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Pam3CSK4 enhances adaptive immune responses to recombinant *Mycobacterium bovis* bacille Calmette-Guérin expressing *Plasmodium falciparum C*-terminus merozoite surface protein-1

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ABSTRACT

Objective: To determine the effects of toll-like receptor 2 (TLR-2) agonist, Pam3CSK4, on cellular and humoral immune response 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) received intraperitoneal phosphate buffered saline T80 (PBS-T80), BCG or rBCG in the presence or absence of Pam3CSK4. Enzyme-linked immunosorbent assay was carried out to measure serum total IgG, IgG1, IgG2a, and IgG2b production. Spleens were also harvested and splenocytes were co-cultured with rBCG antigen for *in vitro* determination of IL-4 and IFN-γ *via* enzyme-linked immunosorbent assay.

Results: The production of total IgG and the isotype IgG1, IgG2a and IgG2b was significantly higher in rBCG-immunised mice than in the BCG and PBS-T80-immunised mice, and Pam3CSK4 further enhanced their productions. A similar pattern was also observed in IFN-γ production. Moreover, there was no significant difference in IL-4 production in all groups either in the presence or absence of Pam3CSK4.

Conclusions: We present evidence of the adjuvant effects of TLR-2 agonist in enhancing the production of total IgG, IgG1, IgG2a, IgG2b, as well as IFN-γ in response to rBCG. However, the presence or absence of Pam3CSK4 had no effect on IL-4 production.

1. Introduction

Malaria is a disease of great public health importance, with high mortality and morbidity, which puts an estimated 3.4 billion people at risk, with more than 215 million reported cases and 445 000 annual death, of mostly children and pregnant women in sub-Saharan Africa[1]. Malaria is caused by one or more of the *Plasmodium* parasite: *Plasmodium falciparum* (*P. falciparum*), *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale* and *Plasmodium knowlesi*, and transmitted *via* the bite of infective female *Anopheline* mosquitos.

The present strategies of malaria control are failing, which are

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based on early detection and prompt treatment of the infections through the use of artemisinin-based combination therapy as well as mosquito vector control[1-4]. In fact, *P. falciparum* has developed resistance to most of the antimalarial drugs currently in use[3,5]. To convert this menace, a number of vaccines are being developed through modalities which include the use of recombinant *Mycobacterium bovis* bacillus Calmette-Guerin (rBCG) to express malaria epitopes such as the merozoite surface protein (MSP)[6-8].

BCG is sufficiently immunogenic, well-tolerated and has an acceptable safety profile[9,10]. It readily elicits both innate and adaptive immune responses in immunised persons, leading to IFN-γ and IgG, IgG1 and IgG2a production[11-13].

Our recombinant BCG clone expressing the MSP-1C of *P. falciparum* generated immune responses by utilizing toll-like receptors 2 (TLR-2) which required a long time to achieve[14]. TLR-2 has been shown to be important in malaria immunity[15]. TLRs, a family of trans-membrane pattern recognition receptors, are present in immune cells such as monocytes, macrophages, and dendritic cells, and play a crucial role in the identification of pathogen-associated molecular patterns[16]. Each of the 13 TLRs identified in mammals possesses a unique pattern of expression, cellular localization, and signaling pathway, resulting in different immune reactions facilitated through a number of adapter proteins[17,18]. To induce a more robust vaccine-induced immune responses and maintain protection against pathogens such as malaria, TLR ligands are being used as adjuvants[19]. This study was conducted to determine the effect of TLR-2 agonist, Pam3CSK4, in enhancing serum immunoglobulin and splenic cytokine production in response to the rBCG clone.

2. Materials and methods

2.1. Ethics

All animal procedures were conducted based on the USA NIH guideline on animal study and approved by the Universiti Sains Malaysia (USM) animal ethics committee. Approval No: USM/ Animal Ethics Approval/2016/ (104) (801) was obtained on the 30th of November 2016.

