CD4⁺CD25⁺Foxp3⁺ regulatory T cells prevent the development of Th1 immune response by inhibition of dendritic cell function during the early stage of *Plasmodium yoelii* infection in susceptible BALB/c mice

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Abstract: Protective immunity against murine malaria infection depends largely on the establishment of effective Th1 immune response during the early stages of infection. Experimental data suggest that the death of *Plasmodium yoelii* 17XL (*P.y* 17XL) susceptible BALB/c mice results from the suppression of Th1 immune response mediated by CD4⁺CD25⁺Foxp3⁺regulatory T cells (Tregs). However, the mechanism by which Tregs regulate Th1 immune response is poorly understood. Since immunity is initiated by dendritic cells (DCs), we analysed DC responses to *P.y* 17XL in control and Treg-depleted BALB/c mice. Myeloid DC proliferation, phenotypic maturation and interleukin-12 (IL-12) production were strongly inhibited in control BALB/c mice. In contrast, plasmacytoid DC proliferation and IL-10 production were strongly enhanced in control BALB/c mice. *In-vivo* depletion of Tregs resulted in significantly reversed inhibition of DC response, which may contribute to the establishment of Th1 immune response, indicating that Tregs contribute to the suppression of Th1 immune response during malaria. These findings suggest Tregs contribute to prevent Th1 immune response establishment during the early stage of *P.y* 17XL infection by inhibiting DC response.

Key words: *Plasmodium yoelii* 17XL, Tregs, dendritic cells, DCs, Th1 immune response

Malaria caused by protozoan parasites of the genus *Plasmodium* is still one of the most life-threatening infectious diseases. The disease is also important in Asia, Latin America and Sub-Saharan Africa, resulting in 350–500 million clinical cases and more than 1 million deaths every year, mostly affecting children under 5 years of age (Sachs and Malaney 2002, WHO and UNICEF 2005). Generation of effector T cells is crucial for the development of protective immunity against malaria (Jacobs et al. 1996, Malhotra et al. 2005, Beeson et al. 2008). Effective Th1 immune response in the early stage of infection critically influences the later development and final outcome of malaria infection (Jacobs et al. 1996, Malhotra et al. 2005). Meanwhile, malaria infections are characterised by immune suppression in which CD4⁺CD25⁺Foxp3⁺ regulatory T cells (Tregs) are involved. Up-regulation of Tregs correlates with rapid parasite growth and inhibition of Th1 immune response during malaria infection (Hisaeda et al. 2004, Walther et al. 2005). Although Th1 immune response development is required to control parasite replication and promote clearance of infected erythrocytes, a better understanding of immune regulation mechanisms in Th1 immune response establishment during malaria infection is important for the effective control of malaria.

Tregs are a T cell subgroup that has immune-regulatory effects. Tregs are widely accepted as potent suppressors and play important roles in immunotolerance, autoimmunity and transplantation (Sakaguchi et al. 1995, Shevach 2002, Martin et al. 2004, Rouse and Suvas 2004, Lee et al. 2005). The immune-suppressive activity of Tregs can be mediated by cell-contact-dependent mechanisms and by the secretion of suppressive cytokines such as TGF- β and IL-10 (Thornton and Shevach 1998, Asseman et al. 1999, Nakamura et al. 2001). In malaria, Tregs expand during infection with *Plasmodium yoelii* 17XL (*P.y* 17XL) and have been shown to inhibit the development of Th1 immune response, responsible for death in susceptible BALB/c mice (Hisaeda et al. 2004, Wu et al. 2007). Data provided on *Plasmodium falciparum* infections in humans correlate the expansion of natural Tregs with higher parasite multiplication rates (Walther et al. 2005). Depletion of Tregs results in a comparable delay in the onset of peak parasitaemia during *Plasmodium berghei* NK65 infection in mice (Long et al. 2003) and allows BALB/c mice to control otherwise lethal infections in the *P.y* 17XL infection model (Hisaeda et al. 2004). Altogether, these observations suggest Tregs have been implicated as major regulators of Th1 immune response establishment during

early *Plasmodium* infections, but their mechanisms of action remain undefined.

