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Different doses of vitamin C supplementation enhances the Th1 immune response to early *Plasmodium yoelii* 17XL infection in BALB/c mice

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ABSTRACT

Vitamin C (ascorbate) is maintained at high levels in most immune cells and can affect many aspects of the immune response. Here, we evaluated the effect of vitamin C supplementation on the immune response to *Plasmodium yoelii* 17XL (*P. yoelii* 17XL) infection in BALB/c mice. Two orally administered doses (25 mg/kg/day and 250 mg/kg/day) of vitamin C significantly reduced levels of parasitemia during the early stages of *P. yoelii* 17XL infection. The numbers of activated Th1 cells and macrophages in the groups receiving vitamin C supplementation were both higher than those in the untreated group. Meanwhile, vitamin C administration reduced the levels of tumor necrosis factor α secreted by splenocytes. Vitamin C also regulated the protective antimalarial immune response by increasing the number of plasmacytoid dendritic cells, as well as the expression of dendritic cell maturation markers, such as major histocompatibility complex class II and cluster of differentiation 86. In conclusion, the doses of vitamin C (25 mg/kg/day, 250 mg/kg/day) during the early stages of malaria infection may better enhance host protective immunity, but have no dose dependence.

1. Introduction

Malaria remains one of the most devastating parasitic diseases in tropical and subtropical regions today. Although the incidence of malaria showed a global reduction, from 262 million in 2000 to 214 million in 2015, it accounts for ~25% of pediatric deaths in endemic areas of Africa [1–3]. Malaria eradication remains difficult, mainly due to the lack of an efficacious vaccine, the emergence of insecticide resistance in *Anopheles* mosquito vectors, and an increase in drug-resistant parasites [4,5]. Thus, there is an urgency to develop new therapeutic approaches to overcome infection of malarial parasite.

A series of studies have proved that the control of anemia and inflammation response mediated by cytokines and effector cells is of major importance for survival to rodent blood malaria infection. Accumulated data have indicated that $CD4^+$T cells are essential for the control of blood-stage malaria infection. In the early stage of infection, Th1 immune responses can control the outbreak proliferation of *Plasmodium* parasites by enhancing $CD4^+$ T cell activity and the phagocytic activity of macrophages (Mφ) through the production of TNF-α and NO [6–10]. In addition, $CD4^+$T-cell subsets with distinctive characteristics and transcriptional programs are governed predominantly by signals derived from antigen presenting cells (APC) and the microenvironment at the time of $CD4^+$ T-cell activation. Dendritic cells (DCs) are necessary for effective priming of the T-cell response in malaria [11]. These studies suggest that protective immunity, i.e. Th1 immune response via DC induction correlated with low morbidity and mortality during *plasmodium* infection.

It has long been recognized that populations residing in malaria-endemic areas generally live under conditions of poor nutritional status. In particular, children and pregnant women, the groups at the highest risk with regards to the adverse effects of malaria, are most affected by poor nutrition [12]. Energy and nutrient supplies are especially important for the immune system due to the high turnover rate of many immune cells, leading to a higher substrate requirement compared to most other body systems [13]. Thus, it can be reasonably to inferred that nutritional status strongly influences the progress of malarial infection.

Vitamin C (L-Ascorbic acid) is an essential nutrient for humans because it cannot be synthesized in the body. Vitamin C has profound effects on both cellular proliferation and differentiation, and is essential

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for the optimal function of the immune system [14–16]. Inadequacy and clinical deficiency of vitamin C may lead to an impaired immune response with altered resistance to infection, impaired growth, and weakened collagenous structures with delayed wound healing [17]. Clinical research has demonstrated that reduced concentrations of serum vitamin C were correlated with the exacerbation of parasitemia levels among children with *Plasmodium falciparum* malaria [18]. Consistently, animal studies have demonstrated lower mortality from infectious and parasitic diseases after vitamin C supplementation [19–21]. Besides what was discussed above, vitamin C is proposed to have an important physiological role as an effective in vivo antioxidant. *Plasmodium* infection causes an immune response resulting in the activation of Mφ leading to the production and release of reactive oxygen species (ROS). Since malaria infection imposes tremendous oxidative stress on the host, a promising strategy to improve the outcome of *plasmodium* infection is to supplement vitamin C or similar antioxidant in populations living malaria area [22].

In light of these data, we chose two different doses of vitamin C: 25 mg/kg/day or 250 mg/kg/day. we hypothesized that vitamin C supplementation may regulate the nature of the host immune response to malarial infection. Accordingly, in this study we compared the patterns of Th1 immune response, the levels of inflammatory associated cytokines production, the function and activation of DCs and Mφs in mice infected with *Plasmodium yoelii* 17XL (*P. yoelii* 17XL) following treatment with two doses of vitamin C to determine the effects of vitamin C on the protective immune response.

2. Materials and methods

2.1. Mice, parasite, and P. yoelii 17XL infection

Female 6–8 week-old BALB/c mice were purchased from the Beijing Animal Institute. *P. yoelii* 17XL was kindly provided by Dr. Motomi Torii (Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan). Infections were initiated by intraperitoneal (i.p.) injection of 1×10^5 *P. yoelii* 17XL-parasitized erythrocytes into BALB/c mice. Parasitemia was monitored by light microscopy examination of Giemsa-stained thin blood smears taken from a tail vein. Mortality was monitored daily. All experiments were performed in compliance with the local animal ethics committee.

