Jpn. J. Infect. Dis., 67, 436–440, 2014

Original Article

Effect of Host Factors on Neutrophil Functions in Response to *Burkholderia pseudomallei* in Healthy Thai Subjects

Pornpun Saengmuang†, Chidchamai Kewcharoenwong†, Patcharaporn Tippayawat, Arnone Nithichanon, Surachat Buddhisa, and Ganjana Lertmemongkolchai*

The Centre for Research & Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand

(Received November 18, 2013. Accepted March 7, 2014)

SUMMARY: *Burkholderia pseudomallei* is an intracellular pathogenic bacterium that causes melioidosis in humans. On infection, neutrophils eliminate the majority of intracellular *B. pseudomallei*. Previous reports on the risk factors for melioidosis have shown that host factors, particularly age and diabetes mellitus, increase susceptibility to *B. pseudomallei*; however, whether these factors influence neutrophil functions in response to infection remains unknown. In this study, whole blood samples were collected from healthy Thai blood donors and co-cultured with *B. pseudomallei*, and phagocytic and respiratory burst functions of neutrophils were then measured by flow cytometry. The results show reduced neutrophil functions in older donors or those with poor glycemic control. Furthermore, the levels of antibody against *B. pseudomallei* showed a positive correlation with neutrophil functions. This study therefore indicated the importance of age, glycemic control, and antibody levels in the activity of neutrophils in melioidosis.

INTRODUCTION

Melioidosis is a serious infection caused by the soil-dwelling the gram-negative bacterium *Burkholderia pseudomallei*. Infection occurs through subcutaneous inoculation, inhalation, or ingestion of contaminated soil or surface water. This disease is endemic in Southeast Asia and northern Australia and accounts for 20% of the cases of community-acquired septicemia in northeast Thailand, where it is associated with a mortality rate of 50% (1,2). A number of risk factors for melioidosis have been defined in several studies (4,5). Melioidosis has been reported to be more common among in males in northeast Thailand, with highest incidence in the age group of 55–64 years (3). Moreover, a high incidence of melioidosis has been reported in patients with diabetes mellitus (DM) (4), with up to 60% of these patients having pre-existing or newly-diagnosed type 2 DM (5).

The duration of conventional course of treatment for melioidosis is not always sufficient for elimination of infection, and the efficacy of antimicrobial therapy is also likely to be less than expected despite in vitro susceptibility (6). This may be attributable, at least in part, to the ability of *B. pseudomallei* to efficiently invade non-professional phagocytic cells and to multiply and survive in professional phagocytic cells, including macrophages and neutrophils (7). Neutrophils constitute an essential component of the innate immune system in humans and are often the first cells to migrate toward inflammatory lesions, where they exert host defense functions. Neutrophils bind to and ingest bacteria through a process known as phagocytosis, which promotes the production of reactive oxygen species (ROS) and the fusion of cytoplasmic granules with pathogen-containing vacuoles (8). Reduction in neutrophil functions, particularly phagocytosis and ROS production, results in increased susceptibility to infection (9). Diabetic patients with very poor glycemic control show impaired of neutrophil functions, including phagocytosis, migration, apoptosis, and neutrophil extracellular traps, in response to *B. pseudomallei* infection (10,11).

Opsonization, a process where antibodies bind to microorganisms to facilitate their killing by phagocytes, is vital for defense against infection. Our previous study demonstrated that *B. pseudomallei* proteins were recognized in plasma samples from healthy individuals in melioidosis-endemic areas (northeast Thailand) but who had not been previously diagnosed with melioidosis (12). The magnitude of adaptive immune responses showed a correlation with antibody titers against killed *B. pseudomallei* cells, as detected using conventional indirect hemagglutination assays (13); however, no correlation between levels of specific antibodies and neutrophil functions in healthy donors has been reported to date.

In this study, we investigated the effects of host factors responses of neutrophils to *B. pseudomallei* in healthy Thai subjects by examining phagocytic and respiratory burst functions.

