



## The Role of TLR-4 on the Mapkinases Signaling Pathways of Inflammatory Responses against Recombinant BCG Malaria Vaccine Candidate

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### Abstract

*Malaria, a life-threatening disease caused by Plasmodium parasites which have developed resistance to all anti-malaria drugs on the background resistance of the mosquito vector to insecticides, necessitating more intense search for an effective vaccine. A recombinant BCG (rBCG) vaccine candidate expressing the merozoite surface protein 1C (MSP-1C) of Plasmodium falciparum was developed in our laboratory, which generated robust innate and adaptive immune responses that pointed to the likelihood of the role of Toll-like receptor-4 (TLR-4). This study analysed the role TLR-4 attachment of the rBCG to macrophages in eliciting the observed immune responses. Mice (n = 6 per group) were immunised with PBS-T80, parent BCG or rBCG in the presence or absence of a TLR-4 inhibitor; TAK-242 and the effects of TLR-4 on the expression of c-Jun N-terminal kinases 1 and 2 (JNK1/2) and extracellular signal-regulated kinases 1 and 2 (ERK1/2) which are involved in the signalling pathway were analysed through western blot on macrophages harvested from the mice peritoneum. The results obtained showed a significant increase in the expression of the MAPKinases in the group immunised with rBCG compared to BCG and PBS-T80 immunised groups. There was significant inhibition of the JNK1/2 and ERK1/2 expression in the presence of TAK-242 signifying, for the first time, the role of TLR-4 in the phosphorylation of both JNK1/2 and ERK1/2 in the immune response against the vaccine candidate expressing the MSP-1C of P. falciparum. This study highlighted the role of TLR-4 in the phosphorylation of ERK1/2 and JNK1/2 in the immune response against recombinant BCG malaria vaccine candidate*

**Key words:** JNK1/2, ERK1/2, Macrophages, Malaria, MAPKinase, MSP, Recombinant BCG, TLR-4, Vaccine

### INTRODUCTION

An estimated 3.2 billion people (half the world's population) live in areas at risk of malaria transmission in 106 countries and territories with close to half a million people annual death (CDC, 2017a) with an estimated 216 million cases of malaria reported in 91 countries in 2016 (WHO, 2017). Ninety percent of these 216 million cases were in Africa, which is also home to 91% of malaria deaths. Children less than five years of age account for about 70 percent of the malaria deaths, which means a daily loss of about 800 children under the age of five. This translates to a child's death every two minutes (WHO, 2016). The current interventions on malaria are primarily based on prevention, early diagnosis and prompt treatment of cases based on the World Health Organisation (WHO) recommendations. These interventional efforts

against malaria have not yielded the desired objectives, as the malarial parasite has proven to be complex and adaptable and has thus survived for a long time (PATH-MVI, 2011). The world is faced with a background insecticide resistance of the *Anopheles* mosquito to all recommended classes of insecticides (CDC 2017b) complicated by the fact that *P. falciparum* has developed resistance to all classes of medicines used in its treatment (White, 2004; Aung *et al.*, 2017) including treatment failure with artemisinin combination therapy (ACT) (Benjamin, 2017) making the search for an effective vaccine most desirable. Several vaccine candidates are being tried including recombinant *Mycobacterium bovis* bacille Calmette Guerin (rBCG) vaccines. Daly and Long (1993) showed protection in mice inoculated with rBCG MSP-1.15, an rBCG containing a fragment of MSP-1 from *P. yoelii*.

