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Role of toll-like receptor 4 in eliciting adaptive immune responses against recombinant BCG expressing the C-terminus of merozoite surface protein-1 of *Plasmodium falciparum*

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ABSTRACT

Objective: To determine the role of toll-like receptor 4 (TLR-4) in eliciting cellular and humoral immune responses against recombinant *Mycobacterium bovis* bacille Calmette-Guérin (rBCG) expressing the C-terminus of merozoite surface protein-1 of *Plasmodium falciparum*. **Methods:** Six groups of mice (n=6 per group) were injected with phosphate buffered saline T80, BCG or rBCG intraperitoneally, in the presence or absence of a TLR-4 inhibitor; TAK-242. Enzyme-linked immunosorbent assay was carried out for serum total IgG, IgG1, IgG2a and IgG2b determination. Spleens were also harvested and splenocytes cultured for determination of intracellular cytokines; IL-4 and IFN-γ *via* enzyme-linked immunosorbent assay. **Results:** The production of total IgG, and the subclasses IgG1, IgG2a and IgG2b was significantly higher in rBCG-immunised mice than BCG and phosphate buffered saline immunised mice in the absence of TAK-242. A significant rise in total IgG occurred with more booster immunisations. The level of IgG2a was highest, followed by IgG2b, then IgG1. The production of both IL-4 and IFN-γ was also highest in the rBCG immunised groups. These significant rises were inhibited in the presence of TAK-242. **Conclusions:** We present evidence of the role of TLR-4 in the increased production of total IgG, IgG1, IgG2a and IgG2b, as well as IL-4 and IFN-γ in response to our rBCG construct.

1. Introduction

Malaria exposes people living in Africa, South-East Asia and some parts of South America to disease conditions, which not only puts about half the world population at risk of transmission but also causes close to half a million annual death of mostly children and pregnant women *via* severe complications[1-3]. The control of this deadly disease has not been achieved, as both the *Anopheles* mosquito vector and the *Plasmodium* parasite[4,5] continue to develop resistance against all modalities of vector control and treatment, forcing the searchlight towards the development

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of malaria vaccine which includes the use of recombinant *Mycobacterium bovis* bacillus Calmette-Guerin (rBCG) to express malaria epitopes like the merozoite surface protein[5].

Due to its unique safety profile, BCG is used in vaccines for other human pathogens[6,7]. It is easily recognised and rapidly phagocytosed, eliciting specific adaptive responses such as antibody formation and T cell responses[8-11]. *Plasmodium falciparum* (*P. falciparum*) merozoite surface protein (MSP)-1 on the surface of lysed merozoites is used as a blood-stage vaccine candidate to generate protective immunity against malaria with both Th1 and Th2 immune responses in human and animals[12-16]. Our laboratory had earlier constructed a recombinant BCG expressing the MSP-1C of *P. falciparum* which elicited robust cellular and humoral immune responses through a mechanism that had not been studied but thought to be initiated by MSP-1 attachment of merozoites to the specific host receptor[17,18].

Toll-like receptors (TLRs) are a group of pattern recognition receptors which recognise conserved pathogens structures leading to the generation of innate and adaptive immune responses[19,20]. There are 13 functional TLRs numbered 1-13 identified in mammal, with TLR-1, TLR-2, TLR-4, TLR-5, TLR-6, and TLR-11 recognising microbial lipids, lipoproteins, and proteins[21]. The present study was based on the finding that TLR-4 activation is a major pathway involved in a class of malaria[22]. TLR-4 recognises glycosyl phosphatidylinositol from *P. falciparum* and is activated through both the MyD88-dependent and MyD88-independent pathways leading to cytokine release and induction of adaptive immunity such as increased titres of IgG1, IgG2a and IgG2b as well as T cell proliferation leading to the expression of IFN-γ and IL-4[23-25]. Mice express IgG1, IgG2a and IgG2b or IgG2c antibodies depending on strain[26]. Immunisation of mice with *Plasmodium*-derived antigen elicits IgG1, IgG2a and IgG2b[27,28]. Both IgG1 and IgG2 are associated with a reduced risk of malaria, with IgG2a and IgG2b protecting against sporozoite infection[29-31]. In this study, IgG1 was utilised as a representative cytophilic IgG since there is a strong correlation between it and IgG3 while IgG2a and IgG2b were chosen as representative non-cytophilic IgGs[32-34]. The study also investigated the induction of splenic cytokines, IFN-γ and IL-4 as representative Th1 and Th2 cytokines respectively[35]. Both IFN-γ and IL-4 are important in B cell responses against malaria and their levels were elevated in malaria-infection[36,37]. INF-γ is also important in protection against severe malaria anemia and parasite clearance while IL-4 is essential for a balanced Th1/Th2 response during malaria infection[36,38,39].