2.2. BALB/c mice

This study used male BALB/c mice (6–8 weeks) acquired from the Animal Research and Service Centre (ARASC), USM. All animals were housed at the ARASC facility and given free access to food and water.

2.3. Preparation of Mycobacterium bovis BCG and rBCG cultures

A colony of parent BCG (Japan) and rBCG016, constructed previously through assembly PCR technique[20], were grown in a 7H11 agar (Becton Dickinson, USA) supplemented with OADC (Becton Dickinson, USA) and kanamycin (Sigma, USA) 15 mg/mL for rBCG. The culture was incubated at 37 ℃ for 2 weeks. Afterward, the cells were transferred to 7H9 media (Becton Dickinson, USA) supplemented with OADC (Becton Dickinson, USA) and kanamycin (Sigma, USA) for rBCG for 1 week, optical density (OD) (A600) = ~0.8. Colony forming unit (CFU) was determined for both BCG and rBCG using the formula developed by Norazmi and Dale[21], and the pellets were resuspended in Dulbecco's Modified Eagle Medium (Merck,Germany).

2.4. Mice immunisation

A total of 36 male BALB/c mice aged 6-8 weeks were divided into six groups (n=6) in the study. Each mouse received intraperitoneal immunisation with 200 μL phosphate buffered saline, Tween 80 (PBS-T80), 1×10^6 CFU of BCG or 1×10^6 CFU of rBCG in 0.1% PBS-T80 respectively in the presence or absence of 10 μg/mL of TLR-2 agonist, Pam3CSK4, which was given 1 h before each immunisation. This was followed by the same immunisation 4 and 8 weeks after the first immunisation. The mice were closely observed daily 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

Blood was collected from the tail vein of the mice before every immunisation and 4 weeks after the last immunisation, just before sacrifice[22]. Briefly, each mouse was restrained and its tail was sterilised using 70% ethanol. Then using a sterile scalpel, a small incision was made near the tip of the tail without anesthesia and blood was collected in a sterile Microcentrifuge tube. Using sterile gauze, little pressure was applied on the tail to stop the bleeding after blood collection. The blood samples were allowed to clot at 4 ℃ overnight and sera were harvested at the following day by centrifugation at 1 500 × g for 15 min.

2.6. Measurement of mice IgG and IgG subclasses using ELISA

The presence of anti-rBCG protein IgG and IgG subclasses antibodies in the sera of immunised mice was quantified using ELISA. Briefly, 1 μg/mL of purified MSP-1_{19} antigen in carbonate-bicarbonate coating buffer (Na_2CO_3-$NaHCO_3$) was added to each well of a 96-well microplate and incubated overnight at 4 ℃. On the following day, the plate was washed three times for 5 min, blocked with blocking buffer, kept at 37 ℃ for 1 h, and then washed again.

Afterward, 100 μL of the sera diluted in PBS buffer at 1:1 000 were added to the microplate wells in triplicate, incubated at 37 ℃ for 30 min and then washed. A total of 100 μL of horse radish peroxidase conjugated anti-mouse IgG, IgG1, IgG2a, IgG2b antibodies at a 1:10 000 dilution in PBS was added and the plate was incubated at 37 ℃ for 30 min. The plates were washed again, and 100 μL of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) substrate was added and incubated for 15 min at 37 ℃. Finally, the reaction was stopped by the addition of 100 μL of 2 M H_2SO_4 and the OD was determined at 450 nm on a microplate reader.

2.7. Preparation of single cell suspension from spleen

All mice were euthanised four weeks after the last booster immunisation *via* intraperitoneal injection of 1 mL/kg sodium pentobarbital. The spleen was aseptically removed and promptly placed in ice-cold complete RPMI 1640 media. The sterile plunger of a 10 mL syringe was then used in grinding the spleen through a 250 μM mesh filter to obtain single cell suspensions, and the suspensions were centrifuged at 400 × *g* for 5 min. The pellets were washed two times and the red blood cells were lysed by re-suspending the pellet in 5 mL of ACK lysis buffer for 5 min on ice.

