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Immunomodulation of Murine Macrophages RAW264.7 Infected with Mycobacterium Smegmatis

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Abstract

Tuberculosis (TB) remains as a major worldwide health problem which cause more than 1.3 million deaths annually. The search for new vaccines more efficacious than Bacille Calmette- Guèrin (BCG) for TB prevention is regaining importance to control the disease. Mycobacterium smegmatis (Ms), which shares genetic and structural homology with virulent mycobacteria, showed extensive advantages in vaccine vector development. In addition, autophagy has emerged as a major immune mechanism against pathogens in macrophages. The aim of this study is to determine immunomodulation capacity of Ms as a possible vector in development of vaccine candidates against TB. The immunomodulatory capacity of Ms were evaluated in RAW 264.7 murine macrophages cell line. The intracellular uptake were assayed by Ziehl- Neelsen staining and its phagocytic index (PI) were evaluated. The supernatant were used to determine cytokine production (IFN-γ and IL-1β). In general, the results demonstrated the immunomodulation effects of Ms in macrophages. For PI, a significant phagocytosis activity was observed in Ms (61.67 ± 2.84) compared to uninfected group. A significant production of IFN-γ (51.17 ± 20.70) but insignificant IL-1β (370.34 ± 6.23) cytokines were observed compared to uninfected group. The results showed that Ms could induce the immunomodulatory effects on RAW 264.7 murine macrophages. Taken together, this study provides preliminary results of potential evaluation of Ms as a new vector for experimental vaccine development against TB.

Keywords: Tuberculosis, vaccine candidate, BCG, mycobacteria, M. tuberculosis, M. smegmatis, Antigen-85B
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Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* (Mtb) which results to extreme coughing, and fever with chest pain [1]. Due to the uncertain efficacy of the Bacille Calmate-Guerin (BCG) vaccine and the emergence of widely resistant Mtb strains, the promise of *Mycobacterium smegmatis* (Ms) as vaccine candidates for TB prevention has been regaining interest among researchers in recent years [2]. There are several factors that contribute to the outcome of TB infection, such as host genetics, virulence factors, the host's health status and vaccination history [1].

TB kills over a million people all around the globe every year [4]. BCG, a live attenuated *Mycobacterium bovis* strain, is thus relevantly similar in antigenic composition to Mtb and has been used as a vaccine against human TB [5]. There is variation in the efficacy of BCG vaccine use against pulmonary TB and it offers protection only for the serious types of the disease in children [6]. Thus, the urgency of developing new more efficacious vaccine than BCG is needed.

Ms is a non-pathogenic, rapidly developing, commensal strain of the species Mycobacterium and can be used as possible candidate for vaccine vector [7,8]. The present study will be a preliminary study to assess the potential of Ms to combat TB. The aim of this study is to determine immunomodulation capacity of Ms as possible vector in development of vaccine candidates against TB. This study attempts to test the hypothesis that Ms may induce immunomodulation effects, specifically on RAW 264.7 murine macrophage cells.

Materials and Methods

Preparation of RAW 264.7

Mouse macrophage cell line RAW 264.7 (ATCC® TIB-71™) was purchased from American Type Culture Collection (ATCC, USA). The cell line was cultured in complete Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen) medium. The cell was grown at 37oC in a humidified incubator with 5% CO2. DMEM supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate was used in this study. For working media, 45 mL of DMEM, 5 mL of serum and 0.5 mL of antibiotic were mixed together. For infection, only 45 mL of DMEM and 5 mL of serum were used, without antibiotic.

Preparation of Ms and BCG

Ms (mc^2155) strain was obtained from the collection of the Universiti Sains Malaysia, Kubang Kerian Campus, Kelantan, Malaysia, while BCG bacterial stock were obtained from Hospital Sultanah Aminah, Johor Bharu. Prior to infection, a colony of Ms and BCG, supplemented with OADC, was grown in 7H11 agar and incubated at 37°C for 1 week and 2 weeks respectively. The bacteria were inoculated with 10% OADC in 7H9 broth and incubated at 37°C for 24 hours. The bacterial colonies were first pelleted and twice treated with sterile Phosphate- buffered saline (PBS).