2.2. Vitamin C treatment

L-Ascorbic acid was purchased from Sigma–Aldrich (St Louis, MO, USA). It was dissolved in 0.9% saline before use. For animal experiments, *P. yoelii* 17XL-infected mice were orally administered 25 mg/kg or 250 mg/kg vitamin C once a day for four successive days beginning on day two post infection (p.i.). The *P. yoelii* 17XL-infected control group received the same volume of 0.9% NaCl solution without vitamin C supplementation at the same time points. A large number of relevant literature has proved that Endpoints for therapeutics administered systemically to animals, are usually assumed to scale well between species when doses are normalized to body surface area (BSA) (ie, mg/m^2) [23]. In addition, Reagan et al. clarified that the formula for dose translation based on BSA, followed as HED (mg/kg) = Animal dose (mg/kg) multiplled by Animal Km/Human Km. Thus, a mouse dose of 25 mg/kg would be 75 mg/m^2 (2 g/day) in a human/60 kg, which is very close to 90 mg/human that was administered.

2.3. Splenocyte preparation and culture

Splenocyte culture was performed as previously described [24]. Briefly, spleens from normal and infected mice were removed aseptically and pressed through a sterile fine-wire mesh in 10 ml Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 0.12% gentamicin, and 2 mM glutamine. Cell suspensions were collected by centrifugation at 350 ×*g* for 10 min. Erythrocytes were lysed with cold 0.17 M NH_4Cl and washed twice with fresh medium. Spleen cell viability was determined to be > 90% using a trypan blue exclusion assay. Spleen cells were adjusted to a final concentration of 1×10^7 cells ml^{-1} in RPMI 1640 supplemented with 10% heat-inactivated FCS. Aliquots (500 µl $well^{-1}$) of the cell suspensions were incubated in 24-well flat-bottom tissue culture plates (Falcon) in triplicate for 48 h at 37 °C in a humidified 5% CO_2 incubator. Supernatant fractions were collected and stored at −80 °C for cytokine detection.

2.4. Flow cytometric analysis

For each experimental group, at least three mice were infected simultaneously and sacrificed at the indicated times to determine the relative levels of the subsets of splenic dendritic cells [myeloid DCs (mDCs) and plasmacytoid DCs (pDCs)]; the expression of major histocompatibility complex class II (MHCII) and cluster of differentiation 86 (CD86), and intracellular toll-like receptor 9 (TLR9) and TLR4 in $CD11c^+$ DCs; and the numbers of regulatory T cells (Tregs), Mφ, and $CD4^+$–T-bet^+–IFN-γ^+ T cells (IFN-γ, interferon-gamma).

To assess $CD4^+$–T-bet^+–IFN-γ^+ T cells, fresh splenocytes were surface stained with fluorescein isothiocyanate- (FITC-) conjugated anti-CD4 (clone GK1.5) antibodies. After fixation and permeabilization using staining buffer reagents as instructed by the manufacturer, cells were incubated with peridinin chlorophyll- (PerCP-) conjugated anti-T-bet (clone eGio4B10) and phycoerythrin- (PE-) conjugated anti-IFN-γ monoclonal antibodies (mAb; XMG1.2). For the detection of $F4/80^+$ Mφ, splenocytes were stained with FITC-conjugated anti-F4/80 (clone BM8) antibodies. To measure Treg cell percentages, FITC-conjugated anti-CD4 (clone GK1.5) and PE-conjugated anti-CD25 (clone PC61) antibodies were added to spleen cells, which were resuspended in 100 µl phosphate-buffered saline (PBS) supplemented with 1% FCS for surface staining. Then the cells were fixed, permeabilized, and intracytoplasmic staining was performed using allophycocyanin- (APC-) conjugated anti-FOXP3 (clone FJK16s, eBioscience, San Diego, CA) antibodies. To assess DCs, cells were co-stained with FITC-conjugated CD11c mAb (clone HL-3) and PE-conjugated anti-CD11b (clone M1/70), PerCP-conjugated CD45R/B220 (clone RA3-6B2), APC-conjugated anti-MHCII (clone M5/114.15.2), or APC-conjugated anti-CD86 (clone GL1). To assess the expression of TLR9 and TLR4 in $CD11c^+$ DCs, spleen cells were stained with FITC-conjugated CD11c mAb. After fixation and permeabilization using staining buffer reagents as instructed by the manufacturer, cells were incubated with biotinylated anti-TLR9 mAb (clone5G5, Hycult biotech) and PE-conjugated anti-TLR4 streptavidin (eBioscience, San Diego, CA). Unless otherwise indicated, antibodies were purchased from BD Biosciences (San Jose, CA). The cells were then washed twice with PBS containing 1% FCS and suspended in 300 µl PBS. The cells were analyzed in a FACSCalibur™ fluorocytometer using CellQuest software. Viable cells were gated by forward and side scattering patterns.

2.5. Detection of cytokines by enzyme-linked immunosorbent assay

Levels of IFN-γ, TNF-α, and interleukin 10 (IL-10) secreted by cultured splenocytes were measured using commercial enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). The resulting colorimetric change (optical density, OD) was measured in a microplate reader at 450 nm.

2.6. Detection of reactive oxidative species (ROS)

Intracellular ROS in splenocyte was analyzed using the fluorescent probe DCFH-DA, a nonfluorescent compound under normal condition, which is converted into highly fluorescent dichlorofluorescein (DCF) by

cellular peroxides. Splenocytes from *P.y*17XL infected mice were collected and washed twice with medium without serum. Subsequently, 5 μM (final concentration) H2DCFDA (Beyotime, China) in methanol was added to splenocytes for ROS estimation. Samples were incubated for 60 min at 37 °C. Cell-associated fluorescence was monitored on FACS Calibur cytofluorometer with the excitation wavelength at 485 nm and emission wavelength at 530 nm. Data was analyzed by "CellQuest Pro" software, and the differences in the mean fluorescence intensity (MFI) were calculated.