Dendritic cells (DCs) are antigen-presenting cells that play a pivotal role in the initiation of immune responses, as they form a bridge between the innate and adaptive immune responses and possess the unique capacity to initiate primary immune responses via the activation of naïve T cells (Banchereau et al. 2000). Several groups have demonstrated that in response to *Plasmodium chabaudi* AS-infected RBC (iRBC) stimulation, splenic DCs could express up-regulated expression of MHC II and co-stimulatory molecules, and produce elevated level of pro-inflammatory cytokine IL-12, in favour of the naïve CD4⁺T cell proliferation and IFN- γ production (Leisewitz et al. 2004, Ing et al. 2006), suggesting that DCs play an important role in innate immunity during blood-stage malaria and shape an adaptive type 1 immune response necessary for control and rapid resolution of infection. Given the crucial role of DCs in both the innate and adaptive immune responses, appropriate modulation of DC functions may provide some clues to establish the protective immunity to malaria. Our previous study showed that abnormal activation and multiplication of Tregs is a crucial factor for down-regulation of Th1 immune response and death in BALB/c mice during the early stage of *P.y* 17XL infection (Wu et al. 2007). In the present study, we compared the difference in Th1 immune response in BALB/c mice infected with *P.y* 17XL before and after depletion of Tregs and further investigated the characteristics for DC activation leading to priming of CD4⁺ Th1 cell responses, to gain an insight into the role of Tregs in the regulation of Th1 immune response during the early malaria infection. Our results indicated that during the early *P.y* 17XL infection in BALB/c mice, depletion of Tregs resulted in profound changes in the host Th1 immune response and enhanced DC activation, events associated with changed infection course. Our findings provide novel evidence for the *in-vivo* relevance of Tregs down-regulating the activation status of DCs during the innate immune response to blood-stage malaria leading to the inhibition of Th1 immune response.

MATERIALS AND METHODS

Animals. Female 6–8-week-old BALB/c mice were purchased from Beijing Animal Institute and maintained in our Animal House under controlled food, light/darkness cycles and temperature. All procedures of animal manipulation were approved by the University Complutense Institutional Animal Care and Use Committee following Chinese law.

Parasite. The parasite of *P.y* 17XL strain was kindly provided by Dr. Motomi Torii (Department of Molecular Parasitology, Ehime University Graduate School of Medicine, Ehime, Japan) in 1998. Since then it has been maintained in our laboratory by periodical passage in BALB/c mice.

Experimental infection. Infections were initiated by intraperitoneal (i.p.) injection of 1×10^6 *P.y* 17XL-parasitized erythro-

cytes (PE) to BALB/c mice. Parasitaemia was monitored by light microscope examination of Giemsa-stained, thin blood smears. Mortality was checked daily. All experiments were performed in compliance with local animal ethics committee requirements.

Splenocyte preparation and culture. Mice (3 mice/group/time point) were sacrificed on days 0, 3 and 6 p.i. and spleen cell suspensions were prepared as previously described (Cao et al. 1998). Briefly, the spleen from each mouse was removed aseptically and pressed through a sterile fine-wire mesh with 10 ml of RPMI 1640 (Life Technologies, Burlington, Ontario, Canada) supplemented with 5% heat-inactivated foetal calf serum (FCS) (Hyclone Laboratories, Inc.), 25 mmol/l Hepes (Life Technologies), 0.12% gentamicin (Schering, Montreal, Quebec, Canada) and 2 mmol/l glutamine (Life Technologies). Cell suspensions were collected by centrifuging at $350 \times g$ for 10 min. Erythrocytes were lysed with cold 0.17 mol/l NH_4Cl and cells were washed twice with fresh medium. Viability of splenocytes was determined by trypan blue exclusion and was always >90%. Splenocytes were adjusted to a final concentration of 1×10^7 cells/ml in RPMI 1640 supplemented with 10% heat-inactivated FCS. Aliquots (500 μl /well) of the cell suspensions were incubated in 24-well flat-bottom tissue culture plates (FALCON) in triplicate for 48 h at 37°C. Supernatants were collected and stored at –80°C until they were assayed for IFN- γ and NO_2^- levels.