MATERIALS AND METHODS

Subjects: Blood samples were collected from 30

*Corresponding author: Mailing address: Cellular and Molecular Immunology Unit, Centre for Research and Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen 40002, Thailand. Tel: +66 4320 3825, Fax: +66 4320 3826, E-mail: ganja_le@kku.ac.th

† These two authors contributed equally to this work.

healthy Thai donors over 15 years age, including 6 men and 24 women. These donors did not suffer from any chronic disease, passed the standard tests for blood bank donors, and had normal glycosylated hemoglobin A1c (HbA1c) levels (<6.5%). Immunoassays were used for the detection of HbA1c, and the results were reported according to the guidelines of the National Glycohemoglobin Standardization Program. Informed consent was obtained in writing from all subjects, and sample collection and analysis were approved by the Khon Kaen University Ethics Committee for Human Research and Khon Kaen Hospital Ethics Committee (HE470506).

Microorganisms: The *B. pseudomallei* strain K96243, prototype strain whose complete genome sequence has been determined, was grown in Luria-Bertani broth for 18 h at 37°C, followed by centrifugation for harvesting cells and washing twice with phosphate-buffered saline (PBS) of pH 7.4. Viable counts of the bacterium were determined by counting the number of colony-forming units (CFU)/ml prior to fixing with 2% paraformaldehyde at room temperature for 60 min. Following fixation, these cells were washed twice with PBS and stored at −80°C until use.

Labeling of bacteria with fluorescein isothiocyanate: A suspension of *B. pseudomallei* at a cell density of 1×10^8 CFU/ml was washed twice with PBS and incubated with fluorescein isothiocyanate (FITC; 1 μg/ml; Sigma, St. Louis, MO, USA) in the dark at room temperature for 60 min, followed by analysis of fluorescence intensity prior to use. These FITC-labeled bacteria were used for a single time in experiments, following which they were discarded.

Flow cytometry analysis of phagocytic and respiratory burst: Neutrophils from whole blood samples were adjusted to a cell density of 3×10^6 cells/ml, followed by in vitro coculture with FITC-labeled bacteria at multiplicity of infection of 10:1 for 60 min or with phorbol 12-myristate 13-acetate (PMA; 800 ng/ml) for 15 min at 37°C. This was followed by the addition of 25 μl of a 2,800 ng/ml solution of hydroethidine (Sigma) and incubation for 5 min at 37°C. Erythrocytes were lysed with lysis buffer (BD Biosciences, San Jose, CA, USA), followed by 2 washes; the reaction was stopped with 2% paraformaldehyde prior to analysis by flow cytometry (FACSCalibur; BD Biosciences). The number of neutrophils exhibiting phagocytic function in 1 ml samples of whole blood from each individual donor was calculated by multiplying absolute neutrophil counts (determined using automated blood counter) with the percentage of phagocytosis (analyzed by flow cytometry) and dividing the obtained value by 100. Respiratory burst function, which occurs after phagocytosis, was subsequently calculated by multiplying the number of phagocytosing neutrophils (calculated above) by the percentage of respiratory burst (analyzed by flow cytometry) and dividing the obtained value by 100.

Antibody detection by indirect enzyme-linked immunosorbent assay (ELISA): Antigens were extracted with Veronal buffer from clinical isolates of *B. pseudomallei* obtained from septicemia patients and coated onto a 96-well microtiter plate (Nunc Maxisorp, Roskilde, Denmark) by overnight incubation at 4°C. The plate was washed with PBS containing 0.05% Tween20 (PBS-T), followed by the addition of PBS containing 3% bovine serum albumin and incubation for 60 min at 37°C. The plate was then washed with PBS-T, followed by the addition of diluted plasma samples and incubation for 60 min at 37°C. This was followed by washing PBS-T and the subsequent addition of biotinylated goat anti-human IgG and horseradish-peroxidase-conjugated streptavidin (BD Biosciences), followed by incubation for 60 min at room temperature. Finally, the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (BD Biosciences) resulted in color development, and the reaction was stopped within 10 min with the use of 2N H_2SO_4. Absorbance (A) was measured at 450 nm using an ELISA reader (Sunrise, Tecan, Germany), and absorbance index was calculated as follows: ($A_{450\,nm}$ test − $A_{450\,nm}$ uncoated)/$A_{450\,nm}$ uncoated.