2.8. Cell cultures

The suspension was centrifuged at 400 × *g* for 5 min and the pellets were suspended in RPMI 1640. Five million splenocytes per mL were seeded into 96-well flat-bottomed tissue culture plates in 100 μL of complete RPMI 1640 containing 10 μg/mL rBCG antigen at 37 ℃ and 5% CO_2 for 24-72 h. The culture solution was then centrifuged at 1 500 × *g* for 10 min at room temperature and supernatants were used for cytokine determination.

2.9. Measurement of cytokines in splenocyte culture supernatants

ELISA analyses were carried out to estimate IFN-γ and IL-4 concentration. Briefly, 96-well ELISA plates were coated with the capture antibody, sealed and incubated overnight at 4 ℃. On the following day, PBS-T20 was used to wash the plates 5 times, followed by blocking with blocking buffer and incubation for 2 h at room temperature. Culture supernatant and standard were then added to corresponding wells, sealed and incubated for 2 h at room temperature. Subsequently, either anti-mouse IFN-γ or IL-4 was added depending upon the cytokine to be measured. The plates were sealed again and incubated for 2 h at room temperature, after which they were washed and Avidin-horse radish peroxidase solution was added to the wells and incubated for 30 min at room temperature. A substrate solution was then 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 plates were promptly read with a microplate reader at 450 nm to determine cytokine concentration and generate a standard curve.

2.10. Statistical analysis

Statistical analyses were carried out using the Statistical Package of Social Sciences (SPSS) software, version 22. The data were obtained from triplicate experiments (n=3) and presented as the mean ± standard error of the mean (SEM). The data were analysed by one-way analysis of variance (ANOVA) for the cytokines and IgG isotype while repeated measures ANOVA (RM-ANOVA) were used for the repeated vaccine on total IgG analysis. Each analysis was followed by the Bonferroni *post-hoc* test. P<0.05 was considered statistically significant.

3. Results

3.1. Antibody responses to rBCG

The results obtained showed no significant difference in the level of total IgG titre in the mice immunised with PBS-T80 in the presence and absence of Pam3CSK4 before immunisation and after the series of immunisations (Figure 1A). However, in the groups immunised with BCG and rBCG, there was a significant increment in the levels of total IgG after the first immunisation both in the presence and absence of Pam3CSK4 (P < 0.05), which was further enhanced with the second and third immunisations (P < 0.05). The presence of Pam3CSK4 led to a more significant increase in total IgG production in both BCG and rBCG immunised mice groups (P < 0.05). In the presence of Pam3CSK4, the rBCG-immunised mice group produced the highest level of total IgG compared to rBCG alone and both BCG and BCG with Pam3CSK4.

The production of IgG isotype; IgG1, IgG2a, and IgG2b was also evaluated in all mice groups (Figure 1B-D). After the third immunisation, the rBCG antigen stimulated higher IgG1 response compared to both PBS-T80 and BCG-immunised mice groups in the absence of Pam3CSK4. This significant production was further enhanced in the presence of Pam3CSK4 (P < 0.05). Similarly, IgG2a (Figure 1C) and IgG2b (Figure 1D) titers were statistically significantly higher in the rBCG immunised mice in the absence of Pam3CSK4 compared to the PBS-T80 and BCG group (P < 0.05). The presence of Pam3CSK4 further markedly increased IgG2a and IgG2b production in both BCG and rBCG groups, but more significantly in the rBCG group (P < 0.05). Moreover, among the IgG subclasses, production of IgG2a was the highest, followed by IgG1, then IgG2b, indicating the potential of the construct, especially with the agonist, to induce mixed Th1 and Th2 response.