Dendritic cell separation and culture. To obtain DCs, spleens from naïve or infected mice were treated for 30 min at 37°C with 1 mg/ml collagenase IV (Sigma Aldrich). The spleens were then homogenised into single-cell suspension, splenic DCs were isolated using BD IMagTM Mouse Dendritic Cell Enrichment set-DM (Cat. No. 557955) according to manufacturer's instructions. The purified DCs were routinely 70–85% CD11c⁺, as determined by flow cytometry. Viability was assessed by trypan blue dye or labelled with 7-actinomycin D.

Aliquots of 1-ml DC suspensions at a concentration of 1×10^6 cells/ml were cultured in triplicate in 24-well tissue culture plates in complete medium. The cultures of splenic DCs (5×10^5 cells/well) were incubated for 48 h at 37°C in a humidified CO_2 incubator. Supernatants were collected and stored at –80°C until assayed for cytokine levels.

In-vivo depletion of CD4⁺CD25⁺ T cells. Depletion of CD25⁺T cells was carried out by intraperitoneal (i.p.) injection of 0.5 mg of anti-mouse CD25 mAb (PC61; rat IgG1) 1 day prior to and 1 day after infection. The antibody was purified from supernatants from confluent cell cultures of the TIB-222 hybridoma (American Type Culture Collection, Manassas, VA) on a Hi-Trap Protein G column according to the instructions provided by the manufacturer. Depletion efficiency, assessed by FACS analysis of CD4⁺CD25⁺ cells, ranged from 80% to 95%, and the effect was shown to persist for more than 10 days (Cambos et al. 2008). Control mice were treated with purified rat IgG1 (R3-x34, Pharmingen International, Japan).

Flow cytometric analysis. All flow cytometry was performed on a FACSCalibur (BD Biosciences) and analysed with CellQuest software (version 3.3, BD Biosciences). All Abs for FACS were purchased from BD Biosciences.

For analysis of spleen DC subpopulation and phenotype, single-cell suspensions of splenocytes were first blocked with anti-CD16/CD32 (Fc γ III/II receptor; 2.4G2), then stained using a combination of FITC-conjugated anti-mouse CD11c (HL3), PE-conjugated anti-mouse CD11b (M1/70), PerCP-conjugated

anti-mouse CD45R/B220 (RA3-6B2), PE-conjugated anti-mouse MHC II (M5/115.15.2) and PE-conjugated anti-mouse CD80 (16-10A1). Isotype-matched Abs were used as staining controls. The difference of DC activation between Treg-depleted mice and control mice were determined by the numbers of CD11c⁺DCs expressing MHC II or CD80 molecules.

For analysis of natural Tregs, splenocytes were incubated with anti-CD16/CD32 (FcγIII/II receptor; 2.4G2) antibody (Fc block), washed, and then co-stained with FITC-conjugated anti-mouse CD4/L3T4 (GK1.5) and PE-conjugated anti-mouse CD25/IL-2 receptor alpha (IL-1Ra, 7D4) mAbs. The cells were fixed and permeabilised in a fixation/permeabilisation solution and permeabilisation buffer (eBioscience, USA), and were incubated with anti-mouse Foxp3-APC mAb (FJK-16S). The percentages and absolute numbers of CD4⁺CD25⁺Foxp3⁺ T cells within the CD4⁺ population were determined using a forward light scatter/side light scatter (FSC/SSC) gate on lymphocytes and a gate on CD4⁺ T cells.

