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Coenzyme Q_{10} protect mice against inflammatory responses during Experimental cerebral malaria

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Abstract

Malaria is a life threatening infectious diseases transmitted by the bite of infected female Anopheles mosquito and responsible for high morbidity and mortality rates. Cerebral malaria is a complex neurological syndrome, whose pathology is mediated by inflammatory processes triggered by the immune system of the host following infection with *Plasmodium falciparum.* Coenzyme Q10 is an obligatory cofactor in the electron transport chain. The reduced form of Coenzyme Q10 serves as a potent antioxidant additionally; Coenzyme Q10 has been identified as a modulator of gene expression, inflammation and apoptosis. However, the modulatory effects of Coenzyme Q10 *Plasmodium berghei* ANKA infection process and risk occurrence of experimental cerebral malaria (ECM) have not been determined. The aim of this study was to elucidate the putative impact of oral administration of Coenzyme-Q10 on the initiation or regulation of inflammatory immune response in ECM of C57BL/6 mice during *Plasmodium berghei* ANKA (PbA) infection. We observed that oral administration of Coenzyme-Q10 both before and after PbA infection significantly hampered infiltration of inflammatory monocytes into the brain. Furthermore, pro-inflammatory cytokine TNF-α, which is associated with inflammation during ECM, was down-regulated in Coenzyme-Q10 administered mice. Remarkably, Coenzyme-Q10 was very effective in inhibiting dendritic cell differentiation. These data collectively demonstrated the immuno-modulatory function of Coenzyme-Q10 on host inflammatory responses during ECM.

Key words: *Plasmodium berghei* ANKA, Coenzyme Q10, experimental cerebral malaria

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1. Introduction

Malaria is considered a major health burden which continues to claim many lives globally; in 2017 alone there were estimated 219 million cases and 435,000 related deaths, majority of these cases occurred in sub-Saharan Africa (WHO, 2018). Malaria is transmitted by the bites of female anopheles mosquitoes; the major pathogens that are known to cause human disease include *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi* and *P. falciparum*.

The chief malaria complication is cerebral malaria which is caused by *P. falciparum;* and is a severe clinical neurological syndrome that affects pregnant women and children under the age of 5 years (WHO, 2016). Cerebral malaria (CM) is known to cause immune-mediated pathology due to sequestration of parasitized red blood cells in the micro-vasculature and overproduction of inflammatory mediators (Van der Heyde, 2006). However, despite treatment with current adjunct antimalarial drugs being in place, it is estimated that 15-25% children die of the disease (WHO, 2014). Moreover, it has been shown that children who survive from CM develop neurological sequelae, blindness and cognitive impairment (Mishra and Newton, 2009). This presents a big challenge to the management of CM. Despite the importance of the effects of the disease on the CNS, there are no projective diagnostics and treatments after onset of the disease. Furthermore, there is paucity of data on molecular events that contribute to the pathophysiology of the disease.

The use of C57BL/6 mice as a mouse model of experimental cerebral malaria (ECM) is well documented; indeed this mouse model mimics many processes that occur in human cerebral malaria (White *et al.,* 2010; de Souza *et al.,* 2010). Notably, induction of pathology during ECM is linked to pro-inflammatory mediators (Scholfield and Grau, 2005). Specifically, dys-regulation of pro-inflammatory cytokine generation leads to tissue activation and generation of reactive oxygen species, with concomitant breakdown of the blood brain barrier that exacerbates the immune-pathological consequences (Angulo and Fresno, 2002). Nevertheless, a number of factors have complicated the understanding of pathophysiological events that leads to CM, this

includes rapid progression of the diseases in the presence of anti-malaria and relatively few cases of CM are reported. More importantly, CM due to *P. falciparum* infection can be prevented and treated.

Coenzyme-Q10 is an important cofactor in the electron transport and uncoupling proteins (Echtay *et al.,* 2000). Moreover, Coenzyme-Q10 is a powerful antioxidant in both the mitochondria and lipid membranes. The potential impact of Coenzyme-Q10 on gene expression has been identified recently (Grneberg *et al.,* 2005; Sabri *et al.,* 2003; Schmelzer *et al.,* 2009; Lee *et al.,* 2004; Sohet *et al.,* 2009).