2.7. Statistical analysis

Data were presented as the mean ± standard error of the mean (SEM). The statistical significance of each dataset was analyzed by one-way ANOVA or *t*-test, as appropriate. Time-to-event data were statistically analyzed with the Kaplan–Meier (K–M) approach to survival analysis (SPSS 17.0). *P*-values were calibrated using Bonferroni correction and were considered statistically significant if they were < 0.05.

3. Results

3.1. Vitamin C improves the course of P. yoelii 17XL infection in BALB/c mice

We monitored the effects of two different vitamin C dosages on the level of parasitemia and the survival rate (Fig. 1A and B) in *P. yoelii* 17XL-infected BALB/c mice. In the present study, BALB/c mice were infected with *P. yoelii* 17XL, which was shown to be highly virulent (parasitemia: 41%) in most subjects. As expected, parasitemia only reached its highest value at 34.2% and 36% in mice receiving 25 mg/kg/day, 250 mg/kg/day vitamin C treatment, respectively (Fig. 1A); All mice died 13 and 11 days p.i. (Fig. 1B). That said, *P. yoelii* 17XL-infected mice treated with either dose of vitamin C developed only a moderate parasitemia and extended the life time in *P. yoelii* 17XL-infected mice that was significantly lower compared with the control group.

3.2. Vitamin C treatment enhances the Th1 immune response during the early stages of P. yoelii 17XL infection in BALB/c mice

The effect of vitamin C supplementation on the induction of the immune response was investigated in *P. yoelii* 17XL-infected BALB/c mice. Both the percentage and absolute cell number of activated Th1 cells ($CD4^+$–T-bet^+–IFN-γ^+) in the whole spleens of mice from the vitamin C-treated groups were significantly higher than those of mice from the infected but untreated group in a dose-dependent manner (Fig. 2A–C). At the same time, we also quantified the percentage and absolute cell number of Mφ in the spleen for each group. Compared with the infected/untreated group, the absolute cell numbers of Mφ in the vitamin C-treated groups (25 or 250 mg/kg/day) were significantly increased on day 3 and 5 p.i., respectively ($P < 0.05$; Fig. 2D–F), but there was no statistical difference between 25 mg/kg/day and 250 mg/kg/day vitamin C-treated group. In addition, as effector molecules of immune response, cytokines and ROS production in splenocytes and were detected. The level of IFN-γ secreted in mice receiving vitamin C treatment increased on day 5 p.i in 250 mg/kg VC treatment group (Fig. 3A). However, vitamin C treatment obviously down-regulated secretion of TNF-α and IL-6 and almost reached normal levels (Fig. 3B, C). As the result of inflammatory reactions, the level of ROS production increased in infected mice, whereas vitamin C treatment (250 mg/kg) just changed as the result of that (Fig. 3D–F), but wasn't correlate with doses of vitamin C treatment.

3.3. Vitamin C treatment stimulates the activation of DCs in P. yoelii 17XL-infected BALB/c mice

We next examined the effects of vitamin C supplementation on the regulation of DC function. The percentage and absolute cell numbers of mDCs and pDCs, as well as MHCII-, CD86- expressing DCs, increased in all groups on day 5 p.i. It is noteworthy that the lower dose of vitamin C (25 mg/kg/day) treatment showed preferable results including activation and mature of DCs compared with the higher dose of vitamin C group (250 mg/kg/day); Compared with in 250 mg/kg VC treatment group, CD68-expression DCs in 25 mg/kg VC treatment group obviously was increased ($P < 0.01$; Fig. 4A–L). In addition, the expressions of TLR4 and TLR9 (Fig. 5A–C; Fig. 5D–F) on DCs in the three groups were increased on day 5 p.i., but there was no difference among the three groups.

4. Discussion

In this study, we demonstrated that vitamin C supplementation can enhance the Th1 immune response to *P. yoelii* 17XL infection in BALB/C mice. The data showed that the dose (250 mg/kg/day) of vitamin C had no obviously difference effect on protective immune response compared with the dose (25 mg/kg/day) of vitamin C. But significantly lower parasitemia was found in higher dose group than those in lower dose and control group during day 5 to 13 p.i. The biokinetic association between the vitamin C dose and immune cell prevalence underlines the specific function of vitamin C with regards to the cellular immune response. Potential regulatory mechanisms of vitamin C are correlated with an enhanced Th1 response through modification of the number and function of DCs.

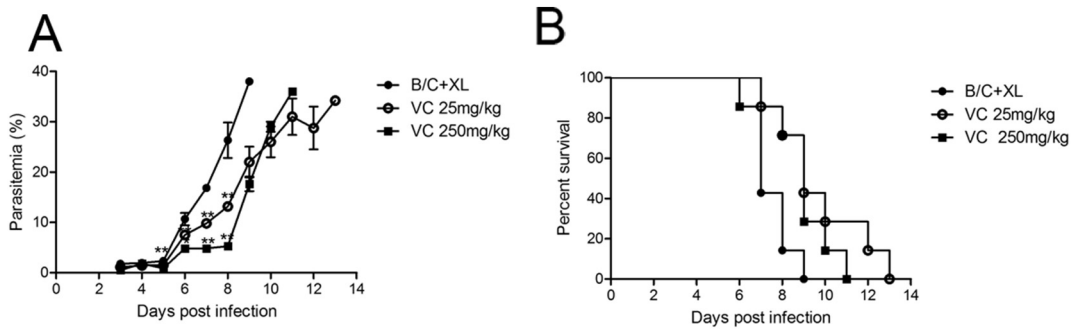

Fig. 1. The effect of vitamin C (VC) supplementation following infection with *P. yoelii* 17XL. The parasitemia (A) and survival curves (B) of *P. yoelii* 17XL infection in BALB/C mice are shown. Two groups of mice were treated with different doses of vitamin C (25 or 250 mg/kg/day), and an infected but untreated control group was established. B/C + XL: BALB/c mice infected with *P. yoelii* 17XL. VC 25 mg/kg, VC 250 mg/kg: daily vitamin C administration for four successive days beginning 2 days after *P. yoelii* 17XL infection. Results are from one representative experiment of three independent experiments. Five mice were used per group in each experiment. ** indicates $P < 0.01$.

