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Oral administration of vitamin D and importance in prevention of cerebral malaria

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ABSTRACT

Cerebral malaria (CM) is a serious and fatal malaria-associated syndrome caused by the development of an overwhelming proinflammatory response. Vitamin D (Vit.D; cholecalciferol) has regulatory functions associated with both innate and adaptive immune responses. Prevention is better than cure, in this experiment, we evaluated prophylactic oral Vit.D as a means of preventing CM presentation before infection of C57BL/6 mice with *Plasmodium berghei* ANKA (PbA) by modulating the host proinflammatory response. Mice that were supplemented with oral Vit.D has reduce death rate and ameliorated the integrity of the blood brain barrier. Prophylactic oral vitamin D relieved the symptoms of brain malaria and avoided death, gained valuable time for the diagnosis and treatment post infection. The robust Th1 response was attenuated in the Vit.D + PbA group. Furthermore, T-cell trafficking to the brain was diminished before PbA infection using Vit.D. The results suggest that Vit.D supplementation mediates the development of an anti-inflammatory environment that improves CM severity. In summary, the use of Vit.D as a nutritional supplement in malaria-endemic regions may help reduce the severity and mortality of CM.

1. Introduction

The World Health Organization (WHO) estimates that 216 million cases of malaria occurred globally in 2016, resulting in 445,000 deaths. An estimated 91% of all malaria deaths occurred in the WHO African Region, where children under 5 years old accounted for nearly 90% of these deaths [1]. It is well known that *Plasmodium falciparum* is the most prevalent malaria parasite in sub-Saharan Africa (SSA), which accounted for nearly 99% malaria infections [1]. As one of the most severe complications of the *Plasmodium falciparum* infection [2], cerebral malaria (CM) is a frequent cause of death and neurological disability among children in SSA [3]. More specifically, some studies indicate the median duration of disease onset is around 5 days [4,5]. However, most falciparum infection cases present clinical symptoms within one month or even a few months after exposure [5]. Treatment-seeking behaviour usually depends on the local economic status [6] and the health caregivers' educational level [7]. It causes most patients with *P. falciparum* being admitted after their clinical symptom appearances. Some patients do not even have a definite diagnosis until diagnosed with CM at the time of medico-legal autopsy [8]. Thus, prevention and control strategies aimed at preventing or ameliorating the severity of CM are vital to reduce childhood mortality and morbidity rates associated with *P. falciparum* infection. Furthermore, malaria and malnutrition are the top two causes of childhood morbidity and mortality in sub-Saharan Africa [9]. Some studies indicate that malnutrition has been suggested to influence susceptibility to and severity of malaria [10,11]. Studies of the relationship between malaria and nutrients have been focused on relatively common nutrients and micronutrients, such as vitamin A [12,13], iron [14–16], thiamine (vitamin B1) [17], vitamin D (VD; $1,25(OH)_2D_3$,$1,25D_3$) [18] and zinc [19], as well as protein energy malnutrition [20–22]. These studies demonstrate that nutrition may be an important factor in reducing the malaria morbidity and mortality.

Vit.D is an important micronutrient needed throughout life [23]. Vit.D insufficiency is a serious problem in southern Ethiopia and

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eastern African [24,25], where infectious diseases, such as malaria, HIV, and tuberculosis, are prevalent [26–33]. A recent study showed that Vit.D mediates the regulation of both innate and adaptive immune responses [34]. Specifically, Vit.D inhibits the membrane expression of MHC-II–complexed antigens and costimulatory molecules on antigen-presenting cells (APCs) [35,36], and inhibits the production of IL-12 and IL-23. In addition, Vit.D indirectly polarizes T-cells from Th1 and Th17 cells towards Th2 cells. These roles of Vit.D are of particular importance in the context of CM, which is associated with the development of a Th1 cell-mediated proinflammatory response. Regulatory T cells (Tregs) are critical in mediating immune responses elicited in response to infectious agents, such as viruses, bacteria, and protozoa [37–40]. Furthermore, Tregs help to regulate the polarized immune responses that occur as a result of CM. Therefore, induction of an appropriate and effective immune response to *Plasmodium* spp. after infection is needed for the subsequent control of this pathogen.

In recent study, intramuscular arteether-vitamin D combination may be the potential CM therapy [41]. Our team has also confirmed that oral administration of Vit.D after PbA infection can effectively inhibit the occurrence of experimental cerebral malaria (ECM) in mice [42]. However, the early intervention is critically important to ameliorate disease severity, since about 80% of the deaths take place within 24 h after acute CM patients are admitted [43]. Additionally, vector control, chemoprevention, and vaccination were recognised as the primary preventive measures to combat malarial infections [1]. We have a preliminary discovery of prophylactic oral vitamin D protecting the mice from early motality [42]. However, the preventive protecting mechanism of oral Vit.D is unclear. In this study, we focused on the preventive benefit of Vit.D on the ECM model. The results showed that Vit.D pre-treatment also significantly inhibited the pro-inflammatory immune responses associated with the development of ECM. This will provide a potential and beneficial strategy for the vitamin D prophylactic application and malaria preventive intervention.