Macrophage infection

Ms and BCG cultures were pelleted for 10 minutes at 5000 rpm, then washed in PBS twice. To order to treat with macrophage, clumps of bacteria have been extracted for 5 minutes at high frequency by ultrasonic treatment accompanied with 5 minute centrifugation at 5000 rpm. Thus, the Ms and BCG were resuspended to a final A600:0.5 OD in DMEM without antibiotic. RAW 264.7 murine macrophages were seeded in a 75 mL three culture flask containing $1x10^6$ cells/mL in antibiotic-free media and were incubated at 37°C overnight. Macrophage were contaminated with, or left uninfected by, Ms, BCG, and lipopolysaccharide (LPS) (100 ng/mL). Each culture flask washed with sterile PBS after 4 hours of infection to remove the non-internalized bacteria. The media were then added to the infected macrophages with antibiotics and left at 37°C for 24 hours.

Phagocytic Assay

The amount of Ms and BCG consumed in each cell determined the phagocytic behavior of the macrophages. Ms, BCG and uninfected infection was conducted using the slide chamber. The cells were washed 3 times with sterile PBS after 4 hours of infection to remove uningested bacilli. The chambers were removed, then heated the slides for 2 minutes. Ziehl-Neelsen Carbol-Fuschin stain was sprayed and heated for 5 minutes until fume was developed. The slides were then cleaned under running tap water until the colour turned to pink and with 3% acid alcohol followed for decolorizer. The slides were then treated for 2 minutes with methylene blue before washing away under warm tap water. The slides were examined under light microscope in the immersion of the oil. For a total of 100 macrophages, the phagocytic index (PI) was calculated as the relative population of the Ms and BCG counted.

Cytokines Determination by ELISA

For cytokine output, supernatants from activated cells were analysed. Cytokine quantification in supernatants culture was performed using mouse cytokine ELISA MAX™ Deluxe Collection kit (BioLegend®, USA) according to manufacturer instructions. In the coating buffer, ELISA plates were coated with 100 µl of antibody capture (anti mouse-IFN-γ and anti mouse-IL-1β). The plates have been sealed and incubated overnight at 4°C. Next, with 250 µl cleaning buffer the plates were washed 4 times. The plates were blotted on absorbent paper to remove any residual buffer before being blocked by shaking at room temperature (RT) for 1 hour with 200 µl of 1× assay diluent and incubated. The plates were washed 4 times for 10 minutes after an hour of incubation. Next, the normal protein solutions (mouse recombinant IFN-γ and IL-1β) were added to the correct wells. To produce a standard curve, six duplicate serial dilutions of the top standard were performed. The culture supernatants test sample was applied to suitable wells and incubated with

shaking for 2 hours at RT. The plates were washed 4 times after incubation, and 100 µl of diluted antibody detection (Biotinylated anti-mouse IFN-γ and IL-1β) was added. At RT the plates were incubated for 1 hour and washed 4 times for 10 minutes followed. 100 µl of diluted Avidin-horseradish peroxidase (Avidin-HRP) was subsequently applied to each well and incubated for 30 minutes at RT.

Statictical Analysis

All experiments were performed in at least triplicate, and representative results are presented. Statistical significance was analysed using Prism 6 software (GraphPad Software, Inc., San Diego, CA, USA) by Independent T- Test. The data were expressed as means ± standard deviations. Statistical significance was set to $p<0.05$, $p<0.01$ and $p< 0.001$.

