Granuloma formation in the liver is relatively delayed, although sustained, in BCG-infected mice co-infected with *Plasmodium*

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Received August 20, 2016; Accepted November 2, 2017

DOI: 10.3892/mmr.2018.8836

Abstract. The purpose of the present study was to examine the effects of *Plasmodium* on the process of granuloma formation in Bacille Calmette-Guerin (BCG)-infected mice. Female six-week-old BALB/c mice were co-infected with BCG and *Plasmodium*. The liver index, pathological alterations and quantity of granulomas in the mice were observed when the mice were co-injected with BCG and *Plasmodium*. The expression of inducible nitric oxide synthase (iNOS) was assessed by immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) analysis. In addition, the expression of interleukin (IL)-10 in liver tissues was observed by RT-PCR. Following co-infection with BCG and *Plasmodium*, the swelling of the liver had been slowly restored to normal, and the time required to allow granulomas to subside had prolonged. In addition, the expression of iNOS increased, while the expression of IL-10 gradually decreased in *Plasmodium*-infected mice. It was concluded that the use of *Plasmodium* relatively delayed granuloma formation in livers of BCG-infected mice. In addition, iNOS and IL-10 are involved in this pathogenesis.

Introduction

To date, approximately three billion people have received Bacille Calmette-Guerin (BCG) vaccination (1). BCG may prevent up to 80% of tuberculosis (TB) infections, and the period of efficacy is 15 years; however, its protective effect varies according to geographical variations (2,3). BCG is one of the world's most widely-used and safest vaccines; however, if vaccinating an immunodeficient infant with BCG may cause disseminated or fatal infection (4). The World Health Organization (WHO) recommends that in TB-prevalent countries, all newborn children be vaccinated against tuberculosis (5,6). BCG is a non-toxic cultured bacterium, which is used to prevent tuberculosis infection. A minor reaction may follow inoculation, including aseptic abscesses. The majority of the side effects of BCG immunotherapy appear to be self-limiting (7). The most common visceral involvement is the formation of asymptomatic liver granulomas, termed granulomatous hepatitis (8,9).

Plasmodium merozoites primarily invade the red blood cells of the host, which is where development and reproduction occurs (10). *Plasmodium* infected erythrocytes are able to adhere to the capillary endothelia of the host and escape from the spleen (11,12). Studies have demonstrated that pre-inoculation treatment with BCG improves the strength of the host immune response to malaria (13,14), although the effects of *Plasmodium* following malaria infection in BCG-vaccinated patients remains unclear (15). Therefore, the present study examined the effects of *Plasmodium* infection in BCG-vaccinated patients and the underlying mechanism. BCG-infected BALB/c mice subsequently infected with *Plasmodium* were used to observe the occurrence of hepatic granulomas. The secretion of pro-inflammatory and anti-inflammatory cytokines was analyzed at different time points following infection.

Materials and methods

Bacterial strain, parasites, mice and infections. Mycobacterium bovis BCG, Pasteur strain were grown in Middlebrook 7H9 medium (Difco Laboratories; BD Biosciences, Franklin Lakes, NJ, USA) with 10% albumin dextrose catalase (ADC) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 0.05% Tween 80. Cryopreserved blood pre-treated with *Plasmodium yoelii* (*P. yoelii* 17XNL) was defrosted and used to infect BALB/c mice. A total of 60 female BALB/c mice (6-week-old, 18-23 g) were purchased from Vital River Laboratory Technology Animal Co., Ltd.

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Key words: Bacille Calmette-Guerin, granuloma, *Plasmodium*, inducible nitric oxide synthase, interleukin-10

(Beijing, China) and maintained in specific pathogen-free grade experimental animal facilities at 25˚C with free access to acidified water and food and maintained at 45-70% humidity with a 12-h light/dark cycle. All mice were divided into four groups. The uninfected mice were set as the control group. BCG group mice were intravenously injected with $1x10^7$ colony-forming units (CFU) BCG per mouse. *Plasmodium* group mice were injected intraperitoneally with $1x10^5$ blood cells infected with *P. yoelii* 17XNL. BCG + *Plasmodium* group mice were intravenously injected with $1x10^7$ CFU BCG and $1x10^5$ blood cells infected with *P. yoelii* 17XNL. The kinetics of the infections was followed over 8 weeks. Following treatment mice had free access to water and food and were kept at 45-70% humidity and 25˚C; the housing room was sterilized by UV lights for 12 h and had a 12-h light/dark cycle. Mice were sacrificed at weeks 1, 2, 4, 6 and 8. The present study was approved by the Institutional Animal Care and Use Committee of Pearl Animal Sci. & Tech. Co., Ltd. (Dongguan, China).

