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Differential Gene Expression Profiles of Lung Epithelial Cells Exposed to *Burkholderia pseudomallei* and *Burkholderia thailandensis* during the Initial Phase of Infection

Patimaporn Wongprompitak[1], Stitaya Sirisinha[1,2] and Sansanee C. Chaiyaroj[2]

SUMMARY *Burkholderia pseudomallei* is the causative agent of melioidosis, and its infection usually affects patients' lungs. The organism is a facultative intracellular Gram-negative bacillus commonly found in soil and water in endemic tropical regions. Another closely related *Burkholderia* species found in soil and water is *B. thailandensis*. This bacterium is a non-pathogenic environmental saprophyte. *B. pseudomallei* is considerably more efficient than *B. thailandensis* in host cell invasion and adherence. A previous study by our group demonstrated that after successfully invading cells, there was no difference in the ability to survive and to replicate between both *Burkholderia* species in cultured A549 human lung epithelial cells. In this study, Human Affymetrix GeneChips were used to identify the difference in gene expression profiles of A549 cells after a 2-h exposure to *B. pseudomallei* and *B. thailandensis*. A total of 280 of 22,283 genes were expressed at higher levels in the *B. pseudomallei*-infected cells than in the *B. thailandensis*-infected cells, while 280 genes were expressed at lower levels in the *B. pseudomallei*-infected cells. Approximately 9% of these genes were involved in immune response and apoptosis. Those genes were further selected for gene expression analysis using reverse transcription PCR and/or real-time RT-PCR. The results of RT-PCR and real-time RT-PCR are in accordance with data from the microarray data in that *bcl2* gene expression in the *B. pseudomallei*-infected cells was 2-fold higher than the level in the *B. thailandensis*-infected cells even though no apoptosis was seen in the infected cells. The levels of E-selectin, ICAM-1, IL-11, IRF-1, IL-6, IL-1□, and LIF genes expression in the *B. pseudomallei*-infected cells were 1.5-5 times lower than in the *B. thailandensis*-infected cells. However, both species stimulated the same level of IL-8 production from the tested epithelial cell line, and no difference in the ratio of adherent polymorphonuclear cells (PMNs) to infected A549 cells of both species was observed. Taken together, our results suggest that *B. pseudomallei* manipulates host response in favor of its survival in the host cell, which may explain the more virulent characteristics of *B. pseudomallei* when compared with *B. thailandensis*.

B. pseudomallei is the causative agent of melioidosis, a common infectious disease endemic in Southeast Asia, especially in the northeastern part of Thailand.[1] The clinical manifestations of melioidosis are rather broad, ranging from fulminant septicemia to localized lesions and chronic disease.[2-4] Although every organ in the body may be involved, the lungs are most commonly affected.[5] Acute pulmonary melioidosis is the most common clinical presentation, and patients often have fever with cough as a result of primary lung abscess or secondary to the spread of septicemic.[6] The disease predominantly affects rice farmers and their family members, who

From the [1]Department of Immunology, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand, [2]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand.
Correspondence: Sansanee C. Chaiyaroj
E-mail: scscy@mahidol.ac.th

are thought to come into contact with soil and water in rice fields where *B. pseudomallei* exists as a saprophyte.[7] *B. thailandensis* is a non-pathogenic environmental saprophyte that is genetically, biochemically, and immunologically closely related to *B. pseudomallei*.[8-10] In fact, a few years ago it was considered to be an avirulent biotype of *B. pseudomallei*, as the two organisms are very similar to each other in most phenotypic characteristics except for level of virulence in man and animals.[10]

B. pseudomallei can adhere to and invade a number of mammalian cells.[11,12] Human lung epithelial cells are particularly susceptible following exposure by inhalation.[12] Generally, host cell adhesion and subsequent invasion are essential steps in the pathogenesis of invasive bacterial infections, and interfering with these processes can reduce disease incidence and severity.[13] An early step of host responses to bacterial invasion is the trafficking of leukocytes from the vascular compartment to extravascular tissues.[14] Leukocyte trafficking to surrounding tissue is often initiated by inflammatory stimuli that induce expression of adhesion molecules on the surface of the endothelial cells.[14] Previous findings showed that *B. pseudomallei* could enter both phagocytic and non-phagocytic cells and subsequently escape into the cytosol.[11,12]

Comparative studies of *B. pseudomallei* and *B. thailandensis* are limited. Previous studies showed that virulent *B. pseudomallei* was considerably more efficient than its naturally occurring avirulent counterpart *B. thailandensis* in host cell invasion and adherence.[12] *B. pseudomallei* exhibited an invasive capacity approximately 10-fold higher than *B. thailandensis* and also exhibited a 2-fold higher adherence capacity. However, after successfully invading the cells, both *Burkholderia* species could similarly survive and replicate in the cultured A549 human lung epithelial cells.[12]

In the present study, the ability of *B. pseudomallei* to induce expression of adhesion molecules and cytokine production by cultured A549 human lung epithelial cells was assessed to gain more insight into bacterial virulence and the mechanism of pathogenicity. Gene expression profiles of A549 cells infected with *B. pseudomallei* and *B. thailandensis* were compared. Analysis of the data revealed that the response of A549 cells to *B. pseudomallei* infection was distinct from that of *B. thailandensis* infection. The results showed that compared with *B. thailandensis*, the virulent *B. pseudomallei* could more readily induce expression of some anti-apoptotic genes and at the same time suppress expression of some adhesion molecules and proinflammatory cytokines.