Cytokine ELISA. IFN-γ levels in splenocyte supernatants were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's protocol (R&D Systems, Minneapolis, MN). Levels of IL-12p40 and IL-10 from DC culture supernatants were measured by ELISA kits according to the manufacturer's protocol (R&D Systems). The OD values were read in a microplate reader at 450 nm. The concentration of IFN-γ, IL-12p40 and IL-10 in samples were calculated against the standard curve generated using recombinant cytokine.

NO₂⁻ assay. NO₂⁻ concentration in culture supernatant of cells was assayed by the Griess reaction (Cao et al. 1998). Briefly, 100 μl Griess reagent [equal volumes of 1% (w/v) sulphanilamide (Wako, Osaka, Japan) and 0.1% (w/v) N-1-naphthyl ethylenediamine dihydrochloride (Wako) in 2.5% (w/v) H₃PO₄] for 10 min at room temperature (RT), and the OD at 550 nm was measured with a microplate reader. NO₂⁻ concentration was determined using NaNO₂ as a standard.

Statistics. Data are expressed as mean with standard errors of the mean (SEM). The statistical significance of differences between the groups (i.e. pre-infection vs. post-infection, and Treg-depleted vs. control mice post-infection) was analysed by Student's *t*-test and one- or two-way ANOVA using Prism (GraphPad). Differences were considered significant when *P* < 0.05.

RESULTS

Infection course

Anti-CD25 mAb (PC61) was firstly introduced to *in-vivo* deplete CD4⁺CD25⁺Foxp3⁺ Tregs in BALB/c mice and depletion efficiency was assessed by FACS analysis of Tregs within the CD4⁺T cell population. In response to *P.y* 17XL infection, BALB/c mice showed significantly higher proportion of Tregs in CD4⁺T cells on day 3 and day 6 p.i. (Fig. 1a, b). However, the injection of anti-CD25 mAb resulted in more than 80% reduction of Tregs on day 3 p.i. and these cells were eliminated for more than 70% in number on day 6 p.i. in BALB/c mice (Fig. 1a, b). The proportion of Tregs in CD4⁺T cells popu-

lation derived from Treg-depleted mice was lower than that of control mice (Fig. 1b).

In accordance with previous observations (Wu et al. 2007), *P.y* 17XL-infected control BALB/c mice showed a rapid increase of parasitaemia from day 4 to 6 p.i. with a peak around 50–60% (Fig. 1c) and all mice died within seven days (Fig. 1d). In contrast, parasite-infected Treg-depleted BALB/c mice developed a more gradual increase in parasitaemia which maintained approximately 10–40% by day 4–6 p.i. (Fig. 1c) and a slightly but not significantly prolonged survival time (Fig. 1d). Importantly, the levels of parasitaemia in Treg-depleted mice were significantly lower than those in control BALB/c mice on day 5 p.i. (*P* < 0.01) and day 6 p.i. (*P* < 0.01) (Fig. 1c). These observations indicated that Tregs markedly influenced *P.y* 17XL infection course in BALB/c mice.

Th1 immune responses

Resistance to blood-stage malaria infections depended on the establishment of an early effective Th1 immune response (Jacobs et al. 1996, Malhotra et al. 2005, Wu et al. 2007). Therefore, we sought to investigate whether Tregs played a role in the control of Th1 immune responses to malaria parasites. To that end, we compared the Th1 immune responses, as evaluated by the production of interferon gamma (IFN-γ) and nitric oxide (NO), in the control and Treg-depleted mice. As shown in Fig. 2a, although the production of IFN-γ in cultured splenocyte supernatants from control mice significantly increased on day 3 and 6 p.i., it would not be sufficient to control the rapid and increasing parasitaemia and resultant death (Fig. 1c, d). However, Treg depletion led to a further increase in the IFN-γ secretion after infection with the peak of 630.39 ± 57.31 pg/ml on day 3 p.i. (Fig. 2a), which was correlated with the depressed parasitaemia (Fig. 1c). Consistently, the difference of increased magnitude of NO was also observed in both control and Treg-depleted mice (Fig. 2b). These data, thus, demonstrated Tregs inhibited the development of *P.y* 17XL-specific Th1 immune responses during malaria infection.

