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CRX−527 as a candidate adjuvant in a recombinant BCG−based malaria vaccine

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ABSTRACT

Objective: To investigate the role of CRX-527, a Toll-like receptor 4 agonist, as the possible adjuvant for recombinant *Mycobacterium bovis* Bacillus Calmette-Guerin expressing merozoite surface protein 1C (BCG-MSP-1C).

Methods: The mice were immunized with BCG and BCG-MSP-1C in the presence and absence of CRX-527. The untreated mice (injected with PBS-T80 only) were the negative control. The ability of CRX-527 to enhance IgG and its subclasses, as well as IL-4 and IFN-γ production in the serum and spleen supernatant was evaluated using ELISA.

Results: Mice immunized with BCG-MSP-1C exhibited the highest production of IgGs, IL-4 and IFN-γ after third immunization. In addition, CRX-527 further promoted the production of total IgG and IgG subclasses as well as IFN-γ and IL-4 in the serum and splenocytes of immunized mice.

Conclusions: CRX-527 has the potential as an adjuvant candidate for the candidate vaccines. Further study is needed to verify appropriate dosage for immunization and its efficacy.

KEYWORDS: CRX-527; BCG; BCG-MSP-1C; TLR-4 agonists; Cytokine; IgG; Malaria

1. Introduction

Global malaria cases are rising steadily, from an estimated 227 million cases in 2019 to 241 million cases and 627 000 deaths in 2020[1]. Artemisinin combination therapy is currently considered the gold standard, the most effective treatment for uncomplicated malaria globally[2]. However, the treatment of malaria has remained a challenge even with the drug combination of choice, artemisinin combination therapy. Evidence shows that the malaria parasite *Plasmodium falciparum* (*P. falciparum*) has developed artemisinin resistance[3,4]. In the face of these challenges, and the fact that most malariologists believe that a potent anti-malaria vaccine is desirable for the control of malaria infection[5], the World Health Organization (WHO) in 2021 recommended the use of circumsporozoite protein vaccine RTS,S/AS01 malaria vaccine in the prevention of *P. falciparum* malaria in children living in regions with moderate to high transmission. However, despite reports of safety and reduced disease severity, RTS,S/AS01 does not provide all the answers needed and exhibits some limitations, as it fails to provide extensive sterile immunity besides the need for three boosters to attain a reasonable efficacy[6]. This led to poor immunogenicity that may spare the infectivity of the plasmodium gametocytes, thereby allowing potent parasites to infect mosquitoes, rendering transmissions to remain unchanged, with a consequent continuous

Significance

CRX-527 emerges as a promising adjuvant for vaccine candidates. The present study demonstrates CRX-527 markedly increases intracellular cytokines and IgG production in mice immunized with BCG-MSP-1C, a vaccine candidate. Hence, CRX-527 plays a valuable role in enhancing vaccine effectiveness. Further research involving various dosages of CRX-527 in mice is necessary to determine the appropriate dosage for immunization.

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endemicity. Furthermore, studies have shown that the protection from the RTS,S vaccine is significantly related to the participant's household socioeconomic status (SES), with better protection achieved by those in higher SES households[7]. The difference led to a more profound immunity gap where the disease is more prevalent since 95% of malaria cases and 96% of malaria deaths occur in world-deprived states with low SES[8].

The global picture of a potential treatment failure and the somewhat less-than-desirable results from the WHO-licensed vaccine, RTS,S, mean continuous search for a malaria vaccine is still pertinent. The search for a more potent vaccine remains a priority in the global fight against malaria. Among the approach in malaria vaccine development was the use of recombinant *Mycobacterium bovis* Bacillus Calmette-Guerin (rBCG) to express malaria epitopes, such as the merozoite surface protein (MSP)[9]. MSP1-based vaccines are safe, well-tolerated, and immunogenic[10]. The development of recombinant vaccines against pathogenic diseases, including malaria parasites, using live bacteria, such as BCG, is a trend now[11]. The recombinant strategy incorporates the *C*-terminus of the MSP-1C protein of *P. falciparum* into the genome of the BCG clone to produce the BCG-MSP-1C vaccine that targets malaria blood-stage infections. The vaccine candidate could stimulate cellular and humoral immune reactions.