Statistical analysis: Statistical analysis (one-way analysis of variance and linear regression with 95% confidence interval [CI]) was performed using GraphPad PRISM software (GraphPad, San Diego, CA, USA). A P value of <0.05 was considered statistically significant.

RESULTS

Phagocytotic and respitratory burst functions of neutrophils in defense against *B. pseudomallei*: The present study investigated the association between phagocytic and respiratory burst functions of neutrophils from 30 healthy Thai individuals residing in a melioidosis-endemic area. Whole blood samples were incubated with FITC-labeled *B. pseudomallei*, and phagocytic (FITC-Bp) and respiratory burst (ethidium bromide [EB]) functions of neutrophils were measured by flow cytometry (Figs. 1A, B). The number of neutrophils with respiratory burst showed a positive correlation with the number of cells with phagocytic activity ($P = 0.0001$; Fig. 1C). This data confirmed the ability of neutrophils to respond to *B. pseudomallei* through phagocytosis and respiratory burst.

Reduced neutrophil functions in older individuals: The effect of subject age on neutrophil functions in response to *B. pseudomallei* was subsequently investigated. Healthy subjects were classified into 3 age groups (<35, 35–60, and >60 years). Phagocytic and respiratory burst functions in response to *B. pseudomallei* showed a significant decrease in the age group of >60 years compared with the age group of 35–60 years ($P < 0.05$; Figs. 2A, B). These results suggest impairment in phagocytic and respiratory burst functions of neutrophils in response to *B. pseudomallei* infection in older subjects.

Correlation between HbA1c levels and neutrophil functions: The effect of glycemic control on neutrophil functions was investigated in 13 healthy subjects. HbA1c levels showed a negative correlation with phagocytosis ($P = 0.0436$; Fig. 3A), while no significant correlation was observed between HbA1c levels and the respiratory burst function of neutrophils ($P = 0.0975$; Fig. 3B). These results suggest that the level of glycemic control exerts an influence on phagocytosis of *B. pseudomallei* but not the respiratory burst of neutrophils.