2. Materials and methods

2.1. Mouse model of disease

Female C57BL/6 mice (6–8 weeks old) were purchased from the Beijing Animal Institute. PbA was kindly provided by Dr. Motomi Torii (Dept. of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan). Vit.D (cholecalciferol) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in soybean oil before use. Mice were randomly divided into three groups: mice that were uninfected by PbA, mice that were infected with PbA (PbA group), and then infected with PbA (Vit.D + PbA group). In parallel, the control group animals received the same volume of soybean oil. Infections were initiated by intraperitoneal injection of 1×10^6 PbA pRBCs. Mice in the Vit.D + PbA group were orally administered 50 μg/kg Vit.D before PbA infection once a day for 5 consecutive days. For the mortality and parasitemia experiments, 10 mice were used in each group. Parasitemia was monitored by counting the number of pRBCs per 1000 RBCs by light microscopy examination of Giemsa-stained thin blood smears [44]. Mortality was assessed daily. All experiments were performed in compliance with the local animal ethics committee.

2.2. Determination of cerebral pathology

When PbA-infected mice began presenting with neurological symptoms (usually at 5 days post infection (d.p.i.)), three mice from each group were sacrificed, and the integrity of the blood brain barrier (BBB) assessed. 200-μl 2% (wt/vol) Evans blue solution (Sigma, Lot# MKBH2094V) in phosphate-buffered saline (PBS) was injected intravenously into the mice. One hour later, mice were euthanized and the brains were isolated and incubated in 2 ml formamide for 48 h at 37 °C. The amount of Evans blue in 100 μl of brain tissue extracts was determined by measuring the absorbance at 630 nm [45].

Table 1
Primers sequences.

Target gene or mRNA	Primer 5′-3′	
	Forward	Reverse
β-Actin	GATTACTGCTCTGGCTCCTAGC	GACTCATCGTACTCCTGCTTGC
VCAM-1	CCTCACTTGCAGCACTACGG	CATGGTCAGAACGGACTTGG
ICAM-1	GGCAGCAAGTAGGCAAGGAC	CTGGCGGCTCAGTATCTCCT
CXCL9	CCGAGGCACGATCCACTACA	AGTCCGGATCTAGGCAGGTTTG
CXCL10	GCCGTCATTTTCTGCCTCAT	GCTTCCCTATGGCCCTCATT
CXCR3	AAGTGCCAAAGGCAGAGAAG	AAAGTCCGAGGCATCTAGCA
TNF-α	TATGGCTCAGGGTCCAACTC	CCCATTTGAGTCCTTGATGG
IFN-γ	TGATCCTTTGGACCCTCTGA	ACAGCCATGAGGAAGAGCTG

2.3. Total RNA extraction and real-time PCR

Total RNA was extracted from isolated brains and spleens by the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of total RNA were subjected to DNase I digestion. The total digested RNA was used for the reverse-transcription reaction with the appropriate oligo (dT) primers. One-fifth of the reverse-transcription reaction mixture was used for real-time PCR with primer sets specific for ICAM, VCAM, CXCR3, CXCL9, CXCL10, IFN-γ and TNF-α. PCR was performed with the SYBR Green PCR Master Mix for 40 cycles in an ABI PRISM 7700 apparatus (Applied Biosystems, Foster City, CA, USA). Primer sequences used here are described in Table 1. Threshold values were obtained by using the PE Biosystem software, and mRNA was quantified. The ratio of each target gene to the internal control (β-actin) was determined. Treated values were calculated on the basis of an untreated control (taken as 100%). The specificity of the PCR was confirmed by melt-curve analysis.

2.4. Measurement of cytokines by ELISA

For quantification of cytokines, splenocytes were harvested from mice and adjusted into 1×10^7/ml with RPMI-1640. Aliquots (5×10^6 cells/well) of the cell suspensions were seeded in 24-well flat-bottom tissue culture plates (Falcon) in triplicate in a humidified 5% CO_2 incubator. Levels of IFN-γ, TNF-α, and IL-10 in culture supernatants and plasma samples were measured by enzyme-linked immunosorbent assays (ELISAs) (R&D Systems, Minneapolis, MN, USA). Optical density (OD) values were measured at 450 nm using a microplate reader (Bio-Rad, USA). The concentration of cytokines in each sample was calculated via a standard curve generated using recombinant cytokines.

2.5. Flow cytometry analysis

To measure Th1-type cells ($CD4^+$ T-bet^+ IFN-γ^+), 10^7 fresh splenocytes cultured in RPMI-1640 were stimulated with 50 ng/ml PMA and 1 mM ionomycin (Sigma-Aldrich) in 12-well plates for 5 h at 37 °C and 5% CO_2 in the presence of 1 ml of brefeldin A (BD Biosciences) to inhibit cytokine secretion. Cells were harvested and their surfaces stained with FITC-conjugated anti-CD4 mAb (clone H1.2F3). Cells were fixed and permeabilized with an intracellular fixation kit (eBioscience), in accordance with the manufacturer's instructions. Then, cells were stained with anti-T-bet-PE (clone eBio4B10) and anti-IFN-γ-APC (clone XMG1.2). To identify Tregs, the PMA and ionomycin stimulated splenocytes were incubated with FITC-anti-CD4 and PE-anti-CD25 antibodies in 100 μl of PBS supplemented with 3% FCS. Cells were also stained for intracellular Foxp3 with APC-anti-Foxp3 antibody (clone FJK16s), as described above [46]. To determine the migration of $CD4^+$ and $CD8^+$ T cells to the brain, brain mononuclear cells were isolated from the brains on day 5 postinfection (p.i.) following a published procedure [47].

