Phagocytic activity and pro-inflammatory cytokines production by the murine macrophage cell line J774A.1 stimulated by a recombinant BCG (rBCG) expressing the MSP1-C of *Plasmodium falciparum*

Rapeah, S.[1*], Dhaniah, M.[1], Nurul, A.A.[2] and Norazmi, M.N.[1]
[1]School of Health and [2]Dental Sciences, Universiti Sains Malaysia,16150 Kubang Kerian, Kelantan, Malaysia
*Corresponding author email: rapeah@kck.usm.my
Received 6 February 2010; received in revised form 1 July 2010; accepted 3 July 2010

Abstract. Macrophages are involved in innate immunity against malaria due to their ability to phagocytose infected erythrocytes and produce inflammatory cytokines, which are important for controlling parasite growth during malaria infection. In this study, the ability of a recombinant BCG (rBCG) vaccine expressing the 19-kDa C-terminus of merozoite surface protein-1 (MSP1-C) of *Plasmodium falciparum*, to stimulate the phagocytic activity and secretion of pro-inflammatory cytokines by the macrophage cell line J774A.1 was measured at varying times. The results demonstrate the ability of the rBCG construct to activate the inflammatory action of macrophages, which is important as a first-line of defence in clearing malaria infections.

INTRODUCTION

Malaria continues to be one of the leading causes of death in the developing and under developed countries. The majority of deaths are in children under the age of five (Elliott & Beeson, 2008; Guerra *et al.*, 2008). It is caused by obligate intracellular parasite of the genus *Plasmodium* and is transmitted to humans through the bite of the female *Anopheles* mosquito. Among four species of human malaria parasites, *Plasmodium falciparum* is the most dangerous due to high fatality rate (Angulo & Fresno, 2002). Various anti-malarial drugs such as chloroquine and artemisinin are currently used to control malaria. However, anti-malarial drugs only partially effective in controlling disease due to the increasing problem of drug resistant parasites all over the world. Thus, additional approaches such as vaccination have become a top public health priority for malaria control (Yazdani *et al.*, 2006).

Research aimed at the development of an effective vaccine against malaria has been conducted for years. Advances in biotechnology and in understanding the molecular mechanism as well as the genetic diversity of the parasites have encouraged more research and provided new opportunities for vaccine development against malaria (Genton *et al.*, 2003; Hall *et al.*, 2005; Ferreira & Hartl, 2007). Thousands of novel proteins from different stages of the parasites' life cycle have been studied for their potential as vaccine targets. As a result, several candidate vaccines have been developed and some of them such as RTS,S, have entered clinical trial (Alonso *et al.*, 2005; Sacarlal *et al.*, 2008). However till date, there is still no safe and effective vaccine that has been approved to be implemented worldwide.

Most studies on malaria vaccine development have focused on asexual blood-stage of the parasitic life cycle. At this stage, the merozoites that are released from infected liver cells enter the bloodstream and invade red blood cells (RBC). Here the merozoites establishing a cycle of invasion and replication, which is responsible for all clinical illness of malaria (Harris *et al.*, 2005). Thus, development of a blood-stage vaccine can generate antibodies that inhibit parasite invasion of red blood cells as well as reduce the complications of the disease (Wipasa, 2002). Several surface proteins of the merozoite have been studied as candidate antigens in vaccine development against blood-stage malarial infection. Of these, the C-terminus of merozoite surface protein-1 (MSP-1C) is one of the most extensively studied. It is a 19-kDa fragment of merozoite surface protein comprising two epidermal growth factor (EGF) domains that are synthesised through a proteolytic process from ~200 kDa molecule during merozoite development. This fragment is bound to the merozoite plasma membrane via its glycosyl phosphatidylinositol (GPI) anchor. It is the only part of the MSP1 complex to be carried inside the erythrocyte during invasion while the remaining fragments are shed as a soluble complex (Blackman *et al.*, 1990; Harris *et al.*, 2005). This region is functionally conserved across species of the genus *Plasmodium* (O'Donnell *et al.*, 2000). MSP-1C is important for erythrocyte invasion and immune responses against the parasite. Antibodies to MSP1-1C have been reported to be associated with protection from symptomatic disease in animals (Hirunpetcharat *et al.*, 1997; Perera *et al.*, 1998; Wan Omar *et al.*, 2007) as well as in human (O'Donnell *et al.*, 2001; King *et al.*, 2002; John *et al.*, 2004).