The activation of humoral and cellular immune responses has been recognized in acquiring immunity to malaria[12]. Immunoglobulins (Igs) are antibodies that specifically recognize and bind to particular antigens and destroy them during an immune response. Naturally acquired immunity against malaria is achieved with sufficient production of antibodies against merozoite antigens, leading to reduced parasitemia and improved clinical symptoms in patients[13]. Meanwhile, for the initiation and maintenance of robust innate immunity, a prerequisite of the activation of adaptive immunity, Toll-like receptors (TLRs) are involved. There are 13 member families of pattern recognition receptors that recognize conserved pathogens structures, leading to the generation of innate and adaptive immune responses[14]. Among the family of TLRs, TLR4 is the major pathway involved in malaria immunity[15]. This TLR recognizes glycosyl phosphatidylinositol from *P. falciparum* and is activated through the MyD88-dependent and MyD88-independent pathways, which leads to the cytokine release and induction of adaptive immunity, *i.e.*, increased levels of IgG1, IgG2a, and IgG2b, besides T cell proliferation, leading to the expression of IFN-γ and IL-4[16].

The synthesis of immunoglobulins such as IgG, induced by both Th1 and Th2 cell types, plays an important role in eradicating malaria parasites and establishing short-lived immunity[17]. Hence, the development of this naturally acquired immunity to human malaria occurs inefficiently, and the protection is only achieved after several repetitive exposures to the parasite[18,19]. Many studies that measure total IgG against malaria have been conducted, with very few analyzing the IgG subclass[20]. IgG subclasses play a vital role in malaria immunity, with cytophilic antibodies (IgG1 and IgG3) being the most important[21]. The non-cytophilic IgGs (IgG2 and IgG4), though not as important as the cytophilic antibodies, also play a significant role in developing effective immunity against malaria[22]. Likewise, the MSP1-based modality, a recombinant vaccine candidate that targets malaria blood-stage infections, was produced by our laboratory. The *C*-terminus of the merozoites surface protein-1 (MSP-1C) of the *P. falciparum* was engineered into the genome of the BCG clone to produce the BCG-MSP-1C vaccine. The vaccine candidate can stimulate higher protection against merozoite invasion and is also capable of stimulating a significant innate and acquired immune response in macrophages and animals[23]. This candidate elicited increased production of total IgG, IgG1, IgG2a, and IgG2b, indicating robust humoral responses.

Cellular immune responses to malaria involving the secretion of cytokines are also important in eliciting protection[24,25]. Intracellular cytokines such as IFN-γ and IL-4 are important in reducing inflammation and eliminating malaria parasites[26,27]. IL-4 plays an important role in the establishment of Th2 responses and suppressing the Th1 effector mechanism mediated by IFN-γ by inducing inducible nitric oxide synthase (iNOS)[28]. IL-4 can block the synthesis of nitric oxide (NO) by increasing the expression of arginase, creating a different pathway for the metabolism of *L*-arginase, the precursor to NO[29]. The vaccine candidate was further evaluated for its adaptive immune elicitation, *i.e.*, acute immune response activation, which increased the production of IL-4 and IFN-γ.

Vaccines can enhance immunogenicity when coupled with adjuvants[30]. A study of our vaccine candidate showed significant adjuvant effects of a TLR-2 agonist, Pam3CSK4[31]. The study explored the possibility of enhanced immunomodulatory effects of the vaccine candidate on BCG-MSP-1C by a TLR4 agonist, CRX-527.

2. Materials and methods

2.1. BCG and BCG–MSP–1C strain

The BCG and BCG-MSP-1C strains were grown on 7H11 agar (Becton Dickinson, USA) for 2 weeks at 37℃, transferred to 7H9 broth (Becton Dickinson, USA), and cultured for 1 week at 37℃ with agitation (200 rpm). The purity of the culture was evaluated by Zhiel-Neelsen staining.

2.2. CRX–527 and lipopolysaccharides (LPS)

A 1 mg/mL stock solution of each CRX 527 and LPS was prepared by dissolving 1 mg crystal CRX-527 and LPS into 1 mL of sterile PBS 1× buffer for animal studies. The stock solution was stored at –20℃ until further use.