2.6. Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEMs). Differences in survival were assessed by the Kaplan-Meier (K-M) test. For comparisons between two groups, statistical significance was analyzed by either a *t*-test or a Mann-Whitney test, depending on the normality of the data. For comparisons between three or more groups, statistical significance was determined by the one-way ANOVA test. Data were analyzed with the GraphPad Prism software package (version 6.01). A *P*-value < 0.05 was considered statistically significant.

3. Results

3.1. Parasitemia and survival analyses

To assess the role of Vit.D treatment in the development of CM, C57BL/6 mice were administered 50 μg/kg Vit.D orally before PbA infection. In parallel, non-infected controls were treated with soybean oil only at each time point. Regardless of Vit.D treatment, PbA-infected susceptible mice typically developed CM symptoms by 5 d.p.i. Mice in the PbA group developed CM and succumbed to infection by 6 to 11 d.p.i. (Fig. 1B), with a parasitemia of ~12% on d9 ($P < 0.05$ compared with Vit.D + PbA group) (Fig. 1A). In contrast to disease presentation in the PbA group, mice in the Vit.D + PbA group were completely resistant to CM and all mice survive until d15 ($P < 0.05$ compared with PbA group, Fig. 1A, B). These results indicate that oral Vit.D protected C57BL/6 mice from CM after PbA infection.

3.2. Vit.D treatment reduces leukocyte adhesion and micro hemorrhaging in brain vessels

CM is associated with alterations of the BBB integrity, resulting in the extravasation of pRBCs into the brain parenchyma. At 5 d.p.i., a loss of BBB integrity was evident in mice in the PbA group, whereas the BBB was intact in mice in the Vit.D + PbA and control groups, based on the similar pattern of dye extravasation in these two groups ($P < 0.05$, Fig. 2). These results suggest that the BBB integrity was better preserved in mice in the Vit.D + PbA group compared to the PbA group after infection.

3.3. Vit.D treatment suppresses CM-associated pro-inflammatory immune responses in the brain

Previous studies in humans and mice independently highlighted the roles of cytokines and chemokines in progression and pathogenesis of CM. IFN-γ and TNF-α are proinflammatory cytokines that contribute to CM pathogenesis. The levels of chemokines, such as CXCL9 and CXCL10, also have been shown to be elevated during CM [48].

To determine whether Vit.D pre-treatment prevents ECM by altering chemokine and cytokine production, we examined the cytokines and/or chemokines by RT-PCR or ELISA in all treatment groups. Although the chemokine levels were not always significantly different between the Vit.D + PbA and PbA groups, Vit.D treatment before infection reduced the mRNA expression levels of IFN-γ (Fig. 4A), TNF-α (Fig. 4B), ICAM-1 (Fig. 3A), VCAM-1 (Fig. 3B), CXCR3 (Fig. 3C), CXCL9 (Fig. 3D), and CXCL10 (Fig. 3E) in the brains of infected mice at 5 d.p.i. Similarly, the serum levels of TNF-α and IFN-γ were decreased in animals in the Vit.D + PbA group at 5 d.p.i. compared to the PbA group ($P < 0.05$ or $P < 0.01$, *t*-test; Fig. 5A, B).

3.4. Vit.D treatment reduces T-cell trafficking in CM

The presentation of CM is partly dependent on the T-cell response, as the trafficking of T cells to the brain is central to the development of CM pathogenesis. The IFN-γ–inducible chemokines CXCL9 and CXCL10 recruit $CD4^+$ and $CD8^+$ T cells to the brain via CXCR3 on the T-cell surface. As Vit.D treatment reduced chemokine expression levels, we next determined whether Vit.D treatment reduced T-cell trafficking to the brain, by isolating and quantifying brain mononuclear cells on 5 d.p.i. An absolute number of$CD4^+$ and $CD8^+$ T cells were higher ($P < 0.05$ or $P < 0.01$) in the brains of mice in the PbA group compared to levels observed in mice from the control or Vit.D + PbA group (Fig. 3F). These data demonstrate that Vit.D pre-treatment reduces T-cell trafficking to the brain during CM.

3.5. Vit.D pretreatment reduces Th1 immune responses

Malaria research has demonstrated the significant impact played by Th1-mediated proinflammatory responses elicited in response to *Plasmodium* infections that progress to CM [49–51]. We hypothesized that a reduced incidence of CM development in Vit.D-treated mice was likely associated with an altered Th1 response. To test our hypothesis, we quantified the percentages of splenic Th1 cells ($CD4^+$T-bet$^+$IFN-γ^+) (Fig. 6A, B), as well as IFN-γ (Fig. 5D) and TNF-α (Fig. 5E) levels in splenocyte cultured supernatants from mice in the Vit.D + PbA and PbA groups at 3 and 5 d.p.i. IFN-γ and TNF-α levels were elevated on 5 d.p.i. in the PbA group ($P < 0.01$), which also presented an increased frequency of the $CD4^+$T-bet$^+$IFN-γ^+ T-cell population. However, compared to normal mice, no discernable expansion of the $CD4^+$T-bet$^+$IFN-γ^+ T-cell population or proinflammatory cytokine production was observed in the Vit.D + PbA group. Vit.D administration before infection reduced the mRNA expression levels of IFN-γ (Fig. 4C) and TNF-α (Fig. 4D) in the spleens of infected mice at 3 d.p.i. Vit.D pretreatment significantly ($P < 0.01$) inhibited the expansion of Th1 cells and proinflammatory cytokine secretion.

