&Cal1 for two individual separate experiments, mice were (n=8/each dose) given infection with *pbA* intraperitonially followed by caudal vein injection of different doses of antibody against lymphotoxin and z-VAD-fmk & Ac-DEVD-CHO from third daily once till 7th day. Graphs showing the survival analysis (% survival probability) of *pbA* infected control mice and the experimental antibody treated and cas-3&cal1 inhibited mice. As evident from the graphs mice which received antibody against lymphotoxin-α and caspase-3 and calpain1 inhibition survived for 30 and 20 days respectively.

Parasitemia, Blood Brain Barrier disruption and edema (Brain water content)

Results of blood smears from lymphotoxin-α and caspase-3 & calpain1 showed the increased parasitemia levels during *PbA* compared to CM and SA experimental conditions. This indicates that the inhibition has no effect on *plasmodium* life cycle but has effect on the mechanisms that lead to CM pathogenesis during PbA infection. We also studied the status of BBB, as its disruption is the one of the events during ECM. Our Evan's blue extravasation experiment to study BBB disruption showed disruption during CM, and it prevented during inhibition studies. Prevention of BBB breakdown by lymphotoxin-α and cas-3 & cal1 inhibition indicating that these molecules involves in BBB breakdown and thus causes the pathology, and from the survival analysis showed increased survival of mice when lymphotoxin-α is inhibited compared to cas-3 and cal1 inhibition. Indicating the critical role of lymphotoxin-α in regulating the complex pathology and would be clear if other factors involved in CM pathogenesis are addressed. Which we would be looking into in the forth coming studies and gives better understanding of the disease. We also studied oedema by quantifying the brain water content, as it is one of the possible mechanisms that also lead to CM and role of oedema has be reported during CM. Our result showed the increased brain water during CM and is significantly brought down by the inhibition studies.

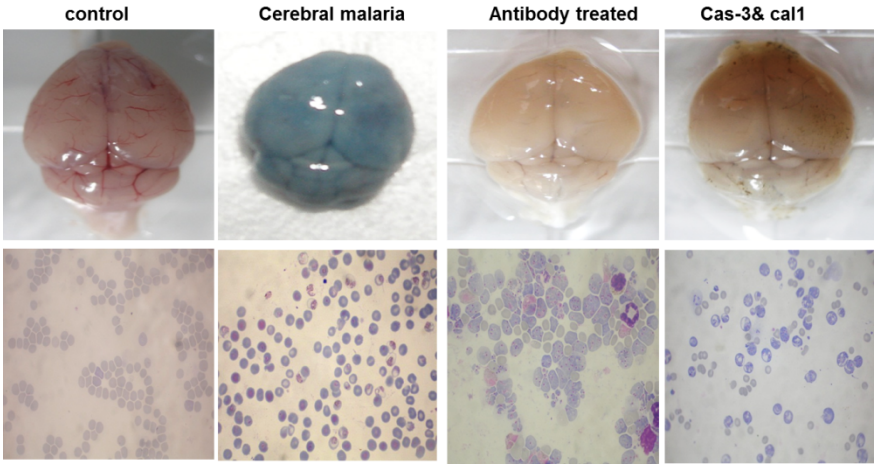


Fig.26. Images of mice brains dissected out after perfusion them with PBS showing the extravasation of Evan's blue only in CM mice brains and respective blood smears showing the infection during different experimental conditions. Indicating the extravasation of Evan's blue only in CM compared to control, Ab(T) and Cas-3&Cal. We can see the normal blood supply in control but significantly less blood supply in Ab(T) treated and Cas-3&Cal1 inhibited as evident from increased parasitemia. Blood Brain Barrier breakdown, the common feature of CM evident from the extravasation of Evan's blue into the brain, implying the parasitemia alone is not the cause

for the CM pathogenesis or there are others dependent on lymphotoxin-α function are essential for the CM pathogenesis. Blood smears magnification 400x.

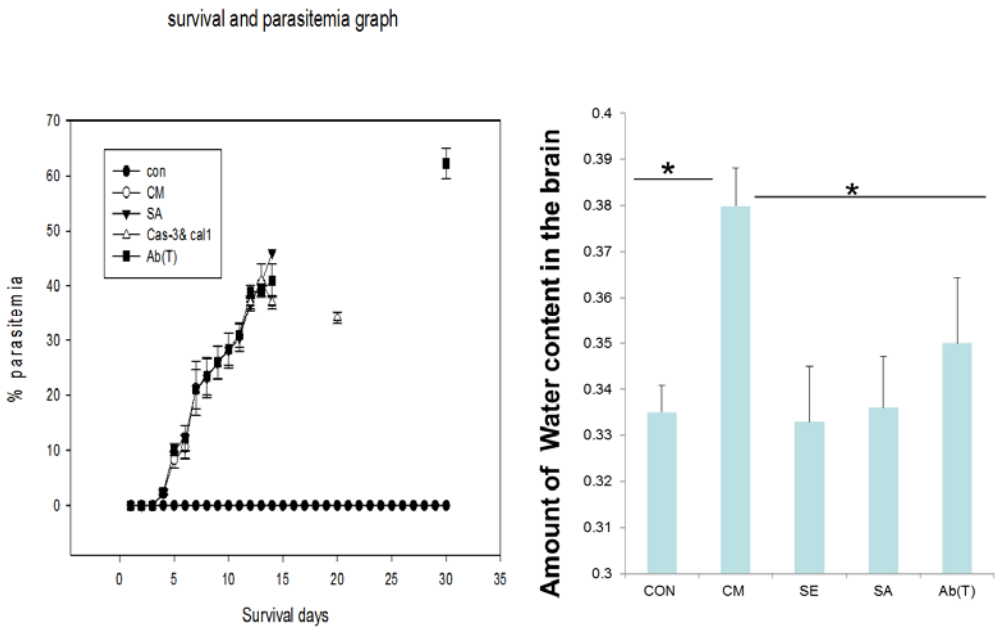


Fig. 27. line graph showing the % parasitemia versus survival probability in days of different experimental conditions mice, indicating CM mice survived only till 7th day with a parasitemia of less than 20%, SA survived for 15 days with a parasitemia around 50%, Cas-3&Cal1 survived for 20 days with parasitemia around 40% and Ab(T) treated mice with above 60% parasitemia. Graph with bars indicating the water content in brain of mice with different experimental conditions, CM mice brain has very significant increase in water content compared to PBS injected control, SE and SA samples. Indicating the oedema in CM and water content is drastically decreased in Ab(T) mice brain samples implying lymphotoxin-α decreased the oedema during *pbA* infection.

As we have reported in the first chapter that CM is caused by the elevated levels of cell death and cytotoxic protease which directly interacts with neuronal cytoskeleton, cleaving them causes neurodegeneration and thus CM. Hence here we studied whether inhibition has any effect on elevated levels of cell death and cytotoxic proteases and the factors that are responsible for their neuronal elevated levels in the brain, their mediated neuronal cell death and ECM.

Lymphotoxin-α and (caspase-3 and calpain1) cas-3&cal1 inhibition decreased the cell death in the brain of PbA mice

We performed the haematoxylin and eosin (H&E) and cresyl violet stain on mice brain sections to study the cell death. Our results showed the decreased or almost no cell death in the lymphotoxin-α inhibited compared to CM mice brain samples.

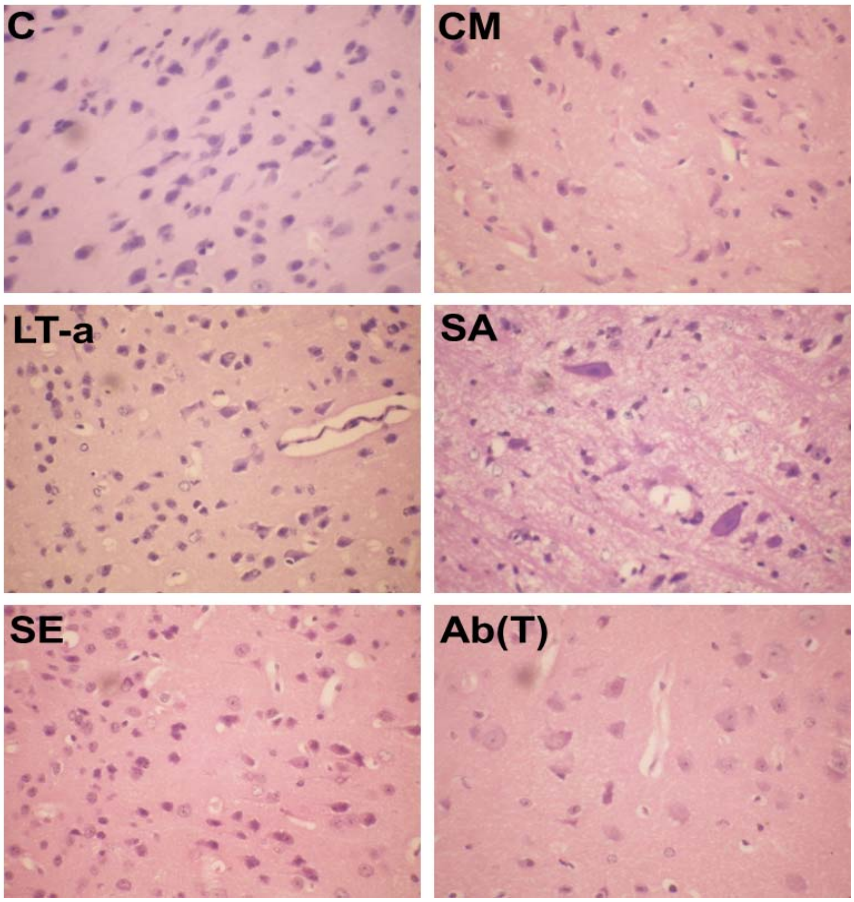


Fig. 28. Hematoxylin and eosin staining of mice brain sections from different experimental conditions showing the increased cell death in CM, SA, LT-α and SE indicated by the extensive vacuolation, detachment of cells from brain parenchyma indicates the necrotic mode of cell death and marginalization of nucleus and pinching of the cells indicates the apoptotic mode of cell death especially in mice treated with LT-α and SE. Crenellation and shrinking of the cells indicate the pyknotic mode of cell death. Enlarged and swollen cells indicative of cell dying of oedema more prominent in SA mice brain samples which is also evident from the patchy type of staining of the entire tissue. We can also see the normal cells and brain parenchyma in Ab(T) mice compared to CM, SE, LT-α treated and SA mice brain samples, indicates the inhibition of lymphotoxin-α decreases the cell death in brain protects the mice from CM.

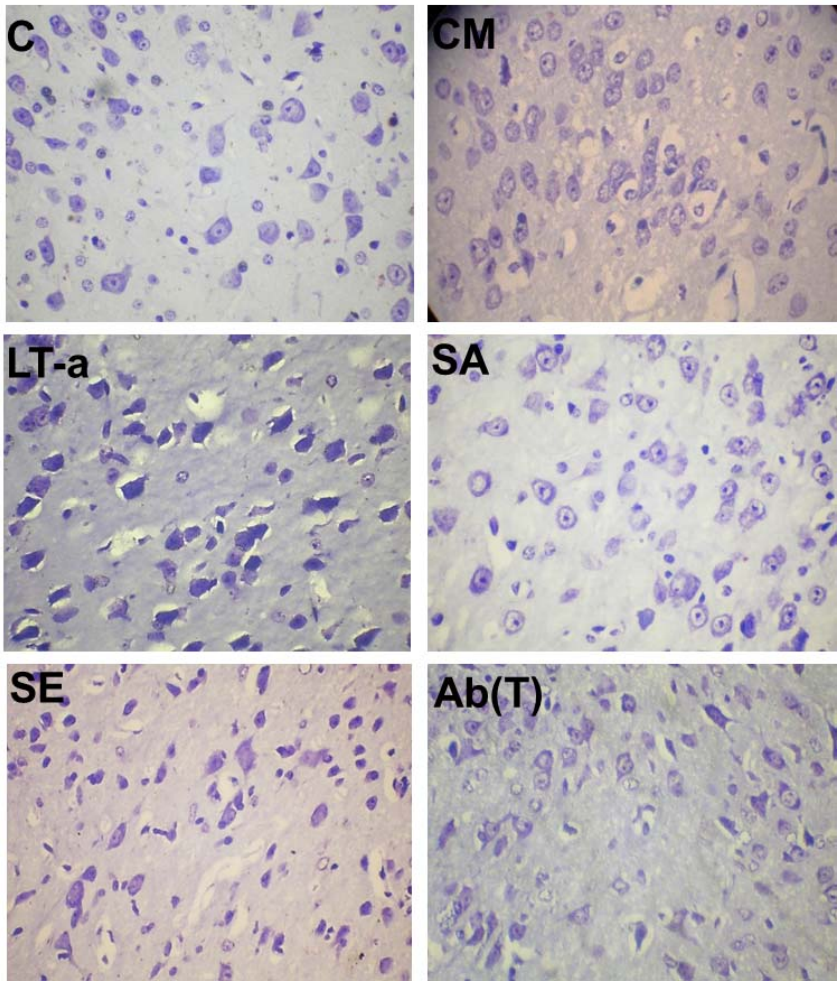


Fig.29. cresyl violet staining of mice brain sections for nissil granules of neuronal cells showing the neuronal cell death indicated by the detachment of cells from brain parenchyma and condensed cells of pyknotic cell death and marginalisation of nucleus in CM mice brain samples. Whereas cells in LT-a treated showing mostly cells are dying by crenelation evident form the darkly stained cells and detachment from surrounding brain matrix. Indicate the mode of cell death lymphotoxin-α is different from that of CM samples. In SA samples cell death appears to be due to edema as the evident from the diffused staining pattern (very light) and vauolation around the cells compared to control, CM, LT-a treated and SE samples. In SE mice brain samples cell death is similar to CM with vacuolation, detachment of cell from brain matrix and crenelated pyknotic cells. Ab(T) treatment decreased the neuronal cell death and vacuolation and staing pattern also similar to SA or control, but there is a cell death compared to control and may be due to hypoxia that arises as less oxygen supply to the brain as a result of increased parasitemia levels surviving in the blood for almost 30 days.