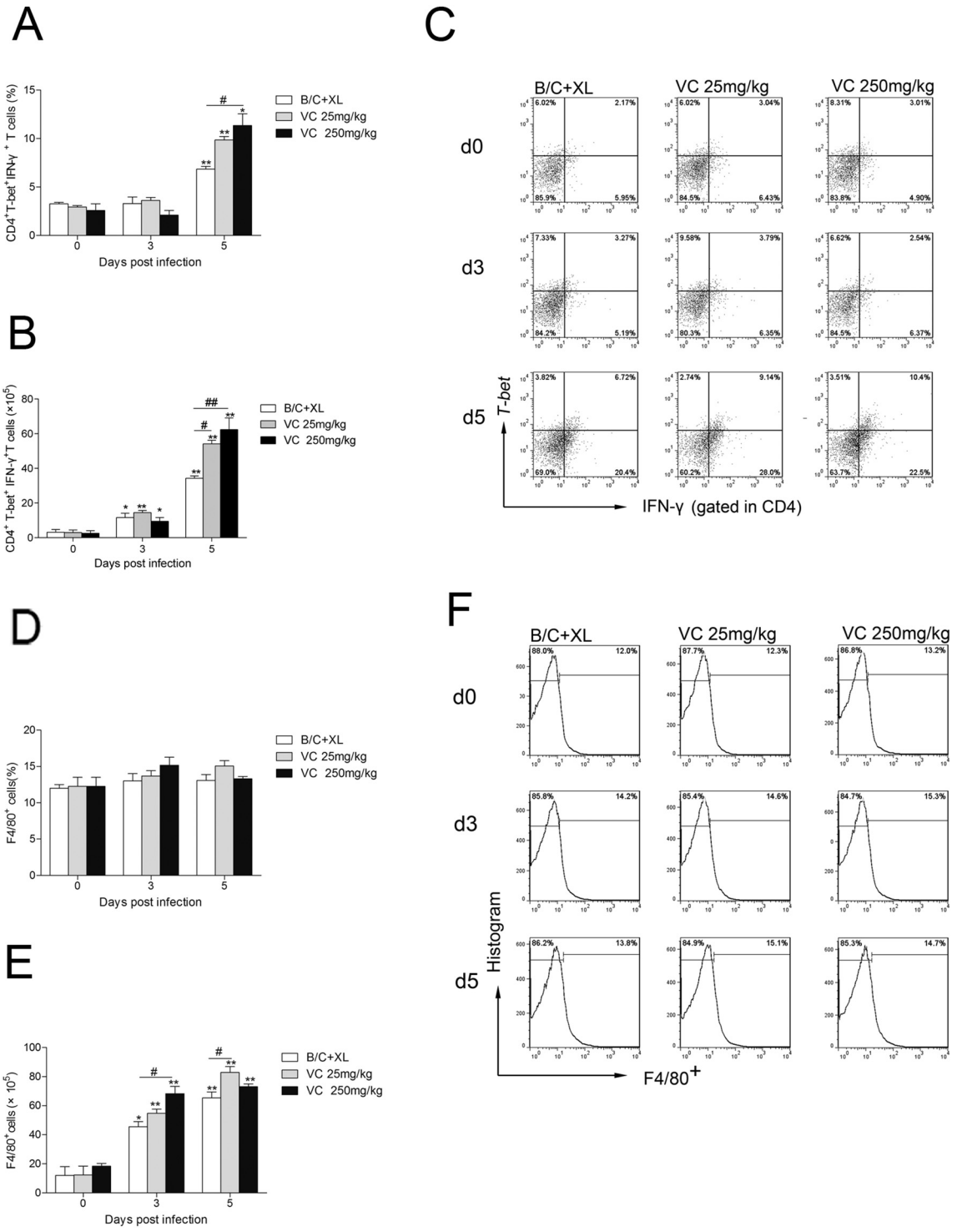

Fig. 2. Vitamin C treatment enhances the Th1 immune response. The proportion, absolute cell number and dot plot of $CD4^+$–T-bet^+–IFN-γ^+ T cells (A–C) and Mφ (D–F) were quantified by flow cytometry. Results are representatives of three independent experiments. At least three mice were used per group in each experiment. Bars represent the mean ± SEM. * indicates $P < 0.05$, and ** indicates $P < 0.01$ between the values in *P. yoelii* 17XL-infected mice and baseline levels (day 0); # indicates $P < 0.05$, and ## indicates $P < 0.01$ between the control and vitamin-C-supplemented mice. △ indicates $P < 0.05$, and △△ indicates $P < 0.01$ between vitamin-C (25 mg/kg/day) and vitamin-C (250 mg/kg/day) mice.* = Calculated value of 'p' by student's *t*-test; # and △ = Calculated value of 'p' by one-way ANON. *P*-values were calibrated using Bonferroni correction.