Phagocytosis of parasites by macrophages has important roles in innate immunity during malaria parasite infection by facilitating the killing of pathogens and priming the adaptive immune response (Stevenson & Riley, 2004; Lee *et al.*, 2007). During this activity, macrophages are activated to acquire microbicidal effector functions and secrete proinflammatory cytokines such as IL-12, TNF-α and IL-1β. This results in inflammation and recruitment of immune cells for the elimination of surrounding pathogens, as well as the release of toxic metabolites such as nitric oxide (NO) which kill the parasites directly and can also combine with superoxide (O_2^-) to form peroxynitrite, a strong bactericidal agent (Mshana *et al.*, 1991; Bogdan, 2001). The anti-malarial activities of IL-12, TNF-α and IL-1β have been previously reported (Yazdani *et al.*, 2006). Administration of IL-12 in mice has been shown to protect against *Plasmodium yoelii* (Sedegah *et al.*, 1994) and blood-stage *Plasmodium chabaudi* (Stevenson *et al.*, 1995; Sam & Stevenson, 1999). In addition, the presence of IL-12 also provides protection from *Plasmodium cynomolgi* infection in the rhesus monkey (Hoffman *et al.*, 1997). TNF-α and IL-1β are also protective against malaria parasite infection by promoting activated macrophages to produce reactive nitrogen and oxygen intermediates, which kill the parasite (Rocket *et al.*, 1991; Tylor-Robinson & Looker, 1998). Furthermore, high levels of TNF-α and IL-1β were detected in the blood of malaria patients and in the spleen of rodent malaria (Kern *et al.*, 1989; Jacobs *et al.*, 1996), thus suggesting that these cytokines are the natural response by host immune system against the pathogens.

The current live vaccine against tuberculosis, *Mycobacterium bovis* Bacille Calmette Guèrin (BCG), has excellent immunoadjuvant properties and can be ingested as well as presented to T cells by professional antigen presenting cells (APCs), such as macrophages. Thus, BCG has been used for years as a live vector for the development of recombinant vaccines for various diseases, including for malaria (Britton & Palendira, 2003).

Previously, we had constructed a recombinant BCG (rBCG) vaccine expressing the MSP1-C of *P. falciparum*. A study conducted in mice showed that the vaccine candidate is capable of inducing

appropriate humoral and cellular immune responses. However, the effects of the construct on innate immune response have not been determined. In this study, the ability of the candidate vaccine to promote phagocytic activity of macrophages and to stimulate the production of inflammatory cytokines such as IL-12, TNF-α and IL-1β were determined.

MATERIAL AND METHODS

Construction of rBCG vaccine expressing the MSP-1C of *P. falciparum*

An rBCG vaccine expressing the MSP-1C of *P. falciparum* was constructed synthetically using assembly PCR (Norazmi *et al.*, 1999). Briefly, the oligonucleotides coding for the composite DNA fragment were mixed at 250 µM each. One microlitre of this mixture was diluted to a final concentration of 2.5 µM as a working solution, and 2.5 µl of the solution was used for the first PCR for gene assembly using the following conditions: denaturation at 95ºC for 1 minute, followed by 55 cycles at 95ºC for 1 minute, 55ºC for 1 minute, 72ºC for 1 minute and a final incubation cycle at 72ºC for 10 minutes. A second PCR was then performed using the following primers: sense 5'-CGGCTAGCATGAACATCAGCCAGCACCAG-3' and antisense 5'-GCTAGCCCTAGGTCAG TTCGACGACGAGCAGAA-3' to amplify the target fragment formed after the first PCR, using the following conditions: denaturation at 95ºC for 1 min, followed by 30 cycles at 95ºC for 1 minute, 60ºC for 1 minute, 72ºC for 1 minute and a final incubation cycle at 72ºC for 10 minutes. The 314 bp PCR product was then cloned into the shuttle vector pNMN013. The shuttle plasmid was transformed into BCG by electroporation.

Preparation of rBCG culture

Mycobacterium bovis BCG (Japan) and rBCG (Japan) were cultured in 7H11 supplemented with OADC and 15 µg/ml kanamycin for rBCG or without kanamycin for BCG and incubated at 37ºC for 2 weeks.

The bacteria were then transferred to 7H9 broth supplemented with OADC and 15 µg/ml kanamycin or without kanamycin for 1 week. For co-culture with macrophages, 10 ml of the BCG/rBCG cultures were centrifuged at 1500 x *g* for 10 minutes. The supernatant was then removed and the pellet was resuspended in 1 ml DMEM.