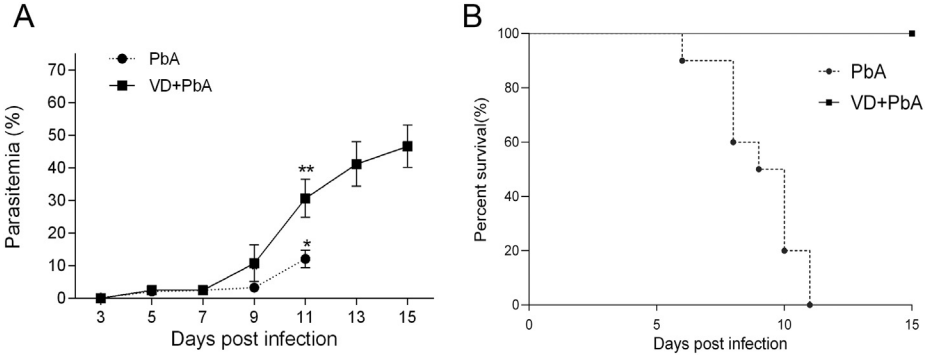

Fig. 1. Vit.D supplementation before PbA infection improves survival. PbA ($n = 10$) and Vit.D + PbA ($n = 10$) groups. Parasitemia levels are expressed as the mean ± SEM.

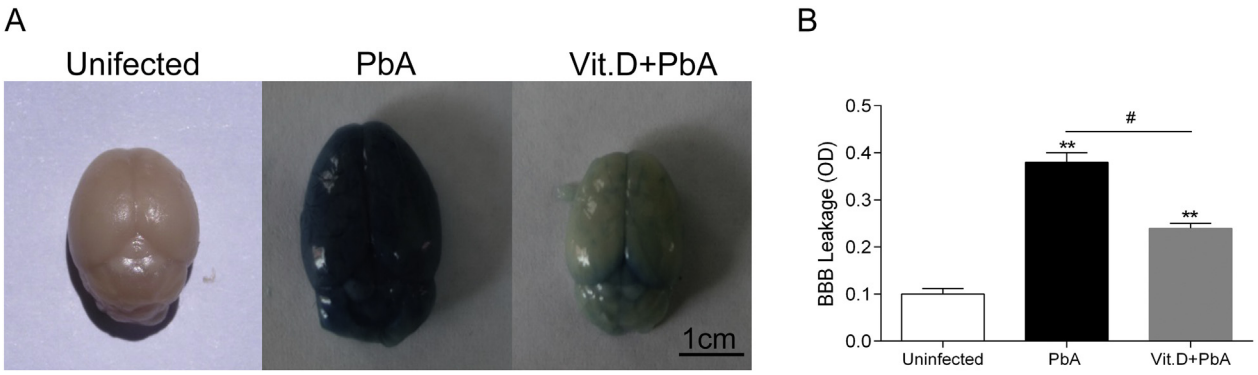

Fig. 2. Vit.D treatment maintains the BBB integrity. Representative images of Evans blue-stained brains from each treatment group. Dye extravasation is shown graphically. Data are expressed as the mean optical density readings at 630 nm (mean ± SEM, $n = 5$). $^{**}P < 0.01$ and $^{\#}P < 0.05$, difference between the PbA and Vit.D + PbA groups (Student's *t*-test).

