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Early Treatment with Chloroquine Inhibits the Immune Response against *Plasmodium yoelii* Infection in Mice

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Chloroquine (CQ), a well-known anti-malarial drug, has long been used for the treatment of autoimmune diseases because of its profound immunomodulatory effects. However, whether this drug modifies anti-malaria immune response is still not clear. Here we studied the immunomodulatory role of CQ in a mouse model of malaria. DBA/2 mice were infected with *Plasmodium yoelii* (*Py*) parasite (intraperitoneal injection of parasitized erythrocytes) and divided into three groups. Two groups received single dose of CQ (gavage administration) at 6 hours after *Py* infection (post-6h) and 3 days after *Py* infection (post-3d), respectively. The third group received saline as control. The course of disease was monitored and the changes of immune response were investigated. It is shown that mice from the post-6h group took longer time to clear the parasites compared with those of the post-3d group. The activation of T helper cells, macrophages, and B cells was significantly suppressed in mice with post-6h CQ treatment as compared with control mice on day 3 and day 5 after infection. In contrast, no such changes were found in mice from the post-3d group. Dendritic cells (DCs) from the post-6h CQ treated mice were less mature as compared with those from control mice as well as those from the post-3d group. Taken together, our data suggest that treatment with CQ early in infection inhibits protective immune response against *Py* infection possibly via mechanisms involving the modulation of DC's function. Our finding provided important information for reasonable use of CQ in malaria chemotherapy.

Keywords: chloroquine; early infection; immune response; immune suppression; *Plasmodium yoelii* 17XL
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Introduction

Malaria remains one of the most prevalent and deadly infectious diseases affecting humans, with over 200 million documented cases worldwide and more than 655,000 deaths per year (World Health Organization 2011). Until recently, there has been no efficacious vaccine for malaria; thus, the use of anti-malarial drugs is still one of the primary methods of controlling this disease. Chloroquine (CQ), synthesized by Hans Andersag in 1934 (Slater 1993), was once the first drug of choice for malaria treatment because it was safe, effective, well-tolerated, and inexpensive (Baird 2004; Vijaykadga et al. 2004; Srivastava et al. 2008; Price et al. 2009; Awab et al. 2010). The extensive use of CQ in malarial epidemic areas was largely reduced since *Plasmodium falciparum* chloroquine resistance was discovered in the late 1950s (Harinasuta et al. 1965). However, CQ is still the drug of choice in areas with *Plasmodium vivax* malaria infection and other areas where CQ remains effective (Baird 2009). In recent years, due to the discovery of agents that can reverse CQ-resistance as well as reports of recovery of CQ sensitivity after its withdrawal, CQ is expected to be reused as first-line antimalarial drug for its unparalleled advantages (Kublin et al. 2003; Mita et al. 2003; Mwai et al. 2009; Kamugisha et al. 2012).

There are several theories as to the mechanism(s) by which CQ conveys antimalarial action. The most accepted one is that CQ interferes with the detoxification processes of malaria parasites within the digestive vacuole. During the erythrocyte stage, malaria parasites utilize host hemo-

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globin for their growth and release heme into the digestive vacuole, because heme is toxic to the parasite. For survival, malaria parasites convert heme into a nontoxic crystalline polymer called hemozoin. This process is blocked by CQ when it accumulates in the digestive vacuole, thus leading to the accumulation of toxic metabolites and subsequent damage or death of the parasite (Thome et al. 2013).

In addition to its anti-malarial action, CQ was found to have immunomodulating effects and has been used for the treatment of autoimmune diseases like rheumatoid arthritis for decades (Meier et al. 2013). The mechanisms involved in CQ immunomodulation have not been fully elucidated; however, current knowledge indicates that CQ exerts its function through both pH-dependent and -independent means. As a weak lipophilic base, CQ enters the lysosome of antigen-presenting cells (APCs) and raises the pH level. A high pH environment affects the function of most lysosomal enzymes and impairs the antigen processing and presentation functions of APCs (Thome et al. 2013). Moreover, it has been found that CQ suppresses intracellular Toll-like receptor (TLR) signaling, especially CpG-induced TLR9 activation (Thome et al. 2014). This effect has been attributed to the inhibition of endosomal acidification by CQ; however, there is evidence that suggests that the interaction between CpG DNA and TLR9 is blocked due to direct binding of CQ to bacterial nucleic acids (Yi et al. 1998). Furthermore, CQ has been proven to prevent lipopolysaccharide-induced pro-inflammatory cytokine secretion via a nonlysosomotropic mechanism (Weber and Levitz 2000).

The course and outcome of malarial disease depends on the complex interaction between malaria parasites and the host immune response. Studies form both human malaria and the mouse model indicate that protective immunity against blood-stage *Plasmodium* infection requires activation of malaria-specific $CD4^+$ T cells and the production of neutralizing antibodies (Stephens et al. 2005). Dendritic cells (DCs) play a critical role in initiating adaptive immunity and shaping the type of T helper (Th) response. Upon infection, DCs internalize *Plasmodium*-infected RBC and are able to process and present malaria antigens. Activated DCs produce cytokines that contribute to the polarization of the CD4 T-cell response (Steinman and Hemmi 2006; Manicassamy and Pulendran 2009). Evidence shows that DC activation involves the engagement of TLR4 and TLR9 by parasite-derived ligands; for example, a parasite protein-DNA complex was reported to be responsible for the induction of inflammatory cytokine responses in DCs (Coban et al. 2005; Wu et al. 2010).