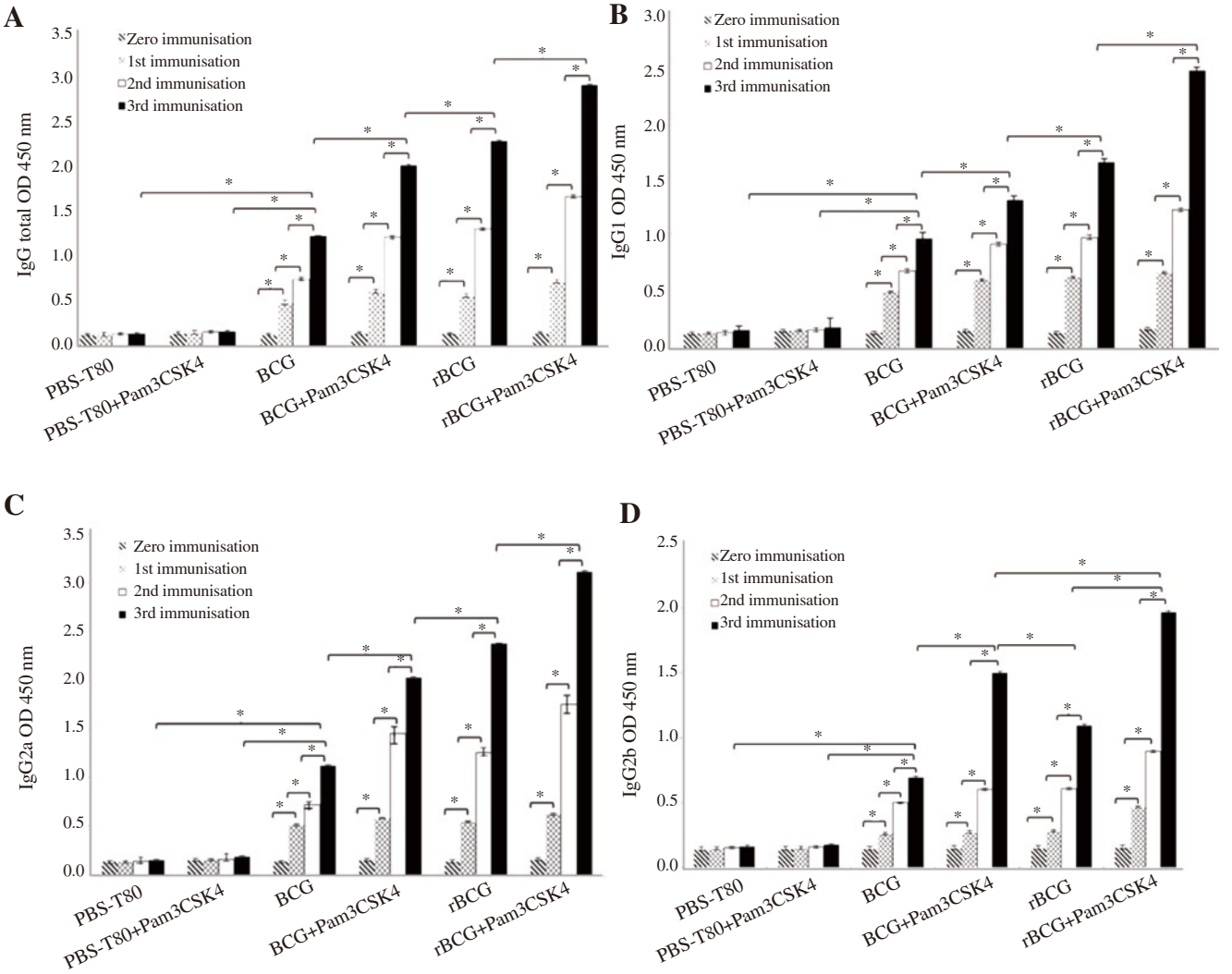

Figure 1. Level of antibodies in the sera. (A) Total IgG and IgG isotype IgG1 (B), IgG2a (C), and IgG2b (D) before immunisation and four weeks after each immunisation. Data are presented as the mean ± SEM for three independent experiments. $^{*}P < 0.05$.

