Available online at www.asric.org
ASRIC Journal on Health Sciences 1 (2022) 7-14

Ubidercarenone Decelerates the Manifestation of Experimental Cerebral Malaria in C57BL/6J Mice by ameliorating the Host Inflammatory Response

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Received 1 July 2022; revised 22 July 2022; accepted 13 August 2022

Abstract

Cerebral Malaria (CM), a highly lethal form of severe malaria is known to cause inflammation that exacerbates pathology in the brain predominantly due to inflammatory immune response as a result of *Plasmodium falciparum* infection. There is limited progress in the development of new approaches for the treatment of CM. Therefore, in this study we investigated whether oral Ubidercarenone (Ubn) can regulate the induction of inflammatory immune response in experimental cerebral malaria (ECM). Three groups of mice were utilized for this study, one group of C57BL/6J mice was used as control; group two was only infected with *Plasmodium berghei ANKA* (PbA) and group three orally supplemented with 200mg/kg ubn followed by infection with PbA. Here we show that Ubn was able to protect majority of mice against ECM. Importantly, Ubn supplementation significantly hampered infiltration of inflammatory monocytes, T cells and cytotoxic granzyme B into the brain. Serum analysis showed a reduction in the levels of TNF-α in Ubn administered mice. Furthermore, Ubn administration resulted in decreased expression of chemokine CCR2 in the brain, leading to reduced levels of activated pathogenic T cells. Notably, anti-inflammatory cytokines IL-10 and IL-22 together with T-regulatory cells, which are associated with protection during ECM, were up-regulated in Ubn treated mice. These results demonstrate that Ubn can assuage PbA-mediated inflammatory responses usually witnesses during ECM.

Keywords: Ubidercarenone; Experimental Cerebral Malaria; Inflammatory Response

1.0 INTRODUCTION

Cerebral malaria (CM), is considered to be a highly fatal neurological syndrome caused by *Plasmodium falciparum* and is characterized by delirium, body ache, fever, coma and ultimately impaired consciousness [1]. Additionally, the pathology that is usually witnessed during CM is mediated by inflammatory processes following *Plasmodium falciparum* infection [2]. Animal models of CM show sequestration of the parasite in microvasculature and the adherence of parasitized erythrocytes in the endothelial lining of the brain [3]. Furthermore, pro-inflammatory mediators which include; infiltrated leukocytes, pro-inflammatory cytokines and chemokines play a major role in the pathogenesis of CM.

Moreover, considerable evidence suggests that overwhelming induction of inflammatory processes is an important trigger mechanism that aids in malaria parasite clearance, but can also play a key role in the development of ECM [4]. Additionally, the situation is further worsened when the generation of pro-inflammatory cytokines overwhelms anti-inflammatory processes during plasmodium infection. Also the indispensable role of infiltrated inflammatory monocytes in the development of immunopathology in ECM has been described [5]. Both innate and adaptive immune systems play an important role during malaria

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infection [6]. Chemokines are important chemo-attractant cytokines that are known to share high degree of sequence homology hence they play a vital role in bridging the innate and the adaptive immune system [7, 8].

Because of the clinical importance of ECM complication, this study was set out to understand whether Ubidercarenone (ubn) can modulate some of the immunological factors that contribute to ECM.

Ubidercarenone is an important bioenergetic factor in the electron transport chain and also a potent antioxidant that has been applied in the treatment of neurodegenerative diseases [9]. More importantly, the reduced form of Ubn when cultured with human fibroblast has been shown to down-regulate the levels of IL-1β and LPS induced TNF-α production from macrophage cells [10]. Likewise, in another study by Schmelzer *et al*. [11] clearly showed that the reduced form of Ubn is a potent anti-inflammatory that acts by decreasing the levels of pro-inflammatory cytokine TNF-α and RANTES and MIP1-α chemokine in human monocyte cell line under LPS challenge [12]. This proven role of Ubn in assuaging inflammation was critical in its application in the current study involving an ECM in C57BL/6J mouse model. The aim of this study was to systematically elucidate the putative impact of ubn on the initiation or regulation of inflammatory immune response in experimental cerebral malaria (ECM). Herein, this study demonstrate that Ubn can protect mice infected with PbA against inflammatory responses that are linked to aggravation of the pathophysiology observed during ECM.