These data suggest that CQ may have a substantial impact on the induction of protective immunity against *Plasmodium* infection when used as anti-malarial agent for malaria treatment and prophylaxis, yet little is known about it. Herein, we investigated the effects of CQ on the immune response to *Plasmodium* infection employing the mouse model of blood-stage malaria. We found that single dose of CQ administered soon after *Plasmodium yoelii* (*Py*) infection suppressed host cellular and humoral immune responses. This effect was associated with poor maturation of DCs. Our data provide evidence for the immunoregulatory role of CQ in malaria, which is critical when considering the use of CQ for malaria treatment and prophylaxis.

Materials and Methods

Mice, parasite, and Py 17XL infection

Female 6- to 8-week-old DBA/2 mice were purchased from the Beijing Animal Institute. Infection was initiated by intraperitoneal (i.p.) injection of 1×10^6 *Py* 17XL parasitized red blood cells per mouse. Mice used in each experiment were matched for age and sex. Parasitemia was monitored by light microscope examination of Giemsa-stained blood smears. Parasitemia was calculated by counting the number of parasite-infected erythrocytes per 1,000 erythrocytes. Mortality was checked daily. All experiments were performed in compliance with local animal ethics committee requirements and approved by the Institutional Animal Ethics Committee in China Medical University.

CQ treatment

CQ was purchased from Shanghai Zhongxin Pharmaceuticals and dissolved in normal saline before use. DBA/2 mice were divided into three groups: the mice in the control group received saline, while the mice in post-6h and post-3d groups received gavage administration of 50 mg/kg of CQ at 6 h after and 3 d after *Py* 17XL infection, respectively.

Splenocyte culture

Splenocyte culture was performed as previously described (Su and Stevenson 2002). Briefly, mouse spleens were removed aseptically and pressed through a sterile fine-wire mesh with 10 ml 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 centrifuging at $350 \times g$ for 10 min. Erythrocytes were lysed with cold 0.17 M NH_4Cl and cells were washed twice with fresh medium. The viability of cells was determined by Trypan Blue exclusion assay and was > 90%. Aliquots (500 μl/well) of the cell suspensions (1×10^7/ml) were incubated in 24-well flat-bottom tissue culture plates 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 and nitric oxide (NO) analysis.

Flow cytometry analysis

To assess DCs, splenocytes collected from mice at different time points post infection (p.i.) were blocked with anti-CD16/32 (FcγIII/II receptor; Clone 2.4G2; BD Biosciences) and then double-stained with FITC-conjugated CD11c mAb (Clone HL3; BD Biosciences), PE-conjugated anti-CD11b (Clone M1/70; BD Biosciences), or PerCP-conjugated anti-CD45R/B220 mAb (Clone RA3-6B2; BD Biosciences) and PE-conjugated anti-MHC II mAb (clone M5/114.15.2; ebioscience). To assess the expression of TLR9 in $CD11c^+$ DCs, after fixation and permeabilization, cells were incubated with biotinylated anti-TLR9 mAb (clone 5G5; Hycult biotech) followed by PE-conjugated streptavidin (BioLegend, San Diego, CA, USA).

To assess $CD4^+$ T cell activation, splenocytes collected from

mice at different time points p.i. were double-stained with FITC-conjugated anti-CD4 (clone H1.2F3; BD Biosciences) and anti-CD69 (clone H1.2F3; BD Biosciences). To analyze macrophages, the splenocytes were stained with FITC-conjugated anti-F4/80 (clone BM8; ebioscience). To measure IgG-secreting B cells, the cells were first surface stained with PerCP-conjugated anti-CD45R/B220 (clone RA3-6B2; BD Biosciences); then they were fixed, permeabilized, and stained with FITC-conjugated anti-IgG (BD Biosciences). After staining, cells were then washed twice with PBS containing 1% FCS and suspended in 300 μl of PBS. Data were analyzed in FACSCalibur flow cytometer using CellQuest software.

Quantification of cytokines and NO production

Levels of IFN-γ, TNF-α, and IL-4 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). To determine NO production, concentrations of NO_2^- in cell supernatants were measured using the Griess reaction.

Statistical analysis

Statistical significance of the differences was analyzed using the Students' *t*-test (SPSS 17.0). A value of $P < 0.05$ was considered significant.