Discussions and Results

Mtb is a very slow developing, acid-fast bacillus [9]. Human beings are the natural host for this pathogen, transmitted through the respiratory tract. The earliest stage started with tubercle bacilli inhalation and then phagocytosed and killed by the macrophages [10]. Alveolar macrophages are capable of inhibiting bacillus development via phagocytosis and engaging in a larger sense of cellular immunity via the process of T-lymphocyte presentation and recruitment [11]. Phagocytic cells, such as macrophages, have a significant role in activating and guiding adaptive T-cell immunity via the presentation of mycobacterial antigens and the production of costimulative signals and cytokines. Eliminating Mtb infection depends mainly on the effectiveness of the interaction between infected macrophages and T-lymphocytes [12]. Therefore, on RAW 264.7 murine macrophages, the current research attempted to evaluate the innate immune activation and immunomodulation effects caused by Ms.

Phagocytic uptake assay was performed to determine the phagocytic activity of macrophages after infection at MOI of 10 with Ms, BCG, and the control (uninfected). The number of bacteria internalized into RAW 264.7 murine macrophages was demonstrated by Ziehl Neelsen (ZN) staining where the presence of acid-fast bacilli was observed as red in colour. Phagocytic activity of RAW264.7 was interpreted as the number of ingested mycobacteria in a total of 100 macrophages counted.

No apparent changes in the morphology of the infected macrophages and the mycobacteria was observed (Figure 1). There is a significant phagocytic index of Ms (61.67 ± 2.84) and BCG (73.33 ± 10.41) when it is individually compared with uninfected macrophages. Macrophages infected with Ms and BCG showed no significant difference (11.67 ± 6.24) in phagocytic index. Overall, phagocytic activity in BCG is slightly higher than Ms infected macrophages.

Since the PI for both organisms were reported as significant, this indicates that Ms and BCG were efficiently phagocytosed by RAW 264.7 murine macrophages after 4 hours co- incubation (Figure 2). This is consistent with previous studies documenting rapidly developing mycobacteria such as Ms being unable to avoid intracellular killing and relatively limited survival in the cellular host environment [12]. Previous study suggested that Ms was effectively destroyed by macrophages as early as 4 hours or no later than 48 hours after infection [13]. Efficient killing of Ms has been observed in various forms of human, murine, and bovine macrophages [14]. Ms' PI also reported being slightly lower than the existing, proven, BCG vaccine. These mean that Ms does not have any pathogenic effects on RAW 264.7. Therefore, in immunocompromised individuals, Ms may be healthy for administration. In addition, earlier study showed that wild type and recombinant Ms offers non-pathogenic effects in natural killer cells or mice deficient in T cells [15].

It is well described that IFN-γ is responsible to regulate autophagy while IL-1β production was regulated by autophagy [16]. Therefore, outlining that the level of expression of costimulatory molecules will regulate the course of infection and the survival of intracellular bacteria is crucial. The bacteria's ability to trigger the expression of these molecules thus decide the activation of the immune response and its fate or survival in the infected host [17,18].

The supernatant of Ms, BCG, LPS stimulated and uninfected macrophages were collected at 24 hours post-infection in order to determine the development of these cytokines modulated by RAW264.7. Next, ELISA was used to test the secreted levels of IFN-γ and IL-1β. The cytokines level produced by Ms, BCG and LPS stimulated macrophages were compared to uninfected macrophages for both cytokines. The production of IFN- γ cytokines in Ms (51.17 ± 20.70) and BCG infected macrophages (80.65 ± 21.28) were elevated, while insignificant number of IFN-γ cytokines were evaluated in LPS stimulated macrophage (17.96 ± 6.40) (Figure 3). There is significant amount of IL- 1β cytokines produced by LPS stimulated macrophages (934.20 ± 4.670), while insignificant production of IL-1β by both Ms (370.34 ± 6.23) and BCG infected (439.20 ± 10.27) were observed (Figure 4).

Results showed remarkable rates of IFN-γ released while IL-1β showed insignificant effects. Many evidences may be clarified about the substantial development of IFN-γ. For example, after antigens have been internalized through phagocytosis (or possibly through autophagic degradation), activated macrophages will show peptide-MHC complexes and express co-stimulatory molecules as a second signal for T-cell activation [19]. The optimum stimulation of the macrophage gradually further increases the development of pro-inflammatory cytokines [20]. However, earlier study also suggested that Ms is processed rapidly by infected cells, altering its cell surface phenotype and modulating the development of potent pro-inflammatory cytokines [21].