As we have shown in the 2nd chapter that during cerebral malaria, there isincreased levels of serum lymphotoxin-α, translocate into the brain and causes cell death and the pathology. It is too simpler in the experimental design to study the exact role or the mechanism of lymphotoxin-α during CM, as this molecule might be helping or making the advantage of all the factors available and thus making the disease more dreadful and

complex. Literature about this molecules shows that it up regulates cell adhesion molecules, chemokine receptors, activation of immune cells and initiation of apoptosis. Here are the possible roles for lymphotoxin-α which might lead to this pathology. 1) One being it up regulates cell adhesion molecules and thus helps in adhesion of pRBCs, clogging of blood vessels and their associated complication. 2) Second being activation of immune cells and up regulation of chemokine receptors lead to infiltration of immune cells and thus may activate the resident immune cells or may directly kills the neuronal cells or may cytotoxic to endothelium and 3) third being it might initiate the cell death through initiation of apoptosis. Therefore studying all these factors during inhibition would give us the better understanding of the mechanisms of lymphotoxin-α in CM pathogenesis and the disease. Hence we started studying whether this inhibition has any effect on lymphotoxin-α level in the brain.

Lymphotoxin-α inhibition decreased the levels of lymphotoxin-α in the brain during PbA infection

Immunofluorescence analysis of lymphotoxin-α showed increased levels in the brains of CM, SE, SA and lymphotoxin-α treated mice, which has been brought down by the antibody treatment. This is also in consistent with the no translocation of lymphotoxin-α into the lipid rafts of mice brain samples in lymphotoxin-α inhibited mice. We found the translocation in the SA mice brain samples might due to the later stages where there occur cerebral symptoms and elevated caspase-3 and calpain1 levels in SA conditions after 11^{th} day of p.i.

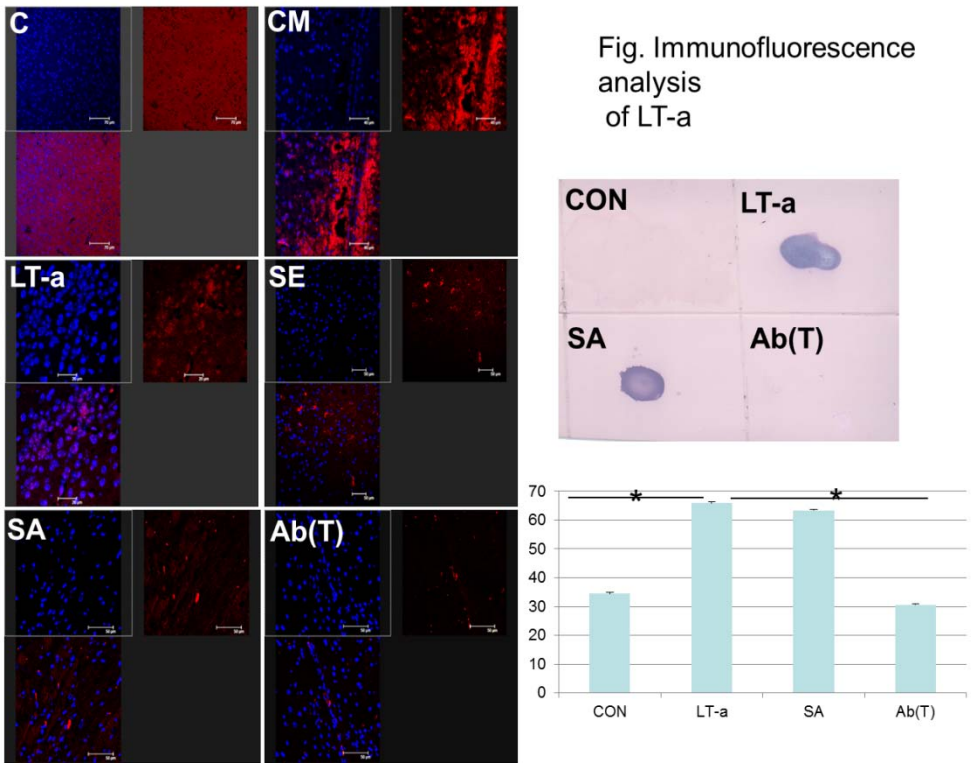


Fig. 30. Immunofluorescence analysis of lymphotoxin-α in the mice brain sections from different experimental conditions showing the significant increase of brain lymphotoxin-α over the control. CM mice brain sections indicated by the increased levels of lymphotoxin-α particularly along the endothelium of blood vessels that may be the reason for the expression of receptors on endothelium which helps in sequestration, BBB disruption and infiltration. Where as in the LT-α treatment, SE increases the lymphotoxin-α all over the brain more prominent from SA and the Ab(T) treatment has drastically decreased the lymphotoxin-α in the brain during *pbA* infection. Dot blot showing the translocation of lymphotoxin-α into the lipid rafts of LT-a treated and SA mice brain samples compared to control. Whereas there is no translocation of lymphotoxin-α in Ab(T) mice brain samples and graph showing the densitometry analysis of the same. Translocation of LT-a in the SA samples might be later/chronic stages where it activates cell death by caspase-3 and calpain1.

As elevated levels of lymphotoxin-α in the brain was decreased during its inhibition, we further studied whether this inhibition prevented the activation of cas-3 and cal1 in the brain of lymphotoxin-α mice. Our Western blot analysis showed decreased the activation of calpain1 and complete prevention of caspase-3 activation and CM, indicating lymphotoxin-α regulates the cell death proteases mediated CM pathogenesis.

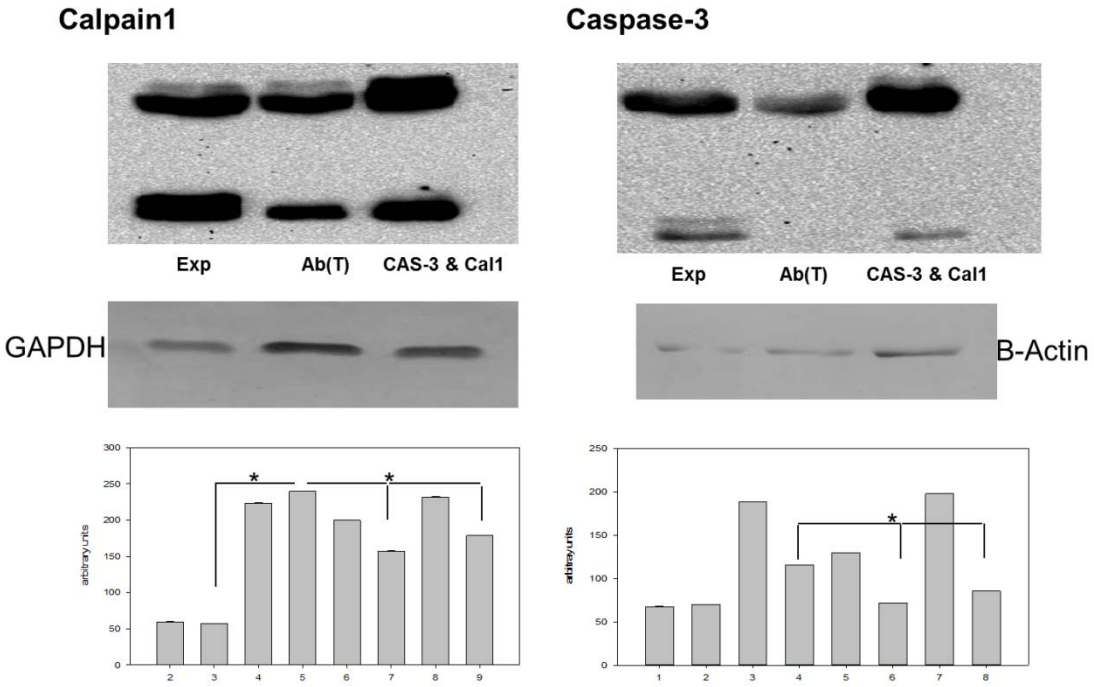


Fig. 31. Western blot analysis showing increased levels of active calpain1 and caspase-3 in CM mice brain samples, lymphotoxin-α and cas-3&cal1 inhibition brought down the active form levels of calpain1 and completely abolished active caspase-3. Bar graphs showing the densitometry analysis indicating the significant decrease of active calpain1 and caspase-3 in lymphotoxin-α and cas-3&cal1 inhibited mice brain samples. $p < .05$ is considered as significant and n=3 individual experiments.

We further studied the status of Hsp70 in different experimental conditions and Western blot result showed increased breakdown of Hsp70 in CM, SE which has been prevented by the lymphotoxin-α inhibition. We didn't find any significant breakdown during SA conditions which distinguishes the condition from CM. This might be explained by the cytotoxic role of CTLs where Gra-b and perforin won't be present to cleave Hsp70.

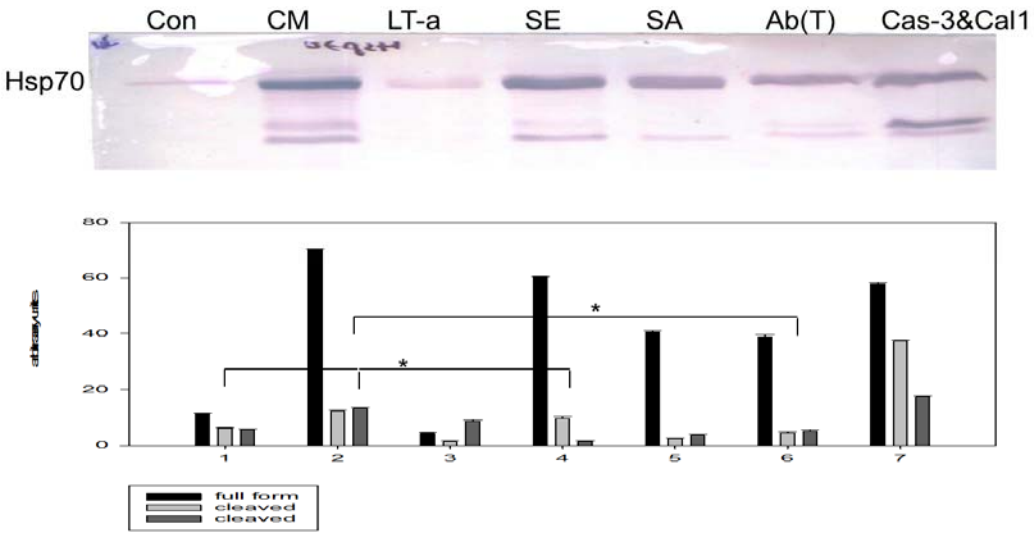


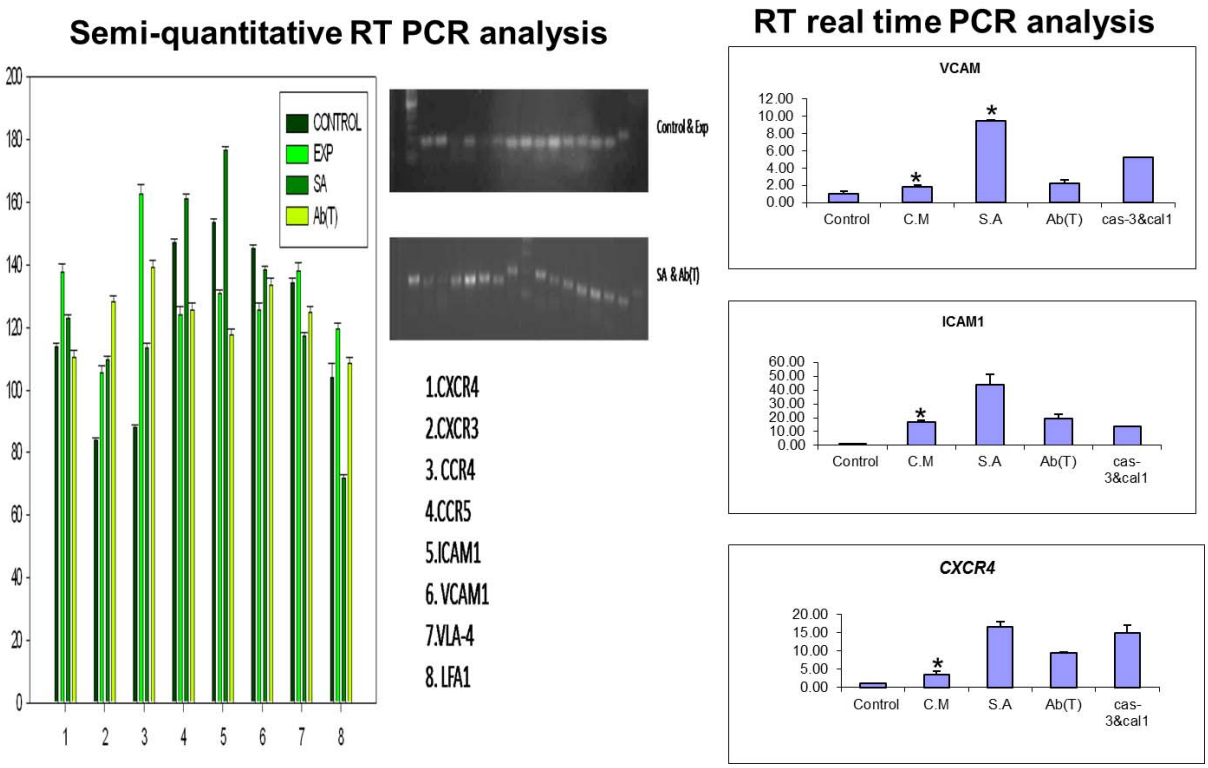
Fig.32. Western blot of Hsp70 in different experimental conditions showing the increased levels of pro and cleaved forms in CM, SE, SA and cas-3&cal1 compared to control. Thus this protein no longer protects the procaspase-3 and prevents its activation. Ab(T) treatment decreased the increased levels of full and cleaved forms of hsp70 compared to CM, implying the Ab(T) treatment prevents the Hsp70 cleavage and thus helps inhibiting the caspase-3 activation and its mediated cell death.