Results

CQ treatment influences the infection course in DBA/2 mice infected with Py 17XL

The usual course for prophylaxis of malaria with CQ covers the full length of exposure plus four additional weeks while the duration of treatment is three to seven days (Amet et al. 2013). To assess the effects of CQ on the anti-malarial immune response, we utilized a single dose of CQ that was not adequate to cure the infection and used it in *Py* 17XL-infected DBA2 mice, who can ordinarily clear the infection without exogenous treatment (Moore et al. 2011). CQ was given to the mice at six hours p.i. (post-6h) and at three days p.i. (post-3d), respectively, to determine the timeframe of its action. As shown in Fig. 1A, parasitized red blood cells could be seen in the peripheral blood on day 3 p.i. at similar levels in all three groups of mice, either with or without CQ treatment. Mice in the control group (treatment with saline) developed a moderate level of parasitemia with a peak value of 29.69 ± 2.77% of erythrocytes infected on day 17 p.i.; the entire course of disease was about 23 days. At the same time, the two groups of mice treated with CQ (post-6h and post-3d) had lower levels of parasitemia and shorter courses of infection, which likely relates to the anti-malarial effect of CQ. However, the post-6h group took longer to recover from the infection than the post-3d group and had a higher rate of erythrocyte infection (20.91 ± 2.64% vs. 16.85 ± 2.54%, respectively). This result indicates that post-6h CQ administration is less effective compared with post-3d application in the treatment of *Py* 17XL infection.

CQ treatment inhibits host cellular immunity

To determine whether there was difference in the protective immune response generated against *Py* 17XL infection between mice given CQ at 6 h p.i. and on 3 d p.i., we quantified the population of activated ($CD4^+CD69^+$) T cells and cytokine production in spleen from mice in each group. CD69 is one of the earliest cell surface antigens expressed by T cells following activation (Sancho et al. 2005). $CD4^+$ T cells from the post-6h group showed a significantly reduced expression of CD69 on day 3 and day 5 p.i. as compared with the control group (Fig. 1B-D). Splenocyte production of pro-inflammatory cytokines such as TNF-α and IFN-γ also reduced in the post- 6h group on day 3 and 5 p.i. (Fig. 1E, F). No such changes were observed in the post-3d group. These data indicate that that there were fewer activated $CD4^+$ T cells and less pro-inflammatory cytokine production in the spleen when CQ treatment was initiated early after infection.

We next assessed the number and function of macrophages that related to pro-inflammatory reaction in the spleen of *Py* 17XL-infected mice. We found that mice from the post-6h group had significantly less $F4/80^+$ macrophages (Fig. 2A-C) in the spleen on day 3 and 5 p.i. We also measured the level of NO in the culture supernatant of splenocytes; NO is produced in large amounts by macrophages after activation and contributes to the killing of parasites (Ahvazi et al. 1995). As expected, splenocytes from mice in the post-6h group had less NO production than that from the control group (Fig. 2D). Thus, mice treated with CQ early after malaria infection exhibit less macrophage activation in the spleen.

The data reported above demonstrate that treatment with CQ soon after malaria infection resulted in suppressed cellular immunity during *Py* 17XL infection. This effect was not likely secondary to the difference in parasitemia, as levels of infection were comparable in all groups on day 3 p.i. (see Fig. 1A).

CQ treatment inhibits host humoral immunity

To investigate whether there is any effect of CQ treatment on humoral immunity against the *Plasmodium* parasite, we analyzed the number of $B220^+IgG^+$ B cells in splenocytes by flow cytometry. The secretion of IgG by B cells depends on Th2 cytokines, such as IL-4. Therefore, the level of this cytokine was also evaluated by collecting supernatants from cultured splenocytes. We found that the percentage (Fig. 3A, B) and the absolute number (Fig. 3C) of IgG-secreting B cells and the level of IL-4 (Fig. 3D) was significantly reduced in the post-6h group on day 8 p.i. compared with the control group, but no significant difference between post-3d CQ and the control group was observed. This result suggests that early treatment with CQ has a suppressive effect on the induction of the Th2 response and the subsequent humoral immunity in *Py* 17XL-infected mice.