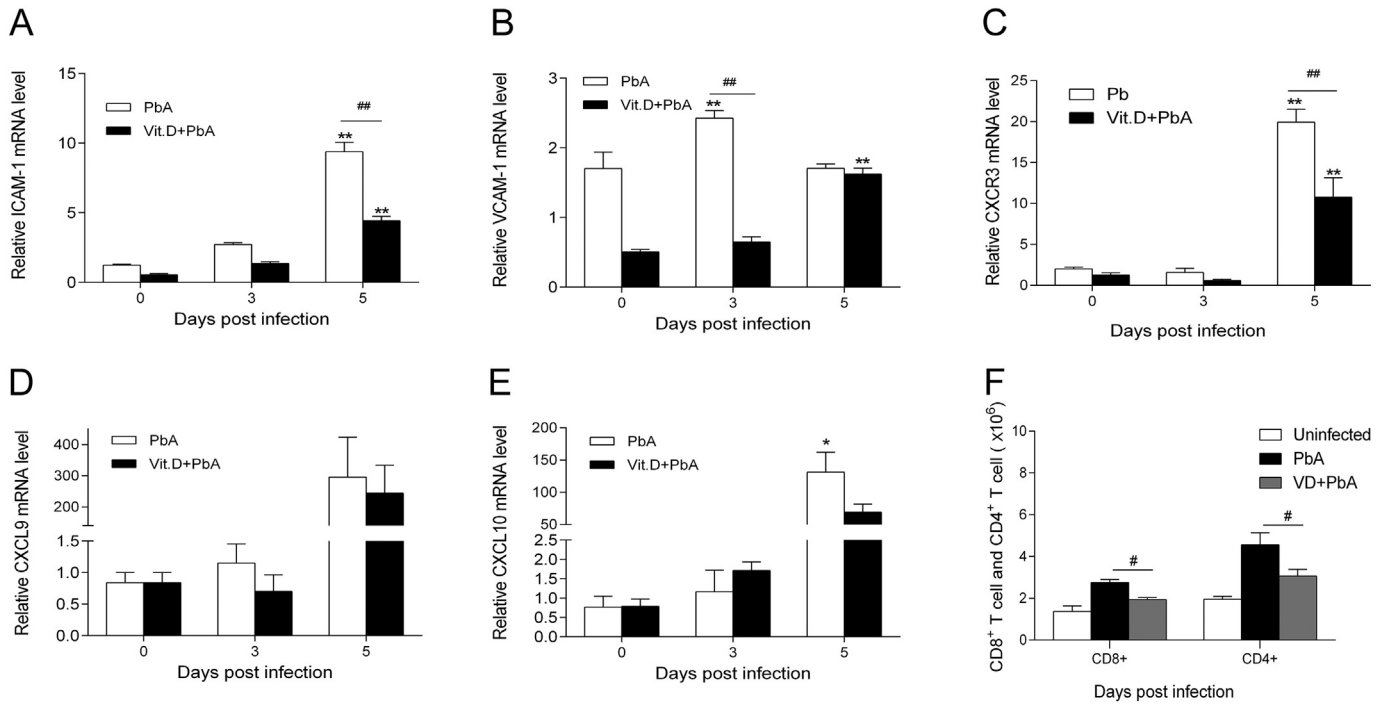

Fig. 3. Vit.D pretreatment alters cytokine profiles. Vit.D supplementation before PbA infection reduced mRNA ICAM and VCAM levels (A and B), and decreased CXCR3, CXCL9 and CXCL10 expression levels (C, D and E) in the brains of PbA-infected mice on 5 d.p.i. The mRNA levels were normalized to β-actin mRNA levels. Fold changes were calculated against levels in uninfected mice. Each experiment was repeated three times ($n = 5$ animals/group). Vit.D supplementation before PbA infection reduced T-cell trafficking to the brain after induction of CM(F). Values represent the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, PbA-infected mice vs. baseline levels on day 0. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, PbA group vs. Vit.D + PbA group.

3.6. Vit.D pretreatment increases Treg levels and IL-10 production

Tregs have an important role in preventing the development of PbA-specific Th1 responses associated with CM progression. In the present study, Treg levels were increased at 3 and 5 d.p.i. in mice in the PbA and Vit.D + PbA groups. At 5 d.p.i., the number of Tregs detected in the Vit.D + PbA group was greater than the number observed in the PbA group ($P < 0.05$, Fig. 7B). Although the IL-10 levels were elevated at 5 d.p.i. in the PbA group compared to the uninfected controls, IL-10 levels were higher in the Vit.D + PbA group animals compared to levels in the PbA group animals ($P < 0.05$), except for a comparison between PbA and Vit.D + PbA at 5 d.p.i. (Fig. 5C, F). This result indicates that the reduced incidence of CM after PbA infection in the Vit.D + PbA group may be due to changes in the number of Tregs and level of IL-10.

4. Discussion

Oral administration of Vit.D or intramuscular arteether and vitamin D co-administration has been studied in many researches to become a potential cerebral malaria therapy after malaria infection [41,42]. In our preliminary study, we found that oral administration of Vit.D can significantly ameliorate the inducible systematic inflammatory responses through reducing the IFN-γ and TNF-α and decreasing the expression of these cytokines in spleen cells. Vit.D can also decrease the expression of CXCL-9 and CXCL-10 in the brain cells. In addition, Vit.D can upregulate the expression levels of regulatory T cells and IL-10 [42]. In this study, we examined the effects of oral Vit.D pretreatment on CM progression after PbA infection of C57BL/6 mice. Vit.D + PbA group mice presented fewer brain inflammatory cell infiltrates and improved vascular integrity compared to the PbA group mice. The robust Th1 response that is commonly observed as a consequence of PbA

