Brief Original Article

The role of TLR-4 in the immunomodulatory effects of recombinant BCG expressing MSP-1C of *Plasmodium falciparum*

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Abstract

Introduction: An earlier constructed recombinant BCG expressing the MSP-1C of *Plasmodium falciparum*, induced inflammatory responses leading to significant production of nitric oxide (NO) alongside higher expression of the enzyme inducible nitric oxide synthase (iNOS) and significant production of the regulatory cytokine, IL-10, indicating significant immunomodulatory effects of the construct. The mechanism of these responses had not been established but is thought to involve toll-like receptor 4 (TLR-4).

Methodology: The present study was carried out to determine the role of TLR-4 on eliciting the immunomodulatory effects of recombinant BCG expressing MSP-1C of *Plasmodium falciparum* leading to the production of NO and IL-10, as well as the expression of iNOS. Six groups of mice (n = 6 per group) were immunised thrice, three weeks apart with intraperitoneal phosphate buffered saline T80 (PBS-T80), BCG or rBCG in the presence or absence of a TLR-4 inhibitor; TAK-242, given one hour prior to each immunisation. Peritoneal macrophages were harvested from the mice and cultured for the determination of NO, iNOS and IL-10 via Griess assay, ELISA and Western blot respectively.

Results: The results showed significant inhibition of the production of NO and IL-10 and the expression of iNOS in all groups of mice in the presence of TAK-242.

Conclusions: These results presented evidence of the role of TLR-4/rBCG attachment mechanism in modulating the production of NO and IL-10 and the expression of iNOS in response to our rBCG-based malaria vaccine candidate expressing MSP-1C of *P. falciparum.*

Introduction

Annually, malaria causes half a million people's death, mostly in developing countries. Its control is elusive as the causative agent, the *Plasmodium* parasite has rendered antimalarials including Artemisinin combination therapies (ACTs) virtually ineffective [1,2]. *Plasmodium* resistance is complicated by *Anopheles* mosquito's resistance to insecticides [3] necessitating an intense search for an effective antimalarial vaccine [4]. A number of ways including the use of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) as a vector of the merozoite surface protein (MSP) are being tried in malaria vaccine researches. BCG, traditionally used as a vaccine against tuberculosis (TB) leads to some level of nonspecific innate immune protection against unrelated, non-TB pathogens [5]. Its qualities of being easily recognised and rapidly phagocytosed [6], informs its manipulation in vaccine development against other diseases such as malaria [7]. In malaria vaccine development, BCG is coupled to agonists such as the 19 kDa C-terminal region of the merozoite surface protein (MSP) [8].

Along this line, our group had earlier developed a recombinant BCG (rBCG) malaria vaccine candidate expressing the MSP-1 of *P. falciparum* which elicited increased nitric oxide (NO) production and significant inducible nitric oxide synthase (iNOS) expression alongside increased inflammatory cytokine release [9] with a possible role of TLR-4 [10]. The current study explored the role of the TLR-4 attachment to MSP-1 in eliciting the production of the pro-inflammatory mediator, NO and its inducing enzyme, iNOS via TAK-242-based inhibition. Due to its importance in malaria immune regulation, IL-10 was also analysed in this study.

Methodology

The rBCG and BCG cultures

A recombinant BCG, constructed earlier in our laboratory and parent BCG (Japan) were separately cultured on a 7H11 agar (Becton Dickinson, Franklin

Lakes, New Jersey, USA) supplemented with oleic acid, albumin, dextrose, and catalase (OADC) (Becton Dickinson, Franklin Lakes, New Jersey, USA), (with 15 µg/mL of kanamycin added to the rBCG culture). Colonies of each culture were later transferred to flasks containing 7H9 broth (Becton Dickinson, USA), supplemented with OADC (with 15 µg/mL of kanamycin for rBCG culture) and observed until an optical density (OD) of approximately 0.8 (A600 ≈ 0.8) was obtained.