A further analysis using rBCG which expressed MSP1<sub>-15</sub> as a fusion protein and  $\alpha$ -k a secretory protein of *M. kansasii* also found that rBCG vaccination induced durable humoral immune response against a *P. yoelii* challenge in immunized C3H/He mice (Sohkichi *et al.*, 1998). Development of immunoprotection associated with antibodies to the 19-kDa Cterminal region (MSP-1<sub>19</sub>) has been shown in both human (Dodoo *et al.*, 2008) and animals (Hirunpetcharat *et al.*, 1997). Our laboratory also used the 19 kDa MSP to construct a recombinant BCG vaccine which elicited higher inflammatory response in macrophages (Dhaniah *et al.*, 2014) with an enhanced phagocytic activity and higher production of cytokines such as TNF- $\alpha$  and IL-1 $\beta$  (Rapeah *et al.*, 2010). The rBCG construct generated not only higher cellular but also humoral immune modulatory activities in animal model (Nurul and Norazmi, 2011) with the generation of a strong protection of more than 70% inhibition of *P. falciparum* 3D7 merozoites invasion of RBCs in mice (Nurul and Norazmi, 2011). The mechanisms of these immunomodulatory effects of our vaccine candidate had not been analysed, hence the need for this study. Immune reactions are initiated by the recognition of pathogen-associated molecular patterns, (PAMPs) by the specialised pattern recognition receptors, (PRRs) like the toll-like receptors (TLRs) (Kumar *et al.*, 2011) located on immune cells such as the macrophages, which effect their functions via phagocytosis, cytokines production and presentation of antigens (den Haan and Kraal, 2012). TLRs on or inside macrophages are essential in phagocytosis which is needed for parasitaemia reduction (Gowda *et al.*, 2011). TLRs are not only important during the generation of innate immune responses, but the cells activated via TLR can also induce adaptive immune responses (Cui *et al.*, 2014). Stimulation of the TLRs leads to maturation of phagosomes and upregulation of various pro-inflammatory cytokines (Medzhitov, 2001; Natarajan *et al.*, 2011; Knobloch *et al.*, 2016) through nuclear factor- $\kappa$ B (NF- $\kappa$ B) pathway as well as other signaling pathways. In acute *P. falciparum* infection TLR4 leads to pro-inflammatory response (McCall *et al.*, 2007) through both MyD88 and TRIF pathways (Bryant *et al.*, 2015) and further downstream via the MAPKinase pathways (Medzhitov and Janeway, 2002). The activated MAPKinases, P38, ERK1/2 and JNK1/2 then translocate to the nucleus, where they phosphorylate target transcription factors, including the activator protein-1 (AP-1) which

lead to the production of pro-inflammatory cytokines (Akira and Takeda, 2004). The activation of these MAPKinases, c-Jun Nterminal kinase 1 and 2 (JNK1/2) and extracellular signal-regulated kinase, 1 and 2 (ERK1/2) during the TLR-4-elicited inflammatory responses against PBS-T80, BCG and rBCG is the subject of this study. The role of TLR-4 in the cascade is investigated by the use of a selective Toll-like receptor 4 inhibitor; TAK-242 which inhibits its intracellular signaling (Matsunaga *et al.*, 2011) by blocking the intracellular domain TIR Toll/IL-1 Receptor of TLR-4 (Takashima *et al.*, 2009).

## MATERIALS AND METHODS

### Ethics

The guidelines of the USM Animal Ethics Committee were followed and its approval sought in all the animal work in this study.

### BALB/c Mice

The BALB/c mice utilised in this study were purchased from the Animal Research and Service Centre (ARASC) Universiti Sains Malaysia and were housed at the facility. The mice were liberally provided with standard laboratory chow and water ad libitum.

**Preparation of BCG and rBCG cultures and Determination of Colony Forming Unit (CFU)** A series of polymerase chain reactions were earlier on carried out in constructing the recombinant BCG expressing the MSP-1C of *P. falciparum* (rBCG) in our laboratory (Nurul and Norazmi, 2011). This and the parent BCG (Japan) were cultured on a 7H11 agar (Becton Dickinson, USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC) (Becton Dickinson, USA) and 15  $\mu$ g/mL of kanamycin (Sigma, USA), for the rBCG at 37°C for 2-3 weeks. A single colony was then aseptically picked from each culture and transferred to flasks containing 10 mL of 7H9 broth (Becton Dickinson, USA) supplemented with OADC with 15  $\mu$ g/mL of kanamycin added to the rBCG culture, for another 2-3 weeks until the optical density (OD) of approximately 0.8 (A<sub>600</sub>  $\approx$  0.8) was obtained. Ten millilitres each of BCG and rBCG were taken and centrifuged in separate tubes at 1500 x g for 10 minutes at room temperature, washed twice with PBS and the pellet resuspended in 1 mL DMEM. The colony forming unit of each sample was then determined from the absorbance reading of a spectrophotometer at 600 nm using the formula described by Norazmi and Dale (1997).