As it is evident from the above results that lymphotoxin-α inhibition protected the mice from CM by decreasing the activation of caspase-3 and calpain1 as evident from the second objective that Gra-b mediated T cell induced neurodegeneration is associated with increased ICAM-1, VCAM-1 and CXCR4 in CM mice brain samples and evident from the earlier reports implicated LT-α in ECM pathogenesis by regulating expression of ICAM-1, VCAM-1 and CXCR3 and T cells kills the neuronal cells by infiltrating in to brain parenchyma with the help of LFA-1 and VLA-4 in auto immune encephalitis. We studied the status of cell adhesion molecules (ICAM-1, VCAM-1) and chemokine receptors (CXCR3, CXCR4, CCR4, CCR5, LFA-1 and VLA-4) or the effect of lymphotoxin-α on these molecules through lymphotoxin-α treatment and its inhibition by anti-lymphotoxin-a antibody treatment.

Effect of lymphotoxin-α on cell adhesion molecules and chemokine receptors in mice brain samples

Our relative quantitative analysis of cell adhesion molecules (ICAM-1 and VCAM-1) and chemokine receptors (CXCR-3, CXCR-4, CCR4, CCR5, VLA4 and LFA-1) showed significant increased levels of all these molecules in SA conditions except the receptor specific for noncytotoxic CD4+ T cells which are protective during CM; LFA1, which is

slightly increased as compared to CM samples. Expression of all these molecules are down regulated by the lymphotoxin-α inhibition, whereas the expression of LFA1 is increased by the lymphotoxin-α inhibition. These results indicate that cell adhesion molecules ICAM-1, VCAM-1 and chemokine receptor CXCR4 are critical for the pathogenesis of CM as they are up regulated only in CM conditions and are down regulated by lymphotoxin-α inhibition.



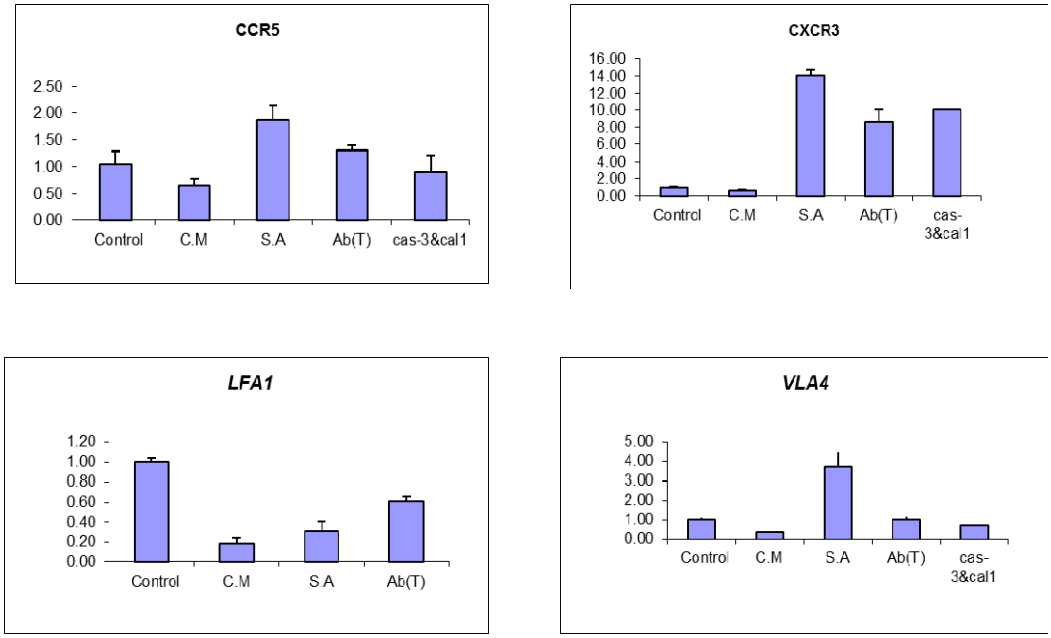


Fig. 33. Graphs showing the relative real time PCR analysis of cell adhesion molecules (ICAM-1 and VCAM-1) and different chemokine receptors (CXCR3, CXCR4, CCR4&5, LFA1 and VLA4) in different experimental conditions. Cell adhesion molecules and all the chemokine receptors are significantly increased in SA mice brain samples compared to control and the other experimental conditions, implying pRBCs might be contributing or the other host factors might be playing a role in inducing the expression in course of infection and all these molecules might not be critical for the CM pathogenesis. Overexpression of ICAM1, VCAM1 and CXCR4 are found to be critical for the CM pathogenesis as their expression is associated with CM and are down regulated by lymphotoxin-α inhibition and protected the mice from CM. This is also evident from the increased levels of LFA1, specific for receptor for protective CD4+ T cells, in Ab(T) treated mice compared to control , CM and SA mice brain samples. VLA4, a specific receptor for cytotoxic CD8+ T cells which are decreased in Ab(T) and thus prevent CM. The values on the y-axis indicate the relative quantitative expression levels in arbitrary units and the values of the experiments are from three individual experiments (n=3) for each sample.

Lymphotoxin-α inhibition decreased the CD8+ cytotoxic T cell infiltration into the brain of PbA infected mice

As the lymphotoxin-α inhibition decreased the expression of cell adhesion molecules and chemokine receptors in the mice brain infected with *PbA*. We further studied the infiltration of T cells as we have hypothesised and reported in our earlier study that during ECM increased cell adhesion molecules and chemokine receptors helps in adherence, sequestration and infiltration of pRBCs and T cells in to the brain and cause pathology. In consistent with the decreased levels of cell adhesion molecules and chemokine receptors, our immunofluorescence analysis showed the decreased infiltration of T cells, especially cytotoxic CD8+ T cells were completely decreased.

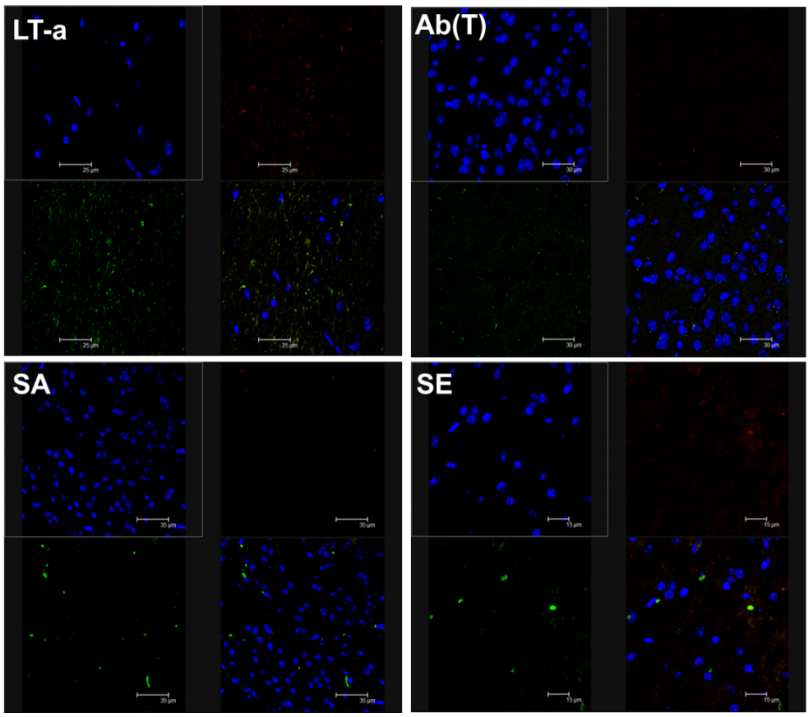


Fig.34. Immunofluorescence analysis mice brain section from different experimental conditions showing the infiltration of T cells into the brain indicated by the increased positivity of sections for FITC labeled CD4+ and APC labeled CD8+ T cells. Both CD4+ and CD8+ T cells are infiltrated in LT-α treated and SE injected mice brain samples, whereas relatively less infiltration of cytotoxic CD8+ T cells in SA mice brain samples compared to CD4+ T cells. CD8+ T cells are critical for CM pathogenesis. This is also in consistent with the decreased CD8+ T cells in the Ab(T) treated mice brain section and their protection from CM.

Status of cytotoxic proteases perforin and Gra-b during lymphotoxin-α and cas-3&cal1 inhibition in mice infected with PbA

From the above results it is evident that during lymphotoxin-α and cas-3&cal1 inhibition of mice infected with *PbA*, there was decrease of cell adhesion molecules, chemokine receptors and infiltration of cytotoxic CD8+ T cells. We have also reported that during ECM infiltrated cytotoxic cells secrete Gra-b and perforin and kill the cells by direct cytotoxicity. Hence we studied the status of perforin and gra-b during lymphotoxin-α and cas-3&cal1 inhibition. Our Western results showed the significant decrease of Gra-b and perforin compared to CM mice brain samples.

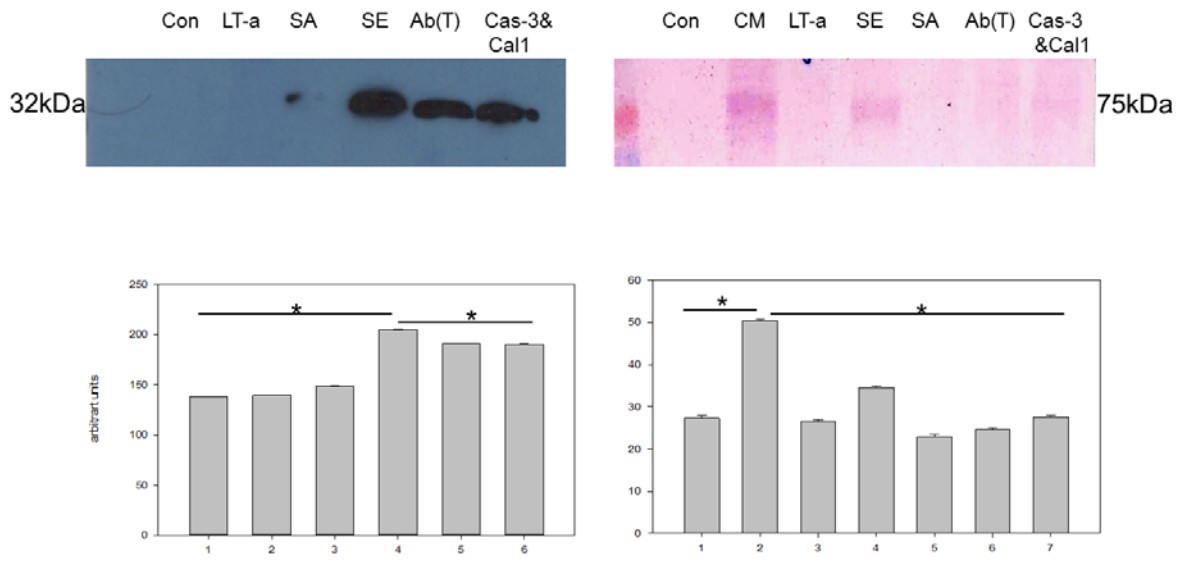


Fig.35. Western blot analysis showing the increased levels of cytotoxic proteases, Gra-b and perforin in different experimental conditions indicating the increased levels of both Gra-b and perforin in CM mice brain samples compared to control and SA mice brain samples. Increased levels of these proteases only in CM, but not in SA and control distinguishes the pathology from control and non-CM SA and these levels are decreased in by Ab(T) treatment. Graphs showing the densitometry analysis of Gra-b and perforin in different experimental conditions, p≤.05 is considered as significant * on the bars of graph by SEM values.

As it is evident from the previous studies that the outcome of the disease depends on the parasite burden, the cytotoxic T cells in the brain and the local secondary stimulus is needed for the T cells to become cytotoxic completely, and our inhibition studies protected the mice from ECM. We studied the status of T parasite burden, T cell infiltration and secondary stimulus (an indicative measure of status of antigen presentation in the brain- class II MHC expression). Our PCR analysis of MSP-1(merozoite surface protein transcript specific for *PbA*) showed the presence of this gene only in the brains of mice which are infected with *PbA*, their sequestration into the brain. There was a significant increase of parasite burden in ECM, SA and is brought down by the lymphotoxin-α inhibition. This was consistent with our decreased cell adhesion molecules which help in the adherence of pRBCs and their sequestration. We also investigated the T cell infiltration by using primers specific for TCR-α, our results showed the increased amount of TCR-α in CM, SA mice compared to control mice and the levels are significantly brought down by lymphotoxin-α inhibition. Cas-3&cal1 inhibition didn't decrease the parasite sequestration but protected mice from CM. This implies that the initiation of cell death signalling by caspase-3 and calpain1 might be the downstream events initiated by the parasite itself or the T cells and is inhibited by the cas-

3&cal1 inhibition even though high parasite burden in the brain of *PbA* infected mice. Our results of antigen presentation an indicative measure of expression of MHC-II in the brain of *PbA* infected mice by semi-quantitative PCR showed the increased presentation in CM and SA mice compared to control. This has been brought down by lymphotoxin-α and cas-3&cal1 inhibition.