Dendritic cell subpopulations

Because Th1 immune response in Treg-depleted BALB/c mice infected with *P.y* 17XL were obviously up-regulated, we assessed whether depletion of Tregs affected the response of DCs following infection in terms of subsets, phenotype and cytokine production. Exposure of DCs to iRBC may lead to expansion of DC subsets that differ in their ability to induce effector CD4⁺T cells (Sponaas et al. 2006, Hisaeda et al. 2008, Wong and Rodriguez 2008). Here, we dynamically monitored the frequency of myeloid (CD11c⁺CD11b⁺) and plasmacytoid (CD11c⁺CD45R/B220⁺) DCs in spleen cells from both groups of *P.y* 17XL-primed mice. As shown in Fig. 3,

Fig. 1. Course of infection with *P.y* 17XL strain of *Plasmodium yoelii* and survival rate in control and Treg-depleted BALB/c mice. Mice were infected intraperitoneally with 10^6 parasitized erythrocytes. (a) Representative dot plots showing CD25 and Foxp3 expression on CD4⁺T cells at various time points (days) after anti-CD25 mAb or isotype control Ab treatment. (b) Absolute numbers of Tregs at various time points after anti-CD25 mAb or isotype control Ab treatment. (c) Parasitaemia was monitored by microscopic evaluation of thin blood films with Giemsa staining. Values represent the mean with SEM ($n = 3$ mice per group). (d) Survival rate were calculated within 8 days post inoculation. Results are representative of three independent experiments. ** $P < 0.01$ versus corresponding values for non-infected mice (0d); ## $P < 0.01$ indicates the comparison between control and Treg-depleted mice.

Fig. 2. IFN- γ and NO productions in splenocyte culture supernatants from control and Treg-depleted BALB/c mice infected with *P.y* 17XL strain of *Plasmodium yoelii*. (a) Parasite-infected mice were dissected on days 3 to 6 and splenocytes were cultured. IFN- γ production was monitored by measuring the amount of IFN- γ in the splenocyte culture supernatants in duplicated wells using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. (b) NO production was monitored by measuring the amount of NO₂⁻ in the splenocyte culture supernatants in duplicated wells by the Griess reaction. Values represent the mean with SEM ($n = 3$ mice per group). Results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus corresponding values for non-infected mice (0d); # $P < 0.05$, ## $P < 0.01$ indicates the comparison between control and Treg-depleted mice.

Fig. 3. Absolute numbers of DC subpopulations in control and Treg-depleted BALB/c mice infected with *P.y* 17XL strain of *Plasmodium yoelii*. Unfractionated splenic cells were stained with antibodies against CD11c, CD11b, CD45R/B220 and were analysed by flow cytometry. **(a)** The bar charts represent the absolute numbers of CD11c⁺CD11b⁺ DCs and **(b)** of CD11c⁺CD45R/B220⁺ DCs from three mice per group. Bars represent the mean with SEM. Results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus corresponding values for non-infected mice (0d); # $P < 0.05$, ## $P < 0.01$ indicates the comparison between control and Treg-depleted mice.

Fig. 4. Maturation profiles of DCs in control and Treg-depleted BALB/c mice following infection with *P.y* 17XL. Unfractionated splenic cells were stained with antibodies against CD11c, MHC II, CD80 and were analysed by flow cytometry. **(a)** The bar charts represent the absolute numbers of DCs expressing MHC II and **(b)** CD80 from three mice per group. Bars represent the mean with SEM. Results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus corresponding values for non-infected mice (0d); # $P < 0.05$, ## $P < 0.01$ indicates the comparison between control and Treg-depleted mice.