Preparation of murine macrophage cell line J774A.1

The J774A.1 mouse macrophage cell line (ATCC, USA) was cultured in DMEM (Sigma, USA) supplemented with 50 µg/ml gentamycin, and 10% fetal bovine serum (Sigma, USA). The cells were grown at 37ºC and 5% CO_2 in a humidified incubator.

Infection of murine macrophage cell line J774A.1 with BCG and rBCG

Macrophages (1×10^6 cells/ml) were incubated with 2×10^7 cfu/ml BCG or rBCG in DMEM supplemented with 50 µg/ml gentamycin, and 10% fetal bovine serum. The cells were incubated for 1 day, 3 days and 5 days in a humidified 5% CO_2 incubator.

Phagocytic assay

For phagocytic analysis, the treated cells were centrifuged at 1000 x *g* for 5 min. The pellet was resuspended in 1 ml PBS. Ten µl of cell suspension was layered on a slide and the slide was stained with Acid Fast Bacilli (AFB) (Sigma, USA). The slide was examined microscopically and the number of phagocytosed mycobacterium was enumerated. The phagocytic index (PI) was calculated as the number of ingested BCG or rBCG per macrophage (Gopinath *et al.*, 2006).

Cytokine assay by ELISA

The pro-inflammatory cytokines, IL-12, TNF-α and IL-1β were determined in culture supernatants of BCG- and rBCG-stimulated macrophages at day 1, day 3 and day 5 using the ELISA Quantikine mouse IL-12, TNF-α and IL-1β kits (R&D Systems, USA) according to the manufacturer's instructions. Basically, 1×10^6 cells/ml macrophages were incubated

with 2×10^7 cfu/ml BCG or rBCG in DMEM supplemented with 50 µg/ml gentamycin, and 10% fetal bovine serum for 1 day, 3 days and 5 days in a humidified 5% CO_2 incubator. 50 µl of the supernatants were incubated in a 96-well microtiter plate (Nunc, Germany) for 2 hours at room temperature. The plate was then washed five times with 400 µl of washing buffer. After washing, 100 µl of mouse IL-12, TNF-α or IL-1β was added to each well and incubated for 2 hours at room temperature. After incubation, the plate was then washed five times with 400 µl of washing buffer before 100 µl of substrate solution was added and the plate was incubated for 30 minutes at room temperature. The reaction was stopped after 30 minutes with stop solution and the optical density (OD) was determined with a microplate reader (Bio-Rad, USA) at 450 nm. Untreated macrophages were used as a control. All cytokine concentrations (in pg/ml) were determined by comparison with the standard curve.

Statistical analysis

All experiments were carried out in triplicate and repeated three times. Data were represented as the arithmetic mean ± standard error mean (S.E.M). Comparison between BCG and rBCG groups was analysed using Student's t-test. The accepted level of significance was $P < 0.05$.

RESULTS

Phagocytic uptake of BCG and rBCG by macrophages

To determine whether incubation time of BCG or rBCG influenced the phagocytic activity of macrophages, the phagocytic capacity of these cells were investigated for 5 days. The phagocytic capacity of BCG- and rBCG-stimulated macrophages increased markedly from day 1 to day 5 post-infection (Figure 1). However, the mean phagocytic index (PI) of cells stimulated with rBCG was statistically
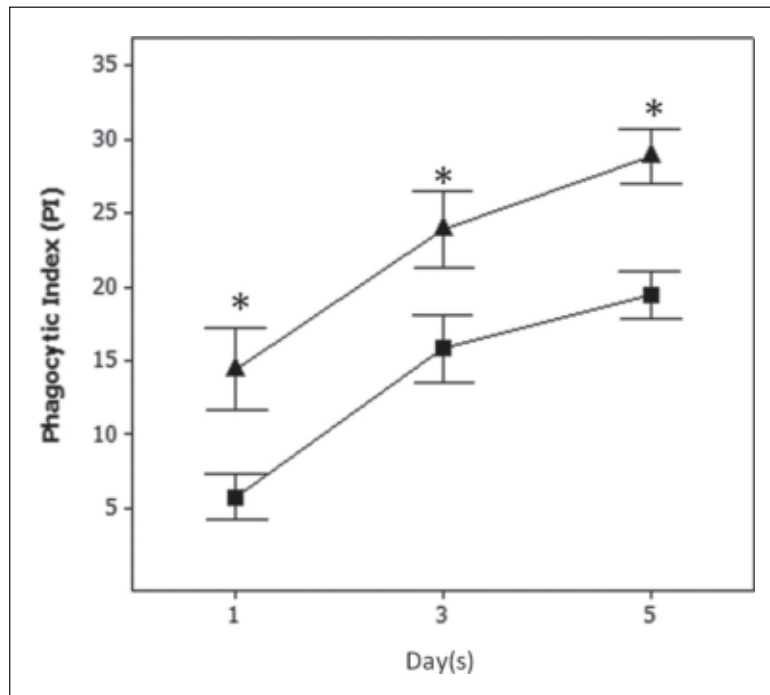