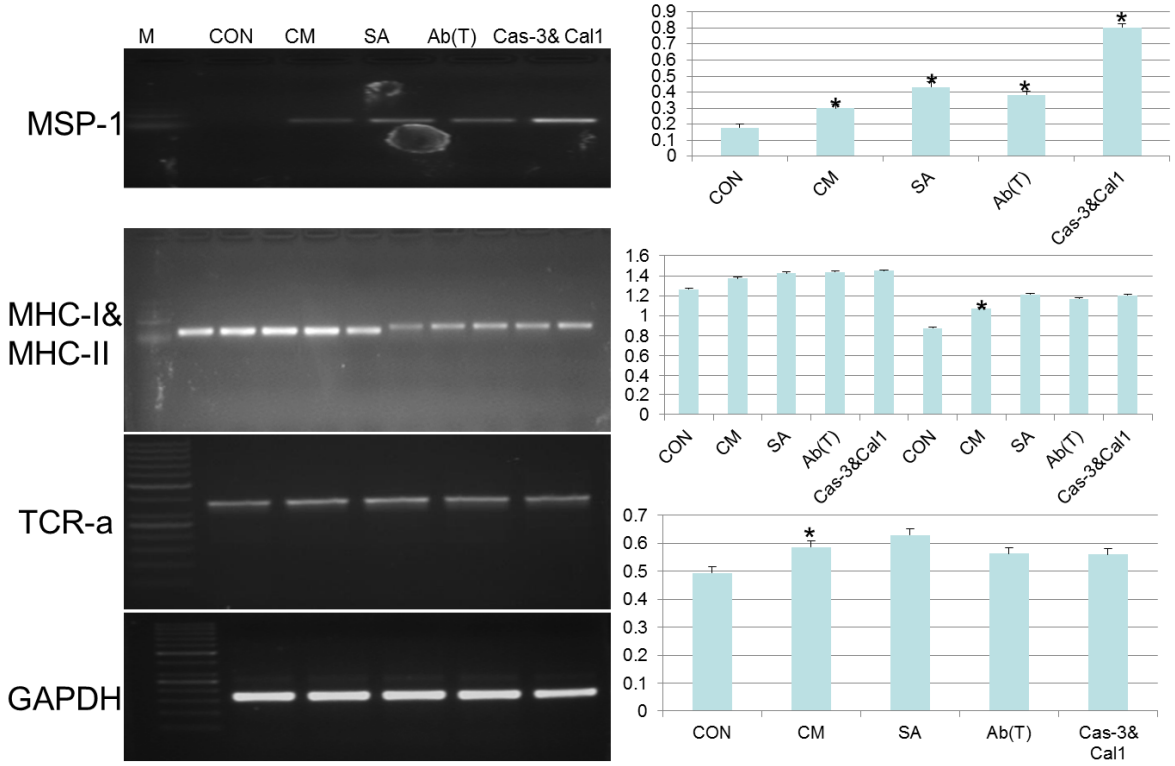


Fig.36. Graphs showing the PCR (MSP-1 and TCR-α) and semi-quantitative PCR analysis of MHC-1&II in the mice brain of different experimental conditions. MSP-1gene presence indicate the *pbA* parasite sequestration in the mice brain during *pbA* infection in SA, CM, Ab(T) and Cas-3&Cal1 inhibition. Significantly very low parasite sequestration in Ab(T) treated mice compared SA and Cas-3&Cal1 inhibited even though Ab(T) mice survived beyond 20 days indicate lymphotoxin-α helps in pRBCs sequestration during *pbA* and thus causes CM. PCR analysis of TCR-α showing the increased levels of T cell infiltration into the brain of CM, SA compared to control and infiltration was greatly reduced by Ab(T) and Cas-3&Cal1 inhibition compared to CM mice brain samples and protected from CM. local secondary stimulus is needed for the T cells to become completely functional and the measure of MHCII is the measure of antigen presentation in the brain. It is evident from the graphs that there is gradual increase in the expression of MHCI compared to MHCII in the brain of different experimental conditions of *pbA* and control. Lymphotoxin-α has no effect on MHCI presentation but significantly decreased the presentation through MHCII and in the MHCII presents antigen to the CD8+ T cells, thus Ab(T) inhibits the activation of cytotoxic CD8+ T cells in the brain and protects mice from CM during *PbA* (capital) infection.

As lymphotoxin-α and cas-3&cal1 has protected the mice from CM by decreasing the expression levels of cell adhesion molecules, chemokine receptors, T cell infiltration, pRBCs sequestration and antigen presentation. We further studied whether all these events lead to decrease the neuronal cell death during ECM. When we studied neuronal cell death in different experimental conditions, we found drastic decrease in neuronal cell death in lymphotoxin-α and cas-3&cal1 inhibited mice compared to CM and SA conditions. Which is also consistent with decreased NF (L) breakdown in lymphotoxin-α and cas-3&cal1 inhibited mice brain samples compared to CM and SA.

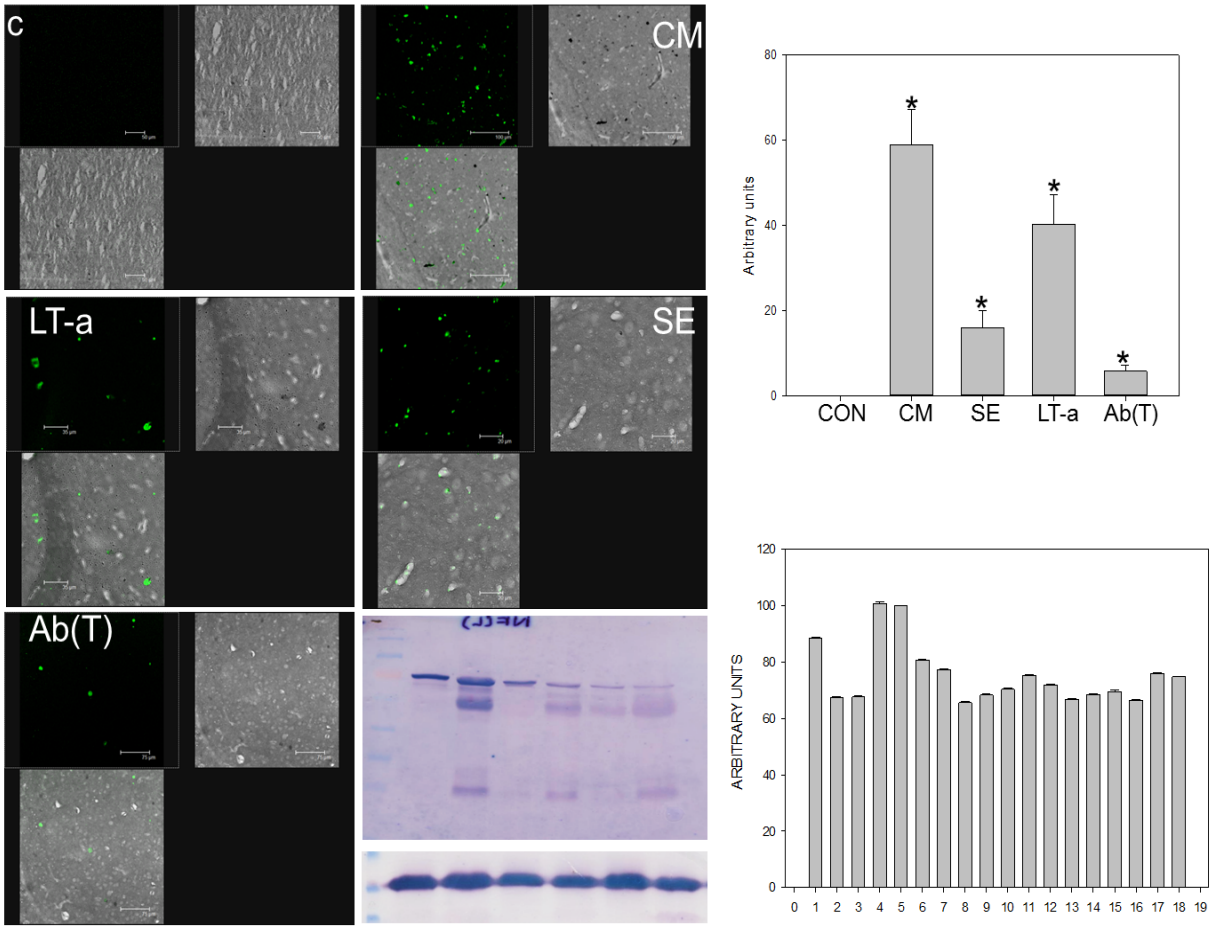


Fig. 37. Immunofluorescence analysis of Fluoro-Jade-b staining for degenerating neurons showing the increased neurodegeneration in CM, LT-α treated, SE injected mice brain samples compared to control and neuronal cell death is decreased by the lymphotoxin-α inhibition. This is also evident from the decreased neurofilament light (NF(L)) protein breakdown and respective cleaved fragments formation in Ab(T) and Cas-3&Cal1 inhibited mice brain samples compared to CM mice brain samples.

Cerebral malaria is a multifactorial disease and the pathology is characterized by brain oedema, infiltration of peripheral immune cells into the brain and neuronal cell death both in animal models and post-mortem human brain samples. Understanding basic pathology of this disease is not possible in humans due to some concrete reasons and the data available from human samples are only from post-mortem, hence events that lead to CM pathogenesis can't be explained clearly. And the assumptions drawn from evidences found in human samples can be studied only using animal models. There are many models available to study the CM however, experimental fatal murine cerebral malaria model is widely accepted and this shows the similar characteristics of CM in humans. This model is developed by infecting C57BL/6 mice with 10^6 PbA (Capital) (*Plasmodium berghei ANKA*) and this is best available model to study the immunopathogenesis of CM. Much of advance in understanding about the disease pathogenesis or towards vaccine development has been achieved by using this model, where knocking down of a molecule which is thought to be critical in the disease pathogenesis or creating the antibodies against critical Plasmodium protein has been studied (Chauhan et al., 2010; Holder and Freeman, 1981; Kumar et al., 2006). But the mechanism by which these cause or prevent the disease is not clearly known and need to be studied extensively. Hence understanding the disease pathogenesis would be a helping hand to look at different ways of preventing the disease.

The pathology of the disease can be explained broadly on two theories, one being hypoxic theory and the second being the cytokine theory. Hypoxic events that lead to cell death are through elevated levels of cell death proteases, caspase-3 and calpain1 which have been shown in many models of hypoxia (Chae et al., 2001; Edelstein et al., 1999; Kim et al., 2007) and cerebral malaria (Idro et al., 2010; Rizzo and Leaver, 2010). Cytokine theory explains up regulation of cell adhesion molecules (Armah et al., 2005) and chemokine receptors (de Kossodo and Grau, 1993) which helps in the infiltration of T cells and pRBCs in to the brain, causes the cell death by direct cytotoxicity of T cells through Gra-b and perforin mediated pathway and activation of caspase-3 and calpain1 by Gra-b. We have reported that these elevated levels of cell death proteases induce neurodegeneration by direct breakdown of cytoskeletal proteins, thus ECM (Eeka et al., 2011a). This is also consistent with the protection of mice from CM when caspase-3 is inhibited or by increasing the expression of anti-apoptotic bcl_2 (Helmers et al., 2008b), this indicates caspase-3 and calpain1 play a critical role in CM pathogenesis. Mice lacking the perforin (Potter et al., 2006) and T

Discussion

cells in which Gra-b has knockdown were protected CM. lymphotoxin-α, an immunomodulator, knockdown also protected mice from CM by decreasing cell adhesion molecules ICAM1, VCAM1 and chemokine receptor CXCR4. Implying not only all these proteases (caspase-3, calpain1, perforin and granzyme-b), cell adhesion molecules (ICAM1 and VCAM1) and chemokine receptor (CXCR4) are essential or interdependent for the outcome of the pathogenesis, but also all these factors might be regulated by the lymphotoxin-α and thus the pathogenesis.

Our results of lymphotoxin-α status studies during ECM showed increased levels of serum lymphotoxin-α and its translocation into the lipid rafts of infected CM mice brain samples compared to PBS injected mice brain samples. Results of lymphotoxin-α and serum from CM mice (SE) injection was lethal to mice within 8 days of post injection (fig.1), hence, we studied whether lethality of mice is through cell death and cytotoxic proteases or by any other mechanisms? Our immunofluorescence (fig.2a) and western blot analysis (fig.2b) showed the increased levels of these proteases in brains of CM, SE (mice injected with serum from CM mice) and SA mice, normal levels in lymphotoxin-α injected mice brain samples. However TUNEL staining showed increased positivity in all the experimental mice brain samples including lymphotoxin-α treated (fig.3) indicating lymphotoxin-α might be causing the cell death by other cell death mechanisms and the CM pathogenesis. Further western blot analysis of cytotoxic protease; Gra-b is significantly increased only in SE injected mice brain samples, whereas perforin is increased significantly in lymphotoxin-α and SE injected mice brain samples compared to control (fig.12). This is also in consistent with increased autophagy in lymphotoxin-α treated and SE injected mice brain samples compared to control and autophagy is more in lymphotoxin-α treated and SE injected when compared to CM indicated by conversion of 18 kDa LC-3 to 16 kDa (data not shown). Thus during CM condition, depending on the genetic makeup of the mice and TCR repertoire (Collette et al., 2004) increased serum lymphotoxin-α levels seems to activate T cells and their infiltration, induce expression of cell adhesion molecules, sequestration of pRBCs, BBB breakdown and cell death as evident from inhibition studies of lymphotoxin-α. Moreover, decreased expression of ICAM1, VCAM1 and CXCR4 (fig.11), lymphotoxin-α levels (fig.8), T cell infiltration (fig.13) and decreased activation of caspase-3 and calpain1 (fig.9) in the brains of *PbA* infected mice. Our relative real time PCR analysis also showed increased levels of chemokine receptors in SA mice brain samples which might be due to the effect of parasite

alone or the other chemokine that are released by the activated immune cells which might not be that crucial for the CM pathogenesis. Consistent with the earlier reports that parasite burden and T cell infiltration are essential for the disease outcome, lymphotoxin-α inhibition decreased parasite sequestration of (fig.14a), T cell infiltration (fig.13&14b) and decreased antigen presentation (fig.14c) the brain of Ab(T) mice. We found very significant sequestration pRBCs and increased antigen presentation compared to CM in the Caspase-3 and calpain11 inhibited mice brain samples but are protected from CM, indicates caspase-3 and calpain1 mediated cell death is crucial for CM pathogenesis and is downstream event of the T cell mediated cell death and the T cell infiltration is mainly due to BBB breakdown rather than their squeezing into brain with the help of expressed specific T cell receptors. This might be the reason for the decreased expression levels of VLA4 and LFA1 in CM mice brain samples compared to control and the increased expression of LFA1 receptors specific for protective CD4+ T cells and significant decrease of VLA4 (fig.11) specific receptor for cytotoxic CD8+ T cells when compared to SA mice brain samples. Thus our results indicate critical role of T cells in initiating neuronal cell death during ECM pathogenesis and BBB breakdown is an important in event for the infiltration of CTLs, their mediated cell death through direct cytotoxicity and cell death signalling through caspase-3 and calpain1. Lymphotoxin-α is the key regulator of all the events that lead to ECM pathogenesis.