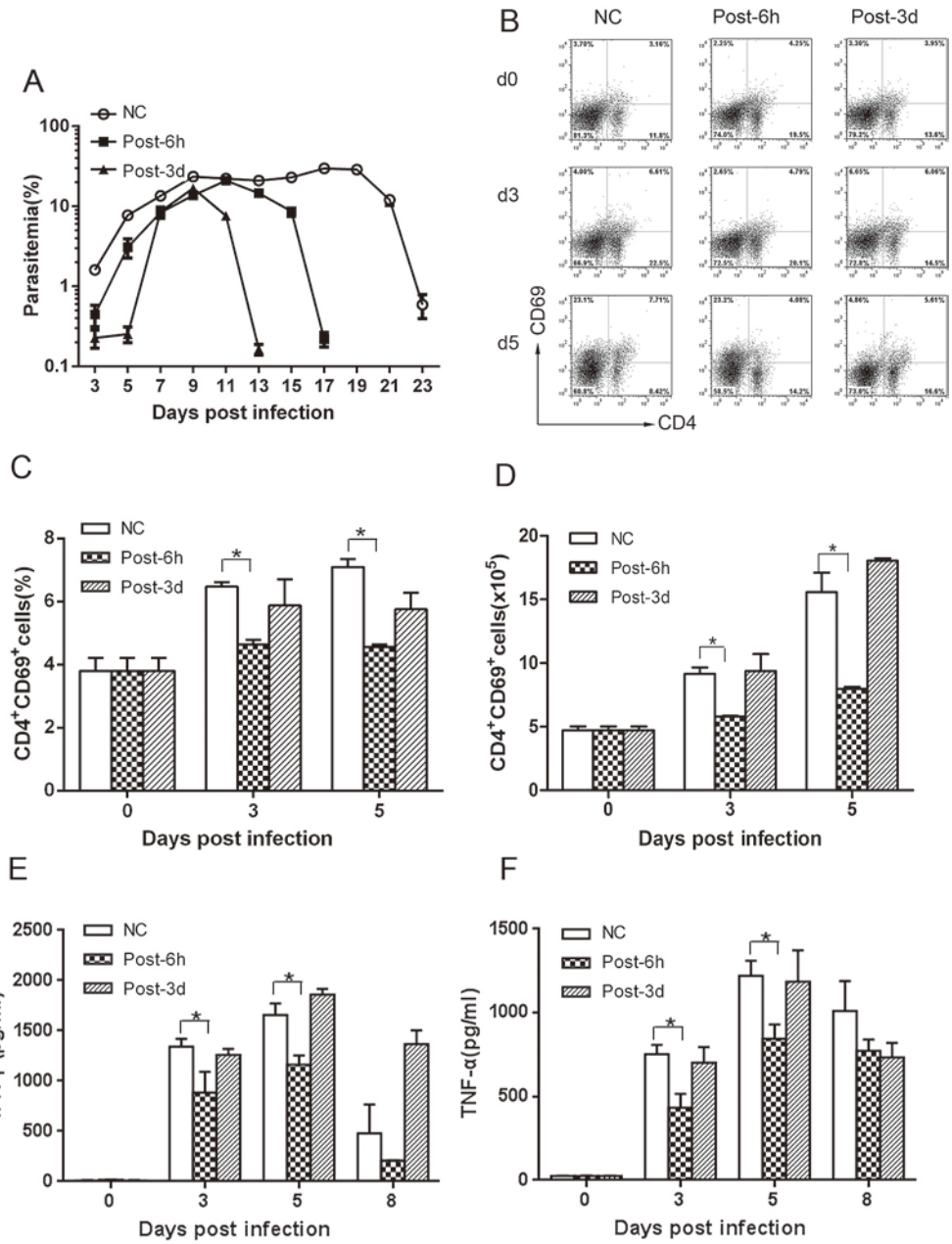

Fig. 1. The effects of CQ treatment on host infection course and cellular immunity. Mice were infected with *Py* 17XL and administrated with 50 mg/kg of CQ at indicated time point after infection. The NC group received normal saline instead of CQ. The infection rate of red blood cells (parasitemia) was monitored via tail vein blood smear (A). The expression of CD69 on splenic T cells was examined by flow cytometry and displayed in dot plot (B). C and D are the statistical graph showing the percentage and the absolute number of $CD4^+CD69^+$ T cells in spleen. Concentrations of cytokine IFN-γ (E) and TNF-α (F) in the culture supernatant of spleen cells were determined by ELISA. Data are presented as mean ± standard error and each group contains at least three mice. Asterisk (*) indicates a statistically significant difference ($P < 0.05$) between the control group and the CQ treatment group. All experiments are repeated more than three times. NC, control group; post-6h, CQ treatment at 6 h after *Py* 17XL infection; post-3d, CQ treatment at 3 d after *Py* 17XL infection.

CQ treatment inhibits activation and maturation of DCs

It is known that CQ inhibits the antigen processing and presentation by DCs, which is a prerequisite for the activation of T lymphocytes (Wykes and Good 2008; Zhu et al. 2012). To clarify whether the decreased cellular and humoral immunity in the post-6h group of CQ treated mice was associated with the hindered maturation and activation of DCs, we assessed the number and phenotype of DC subsets in the spleen. As shown in Fig. 4A-F, *Py* 17XL infection resulted in significant expansion of both $CD11c^+CD11b^+$ DCs (myeloid DC, mDC) and $CD11c^+B220^+$ DCs (plasmatocytoid DC, pDC), in the spleen from control