$$0.1 \text{ OD} = 4 \times 10^6 \text{ CFU/mL}$$

### Mice Immunisation

A total of 36 male BALB/c mice aged 4-6 weeks grouped into six were used for the study. Each group of mice was immunised intraperitoneally three times (3 weeks apart) with 200  $\mu$ L of PBST80,  $2 \times 10^6$  CFU of BCG in 200  $\mu$ L of PBS-T80 or  $2 \times 10^6$  CFU of rBCG in 200  $\mu$ L of PBS-T80 intraperitoneally respectively per mice, in the presence or absence of 0.5 mg/kg of TAK-242 administered intraperitoneally modified from Yao *et al.*, (2013) and Yonglin *et al.*, (2016), one hour before each immunisation and observed for any signs of adverse effects. **Peritoneal Macrophage harvest**

The mice were sacrificed via rapid cervical dislocation three weeks after the last booster and the peritoneal macrophages harvested as described by Ray and Dittel (2010). Briefly, immediately after sacrificing the mouse, each mouse was sprayed with 70% ethanol and mounted on the Styrofoam block. An incision was made on the abdomen using a pair of scissors. Ice-cold sterile 3% FBS and gently injected into the caudal half of the peritoneal cavity using a 27 G needle and the entire body of the mouse was gently shaken and the peritoneum massaged for 10 seconds. A 25 G needle attached to another 10 mL syringe in collecting the fluid which was deposited in tubes kept on ice, after removing the needle from the syringe. This process was repeated, and a plastic Pasteur pipette was finally used to collect the remaining fluid from the cavity. The peritoneal lavage collected was spun at 125 x g at 4°C for 8 minutes and the pellet resuspended in DMEM and cultured at  $1 \times 10^6$  total peritoneal cells/mL for 2 hours at 37°C in a CO<sub>2</sub> incubator after which, non-adherent cells were removed by gentle washing three times with warm PBS and fresh DMEM added and the cells allowed another 2 hours at 37°C in a CO<sub>2</sub> incubator before being scraped using a sterile plastic scraper and transferred into a 15 mL tube and harvested by centrifuging at 125 x g for 5 minutes at 4°C using ice-cold PBS.

### Preparation of peritoneal macrophage cell lysate for protein analysis

The cells placed on ice had PBS aspirated and 100  $\mu$ L of ice-cold RIPA (0.4 M NaCl, 50mM Tris/HEPES pH 7.5, 1% NP-40, 0.1% SDS, 1mM EDTA, 1mM phenylmethylsulfonyl fluoride and 0.05% protease inhibitor) lysis buffer added to the pellet of  $2 \times 10^6$  cells overnight at 4°C. The solution was centrifuged at 1650 x g for 20 minutes the next day, the supernatant aspirated and placed in a fresh tube kept on ice and a bicinchoninic acid (BCA) assay carried out to

determine the total protein concentration before being stored at -80°C until analysis.

### Western blot analysis

The expression of ERK1/2 and JNK1/2 were determined via western blot analysis. The lysed protein was recovered from the -80°C freezer and denatured with Laemmli buffer (62.5 mM Tris pH 6.8, 10% glycerol, 2% SDS, 0.003% bromophenol blue and 5% 2-mercaptoethanol). Electrophoresis of the lysate was carried out on a 10% polyacrylamide gel and then transferred to polyvinylidene difluoride membranes (GE Healthcare). The membranes were blocked for 1 h with 5% blocking solution followed by incubation with rabbit anti-mouse primary antibody for ERK1/2 and JNK1/2 respectively (Abcam, USA) overnight at 4 °C. The incubation with primary antibody was followed by incubation with goat anti-mouse secondary antibody conjugated to HRP for 1 h at 37 °C (Dako). An ECL western blot detection reagent was used to generate chemiluminescence based on manufacturer's recommendation (GE Healthcare). An anti- $\beta$ -actin antibody (Sigma) was used as the control in all cases and intensity of each protein expression was measured using the Image J 1.47 (National Institute of Health, USA) by inverting the film image before the integrated density of each band was measured. The integrated density value (IDV) of each protein was then normalized with that of  $\beta$ -actin to determine the mean relative intensity (MRI).