Summary

Cerebral malaria pathogenesis is poorly understood and is associated with neuronal cell death. The pathology is partly due to the hypoxic conditions and effects of host immune system that arise as a results of 1) due insufficient supply of blood as a result of RBC lyses or blockade of blood vessels pRBCs and RBCs that diminish oxygen supply to the brain. 2) Due to adverse effects of activated immune cells and/or the cytokines released by the immune cells which either may cause the neuronal cell death by direct cytotoxicity, or by activating the resident immune cells or by the cell death signalling initiated by the cytokines. 3) Due to cell death signals initiated by the parasite and 4) and/or the combinatorial effect of all these factors that lead to this complex syndrome.

Events that occur during this pathology are elevated levels of cell death proteases and the neuronal cytoskeletal breakdown mediated by them; this is also evident from the inhibitory studies which protected mice from CM. There was also increased infiltration of immune cells into the brain, particularly T cells, and the role of cytotoxicity of CD8+ T cells is well established as perforin and Granzyme-b knockdown of these cells protected mice from cerebral malaria in mice model. This indicates the interdependence of these cell death proteases (caspase-3 and calpain1) and cytotoxic proteases (perforin and granzyme-b) from cytotoxic T cells in pathogenesis. In this connection lymphotoxin-α, an immune modulator plays a role in formation of secondary lymphoid organs, maturation and maintenance of lymphocytes and also acts against pathogens during infections has shown to prevent CM when it is knocked out. All this implies that lymphotoxin-α may be the key regulator in coordinating the effect of caspase-3 and calpain1 and cytotoxic proteases mediated CM pathogenesis. The possible role of lymphotoxin-α in capase-3 and calapin1 and T cell mediated pathogenesis and as there is no evidence whether these caspase-3 and calpain1 has any role in neuronal cell death, their role in activating cytotoxic proteases or vice versa and if lymphotoxin-α is regulator of these proteases. We framed our objectives to study the

1. Role and mechanism of action of cell death proteases (caspase-3, calpain1 and cathepsin-b) and CTLs in neuronal cell death during CM.

2. Status of lymphotoxin-α and role in neuronal cell death; whether it has any effect on caspase-3 and calpain1 mediated cell death.

3. *In vivo* inhibition of lymphotoxin-α and caspase-3 and calpain1.

We used *plasmodium berghei* ANKA (PbA) induced experimental cerebral malaria (ECM) in C57BL/6 mice as model to study the above objectives and PbA infection , 80-90% of the infected mice develops cerebral symptoms and die by 6-9th day of post infection (p.i) with a parasitemia < 20%. Remaining 10-20% mice die between 10-15th day of p.i are due to severe anaemia with parasitemia >20%. As we find two different conditions, we aimed at studying neuronal cell death during CM condition; we also included severe anaemia samples as non CM severe anaemia (SA) samples to make clear distinction between two disease states of pbA infection. PBS treated mice were taken as control mice brain samples. Our results showed the increased cell death proteases (caspase-3, cathepsin b and calpain1) during CM induce neurodegeneration through neuronal cytoskeletal breakdown by direct interaction. We also found infiltration of CTLs into the brain through up regulation of ICAM-1, VCAM-1 and CXCR4 and these infiltrated T cells kills the neurons through perforin and granzyme-b mediated pathway. Casapse-3 and calpain1 also activates granzyme-b thus aggravates the disease.

Studies on protein levels of lymphotoxin-α showed the increased levels of serum lymphotoxin-α and is translocating into the lipid rafts of CM mice brain samples compared to control which we find only in lipid rafts of CM samples. As we found increased serum lymphotoxin-α and its translocating into the lipid rafts and immunofluorescence analyses showed the increased levels of lymphotoxin-α along the endothelial lining of blood vessels mostly in the brain of CM mice compared to control. Hence, we hypothesised that increased serum lymphotoxin-α might be causing the CM pathogenesis. To test this hypothesis we collected the serum from control and CM mice and injected into the naïve mice and observed the survivality. Here we also included the severe anaemia samples to clearly distinguish the pathology of CM, designated as SA (severe anaemia). A batch of mice where recombinant lymphotoxin-α were given through intra venal and intra peritoneal injection, designates as LT-α (T) (lymphotoxin-α treated). All the mice which received serum from CM, designated as SE mice and lymphotoxin-α were died by 8th day of p.i., brain samples from these SE, SA and LT-α(T) mice were collected and used for histological, protein, expression analysis. Survival analysis indicate serum and lymphotoxin-α are lethal to mice and protein levels showed increased levels of caspase-3 and calpain1 in SA and SE samples when compared to control. We found no difference in caspase-3, calpain1 and LT-α (T) compared to control and no levels of granzyme-b and perforin are present in SA compared to control, but significant

increase was found in SE samples. Indicating the pathology of CM is associated with immune factors along with hypoxic conditions, whereas SA is purely hypoxia mediated and hence not acute. This also throws light on the basic mechanism of pathogenesis and both the conditions are different that largely depend on the immunologic background of the mice. This is also evident from the studies which showed the T cell repertoire determines the susceptibility or resistant to CM towards PbA infection (Collette et al., 2004). But the results of TUNEL assay showed the significant increase of TUNEL positive cells in LT-α (T) brain samples along with SE, SA and CM samples compared to control and will discuss in the next objective the reason for apoptotic cells in LT-α (T) mice brain samples. We also found the increased pro-inflammatory like iNOS, nuclear translocation of NFk-B into nucleus and elevated levels of COX-2 during CM, SE, SA mice brain samples compared to control, but there was no elevated levels of these pro-inflammatory molecules in LT-α (T) mice brain samples. Indicate no inflammatory mediated cell death by lymphotoxin-α treatment.

Hence the lymphotoxin-α treatment and the serum from CM was lethal to mice and elevated levels of caspase-3 and calpain1 in SE injected mice brain samples compared to control and LT-α (T) mice brain samples, but we found increased apoptosis in LT-α (T) compared to control. Further we confirm by inhibiting the lymphotoxin-α (lymphotoxin-α inhibited mice designated as Ab (T) for antibody treatment) during infection and to study whether the caspase-3 and calpain1 activation is downstream and or regulated by lymphotoxin-α, we also inhibited capase-3 and caslpain1 (designated as Cas-3&Cal1) by using specific inhibitors Z-VAD-fmk and Ac-DEVD-CHO respectively by using the methodology described in methods section. Mice were observed for survival analysis and checking the parasitemia levels. From these mice 3 sets of brains from these all these mice were used to estimate the brain water content, and indicative measure of oedema. One mice from each group were given Evan's blue (200 µl of 2% in PBS) intra peritoneally 1 hr. before sacrificing when they are at moribund stage (cerebral symptoms) to study blood brain barrier disruption (extravasation of Evan's blue in to the brain indicate the blood brain barrier disruption). Results of inhibition studies showed the increased survival of mice up to 30 and 20 days of p.i. in Ab(T) and Cas-3 & Cal1 inhibited mice brain samples compared to pbA infected CM and SA mice, with intact blood brain barrier, decreased brain water content compared, decreased cell death and vacuolation compared to pbA infected CM mice brain samples. Further, we found decreased levels of lymphotoxin-α in the brains of Ab(T) mice

brain samples compare to CM, SA and SE injected mice brain samples and no translocation of lymphotoxin-α in the brain of Ab(T) mice brain samples. There was a translocation of lymphotoxin-α in the SA mice brain samples, which might be the reason that induce the caspase-3 and calpain1 mediated neuronal death. But obviously there was no much of T cell infiltration T cells into the brain of SA compared to SE, LT-α(T) and CM samples which makes the mice distinct from CM where involvement of lymphotoxin-α activated and assisted T cells by increasing cell adhesion and chemokine receptors that makes the disease very acute and sudden. PCR analysis showed the decreased T cell infiltration particularly cytotoxic CD8+ T cells (evident from immunofluorescence analysis) even though there was increased pRBCs sequestration in Cas-3 & Cal1 mice brain samples. This is supported by the decreased antigen presentation (MHC-II expression) in Ab (T) mice brain samples which is in consistent with the earlier report indicated the importance of T cells and pRBCs in the brain during PbA infection for CM pathogenesis. Decreased cell death, inhibition of BBB disruption, decreased neuronal cell death in Cas-3&Cal1 mice brain samples even though high infiltration of pRBCs and antigen presentation indicate importance of caspase-3 and calpain1 mediated neuronal cell death that is activated largely by T cell downstream signalling which is again regulated the levels of lymphotoxin-a during PbA infection. Overall our findings shown that during PbA infection depending on the genetic background of mice increased serum levels of lymphotoxin-a induces the expression of cell adhesion molecules and chemokine receptors on endothelium of blood vessels in the brain that assist the entry of pRBCs and activated T cells and secondary stimulation of T cells by antigen presentation. These activated T cells kills the neuronal cells directly and also activate caspase-3 and calpain1 which kills the neuronal cells through cytoskeletal breakdown. Our finds also throws a light on the caspase-3 mediated neuronal cell death through TNFR signalling (Lotocki et al., 2004) (translocation of lymphotoxin-a in to lipid rafts).

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Research Report

Proteolytic breakdown of cytoskeleton induces neurodegeneration during pathology of murine cerebral malaria

Prabhakar Eeka[a,1], Ganta Vijay Chaitanya[b,1], Phanithi Prakash Babu[b,*]

[a]Department of Animal Sciences, School of Life Sciences, University of Hyderabad, India
[b]Department of Biotechnology, School of Life Sciences, University of Hyderabad, India

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ABSTRACT

Fatal murine cerebral malaria is known to induce cellular degeneration by altering cellular morphology and integrity of cell. The morphology and integrity of the cell mainly depends on the cytoskeletal network of the cell. Increased proteolysis of cytoskeletal proteins accompanied by aggravated suicidal proteases activation leads to cellular degeneration. In the present study, we investigated the roles of apoptotic and necrotic cell death proteases, caspase-3, calpain-1 and cathepsin-b in the proteolysis of neuronal cytoskeletal proteins in mouse model of fatal cerebral malaria. We found increased levels of calpain-1, cathepsin-b and caspase-3, with extensive cross talks between these suicidal proteases. Increased levels of these proteases correlated with the enhanced proteolysis of several cytoskeletal proteins including neuronal cytoskeleton proteolytic signature fragments. Further, we also observed that increased levels of these proteases correlated with the appearance of neuronal death that exhibited apo-necrotic continuum. Our results confirm that activation of multiple suicidal proteases, their cross talks and breakdown of the cytoskeletal proteins increase neuronal degeneration and lead to exacerbation of cerebral malaria pathology.

1. Introduction

Cerebral malaria is a clinical manifestation of mental status and coma leading to death of patient within the hours of onset (Looareesuwan, 1992). Cerebral malaria often results in as high as 30% mortality and about 10% of survivors suffer with long and short term neurological problems. The pathophysiology of this disease involves the adherence of the parasitized RBCs (pRBCs) to the activated/inflamed endothelium of the blood vessels in the (Hatabu et al., 2003) brain initiating cell death signaling cascades (Pino et al., 2003b). Increased adherence of pRBC to the vascular wall leads to blockade of blood vessels resulting in diminished oxygen supply leading to hypoxia and disruption of blood brain barrier (Pino et al., 2003a, 2005). Increased BBB permeability facilitates extravasation of peripheral immune cells into brain (Belnoue et al.,

* Corresponding author. Fax: +1 40 23010120.
E-mail addresses: ppbsl@uohyd.ernet.in, prakashbabuphanithi@gmail.com (P.P. Babu).
[1] Equally contributed.

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2002; Bisser et al., 2006; Patnaik et al., 1994). These immune events in the brain promote hyperactivation of resident microglia either directly or through the release of cytokines and toxic metabolites (Deininger et al., 2002). Even though a well defined pathogenesis of CM remains to be understood, recent reports have shown that involvement of various mechanisms including vascular occlusion in the presence of sequestered pRBCs, leukocytes, inflammation, apoptosis/necrosis, platelet activation and angiogenic failure may contribute to the pathogenesis and exacerbation of cerebral malaria (Hunt et al., 2006; Idro et al., 2005, 2006; Jain et al., 2008; Nacher, 2008). Cellular degeneration is one of the leading components in exacerbation of the etiology of cerebral malaria. However, the role of apoptotic and necrotic suicidal proteases, their cross talks and action on various cellular substrates like cytoskeletal proteins remain elusive. Apoptosis and necrosis are tightly regulated processes and can be induced by alterations in several biochemical intermediates, including alterations in high-energy phosphates, intracellular calcium accumulation and reactive oxygen species (Berger and Garnier, 1999). Caspases, calpains and cathepsins are some of the crucial proteases involved in apoptotic and/or necrotic cell deaths (Marks and Berg, 1999; Yakovlev et al., 2001). Calpains and cathepsins can mediate apoptotic or necrotic cell deaths independent of caspases (Liu et al., 2004; Liu et al., 2006; Yamashima, 2000), whereas caspases were specifically involved in executing apoptosis (Riedl and Shi, 2004). However, calpains, cathepsins and caspases share similar substrate specificity and cleave several target proteins like poly-(ADP-ribose) polymerase-1 (PARP-1), post-synaptic density protein-95 (PSD-95), spectrin, neurofilament (NF) and fodrin (Wang, 2000a). Neuronal cytoskeleton breakdown by calpains and caspase-3 has also been shown in many neurological diseases (Stys and Jiang, 2002; Takamure et al., 2005). Axonal injury or neuronal cell death is implicated in many of the neurological diseases including virus induced encephalitis (Strachan et al., 2005), Alzheimer's disease (Higuchi et al., 2005; Marcilhac et al., 2006; Raynaud and Marcilhac, 2006), amylotrophic sclerosis (Locatelli et al., 2007) and cerebral ischemia (Chaitanya et al., 2010a,b; Chaitanya and Babu, 2008; 2009). In the present study, we have investigated the status of these proteases, cross talks between these proteases, proteolysis of cytoskeleton and the ultrastructure of cell morphology during cerebral malaria in the brains of mice infected with *Plasmodium berghei* ANKA.

