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Bacillus Calmette-Guérin-inoculation at different time points influences the outcome of C57BL/6 mice infected with *Plasmodium chabaudi chabaudi* AS

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Abstract: Bacillus Calmette-Guérin (BCG) is an attenuated *Mycobacterium tuberculosis* vaccine. We performed a series of co-infection experiments with BCG-*Plasmodium chabaudi chabaudi* Landau, 1965 AS using C57BL/6 mice to analyse whether BCG can affect the development of protective immunity to infection with *Plasmodium* spp. and the mechanism of this protection. We divided mice into four groups: BCG-inoculation 4 weeks prior to *P. c. chabaudi* AS infection (B-4w-Pc); simultaneous BCG-inoculation and *P. c. chabaudi* AS infection (Pc+B); BCG-inoculation 3 days post *P. c. chabaudi* AS (Pc-3-B) infection; and mono-*P. c. chabaudi* AS infection as control (Pc). The parasitemia level in the B-4w-Pc group was noticeably higher than control group at 6–19 days post infection (dpi). Compared with the control group, the proportion of $CD4^+CD69^+$ T cells was significantly reduced 5, 8 and 12 dpi, but the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was significantly increased in the B-4w-Pc group on 5 and 8 dpi. The B-4w-Pc group also demonstrated reduced levels of IFN-γ and TNF-α on 5 and 8 dpi and significantly elevated level of IL-10 on 12 dpi. There were significantly fewer mDCs ($CD11c^+CD11b^+$) and pDCs ($CD11c^+B220^+$) in the B-4w-Pc group than the control group at all the time points post infection and the expression of MHC II was noticeably reduced on day 8 pi. Our findings confirmed that BCG inoculation prior to *Plasmodium* infection resulted in excessive activation and proliferation of Tregs and upregulation of anti-inflammatory mediators, which inhibited establishment of a Th1-dominant immune response during the early stages of *Plasmodium* infection by inhibiting dendritive cells response. BCG inoculation prior to *P. c. chabaudi* AS infection may contribute to overgrowth of parasites as well as mortality in mice.

Keywords: murine malarial parasite, BCG, co-infection, protective immunity

Malaria and tuberculosis are major causes of morbidity and mortality worldwide. According to the latest estimates from the World Health Organization (WHO), there were about 214 million cases of malaria and an estimated 438 000 deaths in 2015 (World Health Organization 2015). Equally serious is the global burden of tuberculosis. In 2013, an estimated 9 million people developed tuberculosis and 1.5 million died from the disease (World Health Organization 2014). Given the substantial overlap of geographic distribution between endemic regions for these two diseases and especially the large number of individuals with latent tuberculosis living in malaria-endemic regions, co-infection with malaria and tuberculosis is likely to be common (Range et al. 2007, Thapa et al. 2010).

The clinical manifestations of malaria may vary from asymptomatic infection to life-threatening anemia to neurologic involvement. Although adaptive immunity plays a significant role in the development and outcome of disease, other factors from the host and environment likely contribute to the variability in clinical manifestation (Artavanis-Tsakonas et al. 2003). In malaria-tuberculosis co-endemic areas, many children at risk for severe malaria have experienced mycobacterial infections from either exposure to *Mycobacterium tuberculosis*, environmental mycobacteria, or routine vaccination with Bacillus Calmette-Guérin (BCG). However, the modulatory mechanism by which the host immune responses to parasites of *Plasmodium* Marchiafava et Celli, 1885 are modulated by concurrent infections from *M. tuberculosis* or BCG remains poorly understood.

Infection with *Plasmodium chabaudi chabaudi* Landau, 1965 of mice is a model of human malaria. This rodent malaria has many features in common with human disease (Cox et al. 1987, Gilks et al. 1990, Mackinnon et al. 2002)

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and has been used extensively in laboratory research on malaria (Barclay et al. 2012, Schneider et al. 2012, Spence et al. 2012, Cameron et al. 2013, Waisberg et al. 2013). Infection with *P. c. chabaudi* AS is typically self-resolving in C57BL/6 mice; however, it is lethal in A/J mice (Langhorne et al. 2002). The mechanisms for this phenomenon have been extensively investigated (Langhorne et al. 1989, Stevenson et al. 1990, 1995, Stevenson and Tam 1993, Sam and Stevenson 1999) and it is widely accepted that the different outcomes are due to balance and timing of the Th1 and Th2 immune responses (Taylor-Robinson et al. 1993). Our recent data showed that BALB/c mice initiated a Th1/Th2 profile response to *P. c. chabaudi* AS infection, yet DBA/2 mice failed to control the same infection and almost all of them died post-peak parasitemia. Furthermore, we found a higher proportion of Treg cells with elevated Foxp3 expression in DBA/2 than in BALB/c mice, suggesting that the disparity of immune response to *P. c. chabaudi* AS between BALB/c and DBA/2 mice may be associated with suppressive activities of Treg cells (Wang et al. 2013).