2. Materials and methods

2.1. Ethics

All animal work was carried out with the approval of the Universiti Sains Malaysia (USM) Animal Ethics Committee No. (2016(104)(801)) obtained on the 30th of November 2016.

2.2. BALB/c mice

BALB/c mice purchased from the Animal Research and Service Centre (ARASC), USM were housed at the ARASC facility. The mice were provided with standard laboratory chow and water *ad libitum*.

2.3. Preparation of BCG and rBCG cultures

The parent BCG (Japan) and the rBCG016, earlier cloned in the laboratory through a series of polymerase chain reactions were cultured on a 7H11 agar (Becton Dickinson, USA)[40]. The agar was supplemented with oleic acid, albumin, dextrose, and catalase (OADC) (Becton Dickinson, USA) at 37 ℃ in an incubator, with the addition of 15 μg/mL of kanamycin (Sigma, USA) to the rBCG culture for 2-3 weeks and transferred to flasks containing 10 mL of 7H9 broth (Becton Dickinson, USA), with similar supplements for another 2-3 weeks until an optical density (OD) of approximate 0.8 (A600≈0.8) was obtained. Ten millilitres of BCG and rBCG each were centrifuged in separate tubes at 1 500 × *g* for 10 min at room temperature, and the pellets were washed with phosphate buffered saline (PBS), then resuspended in Dulbecco's Modified Eagle Medium and colony forming unit determined using the formula developed by Norazmi[41].

2.4. Mice immunisation

A total of 36 male BALB/c mice aged 4-6 weeks were divided into six groups (n=6) in the study. Each mouse received intraperitoneal immunisations three times, three weeks apart, with 200 μL of PBS-T80, 2×10^6 CFU of BCG or 2×10^6 CFU of rBCG respectively in the presence or absence of 0.5 mg/kg of TLR-4 inhibitor, TAK-242, one hour before each immunisation[42,43]. The mice were closely observed for any signs of adverse effects such as erythema at the site of injection, abnormal movement, decreased activity, decreased feeding or death and none has been found.

2.5. Blood collection

Using a previously described method, blood was collected from the tail of all mice before the first immunisation (pre-immunisation), 3 weeks after 1st immunisation (week 3), 3 weeks after 1st booster immunisation (week 6) and 3 weeks after 2nd booster (week 9) just before sacrifice[44]. In brief, after restraining the mice, the tail was sterilised with 70% ethanol, topical anaesthesia applied and a small cut made on the tail about 1 cm from its tip using a sterile scalpel. A microcentrifuge was placed below the cut region to collect the blood by a gentle massage on the tail. The blood was allowed to clot overnight at 4 ℃ and the sera were harvested at the following day by centrifuging at 1 500 × *g* for 15 min.

2.6. Measurement of total IgG and IgG subclass antibodies by enzyme-linked immunosorbent assay (ELISA)

ELISA was carried out using a 96-well flat bottom plate. Briefly, rBCG was sonicated and the sonicate was diluted in carbonate-bicarbonate coating buffer (Na_2CO_3-$NaHCO_3$) to 1 μg/mL and incubated at 4 ℃ overnight. On the following day, the plate was washed 5 times on a shaker then blocked with blocking buffer and incubated at 37 ℃ for 1 h. The plate was washed and the sera from the six groups of mice diluted to 1:1 000 in blocking buffer were used as the primary antibody and incubated for 2 h at 37 ℃. After washing, secondary antibody; HRP conjugated goat anti-mouse IgG or rabbit anti-mouse IgG1, IgG2a or IgG2b diluted at 1:2 000 in blocking buffer was added to each well and incubated for 1 h at 37 ℃. The plate was washed and 100 μL of 2,2'-azino-di(3ethylbenzotiazolensulfonat) (ABTS; Roche, Germany) substrate was added and incubated in the dark for 30 min at 37 ℃. The reaction was stopped with 100 μL of 2 mol/L H_2SO_4 and the absorbance was measured at 405 nm.