### Statistical Analysis

Statistical package of social sciences (SPSS) software version 22 was used for statistical analyses. All Data shown were representative of 3 experiments; performed in triplicate and presented as mean  $\pm$  standard error of the mean (SEM) and 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

### MAPKinases expression in the peritoneal macrophages of mice immunised with recombinant BCG expressing the MSP-1C of *Plasmodium falciparum*

The MAPKinases are a group of proteins downstream of the receptor-ligand interactions. A number of proteins with diverse functions fall under this category. The present study analysed ERK 1/2 and JNK1/2 as representative MAPKinases for their roles in cytokine production and the subsequent effects that may arise from the release of such cytokines.

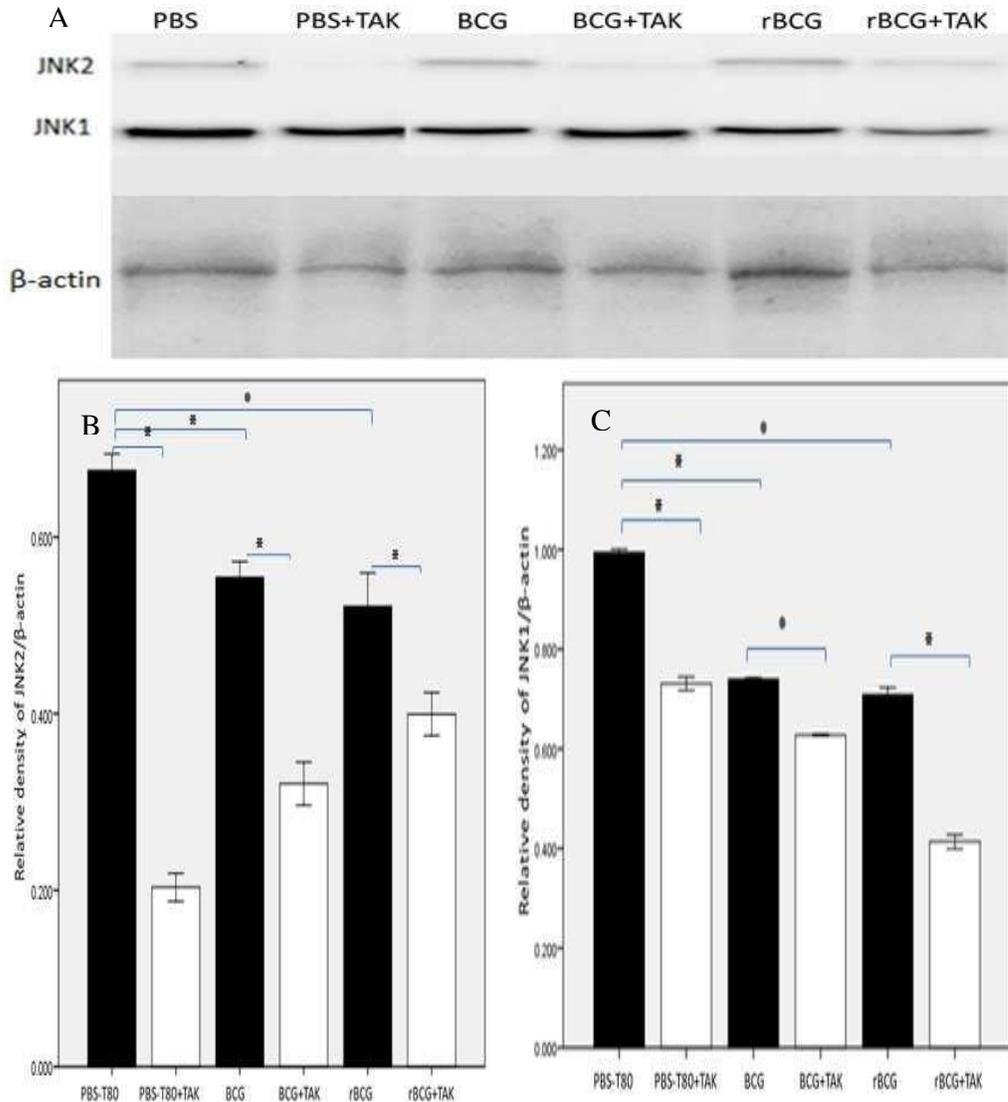
For their roles in the regulation of diverse physiological and immunological processes (András *et al.*, 2016), JNK1/2 were analysed in this study. Two bands were obtained on immunoblotting of the protein obtained from the macrophages harvested from the six groups of mice. These bands were of approximately 46 kDa (the expected size of JNK-1) and 54 kDa (the expected size of JNK-2) (Figure 1A). JNK-1 expression was also significantly higher in the peritoneal macrophage of mice immunised with PBS-T80, BCG or rBCG in the absence of TLR-4 inhibitor, TAK-242  $p < 0.001$ . JNK-1 expression was highest in the intraperitoneal macrophages from mice immunised with PBST80 ( $0.994 \pm 0.002$  MRI), followed by those immunised with BCG ( $0.740 \pm 0.001$  MRI) with the least expression in those immunised with rBCG ( $0.709 \pm 0.003$  MRI). There was significant inhibition of JNK-1 expression in the presence of TAK-242 in the peritoneal macrophage of mice immunised with PBS-T80 ( $p < 0.001$  and mean difference -0.264). JNK-1 expression in the peritoneal macrophages of the group of mice immunised with BCG in the presence of TAK-242 only was also significantly inhibited ( $p < 0.001$  and mean difference -0.113) as was JNK-1 expression which was also significantly inhibited in the group of mice immunised with rBCG in the presence of TAK-242 ( $p < 0.001$  and mean difference -0.295) (Figure 1B).