2.1 MATERIALS AND METHODS

2.2 Ethics statement and Mice

Ethical approval for this study was obtained from the Institute of Primate Research, Kenya. Female C57BL/6J mice (3-4 weeks old) were obtained from Kenya Medical Research Institute (KEMRI), Nairobi. The mice were fed on mice chow and water *ad libitum* and were reared in similar conditions. Mice were assigned randomly with n=5-6 mice per experimental group. Group one (Wild type naïve), Group two (Wild type PbA infected) and group three (Wild type Ubn+PbA).

2.3 Treatment of mice with 200mg/kg Ubidercarenone and infection

Mice were given orally 200mg/kg of Ubn (Zambom Group S.p.A., Italy) for 30 days prior to infection with PbA to the experimental group three and then continued thereafter until mice were sacrificed. *Plasmodium berghei* ANKA was used for this study, in which mice were intravenously infected with $5x10^4$ infected red blood cell (iRBC).

2.4 Euthanization and perfusion of mice

Euthanization was carried out by injecting mice with ketamine (50mg/ml) and Rompun in a ratio of 4:1 intramusculary. Euthanized mice were intracardially perfused with sterile PBS. Brain and spleen samples were extracted and then placed on ice for further preparation.

2.5 Preparation of spleen and brain samples

To obtain single spleen cells suspension, the spleen was perfused with collagenase A, followed by slicing the organ into small pieces and then digestion with collagenase for 30 min at 37 °C. Similarly, after intracardial perfusion of brain samples with 1% sterile PBS, the brains were harvested and single cell suspension prepared as spleen. Cells were counted and were adjusted to 1×10^7 per ml of cells with RPMI medium.

2.6 Cytokine ELISA and flow cytometer

Cytokines were quantified using cytokine-specific sandwich ELISA, for TNF-α, IL-10 and IL-22. ELISA kits were employed and used according to the manufacturers' protocol. The plates were read at 450 nm. After preparation of single cell suspension both splenocytes and brain associated lymphocyte were stained with different antibodies followed by analysis for the expression of CD4, CD8, CD11b, CD11c, CD45, Ly6C, Ly6G, CCR7, CCR2, FoxP3, CTLA-4, CD25 and granzyme B in a FACS Canto II flow cytometer (BD, San Jose, USA). The data was analyzed with FACS Diva® software (Becton Dickinson).

2.7 Statistical analysis

The mean differences between the treatment groups and the controls was determined using the one-way ANOVA followed by Bonferroni test for internal comparison between the different groups. Results are given as mean + SEM with significance level set at $p<0.05$.Data was analysed using Graph pad prism 5.0 statistical software package.

3.1 RESULTS

3.2 Ubidercarenone treated mice have significantly decreased frequency of infiltration of T cells into the brain

In the brain, the frequency of CD3+ and CD8+ T cells was significantly reduced in Ubidercarenone - supplemented mice (Fig. 1A-B). Therefore, these data strongly suggest that Ubidercarenone supplementation can profoundly down-regulate accumulation of these cells in the brain during PbA infection by maintaining a stabile BBB.

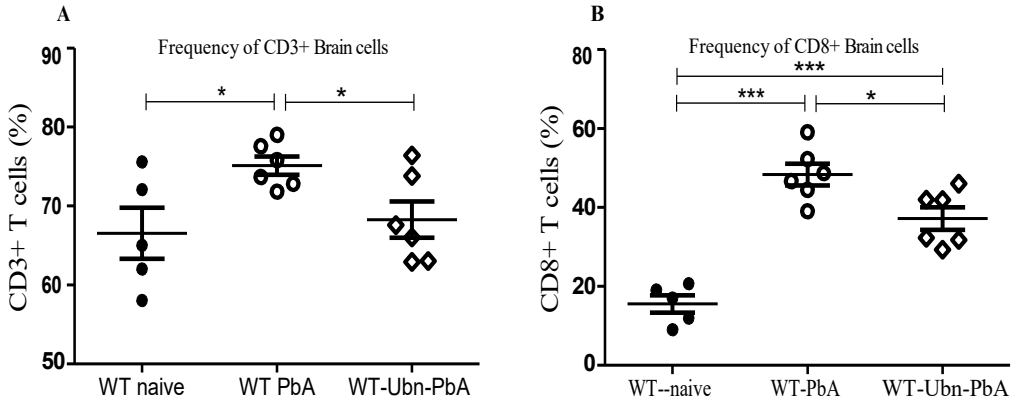

Fig 1: Sequestration of CD3+ and CD8+ T cells in the brain is decreased in Ubidercarenone mice

(A) Percentage of CD3+ T cells in the brain and (B) Percentage of CD8+ T cells in the brain. Percentage of CD3+ and CD8+ T cells calculated as a percentage of infiltrating leukocytes.Percentage of CD3+ and CD8+ T infiltrated leukocyte values were compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: *P ≤ 0.05; ***P ≤0.001).