Histopathological analysis. Following sacrifice, histological examinations were performed. The livers were removed, fixed in 10% formaldehyde solution at 4˚C for 1 week, and then dehydrated. The paraffin sections (5 μm) were cut and hematoxylin and eosin (H&E) staining was performed at room temperature for 2 h. The results of the staining (magnification, x200) were analyzed with an optical inverted microscope (Olympus Corporation, Tokyo, Japan). Simultaneously, the number of granulomas in the liver was determined in the H&E-stained sections (5 sections per mouse, 3 mice per group).

Determination of liver bacterial load. To assess the bacterial load in the liver following co-infection, the mice were sacrificed and the entire liver was homogenized using PBS supplemented with 0.05% Tween 80, and serial dilutions of homogenized liver tissues were plated on 7H11 agar with oleic ADC. The plates were cultured in an incubator at 37˚C with 5% CO_2, and the CFU (copies/mg) were determined at 1, 2, 4, 6 and 8 weeks respectively.

Immunohistochemical analysis. Inflammatory activity in the liver in BCG and *Plasmodium* coinfected mice were evaluated by immunohistochemistry. The liver tissues were cut into 4-μm sections using a cryostat. Inflammatory activity was evaluated via iNOS immunostaining. Briefly, sections were deparaffinized and hydrated, and heated in citrate buffer (0.01 M, Ph 6.0) for 1 min at 100˚C and were then treated with endogenous peroxidase (3% hydrogen peroxide solution) for 20 min at room temperature. Following blocking with 10% goat serum (Beyotime Institute of Biotechnology, Shanghai, China) for another 30 min at room temperature, sections were immunostained with primary antibodies with anti-iNOS (Rabbit monoclonal to iNOS; cat. no. ab178945; 1:500; Abcam, Cambridge, UK) antibody overnight at 4˚C, and subsequently incubated with the secondary antibody (Goat Anti-Rabbit; cat. no. ab6720; 1:1,000; Abcam) for 30 min at room temperature. Sections were then incubated with avidin-biotin-peroxidase complex for 30 min and DAB reagent for 5 min at room temperature. Subsequently, all sections were double stained with hematoxylin and visualized under the microscope (magnification, x10; BX51, Olympus Corporation, Tokyo, Japan), and six fields were selected for statistical analysis.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. RT-PCR was performed as previously described (16). mRNA was extracted from small pieces of liver tissues. First strand cDNA was synthesized from the 1 μg total RNA. Sequences for primers were as follows: For iNOS, forward TCACTGGGACAGCACAGAAT, and reverse TGTGTCTGCAGATGTGCTGA; and for β-actin, forward ACCACACCTTCTACAATGA, and reverse ATAGCACAG CCTGGATAG, and were designed using Primer Premier version 6.0 (Premier Biosoft International, Palo Alto, CA, USA). RT-PCR was carried out in the Applied Biosystems 7300 PCR system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Light Cycler Software version 3 (Roche Applied Science, Penzberg, Germany) was used for the analysis of the results.

Statistical analysis. Data were analyzed using GraphPad version 6.0 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance with least significant difference post hoc tests were used to indicate the significant differences among the different groups. A total of three replicates were performed for each experiment. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Liver index analysis demonstrates that liver granulomas slowly return to normal in co-infected mice. As presented in Fig. 1, from the liver index analysis it was apparent that, due to the co-infection of *Plasmodium* and BCG, the return to normal levels was notably delayed. Treatment with BCG or *Plasmodium* causes swelling of the liver, although this process is transient and the swelling is rapidly reversed (17). In the present study, following co-infection with *Plasmodium* and BCG, the swelling level of the liver returned to normal slowly ($P<0.05$).