2.7. Harvesting of murine spleen and preparation of splenocytes

Mice splenocytes were harvested using a modified method[45,46]. Briefly, mice were sacrificed *via* rapid cervical dislocation, laid on a dissecting board and the skin sterilised with 70% ethanol. Using a set of sterile forceps and scissors, the skin was cut through below the ribcage and the spleen was removed. The spleens from a single group of mice were pooled together and placed in ice-cold complete RPMI medium. A single-cell suspension was made using a metal sieve and the barrel of a 2 mL sterile syringe, and then centrifuged at 400 × *g* for 5 min. The pellet was washed twice with ice-cold RPMI. Ammonium chloride lysis buffer was added and incubated for 5 min on ice, with occasional shaking. The suspension was centrifuged at 400 × *g* for 10 min, washed twice and resuspended in RPMI. Two million splenocytes /mL were then cultured at 37 ℃ and 5% CO_2 for 24 h in a 25 cm^2 flasks in complete RPMI enriched with 25 μg/mL of amphotericin B. The culture solution was then centrifuged at 1 500 × *g* for 10 min at room temperature and the supernatants were used for cytokine determination.

2.8. Assessment of cytokines in splenocyte culture supernatants

ELISA analyses were carried out on the supernatant to estimate IFN-γ and IL-4 concentration. Briefly, a 96-well ELISA plate was coated with capture antibody, sealed and incubated overnight at 4 ℃. On the following day, the plate was washed 5 times with PBS-T20 and blocked with blocking buffer, then incubated for 2 h at room temperature, followed by another 5 washes. Culture supernatant and standard were added into corresponding wells, sealed and incubated for 2 h at room temperature. This was then followed by the addition of anti-mouse IFN-γ or IL-4, sealed and incubated for 2 h at room temperature. Avidin-HRP solution was added to each well after washing and incubated for 30 min at room temperature. A substrate solution was added to the wells and incubated for 5 min at room temperature in the dark. The reaction was stopped by the addition of stop solution and the plate was read with a microplate reader at 450 nm to determine cytokine concentration and generate a standard curve.

2.9. Statistical analysis

Data were analysed using the statistical package of social sciences (SPSS) software version 24. All data were representative of 3 experiments; performed in triplicate and presented as mean ± standard error of the mean (SEM). All the data were analysed by one-way analysis of variance (ANOVA), followed by the Bonferroni *post-hoc* test, except those of repeated vaccine on total IgG analysis where repeated measures ANOVA (RM-ANOVA) was utilised. The *P*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Antibody responses in immunised BALB/c mice

In order to ascertain the role of TLR-4 in humoral immune responses, the levels of total IgG antibodies as well as the IgG isotypes; IgG1, IgG2a, and IgG2b induced by the rBCG in immunised mice at pre-immunisation and 3 weeks after each immunisation were measured using ELISA. In the absence of the TLR-4 inhibitor, TAK-242, the levels of total IgG titre were highest in the mice group immunised with rBCG, which increased with booster immunisations ($P < 0.05$) (Figure 1A), followed by the mice immunised with BCG and then those immunised with PBS-T80. The total IgG production showed a significant increase after the second booster in the BCG immunised mice with no difference in the PBS-T80 immunised mice. Further analysis to determine the role of TLR-4 in total IgG production showed significant inhibition in the presence of TAK-242 across all mice groups ($P < 0.05$).