On the contrary with IFN-γ, there are several reasons on insignificant level of IL-1β being produced by RAW 264.7 murine macrophages when infected with both Ms and BCG. Since IL-1β being regulated by autophagy, it could be due to ineffective autophagy process producing insignificant amount of IL-1β [17]. Other reasons could be

due to the non-pathogenic nature of Ms. Previous study showed that increased IL-1β output ratio was found in pleurisy tuberculosis compared with other forms of TB [22]. Therefore, the argument may be made that an improved IL-1β / IL- 1Ra ratio protects against a more serious tuberculosis presentation.

Conclusions

Taken together, this study concluded that Ms has the capability to induce immunomodulatory effects on RAW 264.7 murine macrophages. Hence, the potential of Ms as vaccine vector candidate could be explored more.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgement

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References

1. Fogel N. Tuberculosis: a disease without boundaries. *Tuberculosis*. 2015;95(5):527.

2. Maura, R. B., Férnandez S, Reyes G, et al. Evaluation of the potential of Mycobacterium smegmatis as vaccine Candidate against tuberculosis by in silico and in vivo studies. *Vaccimonitor*. 2010;19(1):20-16.

3. Paik, S., Kim JK, Chung C, Jo EK. Autophagy: A new strategy for host- directed therapy of tuberculosis. *Virulence*. 2019;10(1):448-459.

4. World Health Organization. Global tuberculosis report 2019. WHO. Published 2019. https://apps.who.int/iris/handle/10665/329368

5. Jagannath C, Lindsey, D. R., Dhandayuthapani S, Xu Y, Hunter Jr RL, Eissa NT. Autophagy enhances the efficacy of BCG vaccine by increasing peptide presentation in mouse dendritic cells. *Nat Med*. 2009;15(3):267.

6. Zumla A, Nahid P, Cole ST. Advances in the development of new tuberculosis drugs and treatment regimens. *Nat Rev Drug Discov*. 2013;12(5):388.

7. Tyagi JS, Sharma D. Mycobacterium smegmatis and tuberculosis. *Trends Microbiol*. 2002;10(2):68-69.

8. Reyrat JM, Kahn D. Mycobacterium smegmatis: an absurd model for tuberculosis? *Trends Microbiol*. 2001;9(10):472-474.

9. Flynn JL. Immunology of tuberculosis and implications in vaccine development. *Tuberculosis*. 2004;84(1-2):93-101.

10. Van Crevel R, Karyadi E, Preyers F, et al. Increased production of interleukin 4 by CD4+ and CD8+ T cells from patients with tuberculosis is related to the presence of pulmonary cavities. *J Infect Dis*. 2000;18(3):1194-1197.

11. Schluger NW, Rom WN. The host immune response to tuberculosis. *Am J Respir Crit Care Med*. 1998;157(3):679-691.

12. Kelley VA, Schorey JS. Mycobacterium's arrest of phagosome maturation in macrophages requires Rab5 activity and accessibility to iron. *Mol Biol Cell*. 2003;14(8):3366-3377.

13. Kadir NA, Sarmiento ME, Acosta A, Norazmi MN. Cellular and humoral immunogenicity of recombinant Mycobacterium smegmatis expressing Ag85B epitopes in mice. *Int J mycobacteriology*,. 2016;5(1):7-13.

14. Jordao L, Bleck CK, Mayorga L, Griffiths G, Anes E. On the killing of mycobacteria by macrophages. *Cell Microbiol*. 2008;10(2):529-548.

15. Young SL, Murphy M, Zhu XW, et al. Cytokine-modified Mycobacterium smegmatis as a novel anticancer immunotherapy. *Int J Cancer*. 2004;112(4):653-660.