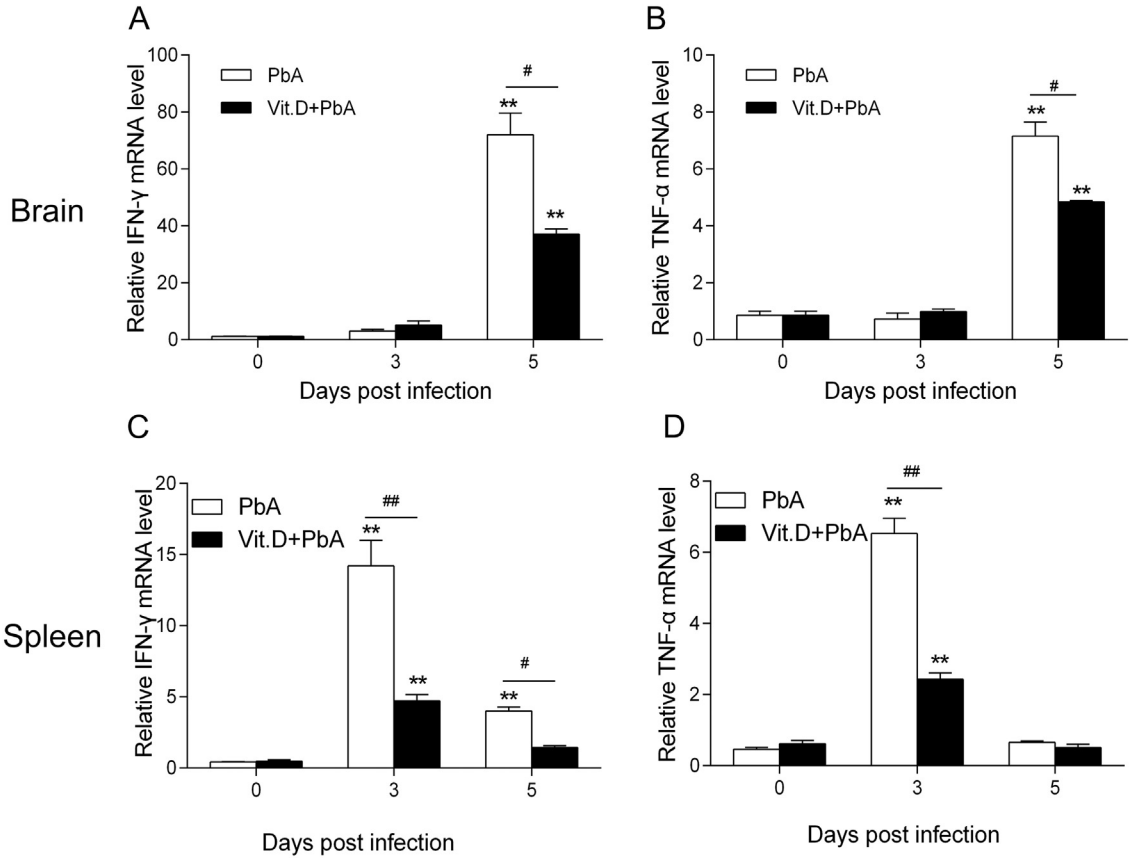

Fig. 4. Vit.D supplementation before PbA infection alters the mRNA levels of IFN-γ and TNF-α in brains (A and B) and spleens (C and D) on 3 and 5 d.p.i. Each experiment was repeated three times ($n = 5$ animals/group). The mRNA levels were normalized to β-actin mRNA levels. Fold changes were calculated against levels in uninfected mice. Values represent the mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, vs. baseline on day 0. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ PbA group vs. Vit.D + PbA group.