Association between antibody levels and neutrophil

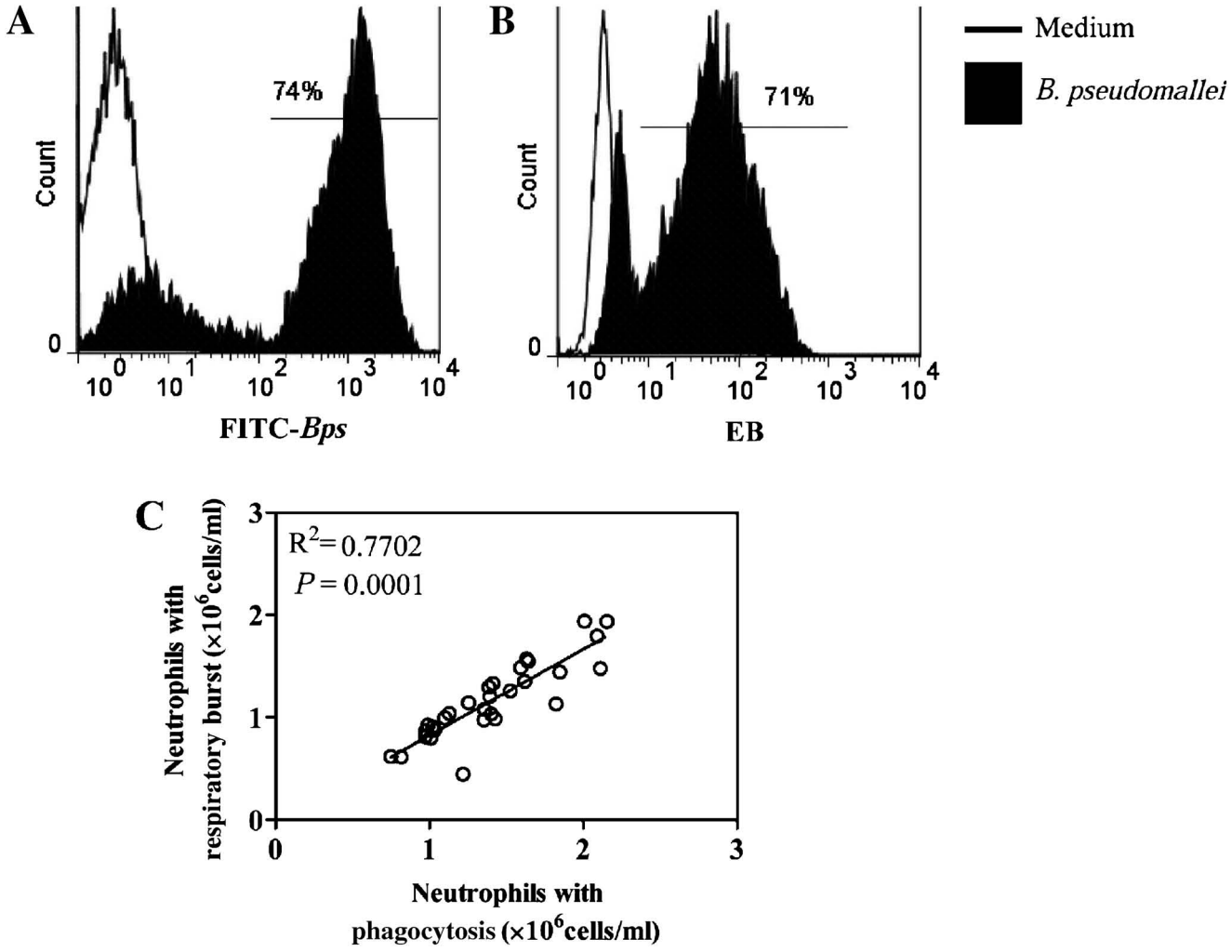

Fig. 1. Ability of human neutrophils to defend against *B. pseudomallei* by phagocytosis and respiratory burst. Whole blood (healthy donors; $n = 30$) containing 3×10^6 cell/ml with neutrophils (ratio 10:1) was stimulated with fluorescein isothiocyanate (FITC)-labeled *B. pseudomallei* for 1 h. Then the neutrophils showing phagocytosis (FITC-Bp) and/or respiratory burst (ethidium bromide-EB) were measured by flow cytometry (A and B, respectively). The correlation between 2 neutrophil functions was analyzed by linear regression with 95% CI (C).