Earlier studies have reported that vaccination with BCG protects mice against *Plasmodium vinckei* Rhodain, 1952, *P. yoelii* Landau et Killick-Kendrick, 1966, *P. berghei* Vincke et Lips, 1948, and *P. c. chabaudi* (Clark et al. 1976, Murphy 1981, Stevenson et al. 1984). The protective factor could be found in the serum of mice pre-infected with BCG and was toxic to both murine and human malarial parasites (Clark et al. 1981, Taverne et al. 1981, 1982, Haidaris et al. 1983, Wozencraft et al. 1984). A later study investigating the mechanism by which BCG can protect hosts against malaria demonstrated that BCG-vaccination could dramatically increase the survival rates of suscep¬tible A/J mice from infection by *P. yoelii* 17XL. Analysis of splenocytes indicated that BCG vaccination biased the immune response toward a Th1 type response (Matsumoto et al. 2000). However, the immunological mechanisms underlying these phenomena have not been well delineated.

With this in mind, in the present study we performed a series of co-infection experiments with BCG-*P. c. chabaudi* AS using C57BL/6 mice in order to elucidate which co-infection phase/phases (BCG-inoculation pre-*P. c. chabaudi* AS infection, simultaneous BCG-inoculation with *P. c. chabaudi* AS infection, and/or BCG-inoculation post-*P. c. chabaudi* AS) can affect the development of protective immunity to *P. c. chabaudi* AS infection. We focused our study on the mechanism of this protection.

MATERIALS AND METHODS

Mice, parasites, experimental infection and BCG-inoculation

Female C57BL/6 mice, aged 6–8 weeks, were purchased from the Shanghai Branch of the Chinese Academy of Sciences, Centre of Zoology. The *Plasmodium c. chabaudi* AS strain was kindly provided by Motomi Torii (Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan). Infections were initiated via intraperitoneal (i.p.) injection of 1×10^6 *P. c. chabaudi* AS-parasitised erythrocytes into C57BL/6 mice. For BCG inoculation, 0.25-mg freeze-dried BCG vaccine vials (Shanghai Institute of Biological Products, China National Biotec Group, Shanghai, China; Lot 2009030901) were reconstituted in 500 µl sterile saline. The required infective dose per mouse was 5×10^4 of BCG, delivered in a volume of 100 µl via intravenous injection into the tail vein. Mice were divided into 4 groups by time of inoculation: mice inoculated with BCG 4 weeks pre-*P. c. chabaudi* AS infection (B-4w-Pc); mice inoculated with BCG and simultaneously infected with *P. c. chabaudi* AS (Pc+B); mice inoculated with BCG 3 days post-*P. c. chabaudi* AS infection (Pc-3-B); and mice infected with mono-*P. c. chabaudi* AS as control (Pc). No mice died as a result of the BCG inoculation. For the parasitemia and survival experiment, 10 mice were used in each group. Parasitemia was monitored at each time point by microscopic examination of Giemsa-stained thin tail blood smears and mortality was monitored daily. For the immunological study, five mice were used per group and each experiment was repeated three times. All experiments were performed in compliance with local animal ethics committee requirements.

Splenocyte culture and quantification of cytokines

Splenocytes were harvested and cultured essentially as previously described (Chen et al. 2009). Aliquots (5×10^6/well) of the cell suspensions were seeded 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 until they were assayed. Levels of IFN-γ, TNF-α and IL-10 in culture supernatants were measured using enzyme linked immunosorbent assays (ELISA) (R&D Systems, Minneapolis, MN, USA).

Flow cytometry analysis

Splenocytes from a portion of each group were collected to determine the relative percentage of Th1 type cells ($CD4^+CD69^+$), Tregs ($CD4^+CD25^+Foxp3^+$), the subsets of splenic myeloid DCs (mDC, $CD11c^+CD11b^+$) and plasmacytoid DCs (pDC, $CD11c^+B220^+$), and the expression of MHC II on DCs ($CD11c^+MHC\ II^+$). Unless otherwise indicated, antibodies were purchased from BD Biosciences. When mouse cells were analysed, the following antibodies were used: FITC-conjugated anti-CD11c (clone HL-3), PE-conjugated anti-CD11b (clone M1/70), PerCP-conjugated anti-B220 (clone RA3-6B2), PE-conjugated anti-MHC II (clone M5/114.15.2, eBioscience), FITC-conjugated anti-CD69 (clone H1.2F3), FITC-conjugated anti-CD4 (clone GK1.5), PE-conjugated anti-CD25 (clone PC61), and APC-conjugated anti-Foxp3 (clone FJK16s, eBioscience). Flow cytometry was performed on a FACS Calibur (BD Biosciences, San Diego, CA, USA) and analysed using the FlowJo software (Treestar, San Carlos, CA, USA).