2.3. Immunization schedule

Forty male BALB/c mice (4-6 weeks), supplied by the Animal Research and Service Centre, Universiti Sains Malaysia, were used in the experiments. All procedures were conducted according to the guidelines and the approval of the Universiti Sains Malaysia Animal Ethics Committee. Eight groups of animals (n = 5 per group) were immunized with 200 µL of PBS-T80 (0.05% PBS-T80), LPS (0.005 mg/mL), BCG (2×10^6 CFU), and BCG-MSP-1C (2×10^6 CFU) in the presence and absence of CRX-527 (0.005 mg/mL) intraperitoneally. A week before the first immunization, the mice were bled from the tail vein to get the pre-immunization blood. The process was repeated for three weeks after each immunization. Essentially, the mice received three immunizations and were bled four times. The blood was centrifuged, and serum was stored at –20℃ until further use.

2.4. Measurement of total IgG and IgG subclass antibodies by ELISA

ELISA plates (MaxiSorp, Nunc) were coated with the BCG-MSP-1C protein antigen in a coating buffer and incubated overnight at 4℃. After several washing steps with PBS-Tween 20, the plates were blocked with the blocking buffer (Roche, Germany) for 90 min at 37℃, followed by several washing steps. Triplicate serum samples (diluted 1∶100 in blocking buffer) were added and incubated for 90 min at 37℃. After several washing steps, the plate was incubated with HRP-conjugated rabbit anti-mouse IgG (Dako, Denmark) at a dilution of 1∶2 000 in the blocking buffer, and goat anti-mouse IgG1, IgG2a, and IgG2b (Abcam, USA) at a dilution of 1∶5 000 in the blocking buffer for 90 min at 37℃. Several washing steps followed before the addition of the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS, Roche, Germany). After 30 min of incubation, the reaction was stopped with 2N H_2SO_4, and the absorbance was read at 405 nm.

2.5. Splenocytes culture

Splenocyte cultures were obtained from the spleen of the mice after they were sacrificed. The splenocytes obtained were cultured in the RPMI 1640 complete medium at 37℃ under a humidified atmosphere of 5% CO_2 for 24 h. The culture was then centrifuged at 1 500 ×g for 10 min at 4℃, and the supernatant was stored at –80℃ until use.

2.6. Intracellular cytokines assay of spleen supernatant and serum

The measurement of intracellular cytokines was done using ELISA. The ELISA plate (MaxiSorp, Nunc) was coated with the capture antibody with a specific antibody for IL-4 and IFN-γ and incubated overnight at 4℃, as described by the manufacturer (Biolegend, UK). The plate was washed four times with the washing buffer and blocked with the 1× assay diluent-A at 37℃ for 1 h with shaking. The plate was washed several times before the triplicate sample (supernatant or serum) and the standard was added and incubated for 2 h at 37℃ with shaking. Several washing steps were done, followed by incubation with the detection antibody solutions at 37℃ for 1 h with shaking. After several washing steps, the plate was incubated with Avidin-HRP at 37℃ for 30 min with shaking. Several washing steps followed before adding tetramethylbenzene (TMB) substrate solutions (100 µL/well). After 20 min of incubation in the dark, the reaction was stopped with 2N H_2SO_4 (50 µL/well), and the absorbance was read at 450 nm.

2.7. Statistical analysis

The result was expressed as mean±SEM and analyzed by one-way ANOVA for the determination of significant differences. P<0.05 was considered significantly different. Statistical analyses were carried out with the SPSS software for Windows (Version: SPSS 26.0).

2.8. Ethical statement

All animal studies complied with the Universiti Sains Malaysia rules and regulations in animal care and use of animals for scientific purposes which was agreed upon and approved by the Universiti Sains Malaysia Institutional Animal Care and Use Committee (USM IACUC) with the approval number (USM/IACUC/2020/ (125) (1099).