Figure 1. Phagocytic Index (PI) of macrophages stimulated with 2×10^7 cfu/ml of BCG (–■–) and rBCG (–▲–) at different days. Data are expressed as mean ± S.E.M. Student's *t*-test was used to determined differences between BCG and rBCG treatments (* $P < 0.05$)

significantly higher ($P < 0.05$) compared to parent BCG vector at all incubation times.

Pro-inflammatory cytokines production by BCG- and rBCG-stimulated macrophages

The expression of inflammatory cytokines, IL-12, TNF-α and IL-1β were also studied over a period of 5 days. Figure 2 shows that TNF-α and IL-1β are produced by macrophages in response to both BCG and rBCG, but the levels and patterns of cytokine production differ in these cells. However, substantial levels of IL-12 were not detectable up to 5 days post-infection in macrophages stimulated with either BCG or rBCG (data not shown).

The concentration of TNF-α secreted by macrophages treated with rBCG was significantly higher than those treated with
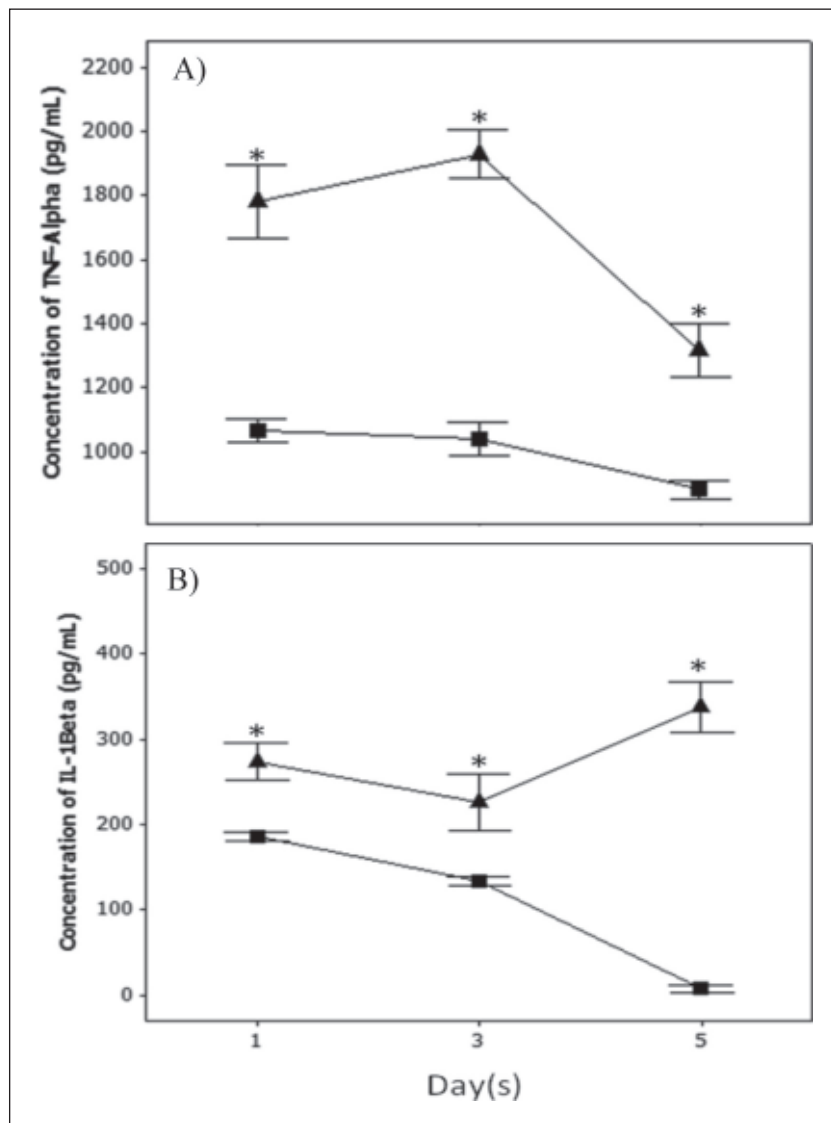