The production of IgG isotype; IgG1, IgG2a, and IgG2b was also evaluated in all mice groups (Figure 1B). In the absence of TAK-242, the rBCG antigen stimulated the highest IgG1 response compared to both control groups. The increase of IgG1, IgG2a, and IgG2b titres was statistically significant in each case ($P < 0.05$), which was the highest in the rBCG immunised mice compared to the BCG and PBS-T80 immunised groups ($P < 0.05$). Similar to the results of total IgG, there were significant differences in the three IgG subclasses of mice immunised with PBS-T80, BCG, and rBCG in the presence of TAK-242 ($P < 0.05$). Interestingly, significant differences were observed in the three IgG isotype ($P < 0.05$), with IgG2a production being the highest, followed by IgG2b, then IgG1 indicating the potential of the construct to induce mixed Th1 and Th2 response.

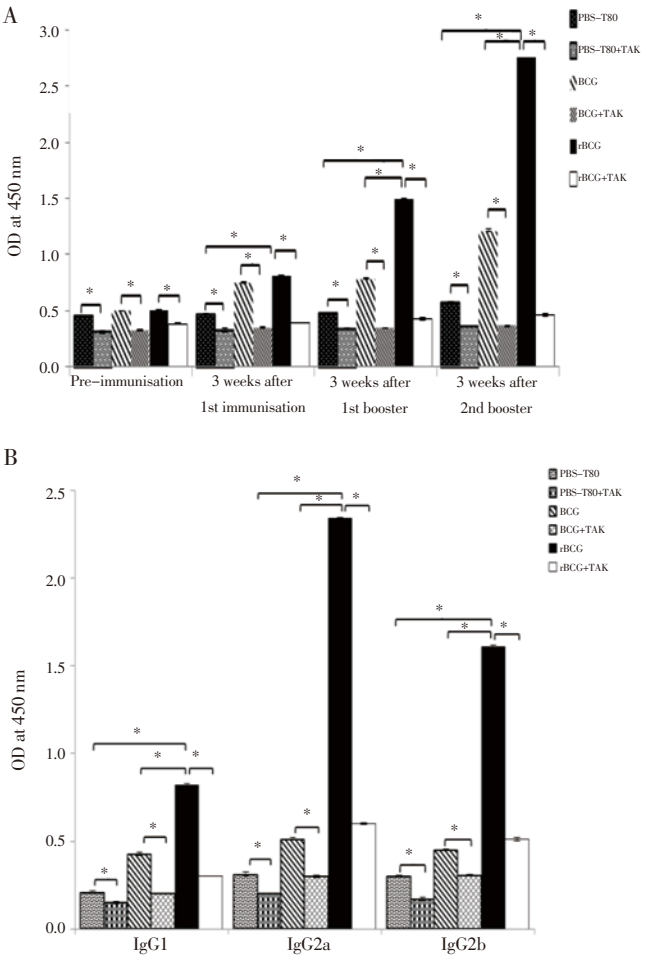

Figure 1. Serum level antibodies in the sera of mice immunised with PBS-T80, BCG or rBCG in the absence or presence of TLR-4 inhibitor.
(A) Total IgG before immunisation and three weeks after each immunisation, and (B) IgG isotype (IgG1, IgG2a, and IgG2b) three weeks after the last booster immunisation. Data are presented as the mean OD of total IgG and IgG isotype ± SEM for three independent experiments, $^{*}P < 0.05$.