2. Results

2.1. CM induces neuronal degeneration in P. berghei ANKA infected mice brains

Neuronal death in the CM brains was observed by staining brain sections with hematoxylin, eosin and cresyl violet. We found an extensive disruption of neuropil and cellular architecture in the brain parenchyma. Increased cellular and tissue vacuolation, crenulated cells and decreased hematoxylin staining were also observed in the cortices of infected mice brain samples over the controls indicating apoptotic mode of cell death. Moreover, we also observed heavily condensed cells with intense hematoxylin staining suggesting pyknotic cell death (Fig. 1a). Cresyl violet staining clearly showed an increased neuronal degeneration as indicated by condensed cell shape, crenulated appearance, pyknosis, intense cresyl violet accumulation, vacuolation and detachment from the brain parenchyma (anoikosis). Extensive tissue disruption in the present study suggests of necrosis in all the infected brain samples (Fig.1b).

2.2. Cerebral malaria results in elevated interactions between activated caspase-3, calpain-1 and cathepsin-b and a mixed apo-necrotic form of neuronal death

Western blot analysis of caspase-3, calpain-1 and cathepsin-b clearly indicated a significant increase in their protein levels in the cortical samples of infected mice over the controls (Fig. 2a–c). Immunohistochemistry of active caspase-3 showed a predominant elevation in the infected mice brain cortices over the controls. Both full form and catalytic fragments of calpain-1 levels were significantly increased in the infected mice brains over the control mice brain cortices. Furthermore, lysosomal protease cathepsin-b levels were also elevated along with active caspase-3 and calpain in the infected mice brain samples. Immunohistochemistry was performed to observe the localization pattern of these proteases in the infected and control mice brain cortical regions. A significant increase in the levels of active caspase-3 cells were observed in the cortical region of infected mice brain over the controls indicating an increase in the apoptotic cell death in the infected mice brain cortices (Fig. 3a). Immunohistochemical analysis of calpain-1 showed a significant increase in the number of calpain-1 positive cells in the infected mice brains over the controls. The elevation of calpain suggests either apoptotic or necrotic modes of cell death chosen by the cell at later stages (Fig. 3b). Further, immunohistochemistry of cathepsin-b also showed a significant increase in the levels of cathepsin-b immunoreactivity in the infected mice brains over the control along with active caspase-3 and calpain-1 (Fig. 3c). This increase correlating with elevated calpain-1 levels indicates that the cell might choose either apoptotic or necrotic modes of cell death. Co-localization studies using triple immunofluorescence was used to study the interactions between these proteases (Fig. 4). We observed some cells in the infected mice brain cortices stained positive for all these three proteases suggesting a tendency for that specific cell to undergo apo-necrotic cell death continuum. Hence, in order to check whether any of the cells in the infected mice brains shows apo-necrotic mode of cell death ultra thin brain sections from infected mice brain were obtained and observed under transmission electron microscope. Ultrastructural analysis showed a mixed type (apo-necrotic) of cell death with morphological features containing both apoptosis and necrosis. We observed marginalization of neuronal nuclei, membrane blebbing, and proapoptotic body formation indicating classical features of apoptosis and mitochondrial swelling, endoplasmic reticulum breakdown, vacuolation and disintegration of nuclear membrane indicating necrotic mode of cell death in a single degenerating neuron (Fig. 5a and b).

2.3. Status of PSD-95, vimentin, NF-L and synaptophysin

Effects of the increased cell death protease levels on cellular substrates like vimentin, PSD-95 and synaptophysin of infected

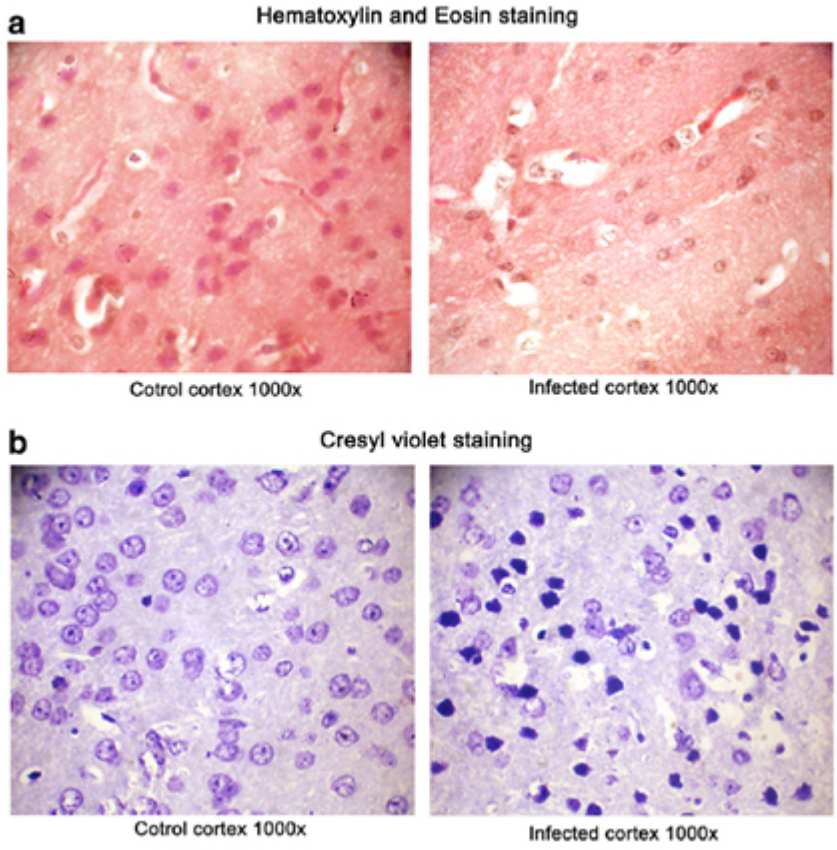


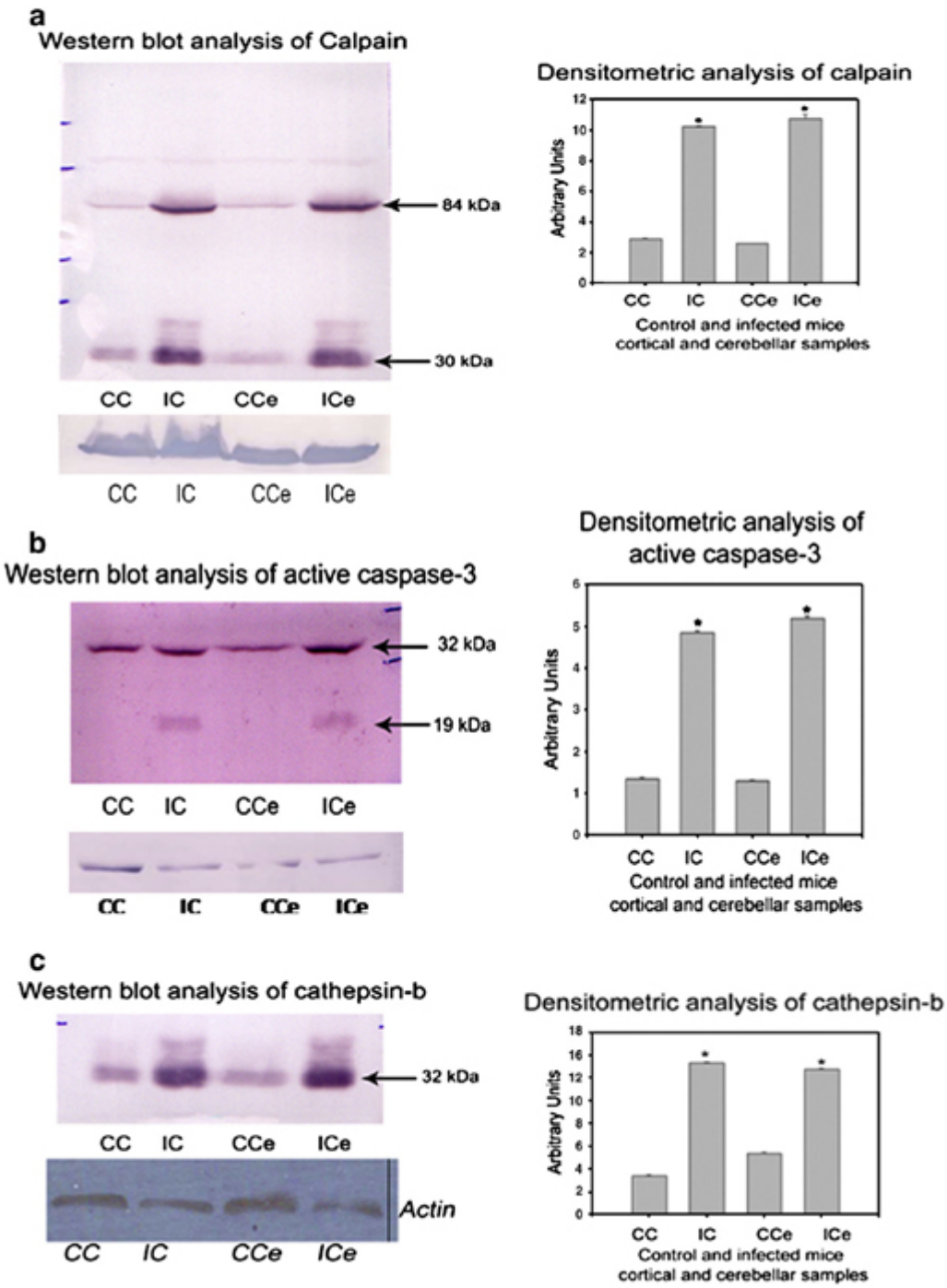
Fig. 1 – Histology of brain tissue sections stained with H&E and cresyl violet. a) Increased cell death in the cortical region of infected mice brain evident by their crenulated appearance, cell shrinkage, vacuolation, detachment from the brain parenchyma, and tissue disruption over the control mice can be observed. Images were obtained at 1000× magnification with Olympus fluorescent microscope. b) Cresyl violet staining was used to identify the status of neurons in the infected mice cortices over the control. Increased neuronal death was observed in the infected mice brain over the control evident by their shrunken, crenulated shape and increased vacuolation and tissue disruption and decreased intensity to stain with cresyl violet. Images were obtained at 1000× magnification with Olympus fluorescent microscope.

mice brains were analyzed by Western blot analysis. Western blot analysis showed a significant increase in the breakdown products of vimentin (Fig. 6a), NF-L (Fig. 6b) and decreased levels of PSD-95 and synaptophysin (Fig. 6c and d). Correlating with increased neuronal death, NF-L was extensively proteolysed to 57 and 53 kDa fragments. Moreover, we observed vimentin breakdown products of 50 and 44 kDa known to be mediated by calpain. We also observed decreased synaptophysin levels in infected over control mice brain cortical samples (Fig. 6d). This might be associated with neuronal degeneration in the infected mice brain cortices over the controls (Fig. 6e). In order to identify the interaction of caspase-3 and calpain with vimentin, PSD-95 and NF-L, we performed co-immunoprecipitation experiments by immunoprecipitating the protein complexes in the cytosol with calpain and caspase antibodies and Western blotting with vimentin, PSD-95 and NF-L antibodies. Significant increase in the binding of vimentin (Fig. 7a), PSD-95 (Fig. 7b) and NF-L (Fig. 7c) with caspase-3 and calpain were observed in the infected mice brain samples over the control mice brains. Negative controls showed no binding of these protein complexes. The increased binding of vimentin, PSD-95 and 7 NF-L correlated with their breakdown and subsequent decrease in the infected samples over the control mice brain samples. The breakdown of these cytoskeletal proteins also correlated with significantly increased cell death in the infected mice brain samples over the controls. Importantly, the decrease in the PSD-95 levels and breakdown of NF-L correlated with the increased neuronal death observed in the cresyl violet staining and neurological deficit of the infected mice.