Accumulating evidence has shown that Coenzyme-Q10, attenuates neuro-inflammation in neurodegenerative diseases toxicity linked to drugs and immune cells (Nyariki *et al.,* 2018; McCarthy *et al.,* 2004; Winkler *et al.,* 2004). However, studies replicating effects of Coenzyme-Q10 on gene expression during experimental cerebral malaria are lacking. Thus, Coenzyme-Q10 appears to be a potent antioxidant to investigate as a potential alternative CM therapeutics on the pathologies that contribute to death in both ECM and CM.

The anti-inflammatory functions of Coenzyme-Q10 especially its inhibitory effect on Nuclear factor kappa B and pro-inflammatory cytokines TNF-α and other mediator have prompted us to examine the role of Coenzyme-Q10 in experimental CM (ECM). In this paper, we investigated the putative impact of Coenzyme-Q10 on ECM and showed that oral administration with Coenzyme-Q10 protected mice from ECM. Oral administration of Coenzyme-Q10 before and after PbA infection rescued mice from the occurrence of ECM. We demonstrate that the protective effect of Coenzyme-Q10 was through the inhibition of host associated pro-inflammatory (IFN-g and TNF-α) and inflammatory monocytes coupled with reduction of dendritic cell maturation and differentiation.

2. Materials and Methods

2.1 Ethics statement and Mice

This study utilized 3-4 weeks old C57BL/6J mice that were purchased from International Livestock Research Institute (ILRI). Approval of all experimental procedures and protocols involving mice were obtained from local regulatory agencies. All experimental procedures and protocols involving mice were reviewed and approved by Institutional review Committee (IRC) of Institute of Primate Research Karen, Kenya. The mice were maintained on mice pellets and water *ad libitum* at room temperature. Wood-chippings were provided as bedding material.

2.2 Experimental design

The mice were randomly divided into three groups each consisting of 5-6 mice. Group one was wild type (WT) naïve control, experimental group two: (WT + Coenzyme-Ql0 + PbA) and experimental group three: (Wild type + PbA alone).

2.3 *Plasmodium berghei ANKA* infection studies

All experiments in this study made use of *Plasmodium berghei* ANKA (Lundie *et al.,* 2008). All experimental mice in group two and three were infected by intravenous (i.v) infection with $5x10^4$ infected red blood cell (iRBC) that was obtained from a donor mice (stock mice) infected with stock solution of PbA, which was stored in liquid nitrogen as iRBC in solution containing 10% glycerol. Parasitaemia in infected mice was monitored daily with 5% Giemsa stained blood smears.

2.3 Treatment of mice with 200mg/kg CoenzymeQ10

Oral administration of 200mg/kg of Coenzyme-Q10 was done daily for one month prior to PbA infection to the experimental group two of mice. Administration of Coenzyme-Q10 was done using a gavage needle. Coenzyme-Q10 powder was prepared by directly dissolving it in olive oil. Coenzyme-Q10 was prepared immediately before use and the solution was protected from light before administration to the animals.

2.4 Parasitaemia determination

From day 2 post infection blood was collected daily from the tail vein and blood smears made. The blood slides were air dried and fixed in absolute methanol. Giemsa staining was made by mixing 1:20 Giemsa stock (Merck kGaA, Darmastadt Germany) and Giemsa buffer pH 7.2. Parasitemia levels were assessed by counting the number of visible parasites in 10 microscopic light fields and the percentage of iRBCs calculated (iRBCs/counted RBCs)* 100.

2.5 Determination of Packed cell volume, body weight and haematological values

Blood was either collected direct from the heart (during euthinization) for full haemogram analysis or from tail for packed cell volume (PCV) determination. Briefly blood was collected from the tail to heparinized capillary tubes which were then sealed with plasticin at one end. The sealed capillaries were centrifuged in a haematocrit centrifuge (Hawksley H England) at 10,000 RPM for five minutes. PCV was read using the micro-haematocrit reader and expressed as percentage of the total blood volume. Full haemogram was analysed using automated Bechman Coulter counter. Body weight of each mouse was determined every two days using the analytical electronic balance (Mettler PM34, DoltaRange®).