3. Results

3.1. Total IgG and IgG subclass antibodies

3.1.1. Antibody responses to CRX-527

Figure 1 shows no significant differences in the levels of total IgG in mice immunized with PBS-T80, LPS, BCG, and BCG-MSP-1C in the presence or absence of CRX-527 before immunization (P > 0.05). After the third immunization, an increase in IgG value was observed in groups of mice immunized with PBS-T80, LPS, BCG, and BCG-MSP-1C in the presence or absence of CRX-527 (P < 0.05). The total IgG in BCG and BCG-MSP-1C-immunized mice in the presence or absence of CRX-527 was moderately higher than that in PBS-T80 and LPS-immunized mice. Moreover, the presence of CRX-527 significantly increased the total IgG production in LPS, BCG and BCG-MSP-1C-immunized mice after the third immunization (P < 0.05).

3.1.2. IgG isotype responses

The production of IgG isotypes: IgG1, IgG2a, and IgG2b was also evaluated in all groups. No significant difference was detected in all levels of IgG isotypes (IgG1, IgG2a, and IgG2b) in mice immunized with PBS-T80, LPS, BCG, and BCG-MSP-1C in the presence or

absence of CRX-527 before immunization (Figure 1).

After the third immunization, the groups treated with BCG and BCG-MSP-1C in the presence or absence of CRX-527 showed significantly higher IgG1 levels than those immunized with PBS-T80 and LPS in the presence or absence of CRX-527 (Figure 1). However, the presence of CRX-527 significantly increased IgG1 production in mice immunized with BCG and BCG-MSP-1C compared with those without CRX-527 ($P < 0.05$). Moreover, after the third immunization, the presence of CRX-527 significantly increased IgG1 production in PBS-T80-immunized mice compared to those immunized without CRX-527 ($P < 0.05$, Figure 1).

A significant increase in IgG2a production in mice immunized with LPS and BCG-MSP-1C in the presence of CRX-527 was detected after the third immunization compared to their counterparts without CRX-527 ($P < 0.05$). The IgG2a response was the highest in mice immunized with BCG and BCG-MSP-1C in the presence of CRX-527 compared to other groups following the third immunization (Figure 1). However, when compared to the PBS-T80 group without CRX-527, other groups showed a significant increase in the IgG2a production except for the PBS-T80 group with CRX-527 ($P < 0.05$, Figure 1).

Similarly, after the third immunization, a significant increase in IgG2b values was noted in mice immunized with BCG and BCG-MSP-1C in the presence of CRX-527 compared to their counterparts without CRX-527 ($P < 0.05$, Figure 1). When compared to the PBS-T80 group without CRX-527, the groups immunized with BCG and BCG-MSP-1C in the presence or absence of CRX-527 showed markedly elevated IgG2b levels ($P < 0.05$, Figure 1). Among the IgG subclasses, the production of IgG2a was the highest, followed by IgG2b and IgG1.

3.2. Intracellular cytokines assay

No significant difference was observed in the IFN-γ production in the serum of mice immunized with PBS-T80, LPS, BCG, and BCG-MSP-1C in the presence or absence of CRX-527 during the pre-immunization period, IFN-γ production increased in almost all groups. However, following the third immunization, IFN-γ production was significantly higher in the serum of mice immunized with BCG and BCG-MSP-1C in the presence of CRX-527 compared