3.3 Ubidercarenone administered mice have significantly decreased Granzyme B production by CD8+ T cells

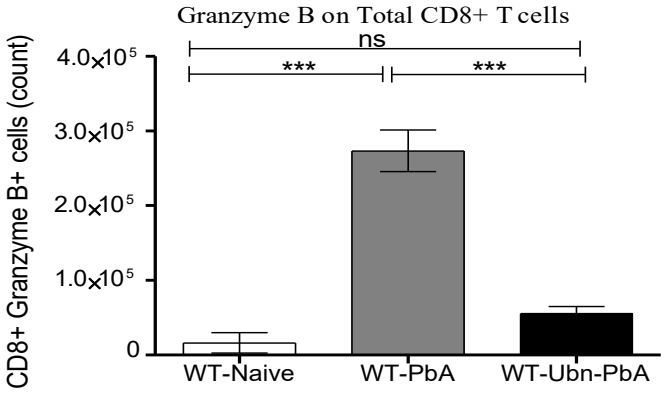

Fig 2: Brain CD8+ T cell production of Granzyme B is altered before and after Co-Q_{10} administration during PbA infection

Absolute number of CD8+ T cells expressing Granzyme-B. Percentage of CD8+ T cells expressing Granzyme B was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: ***P ≤0.001). n=5-6 mice per group.

It was observed that WT PbA-infected mice had an increase in the total cell number of $CD8^{+}$T granzyme B positive cells in comparison to the naive mice whereas the Ubidercarenone administered mice had very few numbers of cells that were producing granzyme B (Fig. 2). These phenomena can be attributed due to a reduction in the total number of CD8+ T cell in the brain.

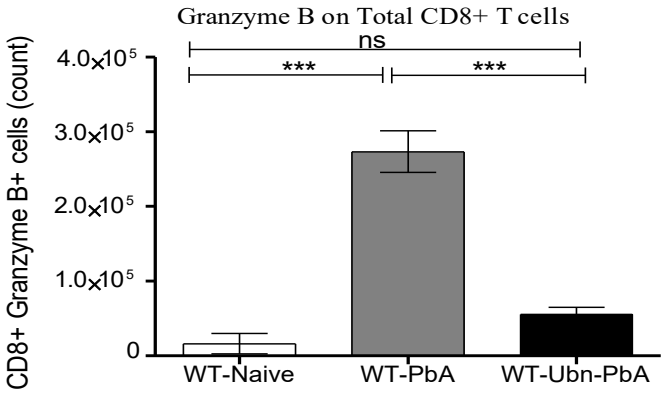

Fig 2: Brain CD8+ T cell production of Granzyme B is altered before and after Co-Q_{10} administration during PbA infection

Absolute number of CD8+ T cells expressing Granzyme-B. Percentage of CD8+ T cells expressing Granzyme B was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: ***P ≤0.001). n=5-6 mice per group.

3.4 Ubidercarenone administration interferes in CD8+T cells CCR2 expression in the brain during PbA-infection

The results shows that Ubidercarenone orally-treated infected mice presented similar CCR-7 to WT PbA-infected mice (Fig. 3A). Interestingly, it was observed that Ubidercarenone orally administered and infected mice had reduced levels of CCR-2 relative to PbA-infected mice (Fig. 3B). Reduction in the CCR-2expression of chemokine receptors in Ubidercarenone administered mice clearly indicates less recruitment of cytotoxic CD8+ T cells in the brain of these mice.

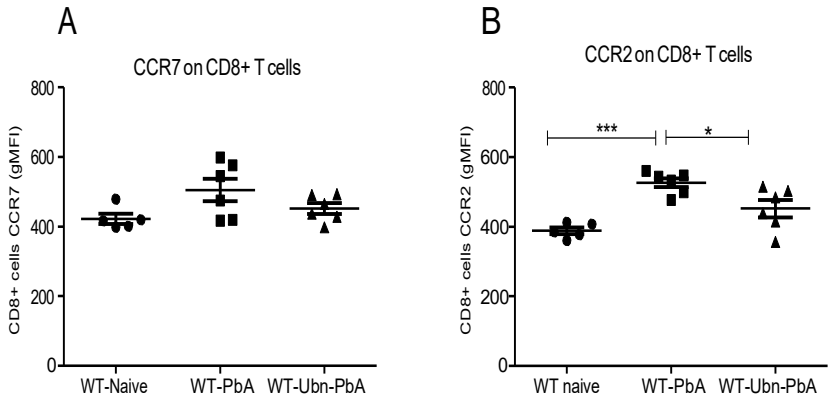

Fig 3: Brains of Ubidercarenone supplemented mice contain reduced number of CD8+T cells phenotypes during PbA infection