2.6 Quantification of cytokine production by splenocytes

Mice were sacrificed on day 6 of ECM; the spleen was harvested followed by preparing the splenocyte culture according to Su *et al.,* (2002). Briefly, the spleen organs from all the experimental treatment groups were harvested aseptically after which they were pressed through a sterile fine-wire mesh with RPMI 1640 medium together with 10% FCS. The splenocytes were then centrifuged at 350 X g for 10 min to make single cell suspensions. Erythrocytes were lysed with cold ammonium-chloride-Tris-buffer (ACT buffer) followed by washing the cells twice with fresh medium. Trypan blue was used to determine the splenocyte viability. Splenocyte were then adjusted to a final concentration of 10 million cells/ml in RPMI 1640 medium mixed with 10% FCS buffer; aliquots of 200 µl of these cells were plated and incubated in 24-well flat-bottom tissue culture plates (Falcon) in triplicate for 24 h at 37°C in a humidified 5% CO2 incubator. Cytokines (IFN-g and TNF) in the culture supernatant and plasma samples were measured by ELISAs.

2.7 Flow cytometry

Mice were sacrificed at day 6 post infections, for flow cytometric measurement of inflammatory monocytes (Ly6C) and dendritic cells (DCs), $1.0x10^5$ cells were re-suspended in 100 µl of FACS buffer and blocked with 2µl of human Fc-block (Sigma Aldrich). To the 96 well plate about $50µl/5x10^4$ cells of the indicated master mix antibodies or appropriate isotype controls were added, gently mixed by vortexing and incubated on ice for 20 min. Additionally, single stained samples were prepared. For this purpose, a small part of each sample was taken and a cell master mix for the brain, liver and spleen cells was made in order to stain it with only one antibody. Thus a single stained sample for each antibody was created. The resulting cells were washed at least twice with FACS buffer by centrifugation followed by discarding the resulting supernatant, approximately 200 µl MACS buffer was added to the cells and then filtered using clean gauze in FACS tubes in order to remove any debris present. The cells were acquired and analyzed using the FACS LSR Fortessa of BD Biosciences. Then the data was analyzed with FACS Diva®/FlowJo software (Becton Dickinson).

2.8 Statistical analysis

One way ANOVA to compare treatment groups with controls Bonferronis post hoc tests was done for internal comparison. T-test was used to compare the percentage of Parasitemia levels. Results were given as mean± with significance set as $p<0.05$. The statistical analyses was done using Graphpad prism version 5 software package.

3.0 Results

3.1 Coenzyme-Q10 administration has no effect on parasitaemia and general body weight during ECM

The effect of Coenzyme-Q10 depends on the dosage and duration of administration. It was observed that blood parasitemia levels were not significantly changed in mice supplemented with Coenzyme-Q10 compared with WT PbA-infected mice (Fig. 1A). This is consistent with other studies that demonstrated no correlation between blood parasitemia levels and survival from ECM (Dende *et al.,* 2015; Gordon *et al.,* 2015). The group of mice receiving Coenzyme-Q10 presented similar change in weight gain as WT infected mice (Fig. 1B).