MATERIALS AND METHODS

Bacterial strains and growth conditions

B. pseudomallei strain 844 (arabinose-negative strain) was originally isolated from a patient admitted to Srinagarind Hospital, Khon Kaen, Thailand. The isolate was identified based on its biochemical characteristics, colony morphology, reaction with polyclonal antibody, and antibiotic sensitivity profiles.[12] *B. thailandensis* was isolated from sandy loam in northeastern Thailand, and the strain demonstrated a more than 10^5-fold decrease in its virulence relative to *B. pseudomallei* 844 in an animal model of acute melioidosis.[12] The bacteria were cultured in trypticase soy broth at 37°C with shaking at 150 rpm. The overnight culture was washed in phosphate-buffered saline (PBS) and adjusted to an appropriate concentration by measurement of the optical density at 650 nm.

Lung epithelial cell and culture condition

A human lung epithelial cell line (A549, ATCC CCL 185) was purchased from American Type Culture Collection (ATCC), Maryland, USA. The cells were maintained in Ham's F-12 (GIBCO-BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO-BRL, New York, USA) and 1% penicillin-streptomycin antibiotic mixture (Sigma, Missouri, USA) at 37°C in a humidified atmosphere of 95% oxygen and 5% carbon dioxide.

RNA extraction and total RNA quantification

After human lung epithelial cells were infected with *B. pseudomallei* or *B. thailandensis* at a multiplicity of infection (MOI) of 10 for 2 hours, RNA was extracted from the epithelial cells using the RNeasy Mini kit (QIAGEN Inc., California, USA) according to the manufacturer's recommenda-

tion. RNA concentration was determined by spectrophotometric measurement at 260 nm. A_{260}/A_{280} ratio was calculated to determine the purity of the RNA, which was allowed to be 1.9-2.1.

Gene expression analysis

Human Genome U133 GeneChip microarrays were purchased from Affymetrix (Affymetrix, California, USA) to examine gene expression profiles of human lung epithelial cells infected with *B. pseudomallei* and *B. thailandensis*. Complementary DNA (cDNA) was synthesized using the Superscript system (Invitrogen, California, USA). Briefly, the first strand of cDNA was synthesized using Superscript-II reverse transcriptase and T7-oligo$(dT)_{24}$ primers. The second strand of cDNA was obtained by using first stranded cDNA, DNA ligase, DNA polymerase-I and RNase-H, followed by T4 DNA polymerase. Then, the double stranded cDNA was used for *in vitro* transcription (IVT) using the Affymetrix IVT kit. The cDNA was transcribed in the presence of biotin-labeled ribonucleotide and T7 RNA polymerase. In each experiment, cDNAs from *B. pseudomallei*- and *B. thailandensis*-infected cells were labeled simultaneously using the same labeling kit. After cleaning up with an RNeasy mini kit (QIAGEN), the biotin-labeled IVT-cRNA was fragmented in a buffer containing 40 mM Tris-acetate (pH 8.0), 100 mM potassium acetate, and 30 mM magnesium acetate at 94°C. Biotin-labeled IVT-cRNA was hybridized to the arrays according to the manufacturer's instruction. After hybridization, the arrays were washed in a GeneChip Fluidics Station 400 with a low stringency wash buffer followed by a high stringency wash buffer. The arrays were then stained with a streptavidin-Cy5 complex (Enzo Life Science Inc., New York, USA). After staining, spot intensities were determined with a GeneChip scanner, which is controlled by GeneChip software (Affymetrix).

GeneChip data analysis

Raw image data quantification and statistical analysis of microarray data were performed with the Affymetrix Microarray Suite Version 5.0 (MAS 5.0). To allow cross-array comparisons, hybridization intensity values were normalized. Up- or down-regulation in each sample were determined using a detection cut-off *p*-value of 0.005. This analysis generated a ".chp" file (with scaled signal intensity, increase/decrease and marginal increase/marginal decrease), which was uploaded to Microsoft Excel for further analysis. GeneSpring Version 7 software (Agilent Technologies, California, USA) was used to normalize the datasets for each individual array using linear regression. Genes with significantly different values of expression between *B. pseudomallei* and *B. thailandensis* infected A549 cells were selected for analysis and confirmation.