Fig. 5. IL-12p40 and IL-10 productions in DC culture supernatants from control and Treg-depleted BALB/c mice infected with *P.y* 17XL strain of *Plasmodium yoelii*. Parasite-infected mice were dissected on days 3 to 6 and splenic DCs were cultured. **(a)** IL-12p40 and **(b)** IL-10 production was monitored by measuring the amount of cytokines in the splenic DC culture supernatants in duplicated wells using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Values represent the mean with SEM ($n = 3$ mice per group). Results are representative of three independent experiments. * $P < 0.05$, ** $P < 0.01$ versus corresponding values for non-infected mice (0d); # $P < 0.05$, ## $P < 0.01$ indicates the comparison between control and Treg-depleted mice.

the subpopulations of DCs increased by a distinct kinetics in both control and Treg-depleted BALB/c mice. The population of splenic CD11c⁺CD11b⁺ DCs significantly increased on day 3 p.i. ($P < 0.01$) and peaked on day 6 p.i. ($P < 0.01$) in Treg-depleted mice, whereas the numbers of CD11c⁺CD11b⁺ DCs in control BALB/c mice were significantly lower than those in Treg-depleted mice on day 3 p.i. ($P < 0.05$) and day 6 p.i. ($P < 0.01$) (Fig. 3a). In contrast, control BALB/c mice showed the proportions of splenic CD11c⁺CD45R/B220⁺ DCs was significantly higher than those in Treg-depleted mice from day 3 p.i. ($P < 0.01$) to day 6 p.i. ($P < 0.01$) (Fig. 3b). These results indicated that Tregs altered the proliferation profiles of DC subpopulations in BALB/c mice following *P.y* 17XL infection.

Dendritic cell maturation profiles

Maturation of DCs is essential for induction of T cell response (Banchereau et al. 2000, Ing et al. 2006). *Plasmodium* infection induced the maturation of DCs characterized by the up-regulation of MHC II and co-stimulatory molecules (Ing et al. 2006). In view of this, we examined and compared the expansion of splenic DCs expressing MHC II and CD80 molecules between control and Treg-depleted BALB/c mice during *P.y* 17XL infection. Both mice showed significantly higher number of DCs expressing MHC II or CD80 molecules following infection compared to naïve mice. However, Treg-depleted mice produced more mature DCs than control mice as demonstrated by the higher populations of cells expressing these molecules from day 3 p.i. ($P < 0.05$) to day 6 p.i. ($P < 0.01$) (Fig. 4). This observation indicated that Tregs selectively impaired the DC phenotypic maturation during the early stage of infection with *P.y* 17XL.

Cytokine secretions

The production of pro-inflammatory and immunoregulatory cytokines by DCs is critical for the generation of downstream innate and adaptive immune responses (Banchereau et al. 2000). IL-12p40 is an important stimulator of the T cell response and plays a critical role in resistance to malaria (Stevenson et al. 1995, Su and Stevenson 2002). Whereas IL-10 exert immunosuppressive activity, and it has also been shown to play anti-inflammatory roles during malaria infection (Kossodo et al. 1997, Brustoski et al. 2005). We thus compared the two cytokine secretion by DCs from control and Treg-depleted BALB/c mice. During the early stage of *P.y* 17XL infection, both control and Treg-depleted mice showed a significantly increase in IL-12p40 production by splenic DCs. However, productions of IL-12p40 in Treg-depleted mice were significantly higher than those in control BALB/c mice on days 3 p.i. ($P < 0.01$) to 6 p.i. ($P < 0.05$) (Fig. 5a). In contrast, the productions of IL-10 markedly increased on day

3 p.i. ($P < 0.01$) and were kept at elevated levels on day 6 p.i. ($P < 0.01$) in control BALB/c mice, whereas such increase was inhibited by the administration of anti-CD25 (Fig. 5b). Taken together, these results indicated that Tregs markedly altered the pattern of pro-inflammatory and immunoregulatory cytokine production by DCs.