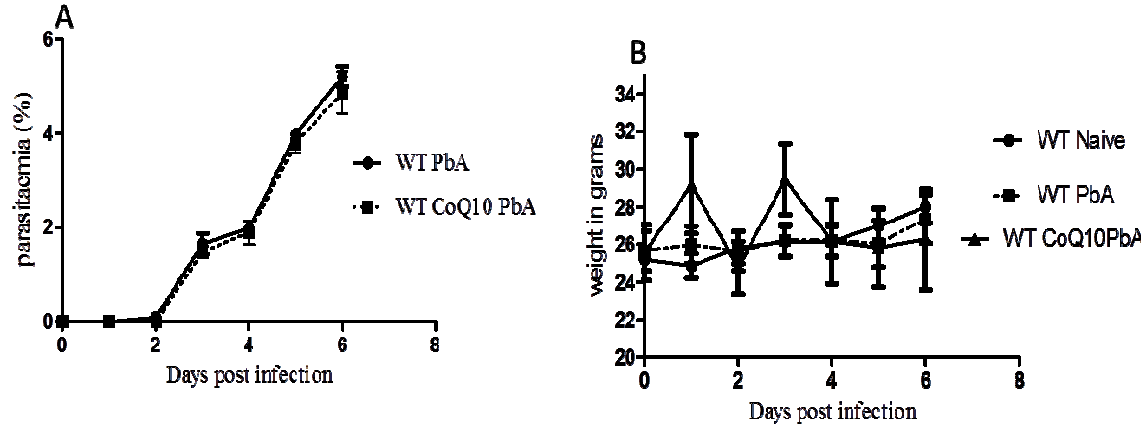

Figure 1: No significant difference in parasitaemia percentage and weight among the treatment groups. (**A**) Parasitemia curve of WT-CoQ_{10}-PbA and WT-PbA mice infected with *P. berghei* ANKA. (**B**) Weight curve of WT naïve, WT PbA and WTCoQ10-PbA mice. N=5-6 mice per group.

3.2 Coenzyme-Q10 supplemented mice have markedly reduced splenic myeloid and plasmocytoid conventional dendritic cells.

Dendritic cells (DCs) play a decisive role that contributes to the events that shapes both the innate immunity and adoptive immune system. After they encounter the pathogen, DCs are activated to initiate and shape the immune responses. DCs are classified as either plasmocytoid dendritic cells (pDCs) or myeloid dendritic cells (mDCs). During infection with *Plasmodium berghei*, cDCs have been shown to induce $CD4^+$ T cell activation. This study by deWalick ruled out the role of pDCs in pathogenesis of ECM. Previous study has also further showed that $CD8\alpha^+$DCs are the major APCs to $CD8^+$T cells leading to priming and proliferation (Lundie, 2011). Therefore, the current study investigated whether supplementation of Coenzyme-Q10 influenced the DC phenotype during PbA infection. Splenic DCs were characterized first as $CD11c^+MHCII^+CD3^-CD19^-$ and then further into $CD8\alpha^+$ and $CD8\alpha^{neg}$ DCs. A decrease in the frequency and total count of mDCs in spleens of Coenzyme-Q10 supplemented PbA-infected mice was observed (Fig. 2A-B). Similarly, the frequency and percentage of pDCs in Coenzyme-Q10 supplemented mice was markedly reduced. (Fig. 2C-D).These data shows that Coenzyme-Q10 inhibits differentiation of splenic DCs during ECM.