Immunisation of Mice

Following the Universiti Sains Malaysia Animal Ethics Committee guidelines, approval [No: USM/Animal Ethics Approval No. 2016 (104) (801)], a total of 36 male BALB/c mice aged 4-6 weeks grouped into six were immunised thrice, three weeks apart. Each mouse received an intraperitoneal (i.p.) injection of 10^6 CFU rBCG in 200 µl phosphate buffered saline containing 0.1% Tween 80 (PBS-T80) while the positive and negative control groups were injected with 10^6 CFU of the parent BCG in 200 µl PBS-T80 and 200 µl PBS-T80 respectively [11] in the presence or absence 0.5 mg/kg of the TLR-4 inhibitor, TAK-242 (Cayman Chemical, Ann Arbor, Michigan, U SA), given intraperitoneally [12], one hour before each immunisation. The mice were observed for signs of adverse effects from the immunisations.

Peritoneal Macrophage harvest

The mice were sacrificed via rapid cervical dislocation, three weeks after the last immunisation. Peritoneal macrophages were then harvested from the mouse as described by Ray and Dittel [13]. Briefly, after sacrifice, each mouse was sprayed with 70% ethanol and mounted on a Styrofoam block on its back. An incision was made on the abdomen, the skin lining lifted then ice-cold sterile PBS (with 3% FBS) gently injected into the peritoneal cavity. The peritoneal lavage was aspirated and spun at 125 x g at 4°C for 8 minutes and 10^6 total peritoneal cells per mL were cultured for 2 hours at 37°C in a CO_2 incubator. Non-adherent cells were washed off and the adherent cells were incubated for another 2 hours. The cells were then gently scraped and harvested by centrifuging at 125 x g for 5 minutes. The supernatant was used for IL-10 and NO estimation while the pellet was used for iNOS Western blot analysis.

IL-10 assay

The effect of TLR-4 on the production of IL-10 was determined by ELISA kits (Bio-Rad Hercules,

California, USA). Briefly, a 96-well ELISA plate was coated with 100 µL of capture anti-IL-10 antibody and incubated overnight at 4°C, then blocked with 0.05% PBS-T20 the following day, followed by the addition of 100 µL of culture supernatant and standard and incubation for 2 hours at room temperature. This was followed by the addition of anti-mouse IL-10 and incubation for 2 hours at room temperature, then 100 µL of Avidin-HRP solution was added and incubated for 30 minutes at room temperature, then washed followed by 100 µL of substrate solution and 15 minutes' incubation in the dark. The reaction was stopped by the addition of 50 µL of stop solution and the OD measured with a microplate reader at 450 nm and a standard curve generated.

Nitric oxide assay

The concentration of NO in the culture supernatant was determined using the Griess Reagent System (Promega, Madison, Wisconsin, USA). Briefly, 50 µL standard and culture supernatants were added to a 96-well plate, followed by 50 µL sulphanilamide solution and the plate was incubated for 10 minutes at room temperature. Fifty µL of N-1-napthylethylenediamine dihydrochloride (NED) solution was then added and incubated for 10 minutes in the dark. The OD was determined at 540 nm using a microplate reader and a standard nitrite curve generated.

Preparation of peritoneal macrophage cell lysate for iNOS analysis

For iNOS expression analysis, the pellet obtained was washed with ice-cold PBS and lysed with Ice-cold RIPA lysis buffer and the lysate centrifuged at 1650 × *g* for 20 minutes. The supernatant was aspirated and placed in a fresh tube kept on ice and the total protein was determined by bicinchoninic acid assay.

Western blot analysis

Gel electrophoresis and transfer to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Chicago, Illinois, USA) were carried out after the cell lysate was denatured with Laemmli buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.003% bromophenol blue and 5% 2-mercaptoethanol). The membrane was then blocked for 1 h with 5% blocking solution followed by incubation with iNOS rabbit anti-mouse antibody (Abcam, USA) overnight at 4 °C. This was followed by incubation with goat anti-mouse antibody conjugated to HRP for 1 h at 37 °C (Dako, Glostrup, Denmark). Chemiluminescence was then generated by an ECL Western blot detection reagent (GE Healthcare,

Chicago, Illinois, USA). The intensity of iNOS expression was measured using the Image J 1.47 (National Institute of Health, USA) and its integrated density value (IDV) normalised with that of β-actin to determine the mean relative intensity (MRI).

Statistical analysis

Statistical analyses were performed 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). The data were analysed by one-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test. The *p*-value of < 0.05 was considered statistically significant.