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Survival analysis was performed using the Kaplan-Meier log-rank test. Statistical significance of the differences was analysed by the *t*-test or one way ANOVA (SPSS 17.0). A value of $P < 0.05$ was considered significant.

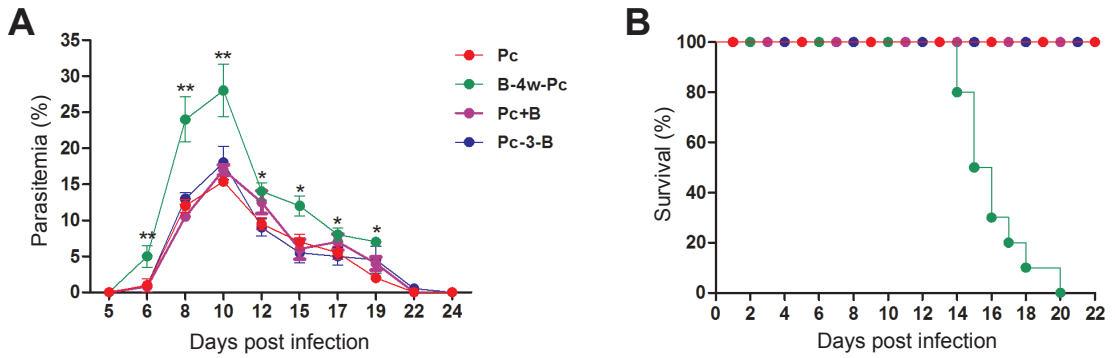

Fig. 1. The effect of BCG-inoculation on the level of parasitemia and survival rate of C57BL/6 mice during infection with *Plasmodium chabaudi chabaudi* Landau, 1965 AS. Parasitemia (**A**) and survival rate (**B**) in C57BL/6 mice after BCG-innoculation 4 weeks prior to infection with *P. c. chabaudi* AS (B-4w-Pc), simultaneous BCG-innoculation and infection with *P. c. chabaudi* AS (Pc+B), BCG-innoculation 3 days post-infection with *P. c. chabaudi* AS (Pc-3-B), and monoinfection with *P. c. chabaudi* AS as control (Pc). A Giemsa-stained thin smear was analysed daily in order to monitor for the presence of parasitemia. Values represent the mean ± SEM (n = 10 mice per group). * indicates $P < 0.05$ and ** indicates $P < 0.01$ between the B-4w-Pc and Pc groups.