Levels of CCR7 (A) and CCR2 (B). Percentage and gMFI of CD8+ T phenotypes was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: *P ≤ 0.05; ***P ≤0.001). n=5-6 mice per group.

3.5 The inflammatory monocyte levels are reduced in the brain of PbA-infected Ubidercarenone supplemented mice

In this study infiltrated inflammatory monocytes were characterized as $Ly6C^{hi}Ly6G^{neg}CD11b^{hi}CD45^{hi}$. The percentage of brain inflammatory monocytes was significantly reduced in Ubidercarenone supplemented mice in comparison with wild type PbA-infected mice (Fig. 4).

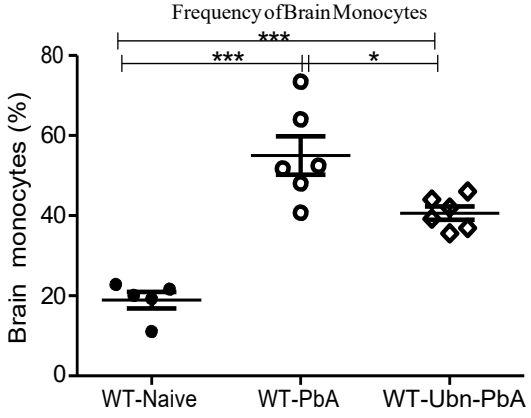

Fig 4: Low amount of inflammatory monocytes in Ubidercarenone supplemented mice

Frequency infiltrating monocytes ($Ly6C^{hi}Ly6G^{neg}CD11b^{hi}CD45^{hi}$). Percentage of monocytes was compared by ANOVA, followed by a Bonferroni posttest (indicated level of significance: *P ≤ 0.05;***P ≤0.001). n=5-6 mice per group.

3.6 Ubidercarenone supplemented mice show a balance in levels of pro-inflammatory and anti-inflammatory cytokine profile at protein level

Mice that suffer from ECM symptoms have been shown to produce excessive amounts of pro-inflammatory cytokines, and Ubidercarenone supplemented mice were found to have markedly reduced serum levels of some of the pro-inflammatory cytokines (Fig. 5A). Whereas, levels of IL-10 and IL-22, anti-inflammatory cytokines were significantly elevated in the infected Ubidercarenone supplemented mice (Fig. 5B–C).

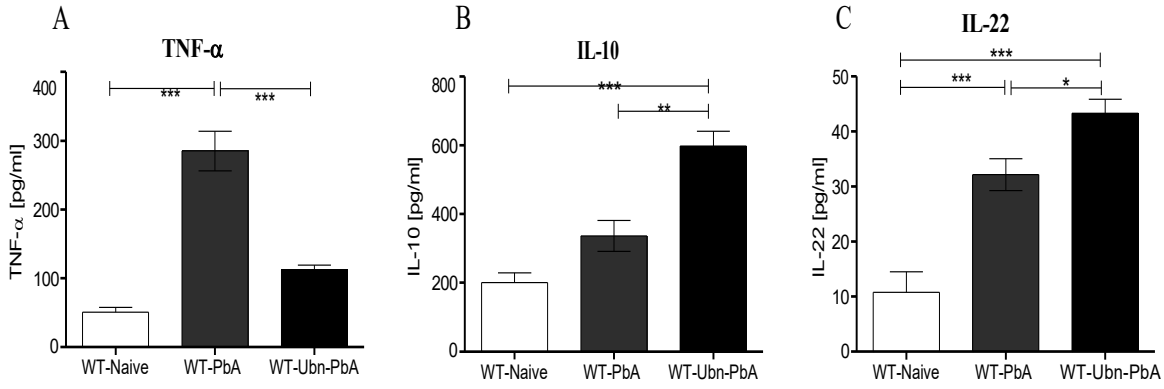

Fig 5: Ubidercarenone protected mice have modulated levels of serum pro-inflammatory and anti-inflammatory cytokines