Like JNK-1, the expression of JNK 2 was also significantly higher in the peritoneal macrophage of mice immunised with PBS-T80, BCG or rBCG in the absence of 0.5 mg/kg of TAK-242,  $p < 0.001$ . Similarly, the expression JNK-2 was also highest in macrophages harvested from mice immunised with PBS-T80 ( $0.675 \pm 0.004$  MRI), followed by those immunised with BCG ( $0.554 \pm 0.004$  MRI) then those immunised with rBCG ( $0.521 \pm 0.009$  MRI) in the absence of TAK-242. JNK-2 expression was also significantly inhibited in the macrophages from the group immunised with PBS-T80 ( $p < 0.001$  and mean difference 0.472), BCG ( $p < 0.001$  and mean difference 0.234) and rBCG and ( $p < 0.001$  and mean difference 0.122) in the absence of TAK-242 (Figure 1C).

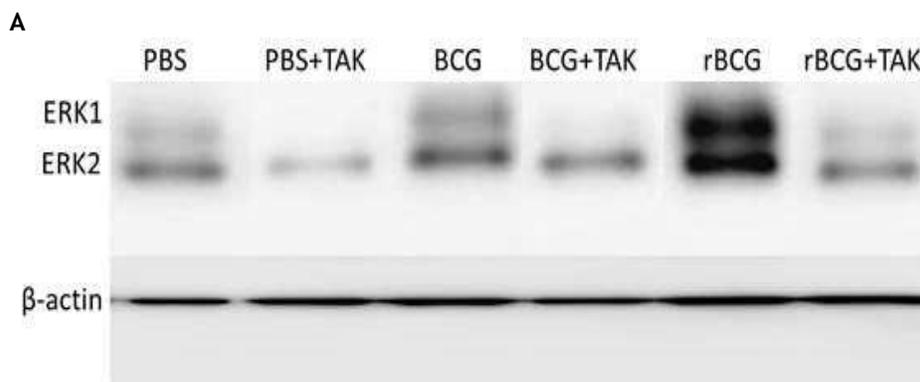
**ERK 1/2 expression in the peritoneal macrophages of mice immunised with recombinant BCG expressing the MSP-1C of *Plasmodium falciparum***

ERK 1 and 2 have been described as having various functions such as cell survival, differentiation, metabolism, proliferation, transcription and death (Roskoski, 2012). These and the fact that they are activated downstream of TLR-4/MyD88 signaling informs their inclusion in this study. A twin band was obtained on immunoblotting of the protein from the macrophages harvested from the six groups of mice. These observed bands were of approximately 44 kDa (the expected size of ERK-1) and 42 kDa (the expected size of ERK-2) (Figures 2A).