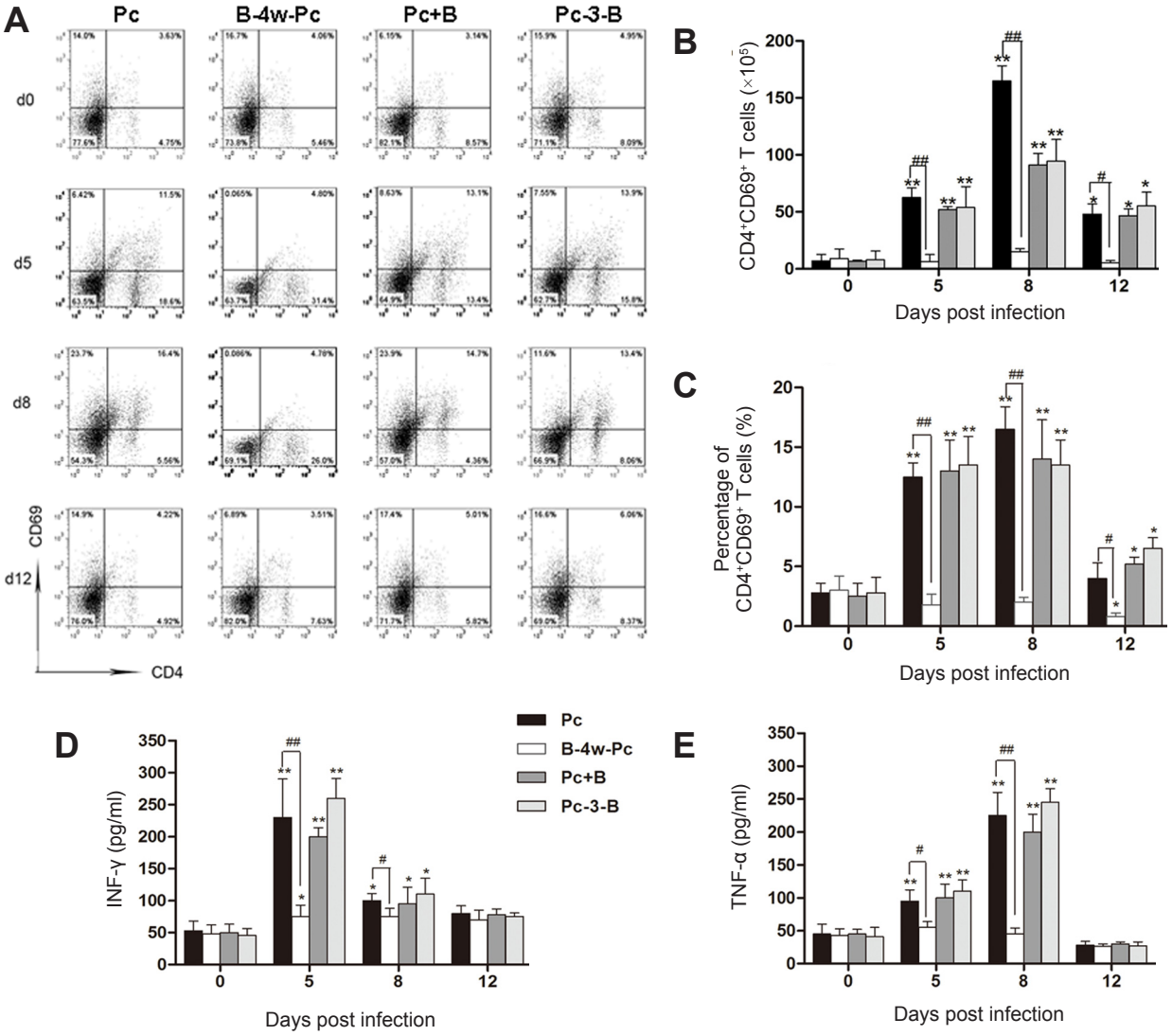

Fig. 2. The effect of BCG-inoculation on the pro-inflammatory immune responses of C57BL/6 mice during infection with *Plasmodium chabaudi chabaudi* Landau, 1965 AS. On day 0 (non-infection), 5, 8 and 12 days post infection, the proportion of $CD4^+CD69^+$ T cells was detected using flow cytometry. Illustration of the gating strategy for identifying splenic $CD4^+CD69^+$T cells using representative dot plots (**A**). Absolute number and proportion of $CD4^+CD69^+$ T cells at various time points post infection (**B** and **C**). Splenic lysates were prepared and the concentrations of IFN-γ (**D**) and TNF-α (**E**) were determined by ELISA. Each experiment was repeated three times. Values represent the mean ± SEM (n = 5 mice per group). * indicates $P < 0.05$ and ** indicates $P < 0.01$ between experimental groups and non-infected mice; # indicates $P < 0.05$ and ## indicates $P < 0.01$ between BCG-innoculated mice and control.