DISCUSSION

It is well known that the establishment of Th1 immune response in early events that occurs in the spleen following *Plasmodium* spp. infection would contribute to the control of parasitaemia and resultant survival of infected host (Jacobs et al. 1996, Malhotra et al. 2005, Wu et al. 2007). Our previous study has established a rodent model in which BALB/c mice are susceptible to *P.y* 17XL infection characterized by an ineffectively elevated Th1 immune response (Wu et al. 2007). Importantly, excessively proliferative Tregs play an immunosuppressive role in establishment of early effective Th1 immune response, leading to the persistent rise of parasitaemia and mortality as a final consequence in susceptible BALB/c mice (Wu et al. 2007). The suppression of Tregs in the malaria infection has been addressed in rodent model by presenting an increased survival associated with higher T cell responsiveness against parasite-derived antigens (Hisaeda et al. 2004). Our results here also demonstrated that Treg depletion resulted in decreased parasitaemia and enhanced Th1 immunity compared with control mice. These findings support the notion that Treg activation could constitute a mechanism utilised by the parasite to evade host immunity.

Successful resistance to blood-stage malaria infections in mice depends on their ability to induce an early effective Th1 immunity characterized by a predominant IFN- γ secretion, which could promote NO production to control parasite growth (Jacobs et al. 1996, Wu et al. 2007, Wang et al. 2009). Tregs expand during infection with the *Plasmodium berghei* ANKA or *P.y* 17XL strain (Nie et al. 2007, Vigário et al. 2007, Wu et al. 2007) and have been shown to inhibit the development of Th1 immune response (Nie et al. 2007, Wu et al. 2007). We found here that the depletion of Tregs had a major positive impact on the production of pro-inflammatory cytokines during *P.y* 17XL infection. Significantly higher levels of IFN- γ and NO were induced in splenocyte culture supernatants from Treg-depleted mice than those in control BALB/c mice on days 3 to 6 p.i. (Fig. 2), indicating that Tregs contributed to the suppression of Th1 immune response during the early phase of *P.y* 17XL infection.

As DCs are capable of initiating an antigen-specific immune response via directly recognizing the antigen and presenting antigen to helper T cells, and shaping the subsequent immune response (Banchereau and Steinman 1998, Ing et al. 2006), in the present study, we performed a comparative analysis of DCs from control and Treg-

depleted BALB/c mice in response to *P.y* 17XL infection, to dissect the role of Tregs in modulating Th1 immune response during early malaria infection. Exposure of DCs to iRBC may lead to expansion of DC subsets that differ in their ability to induce effector CD4⁺ T cells (Sponaas et al. 2006, Wong and Rodriguez 2008). The splenic DC compartment is a heterogeneous population with subsets of cells differing across a spectrum of functions and morphologies (Shortman and Liu 2002), in which myeloid (mDC) and plasmacytoid DC (pDC) can exhibit distinct levels of proliferation under pathogen stimuli (Gilliet et al. 2002). The increase in mDC/pDC ratio is associated with the activation of the Th1 pathway, whereas a decreased ratio is associated with the activation of the Th2 pathway (Rissoan et al. 1999, Liu 2001, Kuwana 2002). Our data showed here that within 6 days of the onset of an experimental blood-stage malaria infection, control BALB/c mice showed obvious multiplication of pDC (CD11c⁺CD45R/B220⁺). In contrast, Tregs depletion resulted in predominant proliferation of mDC (CD11c⁺CD11b⁺). This result is also consistent with findings that the ratio of mDC to pDC was significantly lower, but the level of Tregs was obviously higher in acute *Plasmodium vivax* patients than those of naïve and immune controls (Jangpatarapongsa et al. 2008). We supposed that it is the distinct DC subpopulation proliferation that determines the different functions of DCs in modulating Th1/Th2 polarisation under the presence and absence of Tregs in *P.y* 17XL-infected BALB/c mice. This may suggest that Tregs alter the proliferation pattern of DC subpopulations, which eventually determines the outcome of T cell responses.