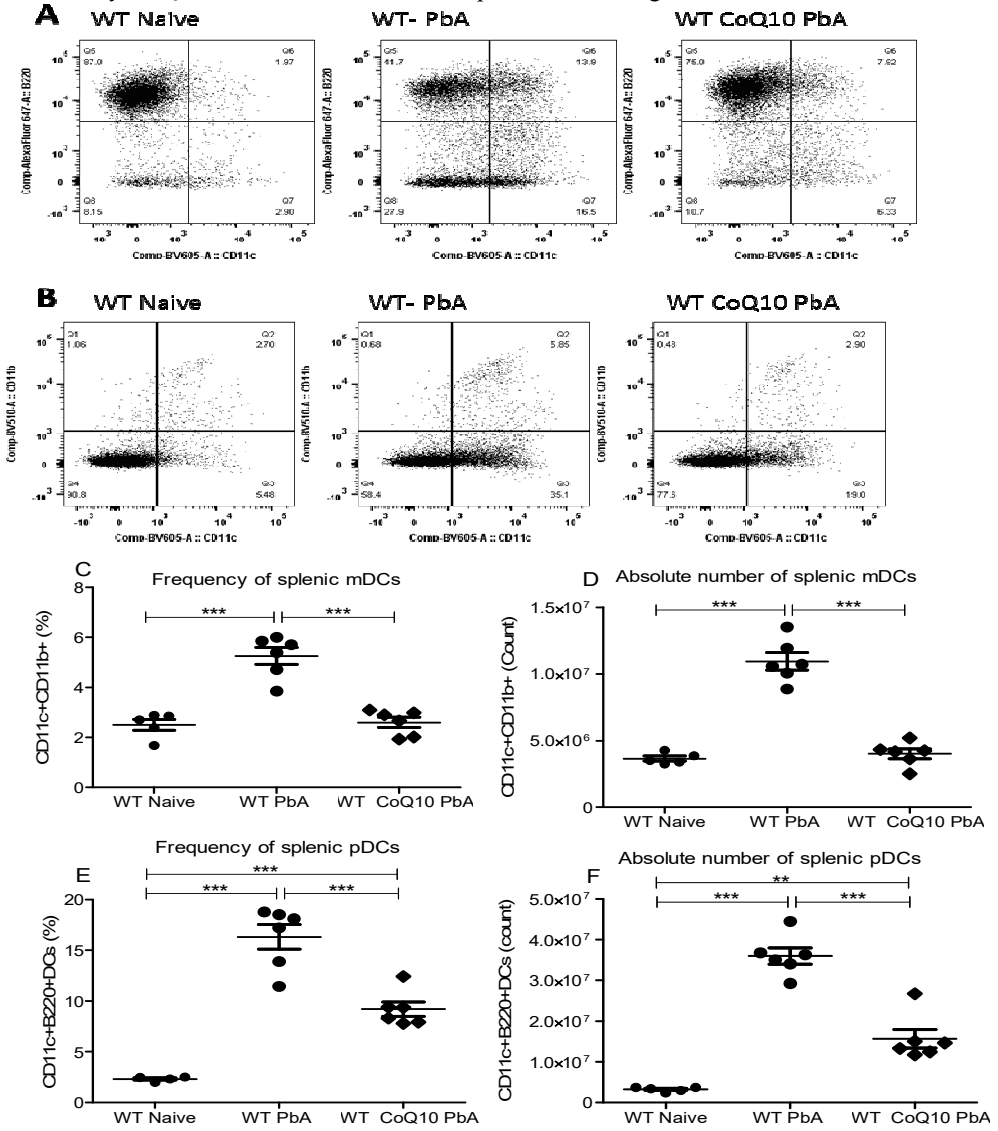

Figure 2: Coenzyme-Q10 supplementation inhibited the expansion of splenic DC subsets
The frequency of spleen CD11c+CD11b+ DCs (mDCs), and CD11c+B220+ DCs (pDCs) were analyzed by flow cytometry. Representative dot plots showing the proportions of brain mDCs and pDCs populations respectively (**A- B**). Bar graphs show the frequencies and absolute numbers of mDCs (**C-D**) and the frequencies and absolute numbers of pDCs populations within the brain. Data are presented as the mean ± SEM (n = 5- 6mice/group).

Results are representatives of three independent experiments. Percentage and total cell count of mDCs and pDCs values were compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: **P ≤ 0.01; ***P ≤0.001). n=5-6 mice per group.

3.3 The inflammatory monocyte levels are reduced in the brain of PbA-infected Coenzyme-Q10 supplemented mice

Infiltrated monocytes have been shown to play an important role in ECM pathogenesis settings (Schumak *et al.,* 2015). Therefore, experiments were conducted to establish the functional significance of oral supplementation of Coenzyme-Q10 on the infiltration of inflammatory monocytes and neutrophils into the brain. Experimental mice in all groups were sacrificed at day 6 p.i. and brain cells associated lymphocytes were isolated. The total number of inflammatory monocytes and neutrophil cells present in the brains of study animals were then quantified flow cytometrically, followed by calculating the percentage and absolute numbers of inflammatory monocytes. Infiltrated inflammatory monocytes and neutrophils were characterized as $Ly6C^{hi}Ly6G^{neg}CD11b^{hi}CD45^{hi}$ and $Ly6G^{hi}Ly6C^{int}CD11b^{hi}CD45^{hi}$ respectively (Fig. 3A). The percentage of brain inflammatory monocytes was significantly reduced in Coenzyme-Q10 supplemented mice in comparison with wild type PbA-infected mice (Fig. 3B). However, the frequency of neutrophil cells present in the brains of Coenzyme-Q10 supplemented and Wild type PbA-infected was comparable (Fig. 3C). Further investigations were done on the phenotype of the infiltrated inflammatory monocytes by looking at the expression of chemokine CCR-2 and adhesion molecule ICAM-1 on their surface. CCR-2 is an inflammatory chemokine with an important role in the recruitment of monocytes to the site of inflammation during infection (Schumak *et al.,* 2015). In comparison to the WT, the expression of CCR2 was decreased among the Coenzyme-Q10 administered group (Fig. 3D). Similarly, an up-regulation of ICAM1 on inflammatory monocytes WT PbA, in comparison to Coenzyme-Q10 group was observed (Fig. 3E).