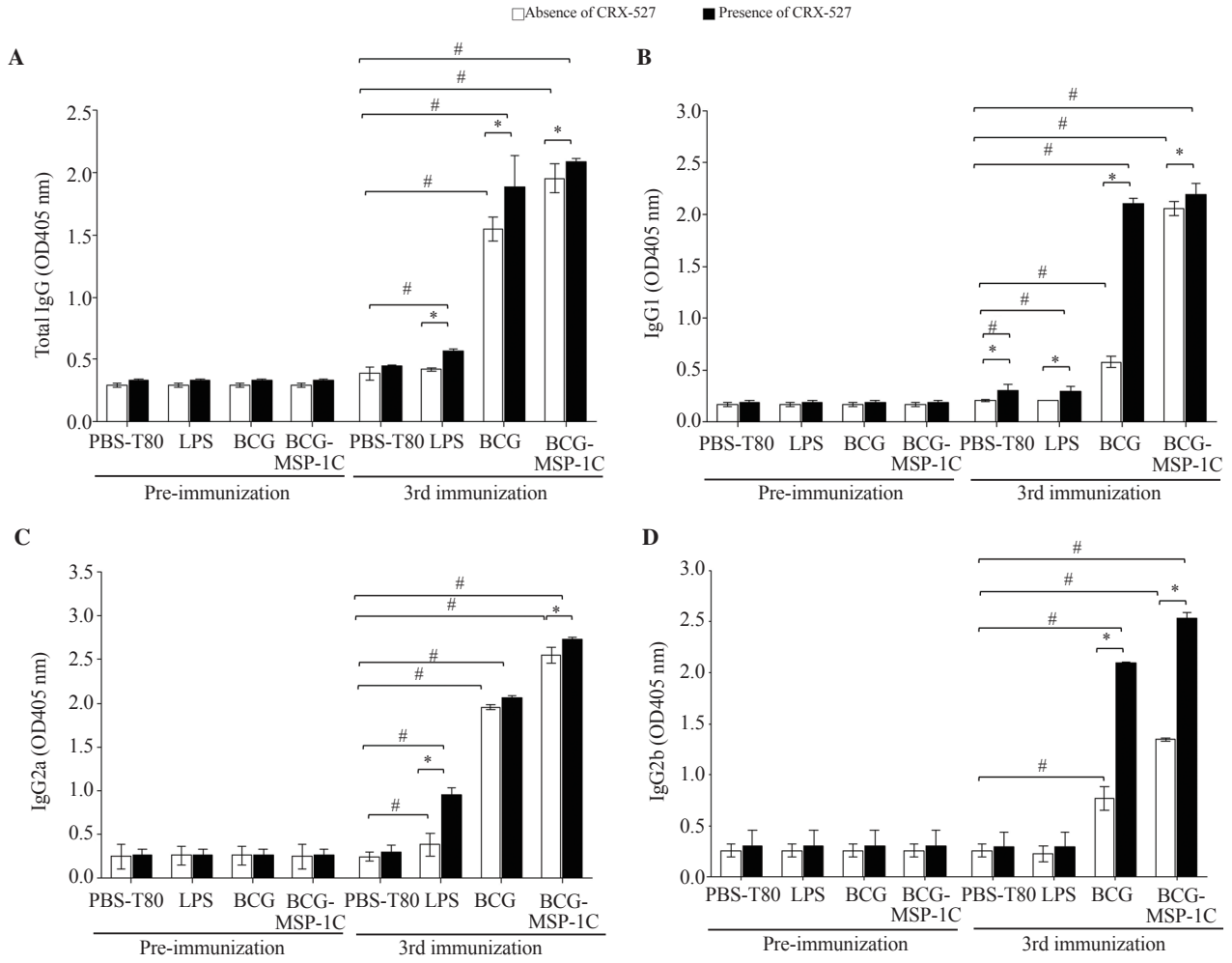

Figure 1. Total IgG (A), IgG1 (B), IgG2a (C) and IgG2b (D) response in mice immunized with PBS-T80, LPS, BCG, and BCG-MSP-1C in the presence or absence of CRX-527. Results are presented as mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared with the counterparts in the absence of CRX-527 and $^{\#}P < 0.05$ compared with the PBS-T80 group without CRX-527.