Serum concentrations levels of TNF-α, IL-22 and IL-10 cytokines (**A-C**). 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

3.7 Ubidercarenone administration increases regulatory T cells in the spleen of mice infected with PbA

The flow cytometric techniques to assess levels of CD4+ T regulatory cells in the spleen was conducted (Fig. 6A). On day 6 of ECM the frequency of CD4+CD25+FoXP3 (T regs) (Fig. 6B) were augmented in Ubidercarenone-supplemented mice in comparison to WT PbA-infected mice. In contrast, the frequency of CTLA-4 was comparable in the two groups of mice (Fig. 6C). These results suggest that Ubidercarenone can also have either direct or indirect influence on T regulatory cells, with a beneficial impact on other T cell effector responses that drive inflammation and pathogenesis in the ECM murine model.

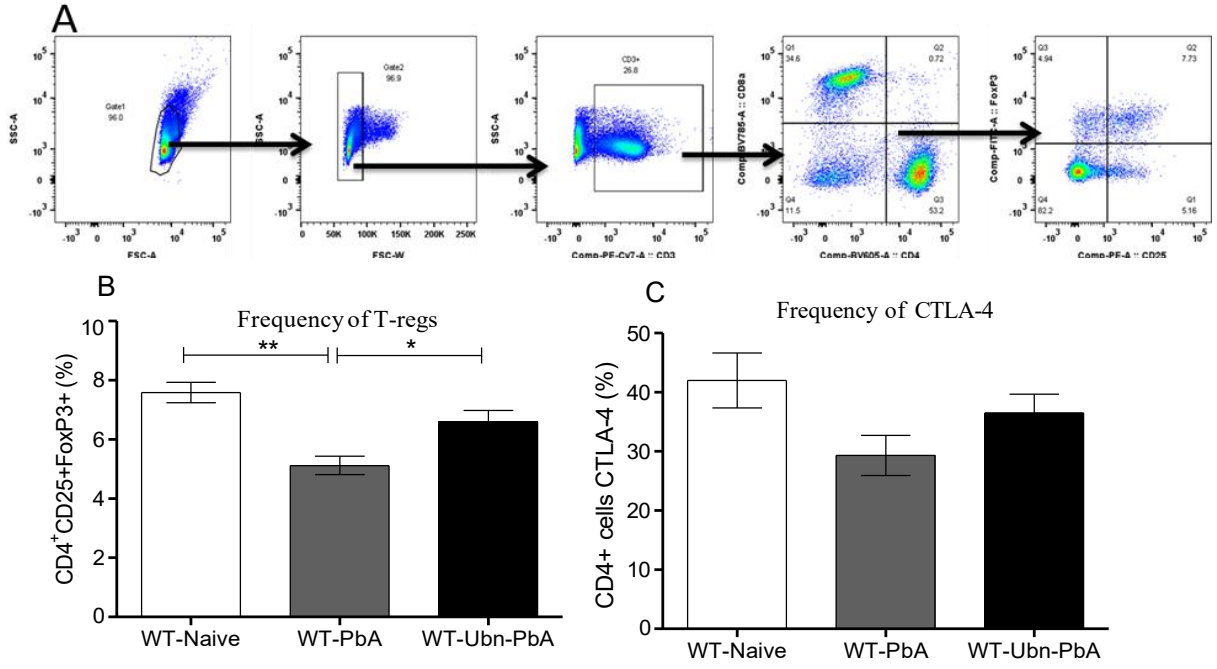

Fig 6: Ubidercarenone supplementation induced the expansion of Tregs during PbA infection

Flow cytometric gating strategy for T regulatory cells characterized as CD3+CD4+CD25+FOXP3+ (A). The frequency of CD4+CD25+FOxP3+ T regs (B) and the percentage (C) of CD4+CTLA+ Tregs were quantified by flow cytometry. The frequencies of T-regs were compared by ANOVA, followed by a Bonferroni posttest. Asterisks indicate significant differences: *P ≤0.05 **P ≤0.01. n=5-6 mice per group