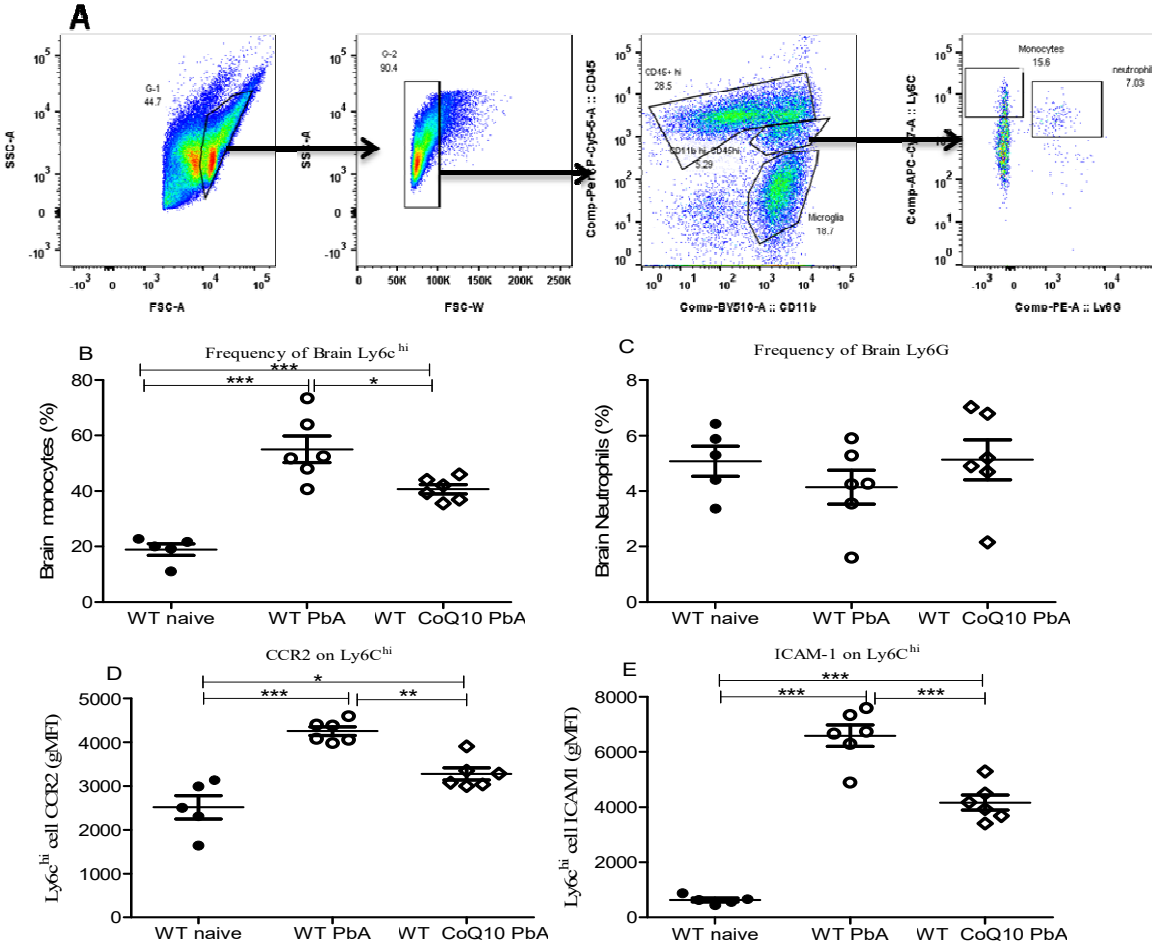

Figure 3: Low amount of inflammatory monocytes in Coenzyme-Q10 supplemented mice
C57BL/6J mice were either un-supplemented or supplemented with Coenzyme-Q10 and then infected with