3. Discussion

Patho-physiological features of cerebral malaria in humans and mouse models include sequestration of pRBCs in the brain vasculature, blood brain barrier breakdown (due to the ischemic

conditions,toxic metabolites or both) (Hatabu et al., 2003; Hunt et al., 2006; Johnson et al., 1993; Medana and Turner, 2006; Pino et al., 2005), elevated immune cell infiltration into the brain (resulting in dampened peripheral immune responses) (Patnaik et al., 1994), cerebral hemorrhages (White and Silamut, 2005), brain edema (Sanni, 2001), degeneration of vascular and non vascular cells of the brain (Medana and Turner, 2007; Pino et al., 2003b; Wiese et al., 2006), increased oxidative load (Becker et al., 2004) and activation of resident immune cells in the brain (glial cells) culminating in increased mortality or persistent



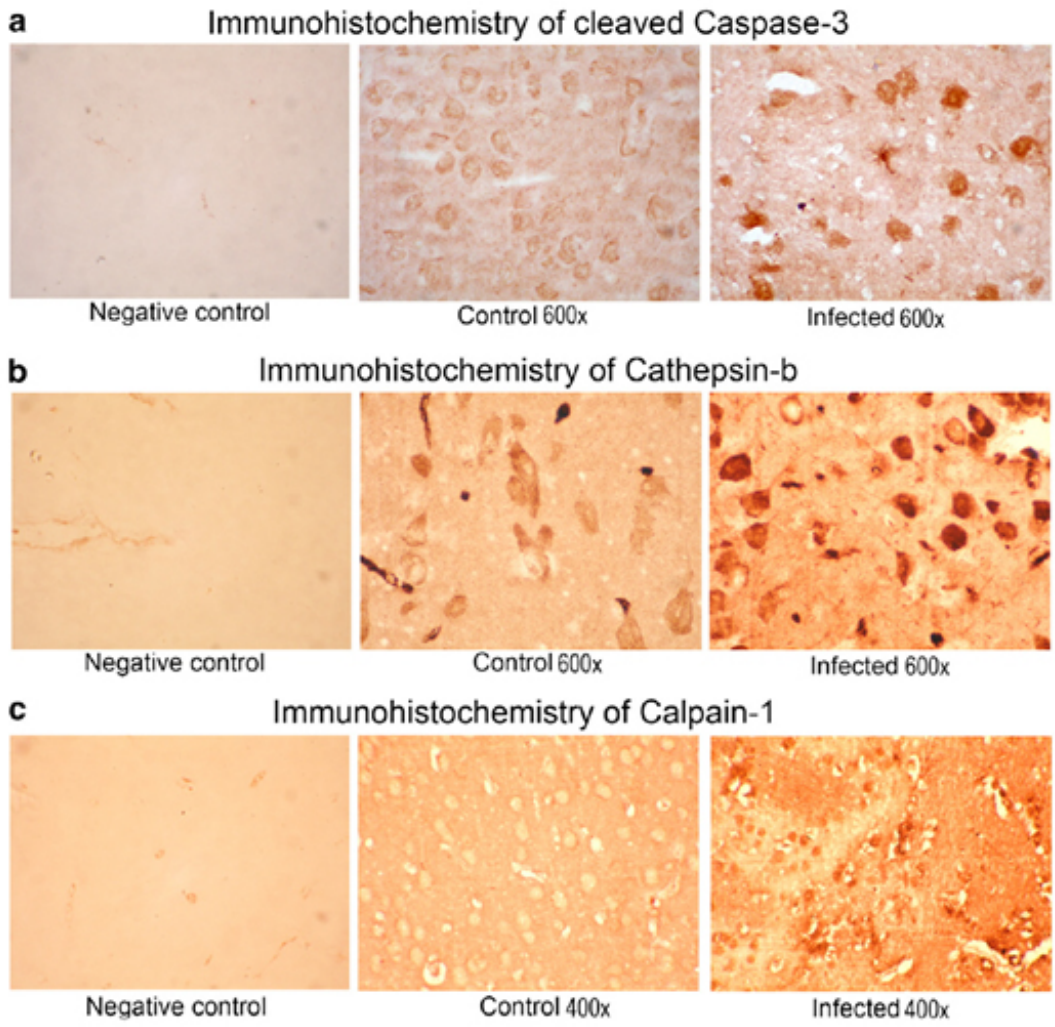


Fig. 3 – Immunohistochemical analysis of cell death proteases. a) Immunohistochemistry of active caspase-3 (antibody specific for active caspase-3 was used) indicated an increase in active caspase-3 in the brains of infected mice over the controls. Magnification 600×. b) Immunohistochemistry of calpain-1 showed a dramatic increase in the intensity of calpain in the infected mice brain cortices over the controls correlating with our previously published results. Magnification 600×. c) Immunohistochemistry of cathepsin-b. Lysosomal protease cathepsin-b was found to be elevated in the infected mice brain cortices over the controls. In addition to its localization in the cells, increased cathepsin-b was also found in the brain parenchyma or brain matrix. Magnification 400×. No immunoreactivity was observed in negative controls in which primary antibody addition was omitted. n=3; 3 parallel sections from each mice brain were used.

neurological deficits. Several hypotheses indicate that cerebral malaria might be a result of increased cytoadherence of pRBC in the brain vasculature leading to focal hypoxic conditions and BBB dysregulation leading to the pathogenesis of the disease (van der Heyde et al., 2006). However, it is becoming well accepted that the pathogenesis is multifactorial.

Fig. 2 – Western blots of calpain, caspase-3 and cathepsin-b. a) Western blot of calpain-1 showed a significant increase in the calpain levels in the infected mice brain cortical regions and an internal control cerebellum samples. Both full length and catalytic fragments (30 kDa) were found to be increased in the cytosolic fraction of infected mice brain samples over the controls. $p<0.05$ is considered to be statistically significant. b) Western blot of caspase-3 showed no difference in the levels of pro-caspase-3 in the in the cytosolic fractions of infected mice brain samples over the controls. However significant increase in the active caspase-3 was observed in the infected over the controls. $p<0.05$ is considered to be statistically significant. c) Western blot of lysosomal protease cathepsin-b was found to be increased in the infected mice brain samples over the control. $p<0.05$ is considered to be statistically significant. $n=5$; each blot was repeated 3 times. Bars represent standard error.

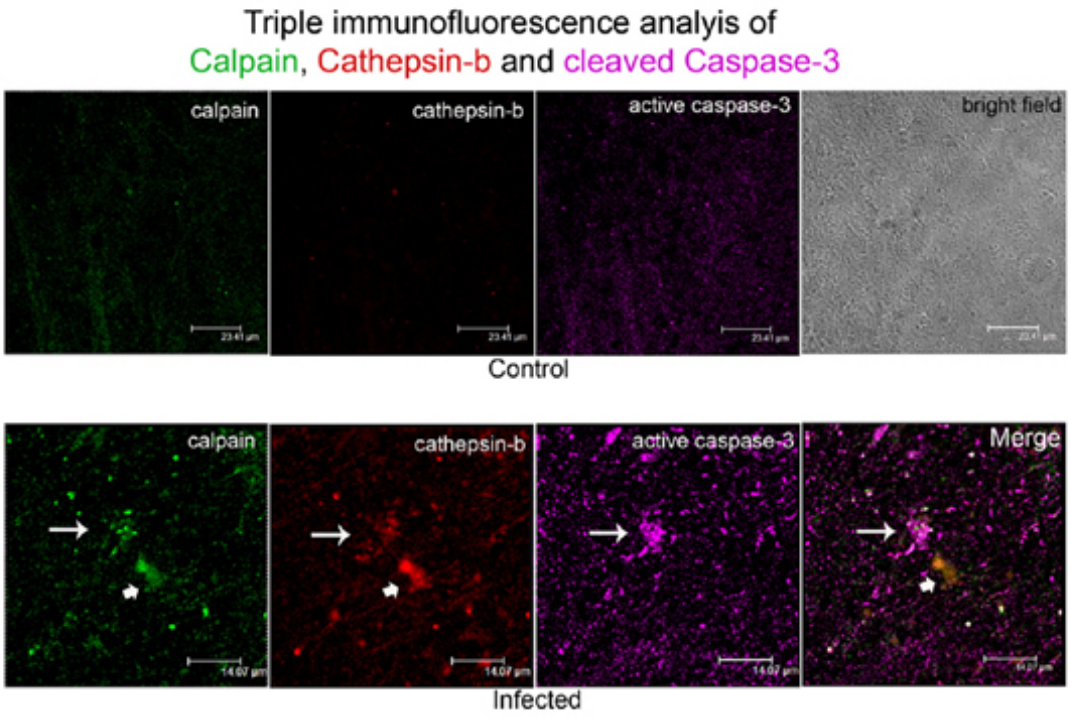


Fig. 4 – Triple immunofluorescence analysis of active caspase-3, calpain-1 and cathepsin-b. Triple immunofluorescence was used to observe the cross-talks between these proteases. Arrow points towards the degenerating cell positive for active caspase-3, calpain-1 and cathepsin-b indicating cross talks among them. Moreover due to the presence of active caspase the resulting cell death might be apoptotic. Cell pointed with arrow head shows the absence of active-caspase-3 and increased presence of calpain-1 and cathepsin-b indicating that the resultant cell death might be necrotic.

In the present study we have observed some important pathological features in CM including elevated suicidal proteases which were involved in apoptotic and necrotic cell deaths. Moreover, in accordance with the previous reports in other models like cerebral ischemia and brain tumor, these proteases were found to interact with each other. Cross talks between suicidal proteases and the ability of these proteases to act independently or in synergy with each other are some of the most troubling phenomena that greatly attenuate therapeutic benefits (Neumar et al., 2003; Rami, 2003). For example, the ability of caspases to cleave calpastatin an endogenous substrate for calpain helps in the activation of calpain (apart from other stimulus like elevated intracellular calcium levels) (Kato et al., 2000). Calpains were known to interact with lysosomes (storage houses of lysosomal proteases) and help in the spillage of cathepsins into the cytosol (apart from acidification of the cell which helps in the activation and spillage cathepsins) (Yamashima et al., 2003). Increased cathepsins levels and activity were shown to be involved in activation of calpains. Once activated calpains and or cathepsins can mediate either apoptotic or necrotic cell deaths and caspases can mediate apoptotic cell death depending on the intensity and nature of the insult.

More importantly, these proteases share similar substrates specificity (with varied intensities) cleaving several cellular substrates (Liu et al., 2006a). PARP-1 is another best known molecule which is cleaved by caspases, calpains, cathepsins and granzymes leading to the production of specific signature fragments like 89 and 21 kDa (caspase specific fragments), 72 and 64 kDa (granzyme-specific fragments), 50 (necrotic fragment produced by cathepsin-b), and 45 (calpain specific fragment) (Froelich et al., 1996; Gobeil et al., 2001; Wang, 2000b; Zhu et al., 2009). However, the conditions and the molecular mechanisms that were involved in a cell choosing the protease required for the specific execution of cell death form still remains to be understood. Hence, it becomes difficult to choose a beneficial therapeutic approach by inhibiting a single suicidal protease. Caspases, calpains and cathepsins are some of the crucial proteases in the cell death machinery. Though they are well known to interact and activate each other, their activation and localization profiles in a temporal, spatial specific manner are still lacking in cerebral malaria. Moreover, their role in mediating either apoptotic or necrotic cell deaths independent or dependent of each other makes it very difficult to understand their downstream signaling events that participate in executing specific cell death. A recent report that also describes the failure of decreasing CM pathology despite inhibiting caspase-3 and calpain-1 is a very good example of highly complex pathology of cerebral malaria (Helmers et al., 2008; Toure et al., 2008).

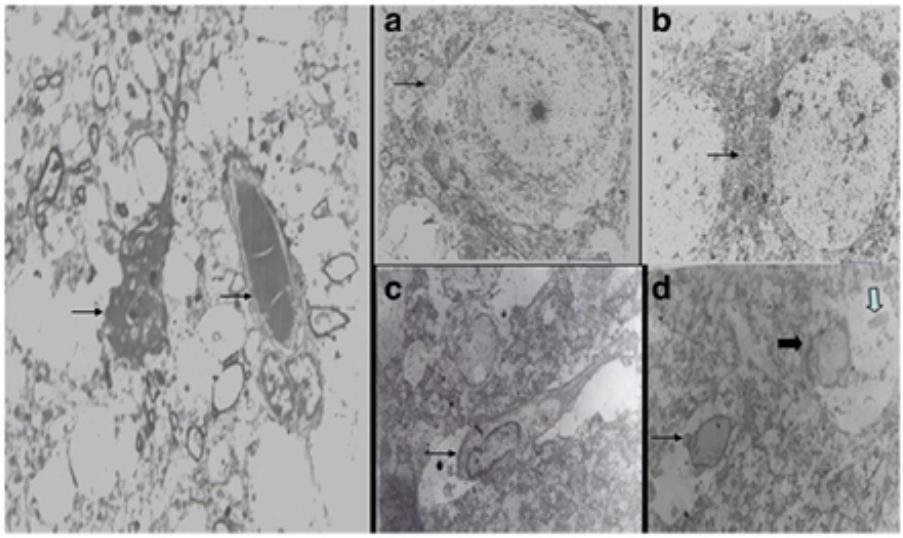


Fig. 5 – TEM photographs of the control brain cortical region showing the intact neuronal cell with intact neuropil and connections with parenchyma tissue. Adjacent right panel showing the oval shaped normal nucleus with intact chromatin. Intact nuclear membrane with normal nuclear pores and intact internuclear tight junctions are shown by arrows (panels a and b). c) Electron micrographs of a neuron that lost its contact with brain parenchyma and degenerating dendrites (arrow). Ultrastructural changes of the neuronal cell of cerebral malaria mice brain cortical region with crenellated neuronal nucleus and apoptotic blebbing formation (thin arrow), endoplasmic reticulum breakage (blue arrow), and swollen edematous nucleus and outernuclear membrane disintegration and vacuolation (thick arrow).We can also observe the internuclear tight junction disintegration in the infected samples(d), suggesting apo-necrotic continuum . Overall magnifications of electron micrographs are 3580×.

Previous reports from our lab showed increased levels of caspase-3, calpain-1 and cathepsin-b in cerebral malaria mice (Shukla et al., 2006) and in rat model of focal cerebral ischemia (Chaitanya and Babu, 2008). In general caspases mediate the cell death through apoptosis by cleaving the cytoskeletal proteins which leads to the shrinkage of cell and eventual death (Fan et al., 2005), whereas calpains execute the cells through necrosis and help in the spillage of cathepsins from lysosomes (storage houses of lysosomal proteases) into the cytosol (apart from acidification of the cell which helps in the activation and spillage cathepsins) (Yamashima et al., 2003) which in turn kills the cells that aggravate the effect. Moreover, the co-localization of these proteases indicates their cross talk during the pathology. The major two types of cell death mechanisms (apoptosis and necrosis) are clearly distinct by their anatomical changes and hence the mode of cell death. Our TEM study showed degenerating neurons exhibiting the features of both necrosis and apoptosis indicating an intermediate stage/type of cell death "apo-necrotic" continuum, which correlated with the elevated levels of these cell death proteases and their cross talk within the same cell undergoing death.