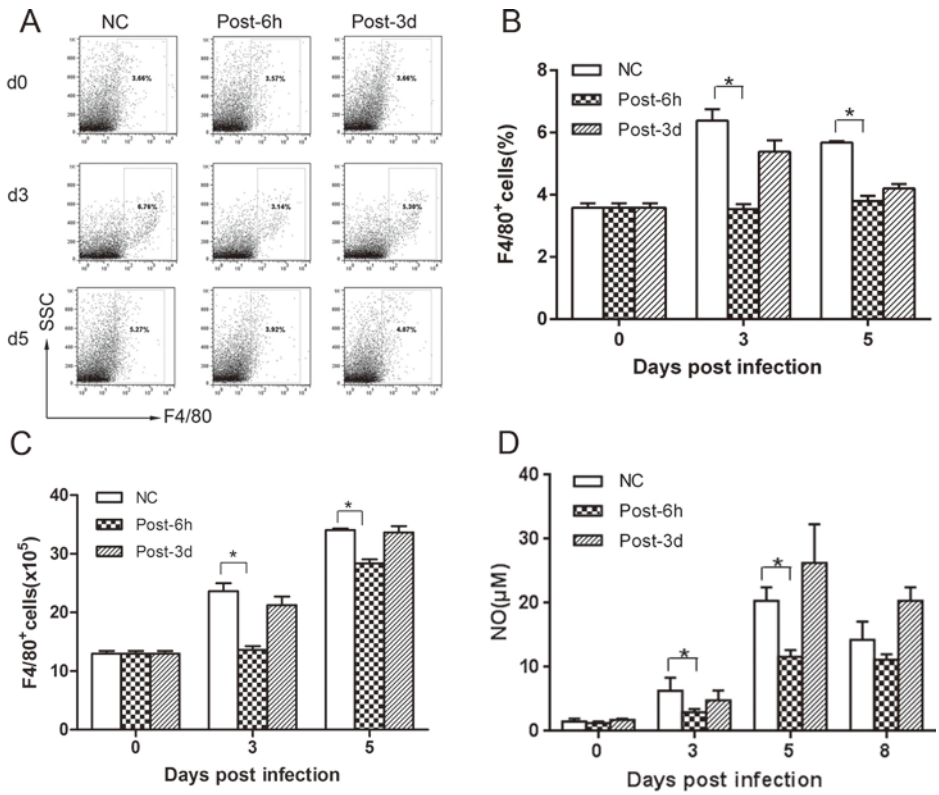

Fig. 2. The effect of CQ treatment on macrophage functions during *Py* 17XL infection. The population of F4/80$^+$ cell in splenocytes was analyzed by flow cytometry and displayed in dot plot (A). Their percentage and absolute number were shown in B and C. The level of NO_2^- in the supernatants of cultured splenocytes from infected mice was determined by Griess reaction. Values represent the mean ± standard error (n = 3 mice/group). Asterisk (*) indicates a statistically significant difference ($P < 0.05$) between the control group and CQ treatment group. The representative data out of three independent experiments with similar results are shown.

mice. However, mice from the post-6h group had a significant reduction in the number of pDCs on day 3 p.i. and a decline in both mDCs and pDCs on day 5 p.i. By contrast, DCs expansion in mice from the post-3d group resembled that of control mice.

Mature DCs exhibit increased expression of major histocompatibility complex (MHC) molecules and co-stimulatory molecules. Fully mature and activated DCs can be identified by the expression of very high levels of MHC molecules II (Reis e Sousa 2006). As shown in Fig. 5A-C, The percentage and the absolute number of the MHC II$^+$ CD11c$^+$ cells in splenocytes from mice of the post-6h group were significantly lower than that from mice of the control group on day 3 and 5 p.i., while there was no difference in this population of cells in mice from the post-3d group. Taken together, our data indicate that the maturation and activation of DCs is suppressed in mice that were administered CQ soon after *Py* 17XL infection, which may contribute to the limited activation of adaptive cellular and humoral immunity in those mice.

CQ decreases TLR9 expression

TLR9 is the one of the most important Toll-like receptors that mediates activation of DC during blood-stage *Plasmodium* infection. It is reported that the engagement of TLR9 with ligands is directly affected by CQ treatment (Zhu et al. 2012). To determine whether TLR9 was involved in dysfunction of DCs in our model, we checked the expression of TLR9 in DCs with intracellular staining. We found that *Py* 17XL infection significantly raised the number of TLR9$^+$CD11c$^+$ DCs in the spleen on day 5 p.i. in the control group and post-3d group (Fig. 5D-F). However, in the post-6h group, CQ treatment resulted in significantly lower TLR9 expression than that of the control group at the same time point. These data showed that early CQ treatment influences the expression of TLR9 in DCs during *Py* 17XL infection. This effect may contribute partially to the inadequate maturation of DC seen in this experiment.

Discussion

Anti-malarial drugs are prescribed to large populations for prophylaxis without screening for the presence of parasitemia (Fidock et al. 2004). It has been given even to certain high-risk groups, such as infants, children, and pregnant women. While CQ may significantly reduce mortality, but whether it will interfere with the host immune system in general or have side effects on the host immune response against malaria infection specifically is currently unknown. Here we demonstrated that a single dose of CQ soon after malaria infection (post-6h group) significantly suppresses