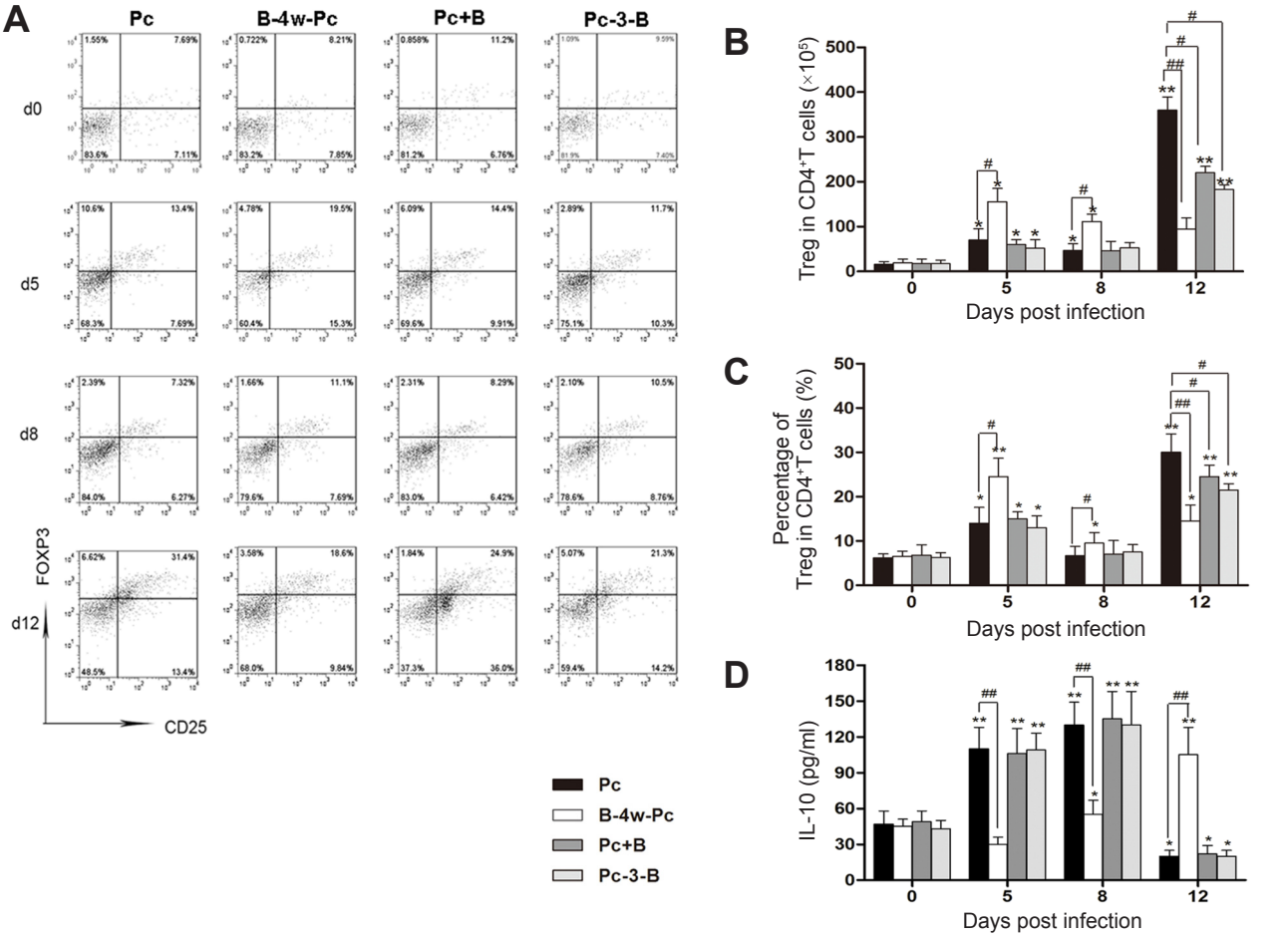

Fig. 3. The effect of BCG-inoculation on Tregs and anti-inflammatory cytokine production in C57BL/6 mice during infection with*Plasmodium chabaudi chabaudi* Landau, 1965 AS. On 0 (non-infection), 5, 8 and 12 days post infection, the proportion of $CD4^+CD25^{+-}$ $Foxp3^+$ T cells was quantified using flow cytometry. Illustration of the gating strategy for identifying splenic $CD4^+CD25^+Foxp3^+$ T cells using representative dot plots (**A**). Absolute number and proportion of $CD4^+CD25^+Foxp3^+$ T at various time points post infection (**B** and **C**). Splenic cell culture suspensions were collected and the level of IL-10 protein was measured by ELISA (**D**). Each experiment was repeated three times. Values represent the mean ± SEM (n = 5 mice per group). * indicates $P < 0.05$ and ** indicates $P < 0.01$ between experimental groups and non-infected mice; # indicates $P < 0.05$ and ## indicates $P < 0.01$ between BCG-innoculated mice and control.

RESULTS

The effect of BCG-inoculation on parasitemia and the survival rate of C57BL/6 mice during infection with *Plasmodium chabaudi chabaudi* AS

To determine whether pre-, post- or concurrent BCG-inoculation affected the survival rate of C57BL/6 mice during infection with *Plasmodium c. chabaudi* AS, parasitemia was monitored at each time point and mortality was monitored daily. The level of parasitemia in the Pc group (control) began to rise on 5 day post infection (dpi), then increased rapidly from 1% to 12% during days 6–8 dpi and reached a mean peak level of 15.4% on days 9, 10 dpi. From that, the parasitemia began to decrease and all mice recovered by 22 dpi. The level of parasitemia in the B-4w-Pc group was noticeably higher than the control group on 6–19 dpi ($P < 0.01$ on 6, 8 and 10 dpi; $P < 0.05$ on 12, 15, 17 and 19 dpi) (Fig. 1A). All mice succumbed to *P. c. chabaudi* AS infection by 20 dpi (Fig. 1B). The susceptibility to infection of the Pc+B and Pc-3-B groups was similar to that of the control group (Fig. 1A,B). These results suggest that it was only the group with an established BCG pre-infection that had a different outcome from the other 3 groups.

The effect of BCG-inoculation on the pro-inflammatory immune responses of C57BL/6 mice during infection with *Plasmodium chabaudi chabaudi* AS

To elucidate whether BCG-inoculation could regulate the pro-inflammatory immune response, which in turn could affect the outcomes of BCG-malaria co-infection, we detected the kinetics of the $CD4^+CD69^+$ T cell response and the concentrations of IFN-γ and TNF-α in different mouse models. The absolute number and proportion of $CD4^+CD69^+$ T cells in the Pc+B and Pc-3-B groups significantly increased on days 5 pi and 8 pi ($P < 0.01$). However, there was a remarkable reduction in the percentage of $CD4^+CD69^+$ T cells on 5, 8 ($P < 0.01$) and 12 dpi ($P < 0.05$) in the B-4w-Pc group as compared to control (Fig. 2B,C). Consistent with these changes in the $CD4^+CD69^+$ T cell population, similar results in the levels of IFN-γ and TNF-α from splenocyte culture supernatants were observed in each groups (Fig. 2D,E).