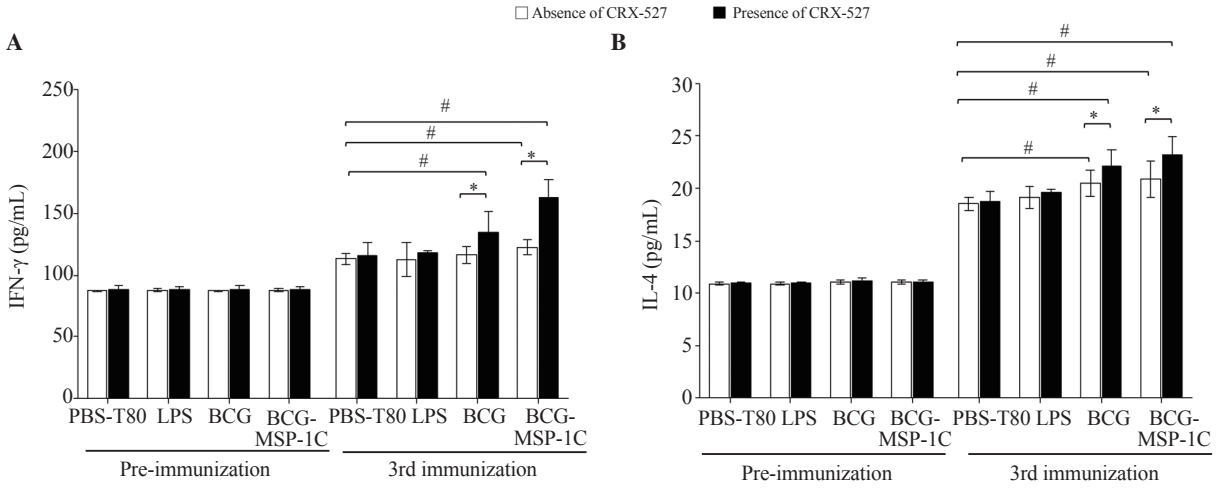

Figure 2. IFN-γ (A) and IL-4 (B) production in the serum of mice immunized with PBS-T80, LPS, BCG and BCG-MSP-1C in the presence or absence of CRX-527. Results are presented as mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared with the counterparts in the absence of CRX-527 and $^{\#}P < 0.05$ compared with the PBS-T80 group without CRX-527.

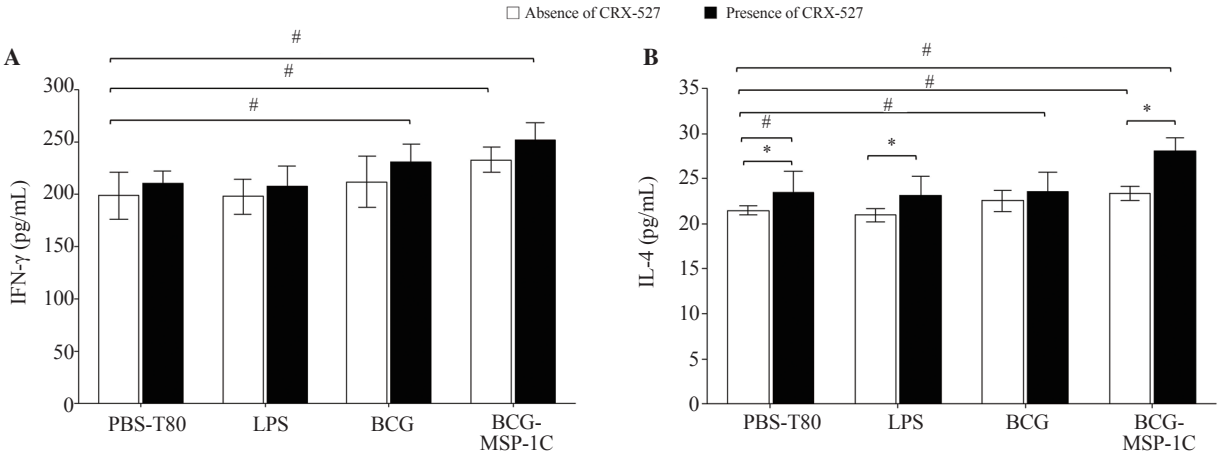

Figure 3. IFN-γ (A) and IL-4 (B) production in the splenocytes of mice immunized with PBS-T80, LPS, BCG and BCG-MSP-1C in the presence or absence of CRX-527. Results are presented as mean ± SEM of three independent experiments. $^{*}P < 0.05$ compared with the counterparts in the absence of CRX-527 and $^{\#}P < 0.05$ compared with the PBS-T80 group without CRX-527.

to the counterparts in the absence of CRX-527 ($P < 0.05$, Figure 2).

The results also showed no significant difference in the production of IL-4 in all groups during the pre-immunization period (Figure 2). However, IL-4 production increased after the third immunization. CRX-527 significantly enhanced IL-4 production in the serum of mice immunized with BCG and BCG-MSP-1C compared to those immunized with BCG and BCG-MSP-1C without CRX-527 ($P < 0.05$).