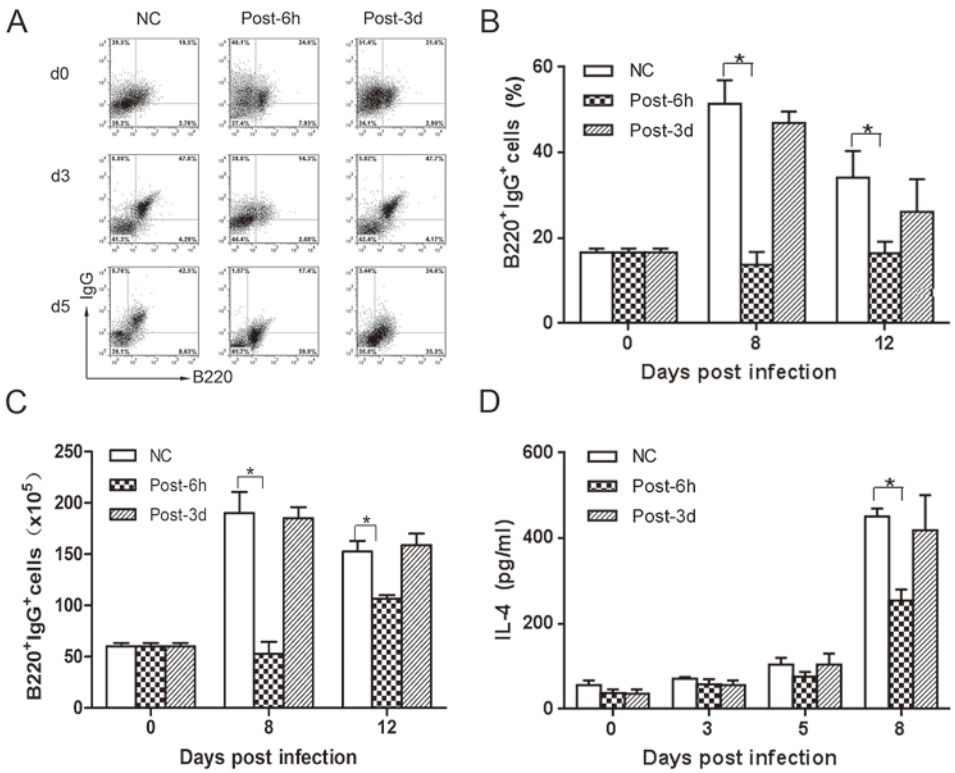

Fig. 3. The effect of CQ treatment on host humoral immunity and Th2 response. The percentage of $B220^{+}IgG^{+}$ B cells in the spleen was evaluated by flow cytometry and shown in dot plot (A) and bar graph (B). The absolute number of these cells was calculated and shown in bar graph (C). The level of IL-4 in the culture supernatant of splenocytes was detected by ELISA (D). Values represent the mean ± standard error (n = 3 mice/group). Asterisk (*) indicates a statistically significant difference ($P < 0.05$) between the control group and CQ treatment group. The results are repeatable in three independent experiments.

both cellular and humoral immunity of the host, possibly through modification of the function of DCs. Our study indicates the importance of reasonable use of CQ for the treatment and prophylaxis of malaria.

There is no doubt that CQ can not only kill malaria parasites directly, but also influence the host immune response to infection. We found in our experiment that single dose of CQ soon after malaria infection (post-6h group) did not affect the initial level of parasitemia compared to the control group, but did shorten the course of disease. This can be explained by the anti-malarial effect of CQ that acts exclusively on the intra-erythrocytic stage of malaria parasite (Yayon et al. 1984). We also found that mice given a single post-6h CQ treatment took more time to clear the infection than those treated with CQ at day 3 p.i. Although this difference could be attributed to the unequal antimalarial efficiency of CQ given at different time points of infection, we could not rule out the possibility that the immune response to infection was differentially modulated by diverse CQ treatment. This possibility was confirmed by our later finding that the protective immune response in the post-6h group of mice was suppressed compared to the control group whereas the post-3d group did not experience a similar immunosuppression. These data suggest that the timing of CQ intervention is critical for its immunomodulatory effect in the mouse malaria model. Only treatment of CQ early after infection results in inhibition of the protective immune response, which implies a potential role for CQ on the function of APCs.

As a component of innate immunity, DCs take up pathogen as soon they encounter them and then process and present the antigen; these events occur during the earliest stage of infection. It is known that CQ affects the antigen processing and presentation of DCs. This effect was repeated in our experiment, in which the expression of class II MHC molecules on DCs was reduced in mice treated with CQ 6 h p.i. We did not observe similar effects of CQ on DCs in mice in the post-3d group; we think this is because antigen processing in DCs takes place before the CQ was introduced at day 3 p.i. This result was consistent with the alterations in the adaptive immune response in this group. Moreover, the contradictory influence of CQ administrated at different time points suggests that CQ's effects on protective immunity against *Plasmodium* infection was largely due to the modulation of DC function at early stages of infection.

There are two major subpopulations of DCs in mice: mDCs and pDCs (Kapsenberg 2003). In our study, the percentage of both mDCs and pDCs was dramatically reduced in the post-6h group. It has been demonstrated that pDCs