4.0 DISCUSSION

This study shows enhanced immuno-modulatory role of Ubidercarenone against pro-inflammatory mediators at cellular and protein levels. Indeed there is ample evidence showing that intravascular accumulation of CD8+ T cells is critical for the development of ECM through production of cytotoxic molecules perforin and granzyme B [13]. Result from this study, shows that Ubidercarenone administration had an impact on T cell degranulation. A decrease in granzyme B secretion in the brains of Ubidercarenone administered mice compared to WT PbA-infected mice was observed from this study. This decrease in Granzyme B secretion may be due to impaired migration and homing of T cells into the brains of Ubidercarenone administered mice. These phenomenon is validated by the fact that degranulation of effector T cells depends on vesicular trafficking [14]. Thus, it is possible that the reduced degranulation of CD8+ T cells within the brains of the Ubidercarenone mice could be triggered by the antioxidant activity of Ubidercarenone and its activity against Granzyme B cytotoxicity. Expression levels of CCR-2 and CCR-7 of brain CD8+ T cells was also investigated in order to shed light on the cause of reduced infiltration of T cells within the brains of Ubidercarenone administered mice. The chemotactic capacity of trafficking of CD8+ T cells was impaired by Ubidercarenone administration. Therefore, reduced accumulation of pathogen specific effector CD8+ T cells can be credited to reduced expression of the chemokine receptors

CCR-2 on the Ubidercarenone administered CD8+ T cells. This is the most probable, since expression of these chemokine receptors is required for T cells trafficking into the brain [15].

One of the mediators that is very critical in initiating and aggravating immune-pathological events during ECM is the infiltration of high levels of inflammatory monocytes to the brain [5]. However, the inflammatory monocytes play a profound role in the destruction and regulation of *P. chabaudi* especially at the blood stage level [16]. Results from the current study, show that levels of inflammatory monocytes ($Ly6C^{hi}Ly6G^{neg}CD11b^{hi}CD45^{hi}$) were down-regulated in the brains of mice that were treated with Ubn. Indeed, the reduced levels of these monocytes in the brains of Ubn treated mice can be associated with improved amelioration of deleterious events during ECM. Also, other studies associates secretion of TNF-α to inflammatory monocytes [17] similar phenotypes have been observed during contact dermatitis [18]; with concomitant functional role of ensuring that pathogenic effector CD8+ T cells are recruited into the brain [5]. Overall, diminished levels of inflammatory monocytes in Ubn receiving mice can also contribute to reduction of CD8+ T cells that are recruited in the brain.

Based on serum, plasma and gene expression data, disruption of the tightly controlled pro-inflammatory as well as the important regulating anti-inflammatory cytokines is a hallmark of CM [4]. Ubidercarenone has been shown to attenuate pro-inflammatory cytokines and chemokines in vitro, due to its anti-inflammatory properties [12]. A marked reduction in levels of the pro-inflammatory cytokine TNF-α in Ubidercarenone administered mice was observed whereas the WT PbA-infected mice exhibited high levels of this pro-inflammatory cytokine. More importantly, IL-22 and IL-10, which are major anti-inflammatory cytokines, were significantly elevated in the infected Ubidercarenone supplemented mice.

A significant portion of immune-regulatory mechanisms is governed by IL-10 cytokine family, which among others includes IL-22 [19]. Moreover, IL-22 has been observed previously to dampen T cell responses in ECM [20]. Because of the pivotal role of anti-inflammatory cytokines IL-10 and IL-22 in inhibiting CM, it therefore implies that elevation of IL-22 and IL-10 would decelerate the inflammatory responses during CM. This was remarkable and first demonstration that Ubidercarenone has the capacity to modulate inflammatory mediators associate with ECM.

Regulatory T cells are highly versatile immune cells that expand during ECM and have been shown to have the capacity to suppress the function of effector T cell responses or B cell activation [21]. Our results show that Ubidercarenone-administered mice had augmented frequency of CD4+CD25+FoXP3 (T-regs) than WT PbA-infected mice. This is despite the fact that we did not observe expansion of CTLA-4 between Ubidercarenone and WT PbA-infected mice. Nonetheless, $CD4^+$ natural regulatory T cells have been shown to protect mice against plasmodium infection through expansion of CTLA-4 [22]. In another study, it was shown that Vitamin D treatment sporadically expands T-reg population and protects the host against ECM [23]. This result therefore suggest that Ubidercarenone can also have either direct or indirect influence on T regulatory cells, with a beneficial impact on other T cell effector responses that drives inflammation and pathogenesis in the ECM murine model.

5.0 CONCLUSION

Ubidercarenone assuage inflammatory response due to Plasmodium parasites resulting in a significant reduction of clinical features associated with PbA infection complication in an experimental mouse model.

6.0 ACKNOWLEDGEMENTS

We wish to acknowledge the pivotal assistance of staff of Institute of Primate Research and the members of Prof. Orina Isaac research team.

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