No significant difference was noted in the IFN-γ production from the splenocytes of mice immunized with PBS-T80, LPS, BCG, and BCG-MSP-1C in the presence of CRX-527 compared with the counterparts without CRX-527 ($P > 0.05$, Figure 3). However, a significant difference in the production of IFN-γ was observed in the BCG group in the presence of CRX-527 as well as the BCG-MSP-1C group with and without CRX-527 when compared to PBS-T80 without CRX-527 ($P < 0.05$).

Moreover, CRX-527 significantly increased IL-4 production in mice immunized with PBS-T80, LPS, and BCG-MSP-1C compared to those without CRX-527 ($P < 0.05$). Meanwhile, compared with the PBS-T80 control group without CRX-527, the groups immunized with PBS-T80 and BCG with CRX-527, as well as BCG-MSP-1C with and without CRX-527 showed significantly increased IL-4 production ($P < 0.05$, Figure 3).

4. Discussion

TLRs serve as an important role in innate recognition and induce an appropriate adaptive immune response against invading organism. TLR4 is a pathogen recognition receptor that is critical for Gram-negative bacteria[32]. A few studies, however, have suggested that TLR4 plays a role in the induction of host immunity against parasite infections including malaria[33].

Using CRX-527 as an agonist for TLR4, this study was conducted

to evaluate the role of TLR4 in modulating adaptive immune response to recombinant BCG expressing the MSP-1C antigen of *P. falciparum*. The results show an increase in total IgG and IgG subclasses in the sera of all inoculated mice after the third immunization in the presence of CRX-527, showing the involvement of TLR4 in promoting the synthesis of total IgG and IgG subclass (IgG1, IgG2a, and IgG2b) response in immunized mice. However, animals immunized with BCG-MSP-1C produced more total IgG and IgG subclass responses than those immunized with the parent BCG clone.

The ability of CRX-527 to stimulate the production of intracellular cytokines in the immunized mice was further explored using ELISA. The production of intracellular cytokines in serum and splenocytes of immunized mice was found to be similar with the result of IgG production. Both IFN-γ and IL-4 production increased dramatically in response to BCG-MSP-1C. The presence of MSP-1C antigen in BCG cells was expected to promote TLR4 binding to the mycobacterium. The binding was further increased with the presence of CRX-527 stimulation. This interaction increased the activation of antigen presenting cells such as macrophages which then presented the degraded mycobacterium to T cells to stimulate T and B cell activation. This study also found that the BCG-MSP-1C clone can induce both humoral and cell-mediated immune responses which supported our prior findings that TLR4 antagonist TAK-242 lowered humoral and cell-mediated immune responses in immunized mice[34].

Cytokines production also indicated the presence of a Th1/Th2 immune response which is important in the elimination of malaria parasite infection. IFN-γ is suggested to be important in the liver and blood of the *P. falciparum* malaria parasite[35]. Meanwhile, IL-4 activates Th2 cells, promoting the production of IgG antibodies which is crucial for combating the malaria parasite. The presence of both Th1 and Th2 responses is crucial in malaria, since initial Th1 response aids in controlling the infection, while a subsequent Th2 response promotes the development of a humoral immune defense, including the production of vital antibodies such as IgG[36]. This combination action ensures a comprehensive and balanced immune defense against the malaria parasite, contributing to the effective clearance of the pathogen and the establishment of long-term immunity.

In conclusion, BCG-MSP-1C uses TLR4 as a ligand to interact with the immune system. The effectiveness can be enhanced by stimulating with CRX-527. This finding is important in preparing future vaccines against malaria parasites which have a very complex life cycle and pathogenesis.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

Both NMZ and RS were responsible for the conception, study design, and content. NMZ performed the experimental studies, data acquisition, data analysis and statistical analysis. RS was the guarantor for this article. The first draft of the manuscript was written by NMZ, MAA and RS. The previous version and revised version of the manuscript were commented by NMZ, RS and MAA. Final manuscript was read and approved by NMZ, MAA and RS.

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