Nutritional deficiencies are frequent in malaria-endemic areas. In fact, 60% of malaria deaths in young children are attributable to undernutrition [25]. However, the relation between malaria infection and the nutrition condition still remains controversial. Some studies considered that vitamin E deficiencies might have protective effects [26], while vitamin A deficiencies likely exacerbate the course of infection [27]. With regard to vitamin C, it is evident that in a separate in vitro study, *P. falciparum* exposed to DHA demonstrated that development of early ring-stage parasites was rapidly interrupted, and that these parasites survived in a dormant form for up to 20 days before resuming normal growth [28,29]. In addition, Ascorbic acid has antioxidant properties and is reported to mop up free radicals. Since malaria infection imposes

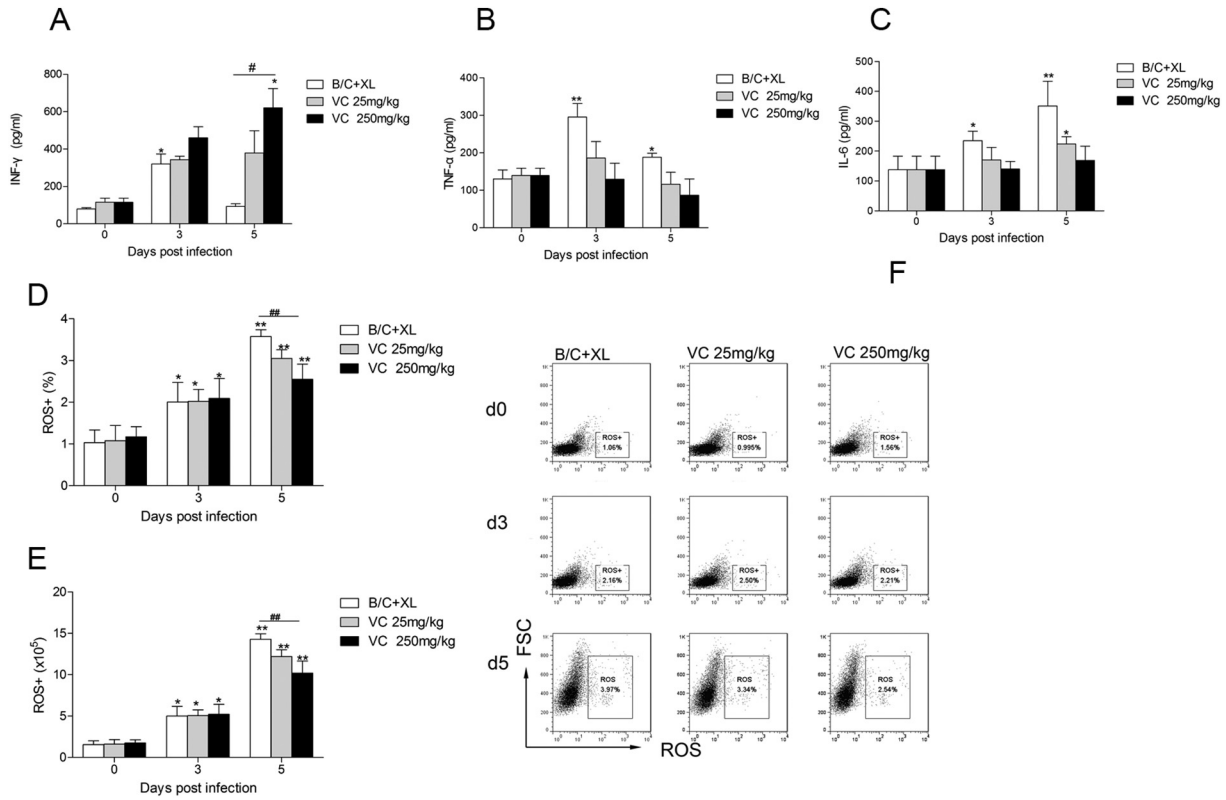

Fig. 3. Vitamin C treatment enhances the Th1 immune response. The concentrations of IFN-γ (A), TNF-α (B) and IL-6 (C) secreted by ex vivo splenocytes were determined by ELISA. The proportion, absolute cell and dot plot of ROS^+ cells were quantified by flow cytometry (D-F). Results are representatives of three independent experiments. At least three mice were used per group in each experiment. Bars represent the mean ± SEM. * indicates $P < 0.05$, and ** indicates $P < 0.01$ between the values in *P. yoelii* 17XL-infected mice and baseline levels (day 0); # indicates $P < 0.05$, and ## indicates $P < 0.01$ between the control and vitamin-C-supplemented mice; △ indicates $P < 0.05$, and △△ indicates $P < 0.01$ between vitamin-C (25 mg/kg/day) and vitamin-C (250 mg/kg/day) mice.* = Calculated value of 'p' by student's t-test; # and △ = Calculated value of 'p' by one-way ANON. P-values were calibrated using Bonferroni correction.

tremendous oxidative stress on the host, the antimalarials are often prescribed with vitamin C or similar antioxidant supplements. Vitamin C may have additional detrimental effects in malaria. Results from an experimental study have shown that concurrent administration of artemether and ascorbic acid compromised the rates of parasite clearance in *P. berghei* malaria infection in mice. This effect was more pronounced at higher doses of ascorbic acid. The high doses of vitamin C by itself could inhibit growth of malarial parasite to some extent [30,31]. Consistent with our results, in animal experiment, we found that *P. yoelii* 17XL-infected mice treated with either dose of vitamin C developed only a moderate parasitemia, which decreased to the lowest on day 6 p.i., and then began to rise. Meanwhile, the life span was just extended in *P. yoelii* 17XL-infected mice treated with high-dose vitamin C (250 mg/kg/day), but all mice still died on day 13 p.i. Vitamin C in repletion dose reduced organ failure in older studies, and recent small controlled studies suggest that vitamin C used in pharmacological doses (6–16 g/day) reduces vasopressor support, fastens recovery from organ failure, and may even reduce mortality [32]. In our studies, we chose doses of vitamin C (25 mg/kg/day, 250 mg/kg/day) total belong to pharmacological doses, and we found that pharmacological doses of vitamin C treatment obtained a better immune-modulating molecule, no concentration dependence.