$5x10^4$ PbA iRBC. At day 6 of ECM, cellular infiltrates from the brains of individual mice were prepared and analysed by FACS. Flow cytometry gating scheme to identify inflammatory monocytes and neutrophil cells is shown for representative animals from WT infected (**A**) gMFI of (**B**) infiltrating monocytes ($Ly6C^{hi}Ly6G^{neg}CD11b^{hi}CD45^{hi}$+); of (**C**) Neutrophils ($Ly6G^{hi}Ly6C^{int}CD11b^{hi}CD45^{hi}$); gMFI of (**D**) CCR2 on monocytes and gMFI of (E) ICAM1 on monocytes. Bars shows mean of each group ± SEM and are representative of at least two independent experiments. Percentage and gMFI of neutrophils, monocytes and its phenotypes was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: *P ≤ 0.05; **P ≤0.01;***P ≤0.001). n=5-6 mice per group.

3.4 Coenzyme-Q10 supplementation abrogates pro-inflammatory cytokine profile at protein level

The genesis of pathology during ECM is linked to immune-pathology component; and disruption of tight regulation of pro-inflammatory and anti-inflammatory cytokines are indicators of cerebral malaria progression (Angulo and Fresno, 2002). Therefore, an assessement was done to determine whether infected WT and Coenzyme-Q10 supplemented mice had differences in the concentration of pro-inflammatory at protein level. Mice that suffer from ECM symptoms have been shown to produce excessive amounts of pro-inflammatory cytokines, and Coenzyme-Q10 supplemented mice were found to have markedly reduced serum levels of some of the pro-inflammatory cytokines (Fig. 4A–B). It was therefore, remarkable that Coenzyme-Q10 supplementation provided some amelioration of inflammation in these mice.

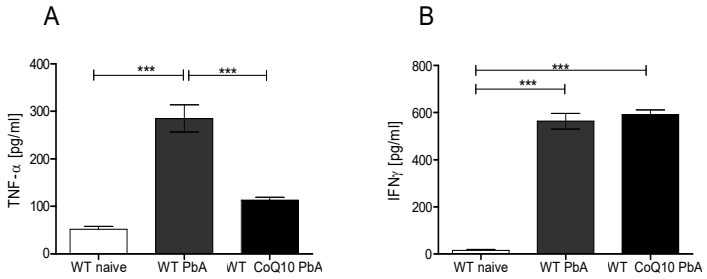

Figure 4: Coenzyme-Q10 protected mice have modulated levels of splenocyte pro-inflammatory cytokines
Indicated groups of mice were infected i.v. with $5x10e^4$ iRBC of PbA. Six days later, animals were sacrificed and the Spleens were isolated to prepare single cell suspensions. 1x10e6 splenocytes were cultured in vitro for 24hr. Splenocytes were stimulated for 4hrs with PMA/Ionomycin in between. Splenocyte concentrations of pro-inflammatory cytokines (**A**-**B**) levels in the supernatant were analysed in the control groups and in PbA iRBC-infected wt and Coenzyme-Q10 PbA-infected mice at d+6 p.i. Cytokine concentrations were determined by sandwich ELISA in the supernatants. Statistics= One Away ANOVA with Bonferroni posttest (indicated level of significance: *P ≤ 0.05; **P ≤0.01;***P ≤0.001). n=5-6 mice per group.

4. Discussion

We report for the first time that oral administration of Coenzyme-Q10 plays a key immune-modulatory role during ECM. Coenzyme-Q10 is a vital anti-oxidant with clinical application especially in the treatment of neurodegenerative and cardiovascular disorders (Spindler *et al.,* 2009; McCarthy *et al.,* 2004; Winkler *et al.,* 2004). Furthermore, previous studies have shown that Coenzyme-Q10 has the capacity to stimulate phagocytic activities and immune responses in rats and mice (Bliznakov *et al.,* 1972). Thus, we were interested in elucidating the immune-modulatory role during ECM.

The Coenzyme-Q10 administered mice did not display any clinical symptoms associated with cerebral malaria, such as limb paralysis coma and convulsion. However, blood parasitemia levels were similar in mice supplemented with or without Coenzyme-Q10. This observation has been observed previously whereby there was no correlation between blood parasitemia levels and survival from ECM (Dende *et al.,* 2015; Gordon *et al.,* 2015). Likewise, the group of mice receiving Coenzyme-Q10 presented similar change in weight gain as WT infected mice.