Like in the case of other proteins evaluated, when the harvested macrophages were evaluated, ERK-1 expression was found to be significantly higher in the peritoneal macrophage of mice immunised with PBS-T80, BCG or rBCG in the absence of TLR-4 inhibitor, TAK-242,  $p < 0.001$ . Unlike JNK, in the absence of TAK-242 ERK1 expression was highest in the macrophages obtained from mice immunised with rBCG ( $1.736 \pm 0.018$  MRI). Then the group immunised with BCG group ( $0.802 \pm 0.009$  MRI) and the lowest expression was in the peritoneal macrophages in the mice immunised with PBST80 ( $0.640 \pm 0.004$  MRI). ERK-1 expression was significantly inhibited in the peritoneal macrophages of mice immunised with PBS-T80,  $p < 0.001$  and mean difference -0.226), then the group of mice immunised with BCG,  $p < 0.001$  and mean difference -0.472) and lowest expression in the group of mice immunised with rBCG,  $p < 0.001$  and mean difference -1.297 in the presence of TAK-242 (Figure 2B). The expression of ERK-2 was similar to that of ERK-1, as it was also significantly higher in the peritoneal macrophage of mice immunised with PBS-T80, BCG or rBCG in the absence of TLR-4 inhibitor, TAK-242,  $p < 0.001$ . In the absence of TAK-242 ERK-2 expression was highest in the peritoneal macrophages from the group of mice immunised with rBCG ( $1.500 \pm 0.006$  MRI) then the group of mice immunised with BCG ( $0.730 \pm 0.004$  MRI) and the group of mice immunised with PBS-T80 ( $0.530 \pm 0.009$  MRI). ERK-2 expression was significantly inhibited in macrophages from the group immunised with PBS-T80,  $p < 0.001$  and mean difference -0.213, then the macrophages of the group of mice immunised with BCG,  $p < 0.001$  and mean difference -0.215 and the group mice immunised with rBCG  $p < 0.001$  and mean difference -0.941 in the presence of TAK-242 (Figure 3C).