Vitamin C is an essential antioxidant scavenging free radicals and enzymatic co-factor for physiological reactions such as hormone production, collagen synthesis and immune potentiation [33–36]. T cells are one of the main players in acquired immunity and have been reported to be influenced by in vivo vitamin C supplementation [37]. Many reports also considered that mega-dose vitamin C administration shifted the overall immune response towards Th1 [38]. In vitro vitamin C–treated DCs rendered the immune response towards Th1 [39]. In addition, a previous study has demonstrated that the resolution of malarial infection depends on the effective establishment of a Th1 immune response [40]. In the early stage of infection Th1 immune responses can control the proliferation of *Plasmodium* parasites by enhancing $CD4^+$ T cell activity and the phagocytic activity of Mφ through the production of TNF-α and nitric oxide [6–10]. Interesting, in our experimental animal models, results have been consistently similar as others have reported. Our data showed that the numbers of activated Th1 cells in the two vitamin-C-treated groups were higher than in infected/untreated mice on day 5 p.i. At the same time, we also found that the numbers of Mφ increased in vitamin C treatment groups on day 3 p.i. and day 5 p.i., respectively, compared with the mice in the infected/untreated group; however, the increases in Mφ numbers were not proportional to the increased numbers of Th1 cells. These results indicated that a Th1-skewed immune response can be effectively established in vitamin C-treated mice. As an important cytokine in Th1 immune responses, the level of IFN-γ production obviously induced by vitamin C treatment in *P. yoelii* 17XL-infected mice. However, it is worth noting that the levels of TNF-α and IL-6 were significantly lower in splenocytes following vitamin C supplementation at both dosage levels compared to control mice. Other researchers have found that high serum levels of TNF-α and IL-6 are associated with disease severity [39,40]. *Plasmodium* stimulates a number of signaling pathways including NF-κB, and upregulate the transcription of the mRNA of inflammatory cytokines such as IL-6, TNF-α, and IL-1. TNF-α and IL-1 in turn can activate transcription factors to synthesize IL-6 [41]. In addition, IL-6 constitutes a key regulator of $CD4^+$T cell differentiation. It maintains the balance between Th1 and Th2 effector functions, inhibiting Th1 differentiation through interfering with IFN-γ production, and promoting Th2 differentiation [42]. It is an exciting that vitamin C