3.2. In vitro production of cytokines from splenocyte

There was a significant increase in production of IFN- γ in the splenocytes of both BCG and rBCG immunised mice groups compared to PBS-T80 group after third immunisation ($P < 0.05$). IFN- γ production was significantly enhanced in the presence of Pam3CSK4 in all mice groups ($P < 0.05$) (Figure 2A). Moreover, no significant differences were found in IL-4 among three immunised groups in the presence and absence of Pam3CSK4 (Figure 2B).

4. Discussion

TLRs provide an effective bridge between innate and adaptive immunity[23]. Their agonists act as enhancers of both the innate and adaptive immune responses and have been used in vaccines against diseases such as HIV[24–27]. Vaccine adjuvants play an important role as synergistic inducers of adaptive immune responses through T cell or humoral immunity[24]. TLR-2 agonists elicit enhanced B cell activation through increased expression of surface receptors, cytokine secretion and proliferation[28]. In this study, we analysed the synergistic effects of TLR-2 agonist, Pam3CSK4 on cellular and humoral adaptive immune responses to our rBCG construct which had earlier elicited robust adaptive responses[29]. Earlier results pointed out the role of TLR-2 in inducing innate immune responses by the construct[14]. The use of effective adjuvants that activate the innate immune response is essential in the production of a potent and durable antibody response to vaccination, since innate immunity elicited *via* TLRs is a prelude to eliciting adaptive immune responses[30,31]. TLR-2 and its agonist, Pam3CSK4 are classical examples of inducers of humoral and cellular immune response[32].

Our results showed that mice immunised with rBCG had a gradual increase in the level of IgG antibody, with subsequent immunisations, compared to the controls. This was similar to the

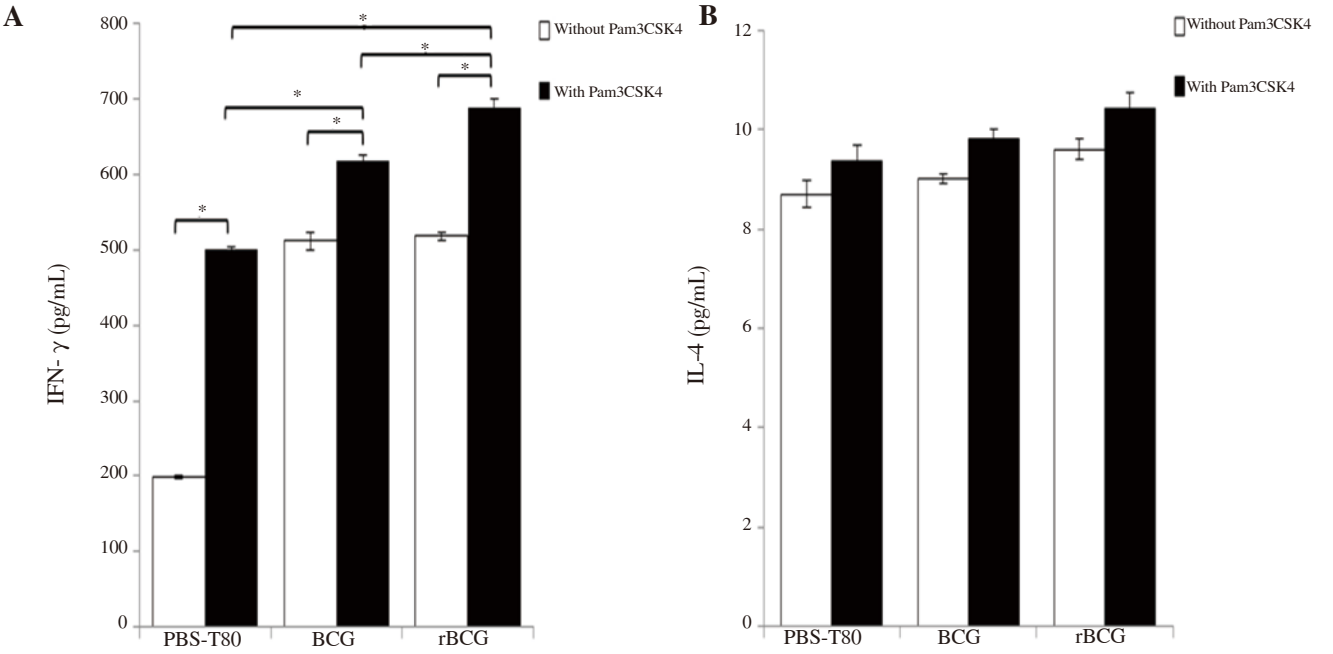

Figure 2. Effects of TLR-2 agonist on IFN- γ (A) and IL-4 (B) production in splenocytes. Data are presented as the mean ± SEM for three independent experiments. $^{*}P < 0.05$.