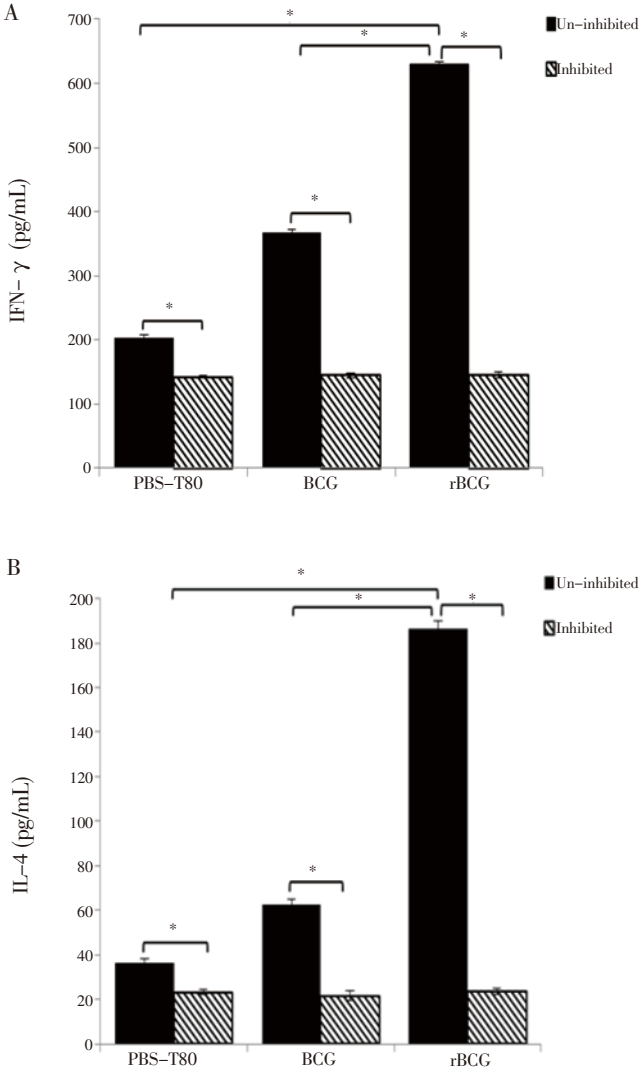

Figure 2. Effects of TLR4 inhibition on IFN- γ (A) and IL-4 (B) production by splenocytes of mice immunised with PBS-T80, BCG or rBCG in the absence or presence of TLR-4 inhibitor.
Data are presented as the mean concentration of IL-4 and IFN- γ ± SEM for three independent experiments, $^{*}P < 0.05$.

3.2. In vitro production of cytokines from splenocytes after rBCG immunisation

The production of cytokines by splenocytes after rBCG stimulation was analysed. In the absence of TLR-4 inhibitor, TAK-242, there was a production of IFN- γ by the splenocytes of rBCG immunised mice, which was 1.5 fold compared to BCG immunised mice and more than twofold compared to PBS-T80 immunised mice group ($P < 0.05$). The IFN- γ production was significantly inhibited in the presence of TAK-242 in all mice groups ($P < 0.05$) (Figure 2A).

In the absence of TAK-242, the production of IL-4 by splenocytes was also highest in mice immunised with rBCG, more than threefold BCG and more than fourfold PBS-T80 ($P < 0.05$). Similar to the results of IFN- γ , the presence of TLR-4 inhibitor significantly reduced IL-4 production in splenocytes from all mice immunisation groups ($P < 0.05$) (Figure 2B).

4. Discussion

TLRs are believed to represent the key receptors for the multitude roles of recognition of microbial antigen, activation of macrophages, dendritic and other cells involved with innate immunity, as well as phagocytosis of microbes and the triggering of host defense mechanisms[47]. TLRs act a central role in the link between the innate and adaptive immunity which is activated when the innate immunity fails to contain the invasion by the micro-organism[48,49]. Activation of TLRs leads to the enhancement of the adaptive immunity[50,51]. TLR-4 is activated *via* the MyD88-dependent pathway and induces an inflammatory response during malaria infection leading to enhanced protection against malaria through an interface between innate and adaptive immunity created by cells of the monocyte/macrophage lineage[52-54]. The present study

analysed the role of TLR-4 in eliciting cellular and adaptive immune responses against an rBCG construct which expresses the MSP-1C of *P. falciparum*.