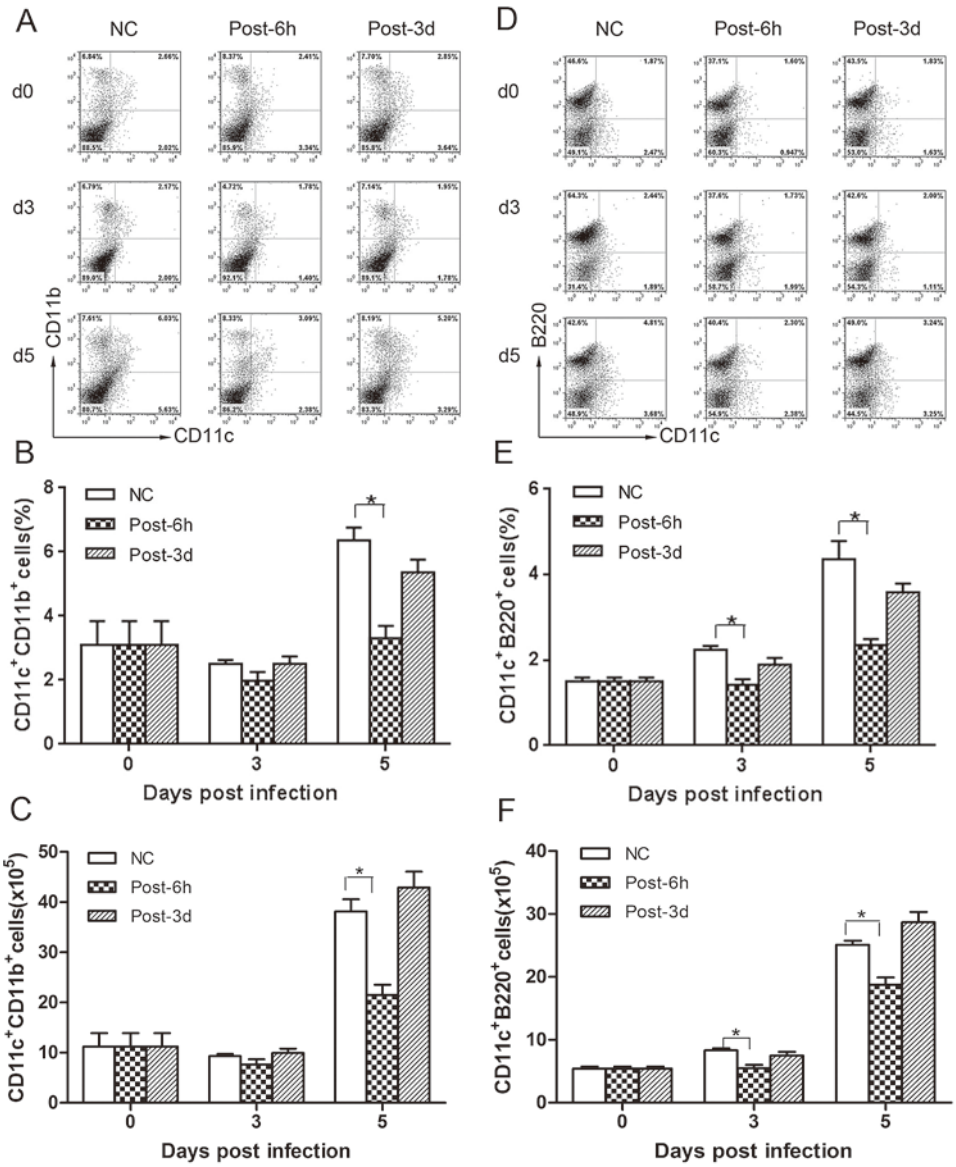

Fig. 4. The effect of CQ treatment on DC subsets during *Py* 17XL infection. The percentage of $CD11c^+CD11b^+$ DCs (A and B) and $CD11c^+B220^+$ DCs (D and E) in the spleen cells was evaluated by flow cytometry and shown in dot plot and bar graph. The absolute number of these cells was calculated and shown (C and F). Data is presented as the mean ± standard error (n = 3 mice/group). Asterisk (*) indicates a statistically significant difference ($P < 0.05$) between the control group and CQ treatment group. Results are representative of three independent experiments.

contribute to B-cell activation and immunoglobulin production through the direct cell-to-cell contact with B cells as well as via pDC-derived soluble factors (Hemmi et al. 2000; Bauer et al. 2001; Kadowaki et al. 2001; Katze et al. 2002; Poeck et al. 2004). Upon exposure to IFN-α, another DC subpopulation, mDCs, can upregulate B-cell activating factor (Litinskiy et al. 2002). Thus, the decreased levels of both DCs may directly cause the negative regulation of IgG-secreting B cells in the post-6h group.

Several reports have indicated that CQ can regulate innate immune functions in a TLR9-dependent manner (He et al. 2004; Hong et al. 2004; Coban et al. 2005; Huang et al. 2005). In addition, early studies in TLR9-deficient mice revealed that TLR9 was not only required for the production of pro-inflammatory cytokines, but also for the induction of Th1-based immune responses and the proliferation of B cells (Liu et al. 2003; He et al. 2004; Iwasaki and Medzhitov 2004). Until now, only TLR2, 4 and 9 have been reported to recognize *Plasmodium* components such as hemozoin, glycosyl phosphatidyl inositol, and DNA-protein complexes leading to the activation of innate immunity (Coban et al. 2005; Krishnegowda et al. 2005; Parroche et al. 2007; Wu et al. 2010). Therefore, we inferred that the remarkable variation in DCs in the post-6h group is probably associated with changes of the function of TLR9. However, although we observed reduced expression of