16. Harris J. Autophagy and cytokines. *Cytokine*. 2011;56(2):140-144.

17. Flores-Batista, V. C. S., Boechat N, Lago PM, Lazzarini LC, Pessanha LR, Almeida AS, Lapa-e-Silva JR. Low expression of antigen- presenting and costimulatory molecules by lung cells from tuberculosis patients. *Brazilian J Med Biol Res*. 2007;40(12):1671-1679.

18. Hubo M, Trinschek B, Kryczanowsky, F. T, A., Steinbrink K, Jonuleit H. Costimulatory molecules on immunogenic versus tolerogenic human dendritic cells. *Front Immunol*. 2013;4:82.

19. Ingolia NT, Murray AW. The ups and downs of modeling the cell cycle. *Curr Biol*. 2004;14(18):R771-R777.

20. Curtsinger JM, Mescher MF. Inflammatory cytokines as a third signal for T cell activation. Current opinion in immunology. *Curr Opin Immunol*. 2010;22(3):333-340.

21. Cheadle EJ, O'Donnell D, Selby PJ, Jackson AM. Closely related mycobacterial strains demonstrate contrasting levels of efficacy as antitumor vaccines and are processed for major histocompatibility complex class I presentation by multiple routes in dendritic cells. *Infect Immun*. 2005;73(2):784-794.

22. Wilkinson RJ, Patel P, Llewelyn M, et al. Influence of polymorphism in the genes for the interleukin (IL)-1 receptor antagonist and IL-1β on tuberculosis. *J Exp Med*. 1999;189(12):1863-1874.

Table and Figure

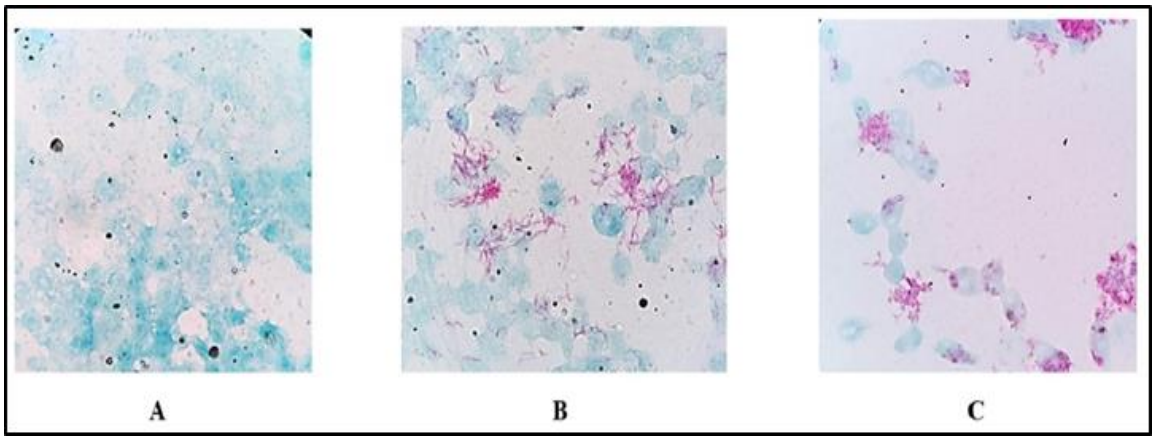

Figure 1: Comparison of macrophage morphology at 100x magnification assayed by ZN staining. (A) Uninfected cells (control). (B)Ms infected cells. (C) BCG infected cells.