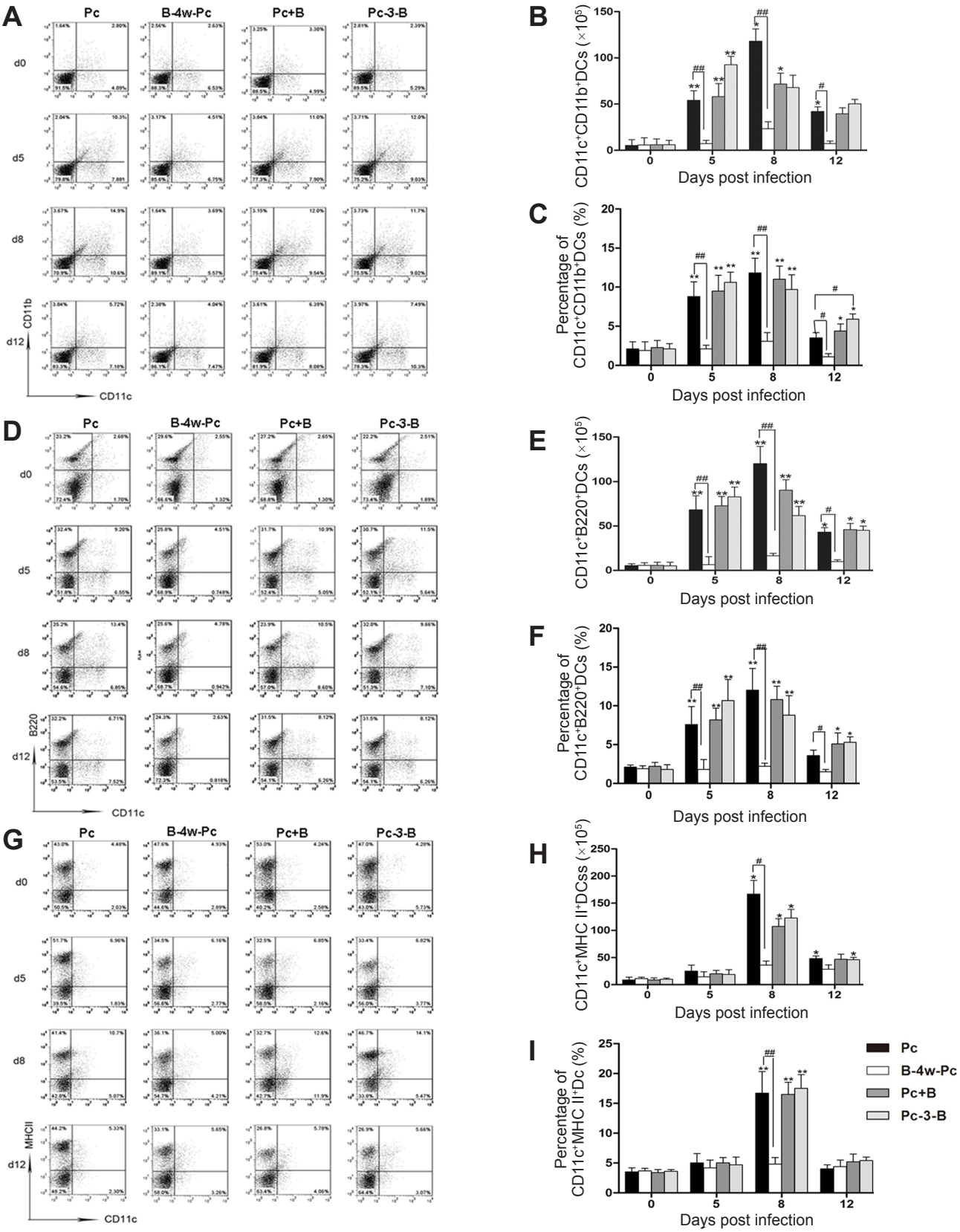

Fig. 4. The effect of BCG-inoculation on DCs of C57BL/6 mice during infection with *Plasmodium chabaudi chabaudi* Landau, 1965 AS. On 0 (non-infection), 5, 8 and 12 days post infection flow cytometry was used to quantify mDC, pDC, and MHC II surface expression on splenic $CD11c^+$ DCs. Illustration of the gating strategy used to identify splenic $CD11c^+CD11b^+$ DCs, $CD11c^+B220^+$ DCs, and $CD11c^+$MHC II^+ DCs using representative dot plots (**A**, **D**, **G**). Absolute number of $CD11c^+CD11b^+$ DCs, $CD11c^+B220^+$ DCs, and $CD11c^+$MHC II^+ DCs at various time points post infection (**B**, **E**, **H**). Proportion of $CD11c^+CD11b^+$ DCs, $CD11c^+B220^+$ DCs and $CD11c^+$MHC II^+ DCs at various time points post infection (**C**, **F**, **I**). Each experiment was repeated three times. Values represent the mean ± SEM (n = 5 mice per group). * indicates $P < 0.05$ and ** indicates $P < 0.01$ between experimental groups and non-infected mice; # indicates $P < 0.05$ and ## indicates $P < 0.01$ between BCG-innoculated mice and control.