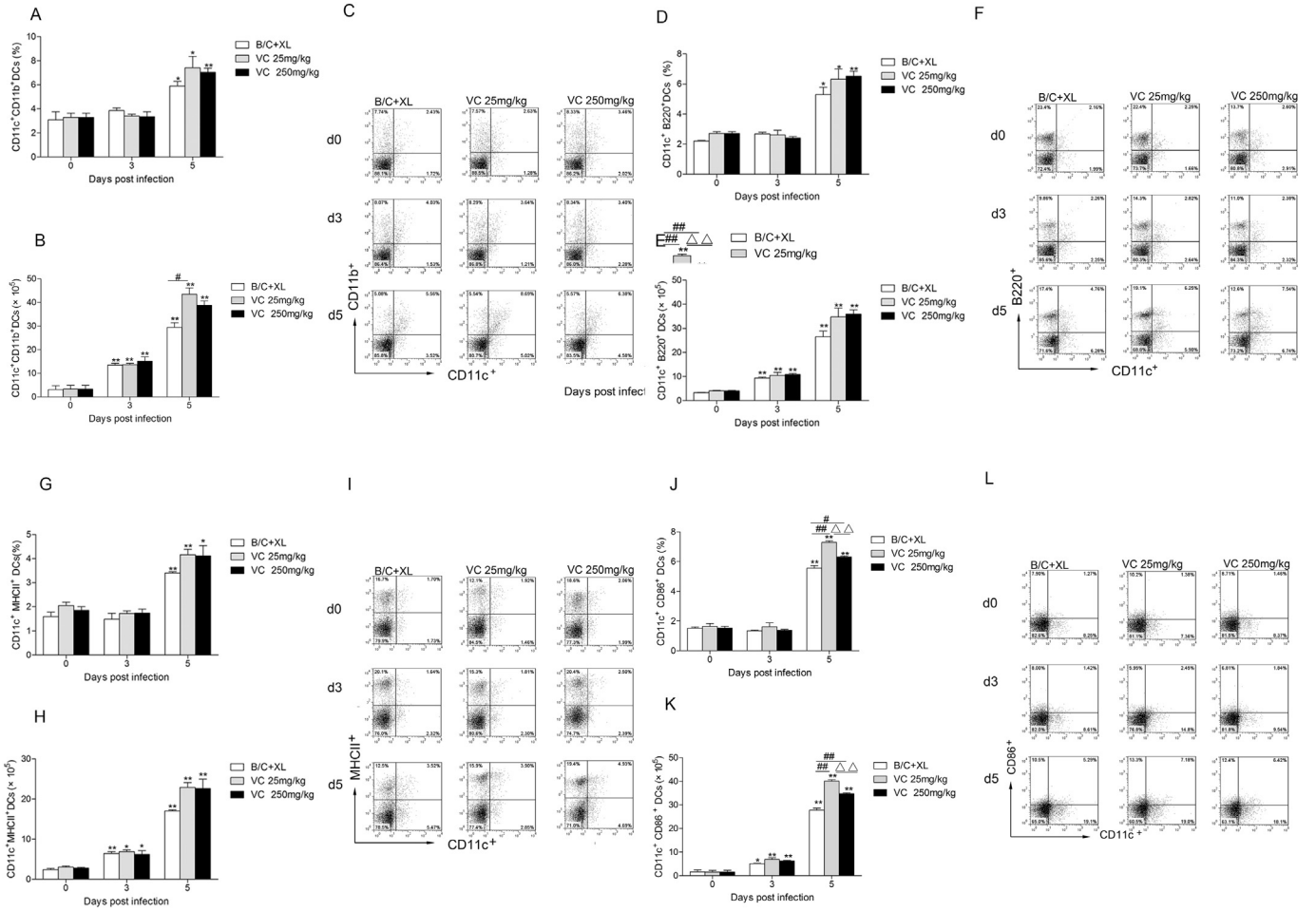

Fig. 4. Vitamin C treatment enhanced the proliferation and activity of DCs. The data represent the proportions, absolute number cell and dot plot of splenic mDCs (A–C) and pDCs (D–F), and the expressions of MHCII (G–I) and CD86 (J–L) in $CD11c^+$ DCs. Results are representatives of three independent experiments. At least three mice were used per group in each experiment. Bars represent the mean values ± SEM. * indicates $P < 0.05$, and ** indicates $P < 0.01$ between the values in *P. yoelii* 17XL-infected mice and the baseline levels (day 0); # indicates $P < 0.05$, and ## indicates $P < 0.01$ between infected/untreated and vitamin-C-supplemented groups of mice; △ indicates $P < 0.05$, and △△ indicates $P < 0.01$ between vitamin-C (25 mg/kg/day) and vitamin-C (250 mg/kg/day) mice.* = Calculated value of 'p' by student's t-test; # and △ = Calculated value of 'p' by one-way ANON. P-values were calibrated using Bonferroni correction.

supplement just down-regulated the secretion of IL-6 and TNF-α, but up-regulated the secretion of IFN-γ, the percentage and activation of DCs and Mφs. Our results demonstrated the beneficial effect of Vitamin C in establishment of Th1 immune response during *P. yoelii* 17XL infection, which was correlated with IFN-γ, TNF-α and IL-6 levels.

In this paper, we have to mention "ROS". Among all antioxidants, ascorbate (reduced vitamin C) is considered to be the most effective water-soluble antioxidant [43]. During the Th1-type response, IFN-γ is probably the most important trigger for high ROS production in Mφs [44]. It appears that high and rapid production of ROS during the acute phase of infection may be beneficial in clearing parasitemia. In contrast, excessive production of ROS can exacerbate malaria pathology such as anemia [45]. In addition, because ROS are formed during T-cell activation and act as a second messenger [46,47], it is could be possible that vitamin C affects T-cell behaviors during activation as an antioxidant. Our results showed that the dynamic of ROS production. Our results showed that the level of ROS production obviously increased on day 3 and 5 p.i in *P. yoelii* 17XL infection compared with control group. However, on day 5 p.i the level of ROS production in 250 mg/kg VC treatment group were lower than those in normal infection group, but higher than those in normal control group. Our results exactly clarified that timely and moderate of ROS production in malaria has a crucial role, which regulates the shift from innate to adaptive immune response or affected the outcome of *plasmodium* infection.

DCs provide a critical link between the innate and adaptive immune responses and are central to T cell activation and differentiation. Kim et al. found through in vitro studies that vitamin C can up-regulate MHCII and CD86 molecules on DCs through the activation of p38 mitogen-activated protein kinases (MAPK) [48]. Consistently, this phenomenon was also observed in animal experiments. Our data showed that vitamin C treatment increased the number of mDCs and pDCs compared with the control group and in 25 mg/kg VC treatment group obviously higher than that of *P. yoelii* 17XL infection group on day 5 p.i.. An increase in expression of MHCII and co-stimulatory molecules has been shown to be a feature of DC maturation [49]. Spaulding et al. describe a novel mechanism of pDC activation in vivo and precise stepwise cell/cell interactions taking place during severe malaria that contribute to immune cell activation and inflammation, and subsequent disease outcomes [50]. In addition, this study found increased expression of MHCII and CD86 in DCs for the mice treated with vitamin C, suggesting enhanced DC maturation and activity, which is essential for the establishment of protective Th1 immunity and a strong immune response [51,52]. TLRs are known to be critical for initiating innate immune responses, but their role in the development of protective immunity to malaria remains poorly understood. Gowda et al found that TLR9 and MyD88 are crucial to this process [53]. However, other studies have concluded that the enhanced expression of TLR4, but not of TLR9, is associated with more complicated malarial disease [54].