findings in other studies which demonstrated a gradual increase in the level of IgG antibody when mice were immunised with rBCG clones[33]. The production of IgG was further increased in the presence of the TLR-2 agonist in a stepwise fashion with each immunisation, similar to what others obtained which showed TLR-2 agonists as enhancers of antibody production[34].

Further analysis showed that immunisation with the rBCG also triggered the production of IgG isotype IgG1, IgG2a and IgG2b with IgG2a being the predominant isotype. The level of IgG2a production was significant after immunisation with rBCG, and this was further enhanced in the presence of TLR-2 agonist, which was similar to vaccination with RSV virosomes that led to a synergistic increase in IgG2a production in the presence of Pam3CSK4[35]. Other studies found similar enhancement of IgG2a in the presence of another TLR-2 agonist, Pam_2CysK_4[36,37]. In line with our findings, Pam3CSK4 led to enhanced IgG2a production with a lesser profound enhancement of IgG1 in response to H3N2 flu vaccine[38]. Our results also showed enhanced IgG2b production in the presence of Pam3CSK4.

This study also analysed the effects of the TLR-2 agonist on cellular adaptive immune responses to the rBCG construct. The production of INF- γ , a representative Th1 cytokine, and IL-4, a representative Th2 cytokine in splenocytes of immunised mice was evaluated. Immunisation of mice with rBCG led to high IFN- γ production in splenocytes which was significantly enhanced in the presence of the TLR-2 agonist. Previous studies reported induced IFN- γ production due to stimulation by TLR-2 ligands such as Pam3CSK4[39]. IFN- γ promotes antibody class-tuning to IgG2a, and it has been linked with protection against severe malaria, while its absence correlates with higher and more prolonged parasitaemia[40]. Contrary to what obtained in IFN- γ , there was no significant difference in the production of IL-4 across the groups and the presence of Pam3CSK4 did not enhance its production significantly. These results were similar to the findings by Barjesteh *et al*, which showed that treatment with TLRs ligands led to significant production of IFN- γ with no effect on IL-4 production[41]. Earlier studies showed that adequate generation of IL-4 helps in the production of IgG1[42].

The results of this study highlighted the synergistic adjuvant effects of TLR-2 agonist, Pam3CSK4, on adaptive immune responses to rBCG expressing the MSP-1C of *P. falciparum*. Adjuvants have the potentials of not only enhancing vaccine immunogenicity but reducing the effective dosage needed to convert the morbidity and mortality of vaccine-preventable diseases[43]. Thus, vaccines that will generate combined humoral and cellular immune responses coupled to an appropriate adjuvant targeting a stage of the *Plasmodium* life cycle are desirable for malaria prevention. This will overcome the intrinsic ability of the *Plasmodium* parasite to escape the immune response of the host as well as its antigenic variation in the multistage life cycle which are among the causes for the slow progress in effective malaria vaccine development[44-46]. This study showed that vaccination with the rBCG coupled with TLR-2 agonist is sufficiently potent to elicit significant humoral and cellular immune responses, which is necessary in order to potentiate the immunomodulatory effects of the rBCG. The absence of the adjuvant required a long time to generate adequate and sustained immune responses[20]. Among the limitations of this study was our inability to carry out a malaria challenge which could have determined the protective role of the immunoglobulins and the splenic cytokines

generated against the rBCG candidate.

Conflict of interest statement

We declare that we do not have any conflict.

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