Histopathological analysis demonstrates that Plasmodium causes the number of granulomas to slowly decrease in BCG-infected mice. As presented in Fig. 2, from a histopathological perspective, it was observed that *Plasmodium* infection did not lead to the formation of granulomas. BCG infection caused the formation of TB-specific granulomas; the number of granulomas reached a peak at week 4 and subsequently subsided. However, following co-infection with *Plasmodium* and BCG, due to the induction of *Plasmodium*, the peak formation of granulomas was delayed until week 6 and subsequently subsided ($P<0.05$).

Expression of iNOS increases and decreases with the formation and disappearance of granulomas in the liver. As demonstrated in Fig. 3, iNOS was highly expressed in the granulomas of the BCG group and BCG + *Plasmodium* co-infected group. In order to further examine whether the expression of iNOS is different among different groups, immunohistochemical staining was performed. As presented in Fig. 4A, it was observed that the *Plasmodium* group had

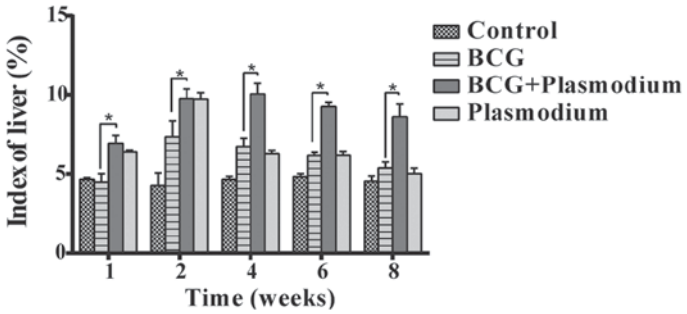

Figure 1. Liver tissues were collected and weighed at weeks 1, 2, 4, 6 and 8, and the liver index was calculated. *P<0.05. BCG, Bacille Calmette-Guerin.