Results

The role of TLR-4 on eliciting the immunomodulatory effects of recombinant BCG expressing MSP-1C of *Plasmodium falciparum* was analysed in six groups of mice (n = 6 per group) immunised with PBS-T80, BCG or rBCG in the presence or absence of TAK-242. Peritoneal macrophages were harvested and cultured. The production of NO and IL-10 were analysed in the culture supernatants via Griess' reaction and ELISA respectively, while the expression of iNOS was analysed via Western blot and the results were as followed.

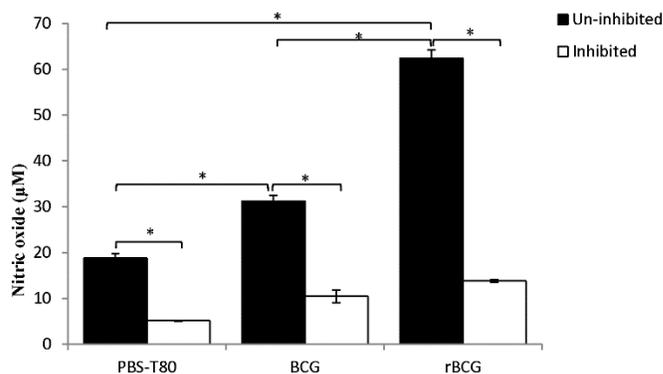*Nitric Oxide (NO) production by peritoneal macrophages of immunised mice*

These results showed significantly higher NO production in the peritoneal macrophages of mice immunised with PBS-T80, BCG and rBCG in the absence of TAK-242, $p < 0.001$ (Figure 1) which was highest in the peritoneal macrophage of mice immunised with rBCG (62.320 ± 0.891 μM), followed by the BCG immunised mice group (31.200 ± 0.923 μM) and lastly, the PBS-T80 immunised group (18.740 ± 0.375 μM). TLR-4 inhibition resulted in significant inhibition of NO production in all groups; PBS-T80 (*p* < 0.001, and mean difference -13.657), BCG (*p* < 0.001, and mean difference -20.779) and rBCG (*p* < 0.001, and mean difference -48.506).

Expression of inducible Nitric Oxide Synthase (iNOS) by peritoneal macrophages of immunised mice

A band of approximately 130 kDa (the expected size of iNOS) was obtained in the immunoblot analysis (Figure 2A). The expression of iNOS was robust in all

mice groups in the absence of TAK 242, $p < 0.001$ (Figure 2B). The expression was the highest in the peritoneal macrophages of mice immunised with rBCG (1.196 ± 0.003 MRI), followed by those immunised with BCG (0.955 ± 0.006 MRI) and the least in the mice immunised with PBS-T80 (0.917 ± 0.003 MRI), in the absence of TAK-242. In the presence of TAK 242, the peritoneal macrophages showed significant inhibition of iNOS expression in the PBS-T80 immunised group (*p* < 0.001, and mean difference -0.706), BCG immunised group (*p* < 0.001, and mean difference -0.670). and rBCG immunised group (*p* < 0.001, and mean difference -0.933).

Figure 1. NO production in peritoneal macrophage of mice immunised with PBS-T80, BCG and rBCG in the presence or absence of TLR-4 inhibitor, TAK-242.

Figure 2. (A) Representative result of iNOS and β-actin protein expression (B) The relative density of iNOS/β-actin in peritoneal macrophages of mice immunised with PBS-T80, BCG, or rBCG in the presence or absence of TLR-4 inhibitor, TAK-242.
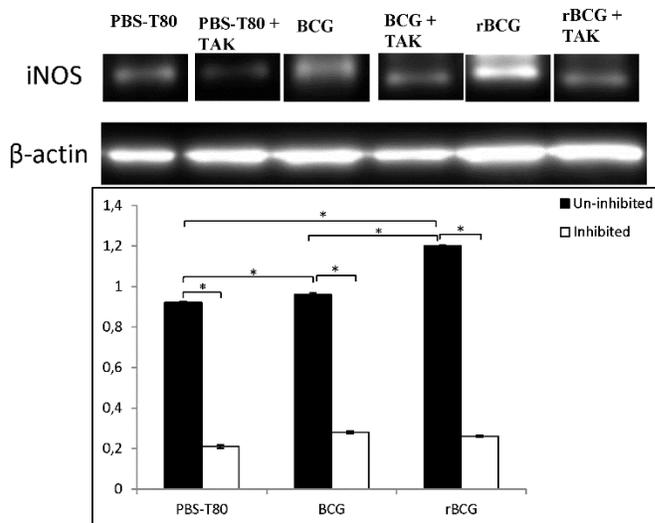
IL-10 production by peritoneal macrophages of immunised mice