We analysed the humoral immune responses to the construct since it has long been shown that stimulation of TLR-4 with agonists such as lipopolysaccharide (LPS) leads to increased production of serum immunoglobulins[55]. Studies by others have earlier found induction of a robust humoral immunity generation by an MSP-1_{19}-based vaccine candidate[56]. The present study showed an increase in the production of total IgG as well as the three IgG isotype; IgG1, IgG2a and IgG2b in agreement with what Matsumoto found which showed a gradual increase in the levels of total IgG in the sera of mice immunised with an rBCG expressing MSP-1[57]. These results further showed that the level of IgG2a was highest, followed by IgG2b and then IgG1 in the group of mice immunised with rBCG indicating the potentials of the candidate to induce mixed Th1 and Th2 response. Other studies have also reported similarly high levels of induction of anti-$MSP1_{19}$ IgG, anti-$MSP1_{42}$ and anti-AMA-1 antibodies of *P. falciparum* among inhabitants of malaria-endemic regions[58,59]. Immunisation with *GAMA* gene DNA vaccine on *Plasmodium berghei* also elicited increased total IgG, IgG1 and IgG2a production[60].

On further evaluation, the role of TLR-4 in eliciting immunoglobulin production was shown by the significant inhibition of total IgG as well as the IgG1, IgG2a, and IgG2b isotype, by the administration of the TLR-4 inhibitor, TAK-242. A similar picture of low-level production of immunoglobulins had earlier been observed in TLR-4-deficient mice in CD8+ DCs with high-level IgG production obtained when nasal administration of hemagglutinin-A protein was carried out on TLR-4^+ mice[61]. In a similar study, Sreenivasulu reported about 40-fold increase in polyreactive IgG level in normal mice injected with LPS and only 2-fold increase in the immunoglobulin in mice deficient in TLR-4[62]. Similarly, another study showed that the levels total IgG, IgG1, and IgG2a produced following immunisation with whole-cell pertussis of mice deficient in TLR-4 were significantly lower compared to wild-type C57BL/6 mice[63]. In the same light, when TLR-4-/- mice were immunised with whole-cell pertussis, there was a significantly lower production of total IgG, IgG1, and IgG2a[63]. Evidently, our results have confirmed the role of TLR-4 in eliciting immunoglobulin production against the rBCG construct in immunised mice.

To investigate the possible role of TLR-4 in the cellular response to the construct, the production of IFN-γ and IL-4 by splenocytes of mice immunised with PBS-T80, BCG or rBCG was analysed. The entire mice groups showed significant production of both IFN-γ and IL-4, with the sera of rBCG immunised group yielding the most significant increase in both IFN-γ and IL-4 compared to the control groups. These results were in line with what obtained in a similar study which found IL-4 and IFN-γ significantly increased when mice were immunised with a glycosyl phosphatidylinositol-anchored micronemal antigen (GAMA) DNA vaccine of *Plasmodium berghei*[60]. Further evaluation showed significant inhibition of the two cytokines in the presence of TAK-242, signifying the importance of TLR-4 in their production. These results were similar one obtained in a mice allergy model, where a significant inhibition of IFN-γ and IL-4 production was found on the administration of TAK-242 to mice exposed to house dust mite[64]. Another study showed that the effect of LPS-induced production of IFN-γ in the experimental autoimmune myositis mouse models was significantly inhibited by TAK-242[65]. Similarly, the production of IL-4 in wild and TLR-4-/- mice immunised with LPS-rich ovalbumin or LPS-free ovalbumin was significantly inhibited in the TLR4-deficient mice[66].

The results from this study highlighted the role of TLR-4 in eliciting adaptive immune responses against rBCG expressing the MSP-1C of *P. falciparum*. However, the results also showed some level of cytokine and immunoglobulin production despite TLR-4 inhibition, indicating that other TLRs may contribute in the recognition of the construct by macrophages. An earlier study with the construct revealed the role of TLR-2 in inducing innate immune responses[67]. This confirmed the findings in clinical malaria which highlighted the importance of a number of TLRs in malaria infection[56]. Studies on other immune cells will help in understanding more on the mechanism of induction of immunomodulatory effects of our malaria vaccine candidate. Further insight into the role of TLR-4 could also be made through the use of TLR-4 inducers. The limitations of this study include our inability to carry out a malaria challenge to determine the protective role of antibodies and cytokines produced in response to the vaccine candidate. Thus, further studies in this regard are required.

Conflict of interest statement

We declare that we do not have any conflict.

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