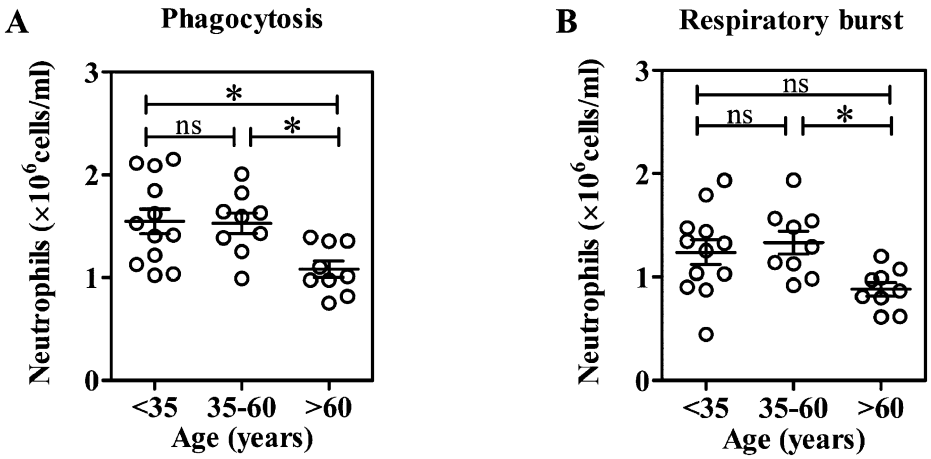

Fig. 2. Activity in response to *B. pseudomallei* by human neutrophils according to donor age group. The numbers of neutrophils with phagocytosis (A) and respiratory burst (B) in 1 ml of whole blood after stimulation with *B. pseudomallei* (healthy donors; $n = 30$) were analyzed and calculated as indicated in Materials and Methods. horizontal lines: mean ± SEM. **P*-value < 0.05. ns, non significant using one way ANOVA test.

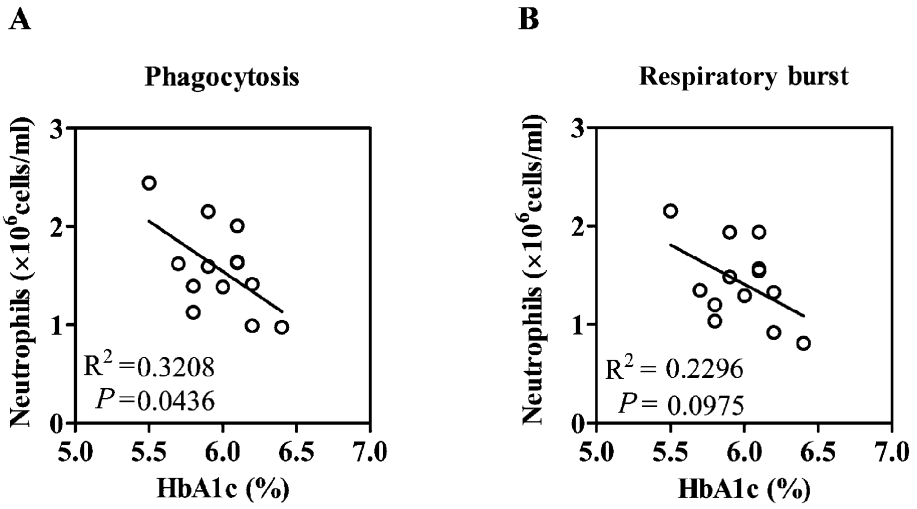

Fig. 3. Correlation of the neutrophil functions and glycemic control. The neutrophils in whole blood (3×10^6 cell/ml) were incubated with FITC-labeled *B. pseudomallei* (multiplicity of infection 10:1 at 37°C for 1 h). Then phagocytosis (A) and respiratory burst (B) of neutrophils were measured by flow cytometry. The correlation between neutrophils functions and glycemic control (% HbA1c) from healthy donors (n = 13) was tested by linear regression with 95% CI.