Stimulation of T cell responses, and more important, the activation of Th1 cells requires both maturation of DCs and secretion of IL-12 (Steinman et al. 1997, Mellman and Steinman 2001, Sher et al. 2003, Cervi et al. 2004). Up-regulations of MHC II and co-stimulatory molecules are characteristic of the maturation of DCs (Steinman et al. 1997, Banchereau and Steinman 1998, Mellman and Steinman 2001, Sher et al. 2003). High expression of MHC II is crucial for DCs to present antigens to CD4⁺ Th cells, and blocking the CD80/CD86 signalling pathway disrupts the Th1/Th2 balance in a *P. chabaudi* AS malaria model (Taylor-Robinson and Smith 1999). We found here that blood-stage *P.y* 17XL infection induced increased proportion of splenic CD11c⁺DCs positive for MHC II and CD80 from control BALB/c mice on days 3 and 6 p.i. However, it is noticeable that the magnitude of increased expressions of these indicators on DCs from control BALB/c mice was less than that from Treg-depleted mice, which was consistent with the establishment of

Th1 immune response. In light of these findings, it seems likely that Tregs inhibit the development of Th1 immune response by impairing DC phenotypic maturation.

In addition to the maturation, activated DCs are also implicated in regulation of Th1/Th2 immune response by producing different cytokines (Ing et al. 2006, Sponaas et al. 2006). It is reported that IL-12 production can be initiated within 2 days of an infection, be required for the release of IFN- γ from Th1 cells, and mediate the protection against parasites infection (Stevenson et al. 1995). On the contrary, immunoregulatory cytokine IL-10 is considered to inhibit protective immune responses to infections with a number of intracellular pathogens, including *Plasmodium* (Omer et al. 2003, Brustoski et al. 2005). We now clearly show that splenic DCs from *P.y* 17XL-infected Treg-depleted mice produced obvious and significantly higher level of IL-12p40 in contrast to the slight production of IL-12p40 from parasite-infected control BALB/c mice. In parallel, IL-10 was markedly produced and kept at elevated levels from day 3 to 6 in splenic DC supernatants from infected control BALB/c mice (Fig. 5a, b), while it was obviously reduced in Treg-depleted mice. This thus raised the possibility that Tregs may be implicated in the induction and regulation of Th1 immune response by modulating the profile of DC cytokine secretion.

In conclusion, the novel finding in this report is the observation of distinct response profiles of splenic DCs during the early stage of infection with *P.y* 17XL in control and Treg-depleted BALB/c mice, which are critically associated with the establishment of Th1 immune response. This also suggests that Tregs are activated by parasites during acute *P.y* 17XL infection, which then play an immunosuppressive role. This immunosuppressive role of Tregs was associated with altered DC subpopulation proliferation profile, impaired DC phenotypic maturation, and reversed balance between pro-inflammatory and immunoregulatory cytokines produced by DCs. Significantly, this is the first report showing that Tregs can contribute to suppression of Th1 immune response establishment during the early stage of *P.y* 17XL infection by shaping DC response.

Acknowledgements. We thank Dr. Motomi Torii (Ehime University Graduate School of Medicine, Ehime, Japan) for his guidance in this research and providing malaria parasite strains of *P.y* 17XL. Critical readings of the manuscript by Dr. Richard Culleton are gratefully acknowledged. This work was supported by grants from the National Natural Science Foundation of China (30800961) and Grant 2007CB513100 from National Basic Research Program in China (973 Program).

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Received 28 April 2009

Accepted 2 July 2009