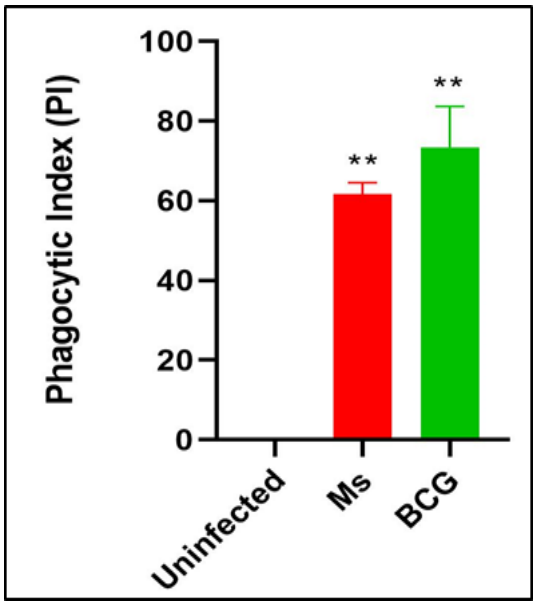

Figure 2: PI of Ms, BCG and uninfected macrophages. The uninfected cells were used as negative control. The data were analysed using T- test and represents as mean PI (± SD). *p< 0.05 and **p < 0.001.

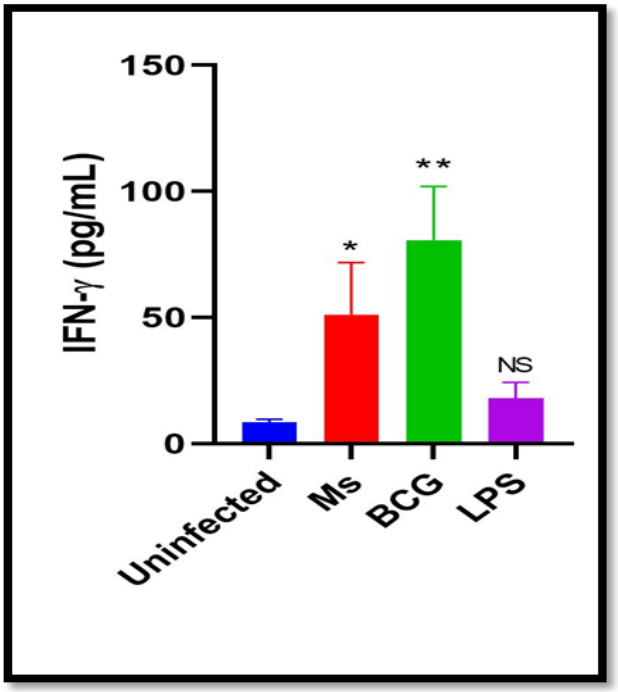

Figure 3: The level of IFN-γ cytokines production. The uninfected cells were used as negative control. The data were analysed using T- test and represents as mean concentration of IFN-γ (± SD). *p <0.05, **p < 0.001 and ns=not significant.

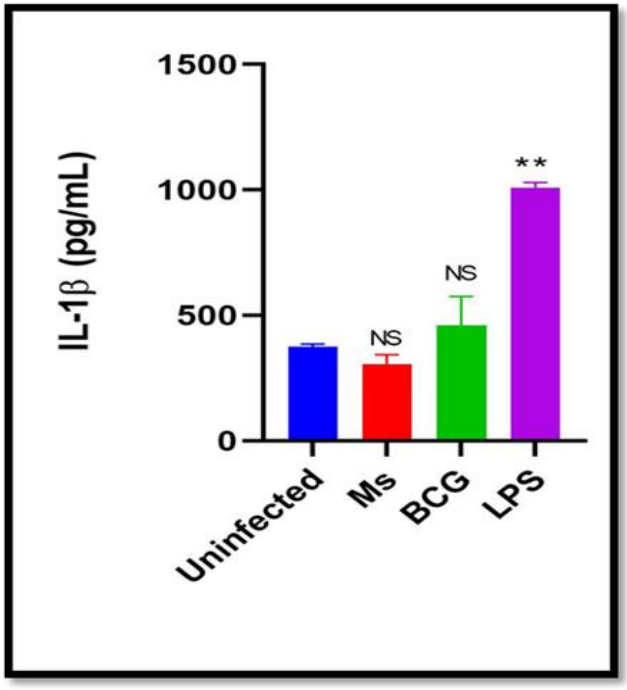

Figure 4: The level of IL-1β cytokines production. The uninfected cells were used as negative control. The data were analysed using T- test and represents as mean concentration of IL-1β (± SD). *p<0.05, **p<0.001 and ns=not significant.