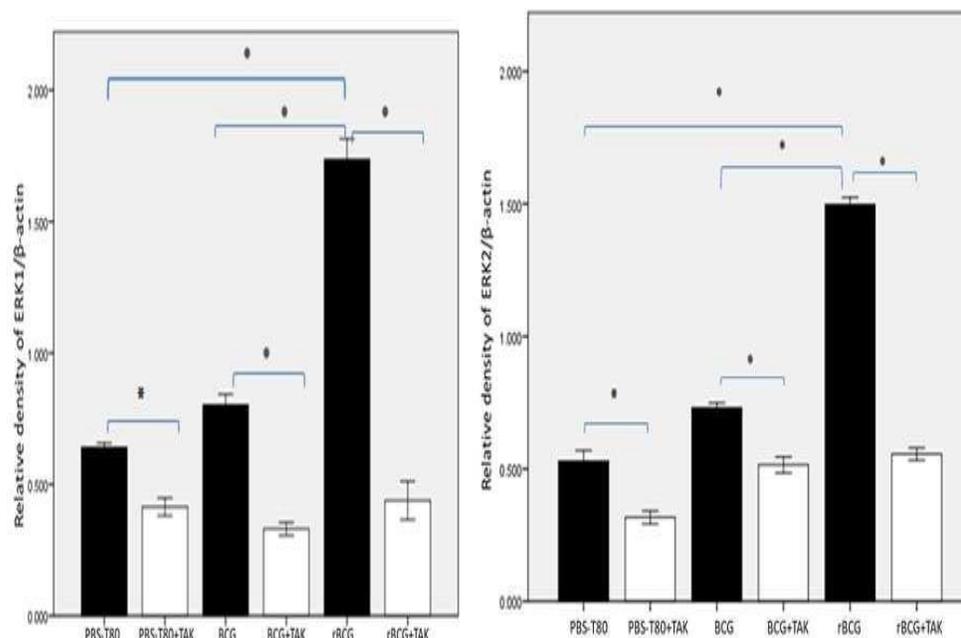


**Figure 1** (A) Representative result of JNK2/1 and β-actin protein expression (B) The relative density of JNK-2/β-actin protein and (C) the relative density of JNK-1/β-actin protein in peritoneal macrophages of mice immunised with PBS-T80, BCG, or rBCG in the presence or absence of TLR-4 inhibitor, TAK-242. Data are presented as mean relative density of JNK1/2/β-actin ± SEM for three independent experiments \**p* < 0.001.



**B**

C



**Figure 2** (A) Representative result of ERK1/2 and  $\beta$ -actin protein expression (B) The relative density of ERK-1/ $\beta$ -actin protein and (C) the relative density of ERK-2/ $\beta$ -actin protein in peritoneal macrophages of mice immunised with PBS-T80, BCG, or rBCG in the presence or absence of TLR-4 inhibitor, TAK-242. Data are presented as mean relative density of ERK1/2/ $\beta$ -actin  $\pm$  SEM for three independent experiments \* $p < 0.001$ .

## DISCUSSION

The MAPKases proteins expressions were evaluated in this study due to their demonstrable effect on the production of cytokines. For example, JNK phosphorylation via the NF- $\kappa$ B pathway leads to the production of TNF- $\alpha$ , IL-6, IL-12 and nitric oxide in macrophages stimulated with *P. falciparum* GPIs (Jianzhong *et al.*, 2005). Likewise, LPS stimulation of macrophages and the subsequent activation of ERK, p38 and JNK MAPKases induce the production of both IL-10 and its mRNA while their inhibition leads to the abolition of IL-10 production (Hugues *et al.*, 2007). TNF $\alpha$  production by leukocytes is also significantly reduced by the inhibition of ERK (Rossana *et al.*, 1996) as well as the inhibition via P38 and JNK inhibition in murine macrophages treated with blueberries

(Chenghui *et al.*, 2011). The role of MAPKases in infections was highlighted by *Influenza A Virus X-31* infection in murine macrophages where it activates MAPKs, JNK 1/2 and ERK1/2 (Cannon, 2014). ERK-1 and ERK-2 are a major regulator of IL-10 induction in macrophages and B-cells (Kaiser *et al.*, 2009; Ruhcha *et al.*, 2018). *Trypanosoma cruzi*- induced ERK activation

elicits macrophages production of IL12 and TNF- $\alpha$  (Ropert *et al.*, 2001).

Interestingly, the results of this study showed increased expression of both JNK1/2 and ERK1/2 in all groups in the absence of TAK-242. Activation of both ERK-1 and JNK1/2 were seen in *enterovirus 71* infection of rhabdomyosarcoma cells (Weifeng *et al.*, 2013) ERK1/2 and JNK in *P. falciparum*-GPI-induced signaling in macrophages (Krishnegowda *et al.*, 2005). In fact, JNK induced the activation of cJun and ERK as well as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  production, which significantly reduced on its inhibition (Lim *et al.*, 2014). It is noteworthy that TLR-4 interaction with its ligand, LPS led to JNK activation and its increased expression (Matsuguchi *et al.*, 2003). In the case of JNK, when its production was evaluated the results showed that the group of mice immunised with PBS-T80 exhibited the highest expression of both JNK-1 and JNK-2 compared to BCG and rBCG immunised groups. A number of studies have shown increased LPS-induced JNK activation (Li *et al.*, 2006; Hussey *et al.*, 2013; Liang *et al.*, 2013), while others reported increased JNK activation in infectious conditions like *enterovirus 71* (Hongjun *et al.*, 2014) *Varicella-zoster* (Markus *et al.*, 2004) and *Porcine*