Cell death in fatal murine cerebral malaria is heterogeneous; in the present study we have observed cells undergoing apoptotic and necrotic cell deaths apart from previously published apo-necrotic continuum. Neuronal apoptosis has been shown to be a main cause for the pathology of cerebral malaria (Wiese et al., 2006). Here we show that neuronal cell death during cerebral malaria involves extensive cleavage of the neuronal cytoskeletal proteins by the cell death proteases belonging to apoptotic and necrotic cell deaths. Cytoskeleton, the filamentous protein and tubules present in the cytosol of the cell are important in maintaining the cell integrity, transport of materials, cell signaling and also for the cell survival (Dillon and Goda, 2005). Through immunofluorescence studies we found the co-localization of the active caspase-3, cathepsin-b and calpain-1 in a single cell undergoing cell death. This indicates the cross talks between these cell death proteases, their synergy in aggravating the cell death and contributing to increased neuronal deficits and or mortality. Our results show that the elevated levels of cell death proteases during cerebral malaria and their action on cytoskeletal proteins might result in increased neuronal death. The decreased intensity of PSD-95, a synaptic density protein, clearly indicates a severe compromise in the neuronal synaptic function which correlates with neuronal filament breakdown and observed neurological deficits in the infected mice over the controls. Moreover, we observed increased interactions of suicidal proteases involved in apoptotic and necrotic cell deaths which correlated with the ultra-structural features of cell undergoing apoptosis, necrosis and apo-necrotic forms of cell death. The inhibition of these proteases during the earlier stages of cerebral symptoms may seem to increase the longevity of the infected, which otherwise leads to sudden onset of un-arousable coma and death. Molecular mechanisms involved in the regulation of these proteases' activation, their involvement in execution of apoptotic or necrotic

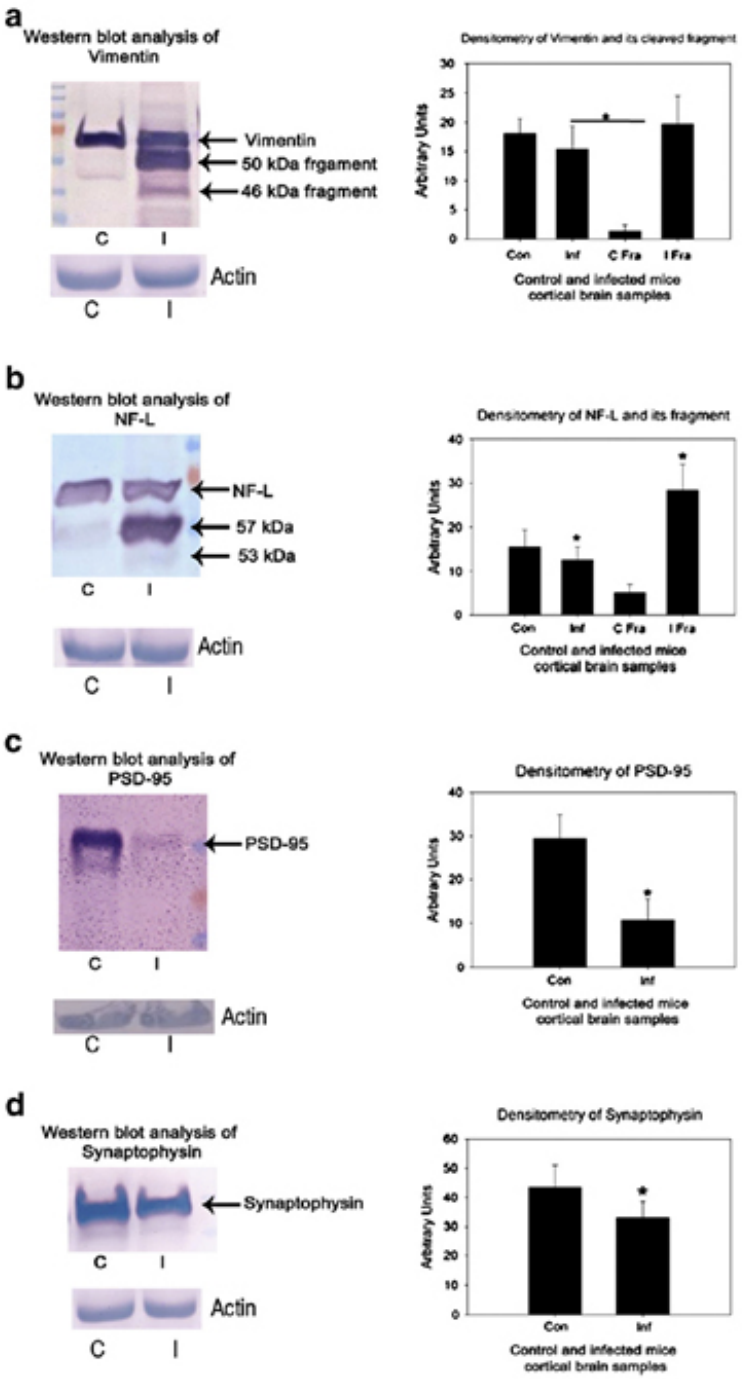


Fig. 6 – Western blots of cytoskeletal proteins. a) Western blot of vimentin. Significantly increased breakdown products of vimentin were observed in the cortical samples of infected mice brains over the controls. The identified breakdown products were having the molecular weight of 50 and 46 kDa. b) Western blot of NF-L. Increased breakdown of neuronal filament was observed in the infected mice brain over the control. The observed molecular weight of NF-L fragments was 57 and 53 (less distinct) kDa. c) Western blot of PSD-95. Significant decrease in the PSD-95 levels was observed in the infected mice brain samples over the controls in the cortical brain samples. Decrease in the PSD-95 levels correlated with the breakdown of NF-L in the infected mice brain samples. d) Western blot of synaptophysin. Decrease in the synaptophysin levels was observed in infected mice cortices over the control mice brain samples. $n=5$; each blot was repeated 3 times. $p<0.05$ was considered to be statistically significant. Bars represent standard error.

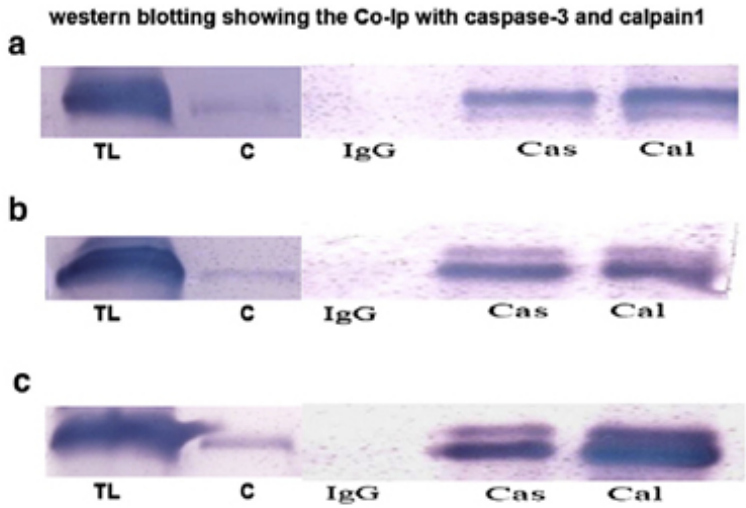


Fig. 7 – Co-immunoprecipitation of vimentin, PSD-95 and NF-L. a) Co-immunoprecipitation of vimentin with caspase-3 and calpain antibodies revealed a significant increase in its binding with the suicidal proteases in the infected mice brain samples correlating with its subsequent breakdown. b) Coimmunoprecipitation of PSD-95 revealed an increase in the binding of calpain and caspase with PSD-95 in the infected mice brain cortical samples correlating with its decreased presence in the Western blots. c) Co17 immunoprecipitation of NF-L indicated an increase in the binding of NF-L with caspase-3 and calpain in the infected mice brain samples. n=5; each blot was repeated 3 times. No binding was observed in the primary antibody omitted negative controls.

or apo-necrotic forms of cell death in a temporal and spatial manner during this pathology need to be addressed. Moreover, these need to be traced out to identify the role of infiltrating immune cells, resident immune cells, hypoxia and the toxins released by the sequestered parasites during the pathology and their link with the activation of suicidal signaling cascades in this pathology.

4. Experimental procedures

4.1. Mice

C57 BL/6J mice were purchased from the national institute of nutrition (NIN) Hyderabad and housed in University of Hyderabad animal housing facility under strict hygienic conditions according to the guidelines of the institutional animal ethical committee (IAEC) and committee for the purpose of control and supervision of experiments on animals (CPCSEA). *P. berghei* ANKA (pbA) parasites were obtained from parasite bank, National Institute of Malaria Research (NIMR), New Delhi, India.

4.2. *Induction of cerebral malaria*

Three to four weeks old C57BL/6J mice were intra-peritoneally injected with 10^6 pbA parasites and the control mice were injected with the same volume of PBS. Infection was monitored by staining blood smears collected via caudal puncture with giemsa and JSB-I/JSB-II. Disease progression was observed by recording the changes in animals' behavioral deficits after infection. ~Eighty five to ninety percent of infected mice showed signs of CM and died between 6 and 15 days of post-infection (PI). CM mice that were completely or partially paralyzed at 6–9 days of PI were sacrificed by an overdose of anesthesia and the brains were isolated. Control and infected mice brains were either 4% paraformaldehyde fixed for histochemical analysis or processed for immunoblot experiments. Mice that died after 9 days were not taken into study as we considered their death was due to severe anemia. The animal procedures employed in this study were approved by IAEC and CPCSEA rules and guidelines.

4.3. Antibodies

Caspase-3 antibody was purchased from cell signaling technology (cat. # 9662), calpain-1 and cathepsins-b were from Chemicon International (Termicula, USA), anti mouse vimentin, a kind gift from Geeta Vemuganti, LVPEI, India. PSD-95 was a gift from Prof. Emmanuel Brouille, synaptophysin was purchased from Santa Cruz (cat. # sc-6926; Santa Cruz Biotechnology, Santa Cruz CA), and secondary antibodies FITC and TRITC conjugated anti mouse and anti rabbit respectively were purchased from Bangalore Genei, India. CY3 was a kind gift from Dr. Siva Kumar, Department of Biochemistry, University of Hyderabad, India.

4.4. Western blots

For Western blot analysis, control and infected mice were killed with an anesthetic overdose. Brains were dissected out (n=5) and immediately snap frozen in liquid nitrogen and were homogenized in the modified radio immunoprecipitation assay buffer containing protease inhibitor 1 mM PMSF and phosphatase inhibitors 10 mM β-glycerophosphate, 10 mM NaF and

0.3 mM Na_3VO_4. The lysates were sonicated and centrifuged, and 50 μg of supernatant was separated on SDS-PAGE, transferred onto nitrocellulose membrane and immunoblotted caspase-3, calpain-1, cathepsin-b, vimentin, PSD-95, NF-L and synaptophysin.

4.5. Co-immunoprecipitation

Control and infected mice brain samples were homogenized in the Nonidet P-40 (NP-40) lysis buffer (50 mM Tris, pH 8.0; 150 mM Nacl; 1% NP-40; complete protease inhibitor). The homogenized samples were briefly sonicated and centrifuged for 10 min at 15,000g at 4 °C. Supernatant was used for the co-immunoprecipitation experiments. Two hundred fifty micrograms of protein from control and infected mice brain lysates (after pre-clearing, with 20 μl of protein A-sepharose for 30 min at room temperature (RT))were used for co-immunoprecipitation experiments. The samples were incubated with calpain or caspase-3 antibody at 4 °C over night followed by washes and incubation with 25 μl of protein-A-sepharose beads for 90 min at RT. Sepharose beads were centrifuged, and boiled in 2X sample buffer. Later protein extracted into the sample buffer was resolved on SDS-PAGE and blotted with antibodies against vimentin (1:1000), PSD-95 (1:1000) and NF-L (1:1000).

4.6. Hematoxylin & eosin and cresyl violet staining

Three micrometers of thick brain tissue sections were processed in xylene and alcohol series and stained with hematoxylin and eosin. The sections were air dried, mounted in DPX and imaged under Olympus microscope. For cresyl violet staining tissue sections were processed as mentioned above and stained with 0.1% cresyl violet for 5 min, alcohol cleared, mounted with DPX and imaged under Olympus microscope.

4.7. Transmission electron microscopy

Cerebral cortices from infected mice brains were transferred into 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post fixed in 2% osmium tetroxide for 2 h. Later they were dehydrated in graded alcohol series, embedded in epoxy resin and 0.5 μm thick sections were obtained using ultra microtome. These sections were toluidine blue stained and examined under light microscope for overall view. Later ultra thin sections were placed on nickel grid and observed under transmission electron microscope.

4.8. Immunohistochemical analysis

For immunohistochemical analysis, 3 μm thick paraffin sections from control and infected mice brains were processed and antigen retrieved in citrate buffer (pH 6.0) for 10 min. The sections were washed (3 times—PBS) and permeabilized in 0.1% Triton-X 100 for 15 min. After permeabilization, sections were washed (3 times—PBS) and endogenous peroxidase was inhibited by incubating sections in 3% H_2O_2. Sections were washed (3 times–PBS) and blocked using 10% normal goat serum for 60 min at RT. The sections were later incubated in primary antibodies against caspase-3 (1:200), calpain-1 (1:200) and cathepsin-b (1:200) overnight at 4 °C, followed by washes in PBS and incubation in the respective biotinylated secondary antibody for 90 min at RT. After secondary antibody incubation, sections were washed (3 times—PBS) and developed using VECTASTAIN Elite ABC kit (Vector Laboratories) with di-amino benzidine (DAB) as substrate. Immunostained sections were imaged using Olympus microscope.

4.9. Triple immunofluorescence protocol

For triple immunofluorescence, sections were processed as mentioned previously. Briefly, control and infected mice brain sections were blocked in 10% goat serum for 60 min at RT and incubated in a cocktail of anti-mouse calpain and anti-rabbit cathepsin-b at 4 °C for overnight. Later sections were washed and incubated in a cocktail of FITC and CY-3 conjugated secondary antibodies for 90 min at RT. Later sections were washed and blocked for another 30 min with 5% goat serum at RT. After blocking, sections were incubated with anti-rabbit active caspase-3 for overnight at 4 °C. Sections were washed and incubated in CY-5 conjugated secondary antibody. After secondary antibody incubation, sections were washed and mounted with 90% glycerol and visualized under Leica confocal microscope.

4.10. Statistics

Western blots were scanned and densitometry was performed using NIH image J software. The values obtained from densitometry were used to check the statistical significance. Unpaired two-tailed *p*-values less than 0.05 (comparison between 2 groups) were considered statistically significant. The values were plotted on sigma plot to obtain bar graphs with standard error.

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