Figure 2. Expression of (A) TNF-α (B) IL-1β in macrophages stimulated with 2 X 10^7 cfu/ml of BCG (–■–) and rBCG (–▲–) at different days as detected by ELISA. Data are from a single experiment performed in triplicate and expressed as mean ± S.E.M. Student's t-test was used to determined differences between BCG and rBCG treatments (* $P < 0.05$)

the parent BCG vector ($P < 0.05$) (Figure 2A). The pattern of TNF-α secretion in rBCG exposed macrophages increased from day 1 (1780 ± 81.6 pg/ml) to day 3 (1927.7 ± 54.2 pg/ml) but decreased rapidly at day 5 post-infection to 1315.1 ± 60.3 pg/ml. In the control BCG vector-stimulated macrophages, the levels of TNF-α secretion decreased persistently from day 1 to day 5 post-infection.

Levels of IL-1β were significantly higher in macrophages stimulated with rBCG compared to BCG controls (Figure 2B). However, the levels of this cytokine decreased from day 1 (273.6 ± 15.7 pg/ml) to day 3 (226.75 ± 23.5 pg/ml), which was followed by a rapid increase to 337.8 ± 21.3 pg/ml at day 5 post-infection. In contrast, IL-1β secretion consistently decreased in cells stimulated with parent BCG vector from day 1 to day 5 post-infection.

DISCUSSION

Macrophages are important in innate immunity during the blood-stage of malaria parasite infection due to their ability to phagocytose infected erythrocytes (Serghides *et al.*, 2003) as well as the production of inflammatory cytokines such as IL-12, TNF-α and IL-1β (Sam & Stevenson, 1999). Activated macrophages can also function as antigen-presenting cells by expressing co-stimulatory molecules such as CD80 and CD86, which are important in the activation of T cells (Lee *et al.*, 2007). This indicates that, macrophage activation participates in both the innate and adaptive immune responses.

In the present study, both BCG and rBCG exhibited the ability to enhance the phagocytic activity of the macrophage as well as the production of TNF-α and IL-1β that has been suggested to be involved in controlling parasite growth in malaria infection. However, the secretion of IL-12 was not detectable in either BCG or rBCG-stimulated macrophages up to 5 days post infection. According to Sam & Stevenson (1999), the secretion of IL-12 by macrophage requires longer incubation

times, particularly longer than 4 days. However, further study is needed to distinguish whether the activated macrophage does not secrete this cytokine, or if secretion occurs after 5 days of incubation. Interestingly, the rBCG-stimulated macrophages produced significant quantities of TNF-α and IL-1β and induced more phagocytic activity to the cells compared to those stimulated by BCG. Our preliminary data suggested that the effect of the rBCG observed in the present study appeared to be associated with NO production. Thus, these findings revealed the promising possibility of parasite clearance by the rBCG-induced macrophage during malaria infection.

BCG in the absence of MSP-1C was also ingested by the macrophages. This might be due to the structure of the lipoarabinomannan in BCG. This protein was suggested to be a potential ligand for the macrophage mannose receptor (Prinzis *et al.*, 1993). Moreover, BCG is also capable of being ingested by macrophages through various macrophage cell surface receptors such as Fc receptors (FcR), complement receptors (CR) and phagocytic pattern recognition receptors (PRR). In the cell, they are either killed by macrophage-derived products, such as nitric oxide, or survive and replicate. Interestingly, when the MSP-1C was cloned into the BCG to produce rBCG, its capacity to induce phagocytosis and production of inflammatory cytokines in stimulated macrophages increased. However, the mechanism by which the presence of MSP-1C in BCG renders rBCG capable of increasing macrophage activation is unknown. One possibility is that the MSP-1C might present on the surface of the BCG and introduce more ligand recognition sites on the macrophage surface, thus inducing more efficient phagocytosis than the parent BCG strain. However, further study is required to understand the actual mechanism underlying this phenomenon.

In summary, the preliminary findings shown here demonstrate that our rBCG construct expressing the MSP-1C of *P. falciparum* is capable of stimulating

significant immunomodulatory effects on the activity of macrophages. These findings may reflect the capacity of our candidate vaccine to influence the ongoing immune response against the parasites.

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