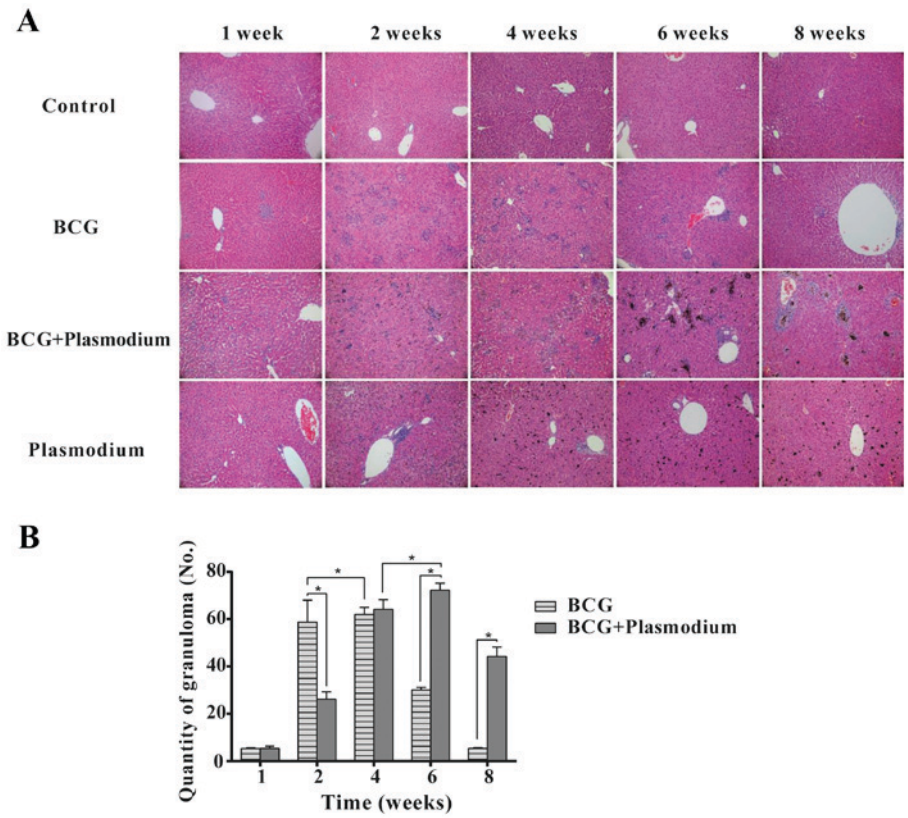

Figure 2. Histopathological analysis (magnification, x200). Liver tissues were collected and sliced into paraffin sections at weeks 1, 2, 4, 6 and 8 for (A) histopathological inspection and (B) the quantity of granulomas. *P<0.05. BCG, Bacille Calmette-Guerin.

decreased expression of iNOS; the expression of iNOS in the BCG group reached a peak at week 4, while in the BCG + *Plasmodium* co-infection group, it reached a peak at week 6 (P<0.05).

Expression of iNOS and IL-10 exhibits reverse trends. The present study aimed to assess why a difference in the expression of iNOS was observed. As exhibited in Figs. 4B and 5, the expression of IL-10 and BCG bacterial load in liver tissues was examined. IL-10 is considered to have immunosuppressive effects. The experimental results demonstrated that the expression of IL-10 was initially high and gradually decreased (*P<0.05), in the *Plasmodium* and BCG + *Plasmodium* groups. There was a higher bacterial load in the coinfected group compared with the BCG group at 2 weeks. A similar significant increase in bacterial load in the liver was observed in the BCG + *Plasmodium* group when compared with the BCG group at 4 weeks.

Discussion

BCG is a mutant strain of *M. bovis*. During the 20 th Century, BCG has been used to vaccinate newborns in order to prevent TB (18). As its safety has been determined, BCG has been used widely around the world; it has officially been recognized as the most safe and effective vaccine against tuberculosis, and it has become one of the vaccinations at birth recommended by the WHO (19,20). BCG is an important approach in the prevention of severe TB infection. The majority of BCG-vaccinated infants do contract an infection, although there remain a small number of infected infants (21,22). BCG infection occurs only in some children; those whose

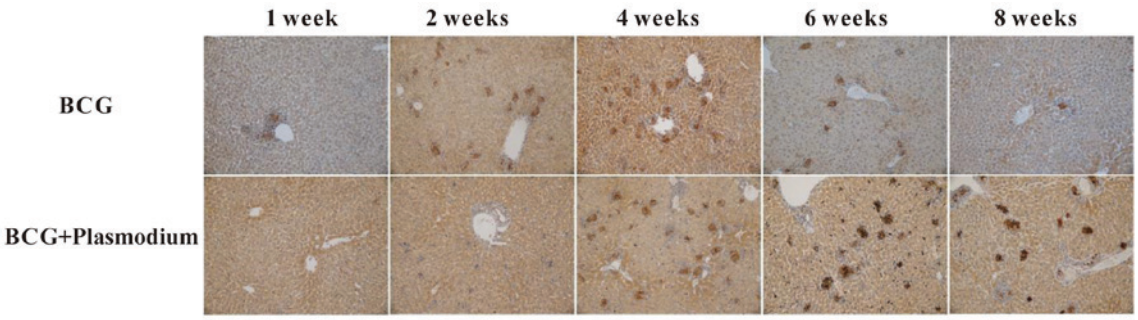

Figure 3. Immunohistochemistry staining for inducible nitric oxide synthase in liver tissues (magnification, x200). BCG, Bacille Calmette-Guerin.