The indispensable role of infiltrated inflammatory monocytes in the development of immunopathology in ECM has been described (Schumak *et al.,* 2015). Others have shown that inflammatory monocytes are critical in the control of blood-stage malaria due to *P. chabaudi* (Sponaas *et al.,* 2009). This control of blood stage infection was reported to be mediated through the generation of ROS and iNOS by these monocytes (Sponaas *et al.,* 2009). In this study, a reduction in brain inflammatory monocytes ($Ly6C^{hi}Ly6G^{neg}CD11b^{hi}CD45^{hi}$) in Coenzyme-Q10 administered mice when compared to controls was observed. Findings from this study provide

compelling evidence that the direct reduction of infiltrated inflammatory monocytes into the brain may be responsible for the observed protection against ECM.

Previously, it has been demonstrated that Ly6C^{hi}CCR2^{pos} inflammatory monocytes are critically involved in the initial stage of Plasmodium blood-stage infection (Schumak *et al.,* 2015). Interestingly, the expression of CCR2 and ICAM-1 on monocytes was decreased in the Coenzyme-Q10 administered group. It is therefore possible that low levels of inflammatory monocytes and there phenotypes in Coenzyme-Q10 administered mice contribute to reduction in brain inflammation, aiding in resistance to ECM pathology witnessed in this study. Nevertheless, inflammatory monocytes are co-producers of TNF-α in animal model of intracerebral hemorrhage (Hammond *et al.,* 2014) and contact dermatitis (Chang and Nakrani*,* 2014); and are responsible for the recruitment of CD8+ T cells into the brain (Schumak *et al.,* 2015).

Dendritic cells (DCs) are classified as either plasmocytoid dendritic cells (pDCs) or myeloid dendritic cells (mDCs). During infection with *Plasmodium berghei*, cDCs have been shown to induce CD4$^+$ T cell activation, whereas pDCs in the pathogenesis of the ECM has been ruled out (He *et al.,* 2014). Furthermore CD8α$^+$DCs are the major antigen presenting cells to CD8$^+$T cells leading to priming and proliferation (Lundie, 2011). DCs are professional APCs that can also cross present antigens that can aggravate the development of ECM. We sought to determine whether supplementation of Coenzyme-Q10 could influence the DC phenotype during PbA infection. Importantly, there was a marked reduction in the accumulation of both mDCs and pDCs in the spleens of the Coenzyme-Q10 administered mice. These data shows that Coenzyme-Q10 either directly or indirectly interferes with differentiation of splenic DCs during ECM. These findings corroborate early observations showing that treatment of PbA-infected mice with vitamin D inhibits splenic DCs differentiation (He *et al.,* 2014).

In a systematic review and meta-analysis, it was putatively demonstrated that exogenous administration of Coenzyme-Q10 significantly decreased genes for inflammation especially TNF-α levels (Zhai *et al.,* 2017). A previous study working with Coenzyme-Q10 reported down-regulation of inflammatory markers associated with multiple sclerosis (Sanoobar *et al.,* 2015). Additionally, in vitro studies demonstrated that Coenzyme-Q10 is a modulator of inflammatory gene expression (Schmelzer *et al.,* 2007). Therefore, in view of the prominent inflammatory responses and antioxidant system failure due to plasmodium infections, employing compounds known to possess potent antioxidant and anti-inflammatory properties in ameliorating severity of the disease seemed logical. In the current investigation, augmentation of brain TNF-α and IFN-g inflammatory genes in PbA-infected mice was not surprising. Interestingly, Coenzyme-Q10 administered PbA-infected mice attenuated these inflammatory genes indicative of less inflammation in the brain of these mice. This was remarkable and first demonstration that Coenzyme-Q10 has the capacity to modulate inflammatory mediators associate with ECM protein level.

Overall, the present study clearly demonstrated that diminished levels of inflammatory monocytes in the brains of Coenzyme-Q10 administered mice could also be an important factor for protection against inflammation. Moreover, Coenzyme-Q10 has the capacity inhibit dendritic cell differentiation and pro-inflammatory cytokines TNF-α, thus protecting these mice against immune-induced inflammation.

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