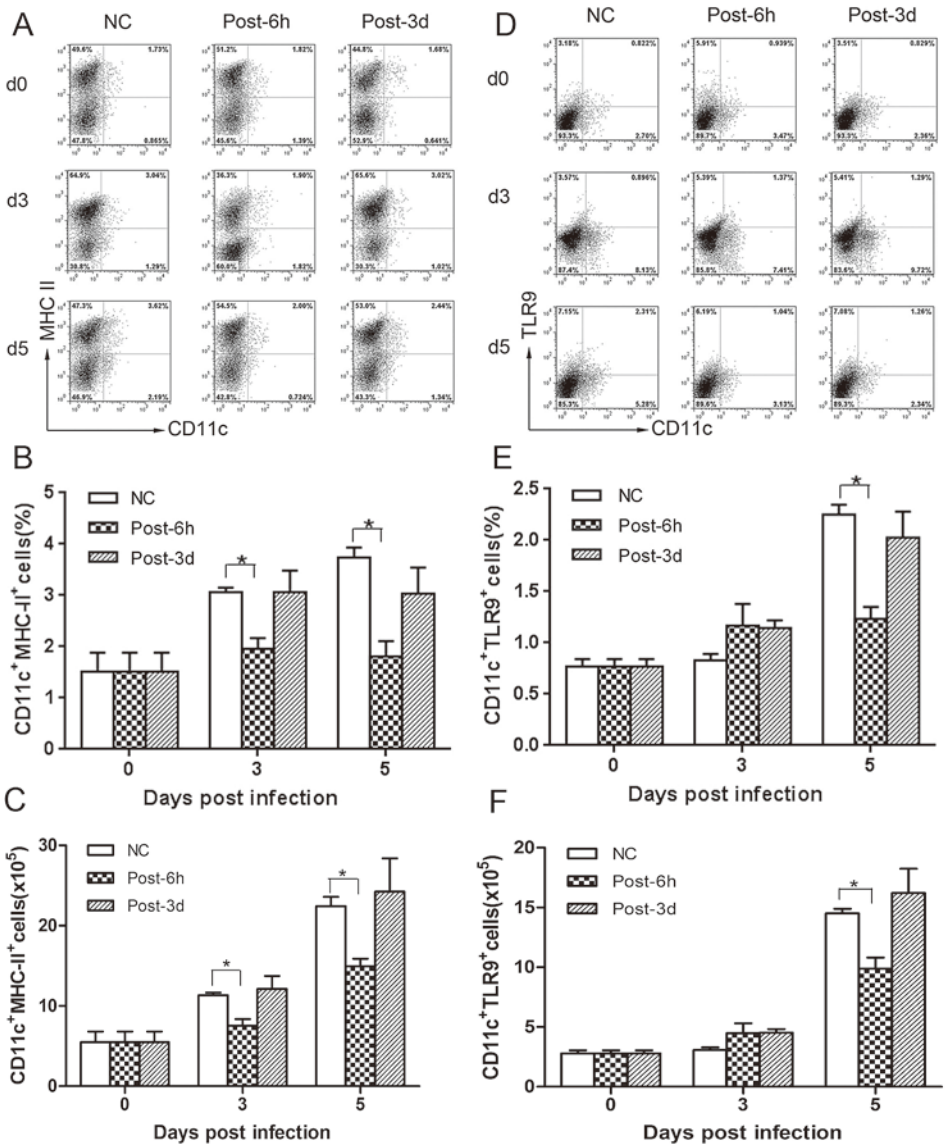

Fig. 5. The effect of CQ treatment on DCs phenotype during *Py* 17XL infection. The expression of MHC II (A) and TLR9 (D) on splenic $CD11c^+$ DCs was evaluated by flow cytometry and displayed in dot plot. The percentage and absolute number of $CD11c^+MHC\ II^+$ (B and C) and $CD11c^+TLR9^+$ DCs (E and F) were shown in bar graph. Data is presented as the mean ± standard error (n = 3 mice/group). Asterisk (*) indicates a statistically significant difference ($P < 0.05$) between the control group and CQ treatment group. Experiments were repeated for 3 times.

TLR9 on DCs in the post-3d group on day 5 p.i., no significant changes were seen in this group on day 3 p.i. when the expression of class II MHC molecules on DC was clearly decreased. One explanation may be that CQ exerts its action on TLR9 signaling mainly by blocking the engagement of the receptor with its ligands but not via downregulation its expression (Yi et al. 1998). The decreased expression of TLR9 on DCs of the post-6h group on day 5 p.i. may be a secondary event following the low maturation of DCs. However, we cannot exclude the contribution of early changes in TLR9 signaling to the inadequate maturation and activation of DCs. More work needs to be done to elucidate the role of TLR9 in the modulation of the anti-malarial immune response by CQ.

Taken together, these data are the first to demonstrate that the effect of CQ on the immune response in *Py* 17XL-infected DBA/2 mice is associated with the time of drug administration. CQ treatment at the very early stages of infection significantly inhibits both cellular and humoral immune responses against malaria, which suggests that CQ may aggravate parasitemia that occurs due to the immune suppression after treatment. Nevertheless, after a critical time point, CQ does not exert its depressive role. Since CQ remains an essential player for the current and future malaria treatment, our discovery provides vital information about the mechanism of CQ action, which has critical implications for malaria therapy. Due to the complexity of immune response against malaria, the immunosuppressive

mechanisms of CQ require further investigation.

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Conflict of Interest

The authors declare no conflict of interest.

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