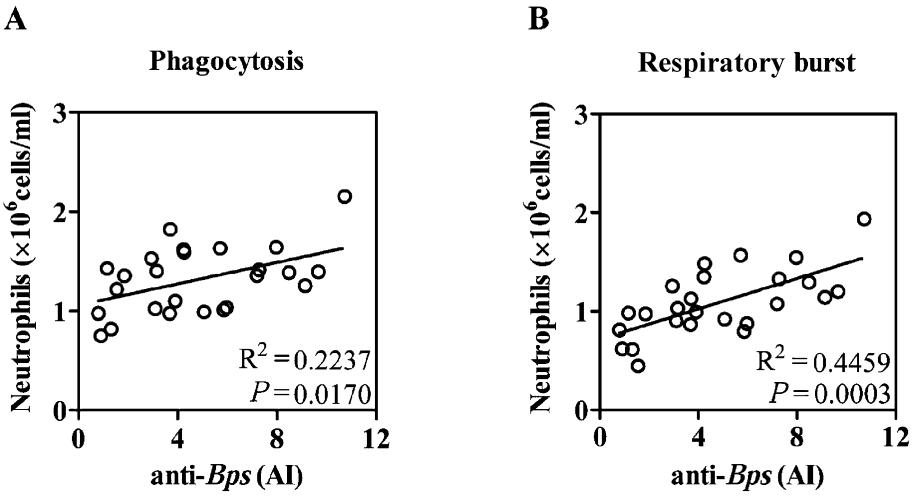

Fig. 4. Correlation between antibody levels to *B. pseudomallei* and neutrophil functions. The antibody levels to *B. pseudomallei* from healthy donors (n = 25) were determined by enzyme-linked immunosorbent assay and presented as absorbance index (AI), and neutrophil functions were analyzed: phagocytosis (A) and respiratory burst (B). The correlation was analyzed by linear regression with 95% CI.

functions: The effect of specific antibodies against *B. pseudomallei* on neutrophil functions was investigated. Plasma samples obtained from 25 healthy Thai individuals residing in a melioidosis-endemic area were assayed for the levels of antibodies against *B. pseudomallei* antigens by indirect ELISA. Interestingly, antibody levels showed a positive correlation with phagocytic (P = 0.0170; Fig. 4A) and respiratory burst (P = 0.0003; Fig. 4B) functions of neutrophils. As control, the extent of respiratory burst induced by PMA was comparable among all individuals (data not shown). These results show that antibody levels contribute to the enhancement of phagocytosis in response to *B. pseudomallei*, in addition to exerting an influence on the bacterial killing functions of neutrophils via respiratory burst.

DISCUSSION

Neutrophils have been previously reported to respond through phagocytosis and respiratory burst to *B. pseudomallei* at lower rates compaied with other gram-negative bacteria such as *Salmonella enterica* serovar Typhimurium and *Escherichia coli*, suggesting that *B. pseudomallei* is likely to exhibit anti-phagocytic activity (11). On the other hand, host factors such as age and HbA1c levels were also found to influence neutrophil functions (2,11,14,15). In the current study, the effects of host factors on responses to neutrophils to *B. pseudomallei* in healthy Thai subjects were evaluated by examining phagocytosis and respiratory burst.

A positive correlation between the number of neutrophils showing phagocytosis and respiratory burst was demonstrated in the current study, which confirmed that neutrophils from 30 healthy Thai individuals ex-

hibited defense ability against *B. pseudomallei* infection. However, the magnitudes of response showed variation among these individuals. Further investigation revealed that neutrophil functions in response to *B. pseudomallei* were significantly decreased in the age group of >60 years, which is consistent with results obtained for *E. coli* (data not shown). Elderly individuals have been previously reported to suffer higher rates of morbidity and mortality from infectious diseases, including melioidosis (11). Moreover, elderly individuals were found to have reduced numbers of neutrophils with decreased functions (data not shown).

Neutrophil functions are affected not only by age but also by glycemic control. In fact, the major risk factor associated with severe melioidosis is DM (5); our previous study demonstrated that neutrophil functions in response to *B. pseudomallei* infection were altered in diabetic patients, particularly ones with poor glycemic control (11). In the current study, the level of HbA1c was found to show a negative correlation with the numbers of phagocytic neutrophils. However, significant differences were not observed in the numbers of neutrophils showing respiratory burst. Therefore, levels of glycemic control in the general population would be useful for assessing susceptibility to this infection.