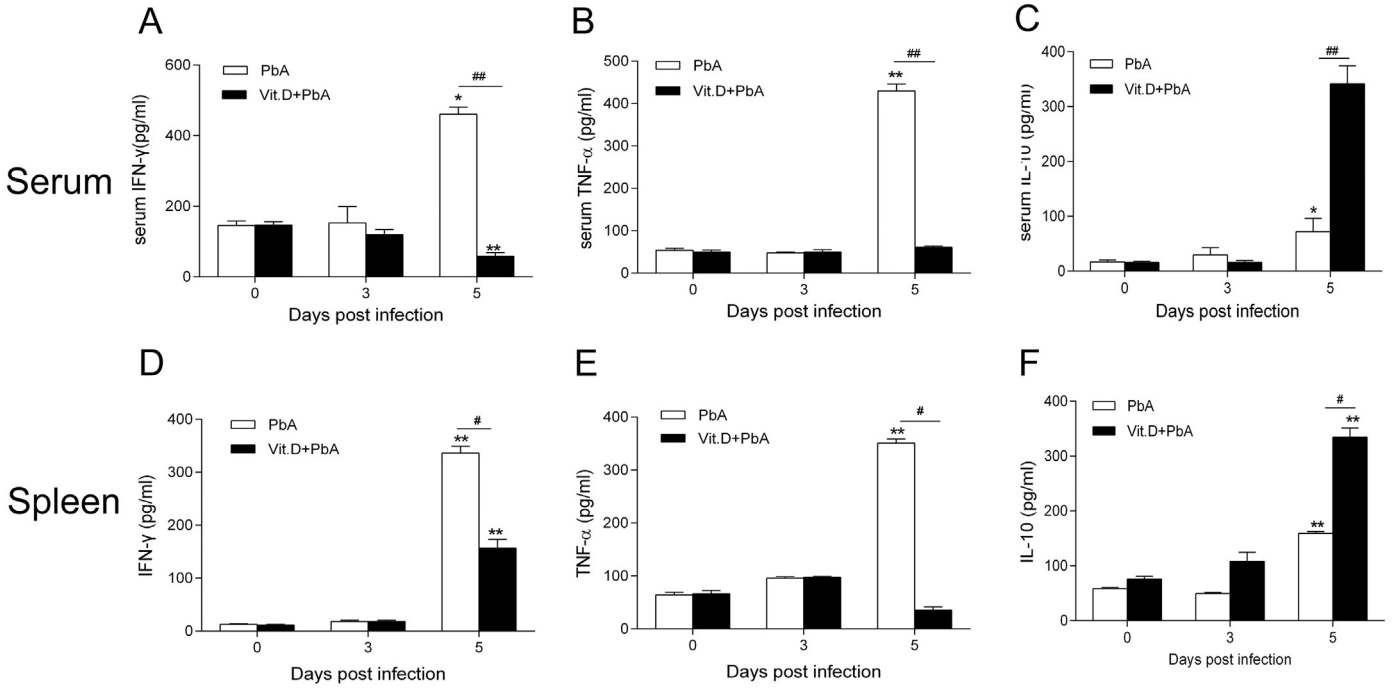

Fig. 5. Vit.D pretreatment reduced expression of proinflammatory cytokines IFN-γ and TNF-α (A and B). Levels of proinflammatory cytokines in the spleen (D and E). Oral Vit.D increased IL-10 (C and F) production in serum and spleen. Each experiment was repeated three times ($n = 5$/group). Values represent mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$, significant differences between the values in PbA-infected mice and the baseline levels on day 0, respectively. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$, significant difference between the PbA and Vit.D + PbA groups.

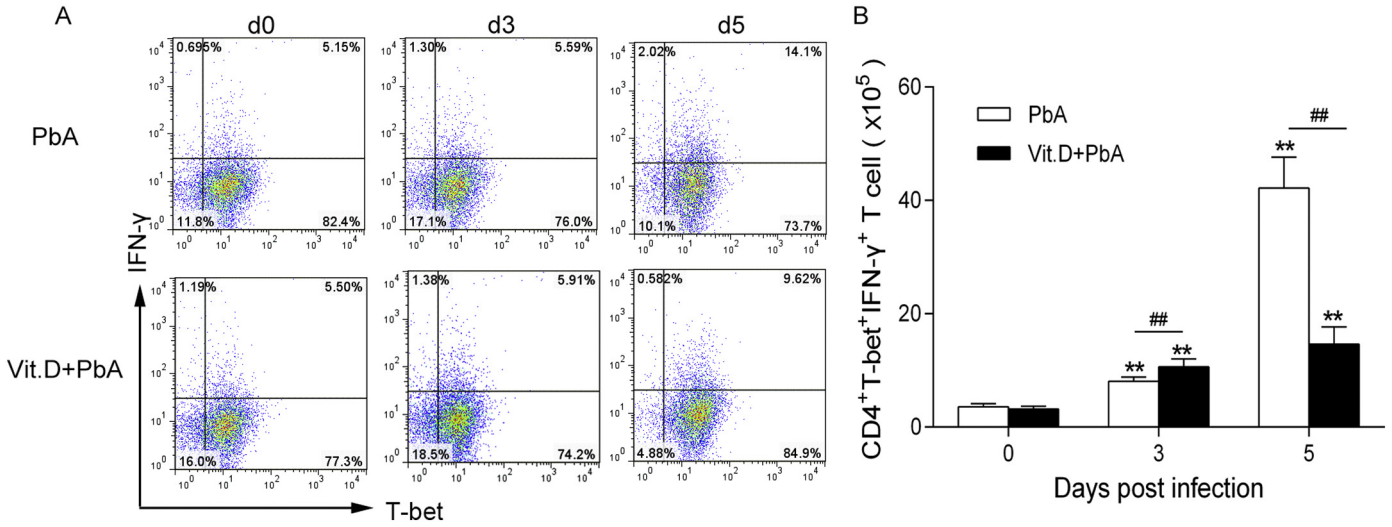

Fig. 6. Vit.D alters the nature of the developing T-cell response. Vit.D supplementation before PbA infection reduced the expansion of CD4 + T-bet + IFN-γ + T cells. Values represent the mean ± SEM ($n = 5$ mice/group). $^{*}P < 0.05$ and $^{**}P < 0.01$, PbA-infected mice at 3 and 5 d.p.i. vs. baseline levels, respectively. $^{\#\#}P < 0.01$, PbA group vs. Vit.D + PbA group.

infection of C57BL/6 mice (and is responsible for mediating progression of CM) was diminished in Vit.D + PbA group mice. Furthermore, T-cell trafficking to the brain was reduced, Tregs and IL-10 levels were unregulated, and the serum and splenic levels of IFN-γ, TNF-α, CXCL9, and CXCL10 were reduced after Vit.D treatment. Taken together, these results indicate that the prophylactic administration of oral Vit.D via a multifactorial process, result in maintenance of the BBB and improve survival. The mechanism of Vit.D pre-treatment for ECM was nearly consistent with our Vit. D post-treatment [42].