The effect of BCG-inoculation on Tregs and anti-inflammatory cytokines in C57BL/6 mice during infection with *Plasmodium chabaudi chabaudi* AS

To analyse the role of Tregs in the response to BCG-malaria co-infection, we first followed the kinetics of Tregs in different groups of mice. As shown in Fig. 3, the absolute number and proportion of $CD4^+CD25^+Foxp3^+$ Tregs in the control group significantly increased on 5 ($P < 0.05$) and 12 dpi ($P < 0.01$) . These values in the B-4w-Pc group were significantly higher on 5 and 8 dpi ($P < 0.05$), but remarkably lower on 12 dpi ($P < 0.01$) as compared to the control group. No significant difference was detected between control and the Pc+B group or Pc-3-B group except on 12 dpi (Fig. 3B,C). We further analysed production of the anti-inflammatory cytokine IL-10 to BCG-malaria co-infection. The production of IL-10 in the B-4w-Pc group was significantly lower on 5 and 8 dpi ($P < 0.01$), but conspicuously higher on 12 dpi ($P < 0.01$) as compared to the control group. There was no significant difference between control and the Pc+B group or Pc-3-B group in the production of IL-10 (Fig. 3D).

The effect of BCG-inoculation on DCs in C57BL/6 mice during infection with *Plasmodium chabaudi chabaudi* AS

To investigate the effects of BCG-inoculation on the regulation of DCs, the absolute number and proportion of mDCs and pDCs were detected by flow cytometry. The proportion of mDCs and pDCs on 5, 8 ($P < 0.01$) and 12 dpi ($P < 0.05$) in the B-4w-Pc group was significantly lower than in the control group. There was no significant difference between control and the Pc+B group or the Pc-3-B group with except for a significant increase in mDCs in the Pc-3-B group on 12 dpi ($P < 0.05$) (Fig. 4B,C,E,F). We further compared the expression of the DC maturation marker, MHC II, in splenic $CD11c^+$ DCs. The expression of MHC II on 8 dpi on DCs in the B-4w-Pc group was significantly lower than that on DCs of the control group ($P < 0.01$), but no significant difference was observed between control and the Pc+B group or the Pc-3-B group at the indicated time points (Fig. 4H,I).

DISCUSSION

The ability to control parasite density has long been recognised as crucial to a favourable outcome during malarial infection (Stevenson and Riley 2004). Previous studies have shown that protective immune responses induced by infection with species of *Plasmodium* can effectively control or eliminate parasites in the host (Taylor-Robinson and Phillips 1994, Couper et al. 2005, Seixas and Ostler 2005, Yazdani et al. 2006). In the present study, the determination of parasitemia from tail blood smears indicated that poor outcome in the B-4w-Pc group was associated with a higher parasite density a day earlier than in the control group. The peak level of parasitemia in the B-4w-Pc group was almost two-fold that of the Pc+B, Pc-3-B and control groups. Coupled with this finding, all mice in the B-4w-Pc group succumbed to infection with *Plasmodium c. chabaudi* AS by day 20 pi. It has been argued that the ability to control parasite density is closely linked to survival. Taken together, these results suggest that BCG-malaria co-infection at different time points may modulate the host immune response and result in the differences in the development of protective immunity to *P. c. chabaudi* AS infection. More importantly, BCG inoculation 4 weeks prior to *P. c. chabaudi* AS infection did not delay or reduce parasitaemia (in fact it encouraged earlier appearance and a higher peak), turning what is otherwise a self-curing model of malaria infection into a fatal one.

Successful resistance to blood-stage malarial infection depends on the ability to induce an early effective Th1 immunity for controlling parasite growth, which is characterised by predominant IFN-γ secretion and effective NO production (Malhotra et al. 2005, Wang et al. 2009, Bueno et al. 2010). A previous study has shown that TNF is toxic to parasites *in vivo* (Taverne et al. 1989) and indicates that the kinetics of TNF release is coordinated with the reduction in parasite density (Stevenson et al. 1992). Mycobacterial infection also potentially induces Th1 immune responses characterised by predominant IFN-γ and TNF-α secretion, which has been confirmed in experiments with animals and clinical practice (Flynn et al. 1993, Keane et al. 2001). Moreover, BCG-inoculation noticeably elevated the survival rates of A/J mice with *P. yoelli* 17XL infection, and shifted the immune response toward Th1 type. In addition, neutralisation of IFN-γ with anti-IFN-γ mAb abrogated this protection and mice then developed fulminating infections (Matsumoto et al. 2000).