The current data revealed that the level of antibodies against *B. pseudomallei* was positively associated with phagocytic and respiratory burst functions of neutrophils. This result is consistent with our previous studies, which showed that levels of antibody against BPSL2765 (OmpA protein) were 10-fold higher in individuals with melioidosis (12). A recent study showed that the levels of specific antibodies that increased against the BPSL2765 peptide effectively enhanced phagocytosis and ROS production in an opsonization and killing assay using *B. pseudomallei* (16). This result suggests that the levels of antibodies are likely to play an important role in the response to *B. pseudomallei* infection.

Taken together, the results obtained imply that age- and glycemic-control-related reduction in neutrophil functions could be partly responsible for increased susceptibility to infection. The association between antibody levels and neutrophil functions presents a unique opportunity for further identification of host factor mechanisms in infection and disease.

Acknowledgments The authors thank Ms. Jeerawan Dhanasen for sample collection and Ms. Vicki Harley for editorial help. This work was supported in part by The Centre for Research & Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Thailand and The Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program and Khon Kaen University (grant PHD/0026/2552).

Conflict of interest None to declare.

REFERENCES

1. Cheng AC, Currie BJ. Melioidosis: Epidemiology, pathophysiology, and management. Clin Microbiol Rev. 2005;18:383-416.
2. White NJ. Melioidosis. Lancet. 2003;361:1715-22.
3. Limmathurotsakul D, Wongratanacheewin S, Teerawattanasook N, et al. Increasing incidence of human melioidosis in Northeast Thailand. Am J Trop Med Hyg. 2010;82:1113-7.
4. Wessels I, Jansen J, Rink L, et al. Immunosenescence of polymorphonuclear neutrophils. Sci World J. 2010;10:145-60.
5. Suputtamongkol Y, Chaowagul W, Chetchotisakd P, et al. Risk factors for melioidosis and bacteremic melioidosis. Clin Infect Dis. 1999;29:408-13.
6. Cheng AC, Stephens DP, Anstey NM, et al. Adjunctive granulocyte colony-stimulating factor for treatment of septic shock due to melioidosis. Clin Infect Dis. 2004;38:32-7.
7. Jones AL, Beveridge TJ, Woods DE. Intracellular survival of *Burkholderia pseudomallei*. Infect Immun. 1996;64:782-90.
8. Nathan C. Neutrophils and immunity: challenges and opportunities. Nat Rev Immunol. 2006;6:173-82.
9. Weiss SJ. Tissue destruction by neutrophils. N Engl J Med. 1989;320:365-76.
10. Riyapa D, Buddhisa S, Korbsrisate S, et al. Neutrophil extracellular traps exhibit antibacterial activity against *Burkholderia pseudomallei* and are influenced by bacterial and host factors. Infect Immun. 2012;80:3921-9.
11. Chanchamroen S, Kewcharoenwong C, Susaengrat W, et al. Human polymorphonuclear neutrophil responses to *Burkholderia pseudomallei* in healthy and diabetic subjects. Infect Immun. 2009;77:456-63.
12. Suwannasaen D, Mahawantung J, Chaowagul W, et al. Human immune responses to *Burkholderia pseudomallei* characterized by protein microarray analysis. J Infect Dis. 2011;203:1002-11.
13. Tippayawat P, Saenwongsa W, Mahawantung J, et al. Phenotypic and functional characterization of human memory T cell responses to *Burkholderia pseudomallei*. PLoS Negl Trop Dis. 2009;3:e407.
14. Saito Y, Takahashi I, Iwane K, et al. The influence of blood glucose on neutrophil function in individuals without diabetes. Luminescence. 2013;28:569-73.
15. Wenisch C, Patruta S, Daxböck F, et al. Effect of age on human neutrophil function. J Leukoc Biol. 2000;67:40-5.
16. Gourlay LJ, Peri C, Ferrer-Navarro M, et al. Exploiting the *Burkholderia pseudomallei* acute phase antigen BPSL2765 for structure-based epitope discovery/design in structural vaccinology. Chem Biol. 2013;20:1147-56.