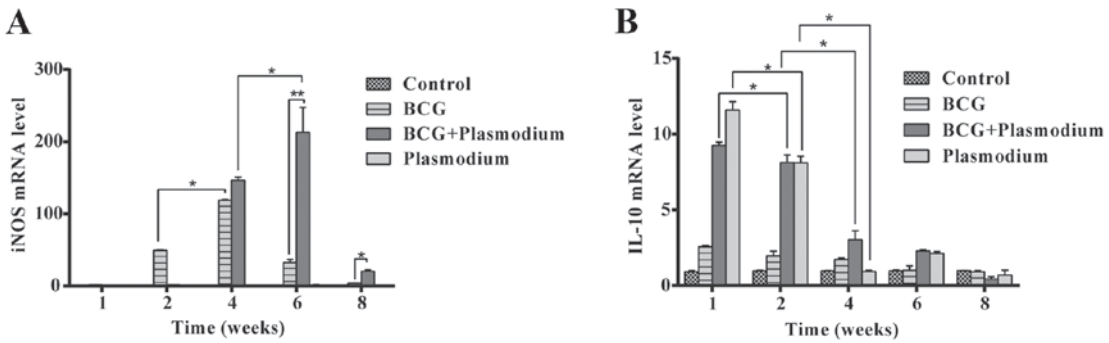

Figure 4. Reverse transcription-polymerase chain reaction analysis. Differences in the mRNA expression of (A) iNOS and (B) IL-10 were examined in liver tissues. *P<0.05; **P<0.01. iNOS, inducible nitric oxide synthase; IL-10, interleukin-10; BCG, Bacille Calmette-Guerin.

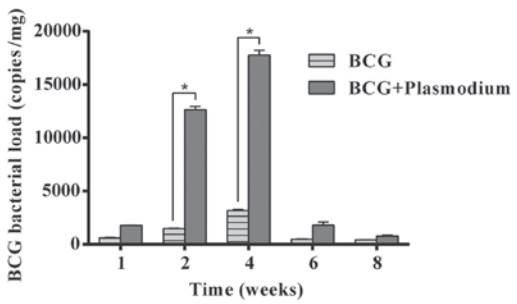

Figure 5. BCG bacterial load in liver tissues of mice infected with BCG or BCG + *Plasmodium* at different post-infection time points. *P<0.05. BCG, Bacille Calmette-Guerin.

host immune defense ability against BCG may be low (23). The infection may lead to the occurrence of granulomatous hepatitis in the liver (24).

Malaria, an insect-borne disease caused by *Plasmodium* infection, is one of the most serious communicable diseases (25,26). The prevention and treatment of malaria faces serious challenges and understanding of the biological characteristics of the malaria parasite and its association with the host immune system remains limited (27). The present study demonstrated that the use of *Plasmodium* in BCG-infected mice generated a high level of iNOS and that granuloma formation was delayed, although sustained.

In order to confirm the findings in the liver tissues of co-infected mice, histopathological analysis was performed (28). The H&E staining images demonstrated that the livers of co-infected mice exhibited extensive granulomas, which were decreased in number in the *Plasmodium* group. Granuloma formation in liver was delayed in BCG-infected mice co-infected with *Plasmodium*, indicating that *Plasmodium* was the primary cause of this phenomenon. The present study suggested that the reason for this was that *Plasmodium* may exert a direct impact on the delayed-type allergic immune response, although the mechanism is not yet clear.

To illustrate the mechanism of action of *Plasmodium* in BCG-infected mice, immunohistochemical and RT-PCR analyses were performed. The results demonstrated that with the formation and reversal of hepatic granulomas, the expression of iNOS increased and decreased, while the expression of IL-10 exhibited the opposite trend. These results demonstrated that iNOS and IL-10 were involved in the pathological process.

In conclusion, the present study provided an innovative examination of the role of *Plasmodium* in BCG granuloma formation. The results of the present study demonstrated that, following co-infection with *Plasmodium* and BCG, the formation of granulomas in liver was relatively delayed, although sustained, in mice.

Acknowledgements

Not applicable.

Funding

The present study was supported by Provincial Science and Technology Project of Guangdong Province (grant nos. 2016A030303056).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XC and LQ designed the present study and approved this submission. BN and JY performed the experiments and wrote the manuscript. MJ performed the immunohistochemical staining. SZ helped to collect data and revised the manuscript.

Ethics approval and consent to participate

The present study was approved by the Institutional Animal Care and Use Committee of Pearl Animal Sci. & Tech. Co., Ltd. (Dongguan, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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