circovirus Type 2 (Li *et al.*, 2009). On the other hand, an evaluation of ERK in this study found robust expression of ERK1/2, which was most abundant in the group of mice immunised with rBCG than in the group immunised with BCG or PBS-T80. This result was similar to the one obtained when gastric epithelial cells were stimulated with *H. pylori* which resulted in robust upregulation in the phosphorylation of ERK1/2 (Shin-ichi *et al.*, 2010). Increased phosphorylation of ERK1/2 was also observed in cells infected with *alphavirus* (Kelsey *et al.*, 2014). A study on *porcine epidemic diarrhoea virus* (PEDV) infection also showed a similar but transient activation of ERK1/2 at the early stage of the infection (Youngnam and Changhee, 2005).

On the possible role of TLR-4 in the expression of these MAPkinases, the results showed TAK242 inhibiting the production of both ERK1/2 and JNK1/2 in all the groups of mice. Results similar to ours have been obtained in TAK-242 inhibition of LPS-induced phosphorylation of JNK and ERK in RAW264.7 cells (Li *et al.*, 2006; Masayuki *et al.*, 2006) and muscle cells (Hussey *et al.*, 2013). The role of TLR-4 was further shown by the decrease in ERK1/2 phosphorylation in minimally oxidized lowdensity lipoprotein-induced cytokine production in wild and TLR4-/- mice (Yury *et al.*, 2005). Furthermore, acute ethanol treatment of murine macrophage inhibited the TLR-4-induced activation of ERK1/2 and blocked TNF $\alpha$  production (Joanna *et al.*, 2005) signifying the role of TLR-4 in the ERK1/2 pathways. *In vitro* and *in vivo* salidroside treatment of macrophages downregulated protein expression of toll-like receptor 4 (TLR4) and CD14 and inhibited LPS-triggered phosphorylation of LPS-activated kinase 1 (TAK1), p38, JNK and ERK as well as inhibited iNOS expression and NO, IL-1 $\beta$  and TNF- $\alpha$  production (Peng *et al.*, 2016). Further similar findings showed TAK-242 inhibition of LPS-induced ERK1/2 activation in cardiac fibroblasts and cardiac myofibroblasts (Pia *et al.*, 2016). A further evaluation of the LPS-induced JNK activation in macrophages showed that the activation was abrogated in TLR-4 null mutation (Muzio *et al.*, 1998) signifying the role of TLR-4 in the activation of this MAPKinase and highlighting the mechanism of TLR-4/macrophage downstream interactions.

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The role of TLR-4 in the expression of ERK was further shown in a study investigating bone environment-induced cytokine production which found that necrotic bone fluid and particles increased ERK1/2 expression in macrophages which was attenuated on treatment with TAK-242 (Naga *et al.*, 2016).

## CONCLUSION

This study has shown the mechanism through which TLR-4 exerts its effects on the signaling pathways downstream of inflammatory responses. Our earlier observed enhanced immunomodulatory effects of our recombinant BCG malaria vaccine construct has now been shown to be due to the increased bonding between the toll-like receptor 4 present on macrophages and the MSP-1C of *plasmodium falciparum* which activates MyD88 which in turn activates of the MAPKinases, JNK1/2 and ERK1/2. The phosphorylation of JNK1/2 and ERK1/2 led to activation of other kinases which resulted in cytokine release and further generation of other immune reactions.

## RECOMMENDATIONS

A further insight into the understanding of the mechanisms of the immunomodulatory effects of our vaccine candidate will be achieved through further studies involving other downstream proteins such as Interleukin-1 receptor-associated kinase-1 and 2 (IRAK1 and 2, TNF receptor-associated factor 6 (TRAF6), transforming growth factor- $\beta$ -activated kinase 1 (TAK1) and the activator protein-1 (AP-1) which lead to the production of pro-inflammatory cytokines.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Author Contributions

M.A.A. performed the research and analysed the data. R.S. designed the experiments and analysed the data. Both authors drafted and approved the final manuscript.

## Acknowledgments

The research was supported by the Fundamental Research Grant Scheme (FRGS) No. 203/PPSK/6171140.

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