The results obtained also showed a robust IL-10 production in all groups of mice in the absence of TAK-242, $p < 0.001$ (Figure 3). The production of IL-10 was significantly higher in the rBCG group (378.807 ± 1.10 pg/mL), followed by the BCG group (268.00 ± 3.539 pg/mL) then the PBS-T80 immunised mice group (216.030 ± 1.734 pg/mL). There was also a significant inhibition in IL-10 production in the peritoneal macrophages of all mice in the presence of TAK-242, with PBS-T80 ($p < 0.001$, and mean difference -48.673), BCG ($p < 0.001$, and mean difference -114.266) and rBCG only ($p < 0.001$, and mean difference -184.232).
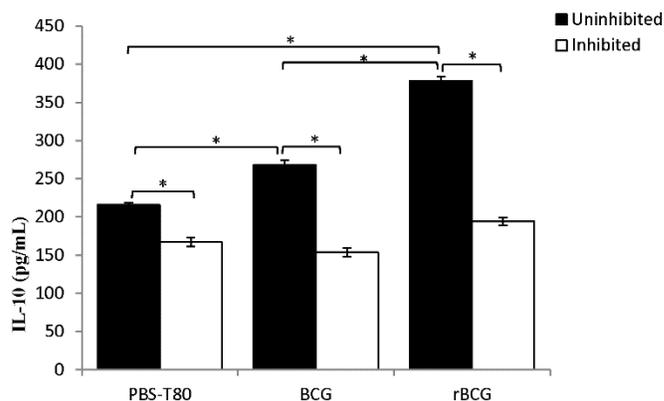
Discussion

This study analysed the role of TLR-4 in the expression of iNOS and the production of NO and IL-10 in mice immunised with recombinant BCG expressing MSP-1C of *Plasmodium falciparum*. The results showed a robust production of the pro-inflammatory mediator, NO and the enzyme iNOS which were highest in the rBCG immunised group. This was similar to their production in J774.1A macrophage cell lines infected with rBCG [9,10]. LPS stimulation of macrophages [14] cardiomyoblasts [15], cardiac myocytes [16] also led to a significant expression of iNOS and NO production. Similarly, rheumatoid arthritis [17,18] and the brain of rabies mouse-models showed increased NO and iNOS mRNA levels [19].

Further analysis showed that the increases in iNOS and NO were both significantly inhibited in the presence of TAK-242. Previous studies by others also showed that the inhibition of TLR-4 led to the inhibition of LPS [20] and megalo-type α-1,6-glucosaccharide-induced NO production [21]. TLR-4 inhibition also decreased immunoexpression of iNOS in the microglia of day old Wistar rats [22] and allografts [23]. It is of note that in this study iNOS expression was significant even in the un-stimulated group, which was contrary to our expectations. Even though some studies had shown iNOS to be constitutively expressed [24] we suggest further studies on the biosynthesis of iNOS to unravel the reasons behind these increased expressions.

This study also analysed the role of TLR-4 in the production of the immunoregulatory cytokine, IL-10 for its role in controlling inflammation through repression of pro-inflammatory cytokines production [25] especially in acute malaria [26] and found it to be highest in the rBCG immunised similar to what obtained in malaria [27]. Similarly, a robust IL-10

Figure 3. IL-10 productions in peritoneal macrophage of mice immunised with PBS-T80, BCG and rBCG in the presence or absence of TLR-4 inhibitor, TAK-242.

production was found in the lung interstitial macrophages of house dust mite challenge [28]. Furthermore, our results showed inhibition of IL-10 production in the presence of TAK-242. A number of studies also found IL-10 inhibition by TAK-242 in LPS challenge [29] and sepsis model [30] studies, indicating the role of TLR-4 in the production of IL-10.

Conclusion

In this study, the role of TLR-4 in modulating the effects of recombinant BCG expressing MSP-1C of *Plasmodium falciparum* via the expression of iNOS and the production of NO and IL-10 was highlighted. These immunomodulatory functions of TLR-4 are important innate immune responses essential in malaria parasite elimination and regulation of inflammation and a pre-requisite in eliciting of the adaptive immune responses.

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