These findings indicate that potentiation of Th1 immune responses plays a protective role in co-infected mice. However, our present data demonstrate an opposite outcome in C57BL/6 mice inoculated with BCG 4 weeks pre-infection with *P. c. chabaudi* AS. We found that BCG-inoculation in the Pc+B and Pc-3-B groups did not result in potentiation of Th1 immune responses compared to the control group. More importantly, the levels of IFN-γ and TNF-α were significantly reduced and Th1 cells, which mediate pro-inflammatory responses were also remarkably lower in the B-4w-Pc group as compared to the other groups. Leisewitz et al. (2008) have reported that BCG-induced death in *P. c. chabaudi* AS infected C57BL/6 mice was associated with an excessively increase of $CD4^+$ expressing IFN-γ; therefore, their work seems to contradict our findings. Our findings support the view that successful resistance to blood-stage malarial infection depends on the ability to induce an early Th1 immune response in order to control parasite growth.

A group has reported on the influence of pre-infection with *Mycobacterium tuberculosis* and subsequent co-infection with *P. yoelii* 17XL in resistant C57BL/6 and susceptible BALB/c mice. This pattern of infection afforded significant protection to C57BL/6 mice, but there was no protection provided to the BALB/c mice. The microarray results suggested a clear trend towards a Th1 response in resistant C57BL/6 mice in contrast to the susceptible BALB/c mice, which was confirmed by cytokine levels in splenocyte culture supernatant (Page et al. 2005). Our previous data have suggested that (i) Treg proliferation is

causally associated with the suppression of Th1 responses during early malarial infection, leading to elevated parasitemia and mortality in mice (Wu et al. 2007, Feng et al. 2012); (ii) Tregs may have an important role in regulating pro-inflammatory Th1 responses in an IL-10-dependent manner and induce $CD4^+$ T cell apoptosis during early rodent malarial infections (Wu et al. 2007, Chen et al. 2009, Zheng et al. 2009); and (iii) Tregs mediate the incidence and outcome of cerebral malaria (CM) in mice infected with *Plasmodium berghei* ANKA by modifying the pro-inflammatory response (Wu et al. 2010). In addition, Tregs have a vital role in modulating Th1/Th2 responses and are involved in the shift of Th1 to Th2 cells (Song et al. 2009).

Together, these findings suggest that the disparity of Th immune responses to infection with *Plasmodium* spp. in mice is controlled by modulation of the host immune-regulatory network, which may play an important role in determining the susceptibility and severity of malaria. In the present study, we found that there was a negative correlation between the dynamic changes of Tregs and the establishment of the pro-inflammatory Th1 response in C57BL/6 mice when BCG-inoculation occurred four weeks prior to infection with *P. c. chabaudi* AS. The increase in Treg proliferation was not correlated to that of IL-10 secretion, which implies that there was no natural and intrinsic relationship between these two biological processes. Our data confirm that an excessive proliferation of Tregs could interfere with the appropriate balance between pro- and anti-inflammatory immune responses, leading to increased parasitemia and mortality in mice.

Dendritive cells (DC) provide a vital bridge between the innate and adaptive immune responses and therefore play an important role in the activation and differentiation of T cells and in the induction of adaptive immune responses (Banchereau and Steinman 1998, Diao et al. 2004). The balance between Th1 and Th2 cell development is related to DC maturation and Th1/Th2 cytokine production (Stevenson and Urban 2006, Wykes et al. 2007, Ing and Stevenson 2009). Similarly, the balance between mDC and pDC subsets renders DCs able to direct the appropriate T-cell response (Liu 2001, Jangpatarapongsa et al. 2008). Up-regulated MHC II and co-stimulatory molecules are characteristic of the DC maturation (Stevenson and Urban 2006, Wykes et al. 2007, Ing and Stevenson 2009). High expression of MHC II is crucial for DCs to present antigens to $CD4^+$ Th cells (Roche and Furuta 2015). In contrast with other studies, we found that BCG-inoculation four weeks prior to infection with *P. c. chabaudi* AS dramatically inhibited the activation and maturation of mDC and pDC and significantly down-modulated expression of the DC maturation marker, MHC II. Our previous findings suggest Tregs prevent Th1 immune response establishment during early stages of infection with *P. yoelii* 17XL by inhibiting DC responses (Zheng et al. 2009).

Taken together, BCG-inoculation four weeks prior to infection with *P. c. chabaudi* AS may inhibit the activation and maturation of DCs by inducing the excessive proliferation of Tregs, which is causally associated with the suppression of a moderate Th1 response and therefore contributes to overgrowth of parasites as well as mortality of the mice.

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