Since Vit.D deficiency is common in hyperendemic areas of tuberculosis and HIV infection [26–33], vitamin D adjunctive therapy for those infected cases has been reported [52,53]. The question related to the effectiveness of Vit.D in preventing pulmonary tuberculosis is currently addressed in Phase 3 trials in Tanzania and Mongolia [52]. Meanwhile, Villamor E once reviewed that adequate levels of Vit.D may better control HIV or opportunistic infections among HIV-infected individuals [53]. As we mentioned, cerebral malaria is a fatal complication of *Plasmodium falciparum* infection. It is prevalent in developing countries, where it brings considerable disease and financial burden to impoverished countries. Olaf MÜller once in his study reported that the median duration of the final episode was around 5 days and nearly 60% children did not receive any definite and effective treatment during those episodes [4]. It suggests that if we give some preventive measures before the onset of malaria infection, can we change the immune system status in advance and then it will ameliorate the progression of CM. To date, many successful intervention strategies have been developed to improve malaria prevention. These strategies include vaccines (i.e., vectored immunoprophylaxis), vector control with insecticide-treated mosquito nets, indoor residual spraying, or long-lasting insecticidal nets, and chemoprevention, such as sulfadoxine-pyrimethamine or mefloquine treatment for pregnant women and amodiaquine plus sulfadoxine-pyrimethamine treatment for children [54–56]. Among the above measures, it is usually ignored that the nutritional support prophylactic treatment may also be potentially recognised as the preventive measure for endemic people.

Vit.D deficiency is a global epidemic that affects populations living beyond latitudes 35° N and 35° S of the equator due to seasonal sun exposure [57]. However, even in areas with abundant sunshine, such as parts of Africa, Vit.D insufficiency remains problematic [5,23]. The observed Vit.D deficiency among Africans may be influenced by low

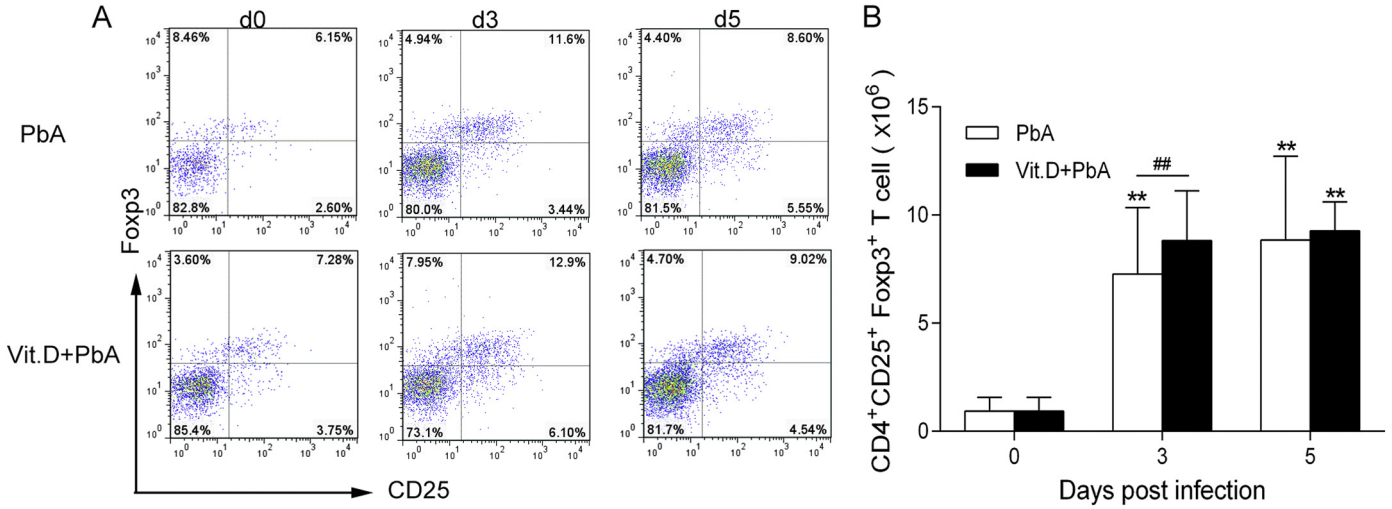

Fig. 7. Vit.D pretreatment increases Treg numbers. At 3 d.p.i., the Vit.D + PbA group showed increased numbers of Tregs compared to the PbA group. Values represent the mean ± SEM ($n = 5$ mice/group). $^{*}P < 0.05$ and $^{**}P < 0.01$, PbA-infected mice at 3 and 5 d.p.i. vs. baseline levels, respectively. $^{\#\#}P < 0.01$, PbA group vs. Vit.D + PbA group.

consumption of the few naturally occurring Vit.D-rich food sources (meat, egg yolk, offal, and oily fish) by people on this continent [58–60]. Some studies indicated that the dark skin may decrease the 25-hydroxyvitaminD [25-(OH)-D_3] [61–64]. The distribution and composition of melanin on the skin change with the ultraviolet radiation [62]. Melanin can effectively absorb and scatter the electromagnetic radiation from visible light to ultraviolet and then competes with 7-dehydrogenated cholesterol for UVB photons [65]. These studies indicate that the genetic characteristics of the blacks do not make them as highly sensitive to the composition of UV-induced Vit.D as the whites, although the blacks live at lower latitude regions where the solar ultraviolet radiation is abundant. Therefore, foodborne Vit.D for Africans becomes very essential and necessary.

In conclusion, prophylactic oral administration of Vit.D prevented CM in mice, suggesting that administration of oral Vit.D could potentially be used to prevent CM symptoms in malaria-endemic regions, such as sub-Saharan Africa. Any prevention strategy for malaria needs to consider the importance of nutrition, especially in children who are the most vulnerable to malnutrition. Data presented in this report suggest that Vit.D supplementation in malaria-endemic regions is likely to have an impact in reducing the disease burden of malaria, in part by decreasing the rates of CM-related mortality.

Acknowledgements

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