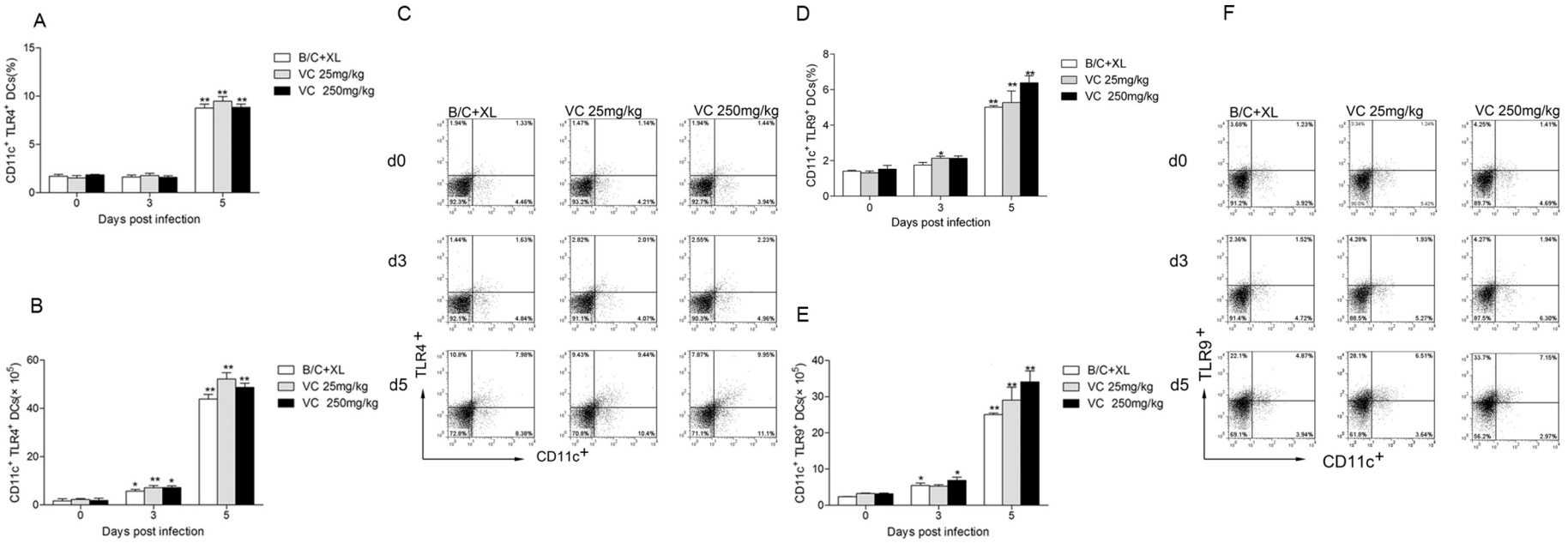

Fig. 5. The proportions, absolute number cell and dot plot of splenic CD11c$^+$ DCs expressing TLR4 (A–C) and TLR9 (D–F). Results are representatives of three independent experiments. At least three mice were used per group in each experiment. Bars represent the mean values ± SEM. * indicates $P < 0.05$, and ** indicates $P < 0.01$ between the values in *P. yoelii* 17XL-infected mice and the baseline levels (day 0); # indicates $P < 0.05$ between infected/untreated and vitamin-C-supplemented groups of mice; △ indicates $P < 0.05$, and △△ indicates $P < 0.01$ between vitamin-C (25 mg/kg/day) and vitamin-C (250 mg/kg/day) mice.* = Calculated value of 'p' by student's t-test; # and △ = Calculated value of 'p' by one-way ANON. P-values were calibrated using Bonferroni correction.

Arama et al. demonstrated that the altered activation status of APC subsets and strongly inhibited TLR responses in peripheral blood may be the reason why children possess higher susceptibility to *P. falciparum* infection. These findings suggest that DCs and TLR signaling may both be important factors in generating a protective immune response to malaria [55]. Here, our data demonstrated that vitamin C supplementation can induce a Th1 protective immune response that correlated with the activation and function of DCs not via TLR4 or TLR9.

In contrast, the activity of the immune system can affect nutrient status, as can be observed in immune diseases, including acquired immunodeficiency syndrome (AIDS) [56–58] and rheumatoid arthritis [59–61]. Accordingly, dietary factors have become increasingly important in the potential treatment of immunological inflammatory diseases [62]. Some experiments considered that vitamin C exerted toxic effects, at least in vitro, when the concentration was high and when it was given before T cell activation. These toxic effects are not thought to be via anti-oxidant effects of vitamin C [37]. Thus, it is important to human being be supplemented with defined daily dose of vitamin C for their lives. The daily requirement of vitamin C is 75–90 mg [63], and many investigators insist beneficial effects of even more dose, up to 1–10 g/day, defined as mega-dose [64,65]. Vitamin C appears to be able to both prevent and treat respiratory and systemic infections. Prophylactic prevention of infection requires dietary vitamin C intakes that provide at least adequate, if not saturating plasma levels (i.e., 100–200 mg/day), which optimize cell and tissue levels. In contrast, treatment of established infections requires significantly higher (gram) doses of the vitamin to compensate for the increased inflammatory response and metabolic demand [66]. At present, we chose two doses of vitamin C (25 and 250 mg/kg) in BALB/c mice. Since vitamin C (250 mg/kg/day) is too high concentration, we didn't observe any side-effects in our experiments. If vitamin C does indeed benefit critically ill patients, there still remain many unanswered questions, such as should we provide repletion or pharmacological doses? Because pharmacological doses are possibly needed to optimize the antioxidant effects of vitamin C, the clinical effect of these doses might be stronger. However, large studies on side effects are not available [32].

4.1. Concluding remark

Vitamin C regulated the protective immune response to *P. yoelii* 17XL infection in BALB/c mice. We highlighted that vitamin C could enhance Th1 immune responses and DC activity during *P. yoelii* 17XL infection in mice, but there was obviously difference between 25 mg/kg/day and 250 mg/kg/day vitamin C treatment group. However, to physiological doses of vitamin C supplement, the vitamin C concentration may be vitally important for the host as, if all essential nutrients are supplied in appropriate amounts, the biochemical and cellular mechanisms that provide the basis of the immune system can work optimally [67]. Older studies showed less organ dysfunction when vitamin C was administered in repletion dose (2–3 g intravenous vitamin C/day). Recent small controlled studies using pharmacological doses (6–16 g/day) suggest that vitamin C reduces vasopressor support and organ dysfunction, and may even decrease mortality [68]. Therefore, it is not surprising that the immunocompetence of an organism is significantly influenced by its intake of micronutrients (vitamins, minerals, and certain fatty acids) [69–71]. As the doses of vitamin C tested here have been proven to be safe, our study provides additional evidence for the potential use of vitamin C to enhance host immunity against malarial infection in malaria-endemic regions.

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