Reverse transcription polymerase chain reaction and real-time reverse transcription polymerase chain reaction

RT-PCR was used to verify the microarray data. Total RNA was extracted from infected cells and was subsequently reverse-transcribed using the Superscript system (Invitrogen, California, USA). The cDNA preparations were stored at -20°C until PCR amplification. The PCR were performed using the following primers: Bcl2 forward primer: 5' GCGGGCATTCAGTGACCTGA 3' and reverse primer: 5' TAAGTGGCCATCCAAGCTGC 3', amplifying a 212 bp fragment; intracellular adhesion molecule (ICAM-1) forward primer: 5' AAAGGATGGCACTTTCCCAC 3' and reverse prime: 5' TTCCCCTCTCATCAGGCTAGAC 3', amplifying a 594 bp fragment; leukemia inhibitory factor (LIF) forward primer: 5' CAGCTCAATGGCAGTGCCAA 3' and reverse primer: 5' GTTCACAGCACACTTCAAGAC 3', amplifying a 477 bp fragment; interferon regulatory factor-1 (IRF-1) forward primer: 5' AGTGACAGCGAGACCCTCTCC 3' and reverse primer: 5' CCCTCAACAGCCAAGTGTGAC 3', amplifying a 239 bp fragment; interleukin-6 (IL-6) forward primer: 5' TTCGGTCCAGTTGCCTCTC 3' and reverse primer: 5' TGGCATTTGTGGTTGGGTCA 3', amplifying a 495 bp fragment. Glyceraldehyde phosphate dehydrogenase (GAPDH) forward primer: 5' ATGGGGAAGGTGAAGGTCG 3' and reverse primer: 5' GGGGTCATTGATGGCAACA 3', amplifying a 265 bp fragment. The optical densities of PCR products were analyzed by agarose gel electrophoresis and gel documentation using BIO-PROFILE image analysis software (Vilber Lourmat, France). The house-keeping gene, GAPDH, was used as an internal control and for normalization.

Real-time reverse transcription polymerase chain reaction was used to measure relative gene expression levels. One step RT-PCR was performed on an ABI Prism 7500 Real Time PCR System using TaqMan 2x Universal PCR master mix, 40x MultiScribe/RNase inhibitor mix, and pre-optimized ABI Assays-on-Demand probes (Applied Biosystems, California, USA) for E-selectin, IL-11, ICAM-1, TNF-α, IL-1β, and GAPDH using the manufacturer's recommended methods. Relative expression was estimated by the threshold cycle (C_T) of the gene of interest normalized to the C_T of GAPDH mRNA.

Detection of interleukin-8 protein

The concentration of IL-8 in the supernatant of *B. pseudomallei*- and *B. thailandensis*-infected A549 cells was measured at 3 hour interval for 12 hours using the Human IL-8 ELISA kit (Pierce Biotechnology, Inc., Illinois, USA). The culture supernatant of TNF-α induced A549 cells was used as a positive control and that of the uninfected cells was used as a negative control. The sensitivity of the assay system was 2 pg/ml.

Isolation of polymorphonuclear cells (PMNs)

Heparinized blood was drawn from healthy donors. PMNs were isolated by Ficoll-Hypaque density gradient separation and dextran sedimentation. Residual erythrocytes were removed by hypotonic lysis, and PMNs were resuspended in Hank's balanced salt solution (HBSS) supplemented with 10% human serum. Isolation yielded 99% PMNs that were 95% viable as measured by Trypan blue exclusion. Ethical approval of this study was obtained from the Faculty of Medicine Ramathibodi Hospital (FWA 00002882, IRB 0000526), Mahidol University.

Adhesion of PMNs to A549 cells

After the lung epithelial cells were infected with *B. pseudomallei* or *B. thailandensis* for 2 hours, PMNs were added to the co-culture and incubated at 37°C for 30 minutes in a humidified 5% CO_2 atmosphere. The unbound PMNs were washed out with PBS. Bound cells were fixed with 2% glutaraldehyde and stained with Giemsa. The number of PMNs bound to 100 cells of A549 cells was determined. The positive control was PMNs adhered to TNF-α treated A549 cells.

Detection of apoptosis in *Burkholderia*-infected A549 cells

A549 lung epithelial cells were seeded at 1 x 10^6 cells per well in a six-well microtiter plate. After being infected with *B. pseudomallei* or *B. thailandensis* at the MOI of 10, the cells were washed and further incubated in the presence of 250 mg/ml of kanamycin for 12 and 24 hours. DNA fragmentation analysis was then performed in parallel with anti-Annexin V-FITC and propidium iodide (BD Biosciences, California, USA) staining using the Annexin V-FITC apoptosis detection kits II protocol. Finally, fluorescence of the stained cells was measured by a flow cytometer (FACSCanto, Becton Dickinson, California, USA).

RESULTS

Expression microarray analysis of epithelial cells infected with *B. pseudomallei* and *B. thailandensis*

The transcriptional response of a human lung epithelial cell line infected with *B. pseudomallei* and *B. thailandensis* was examined by hybridization of cRNA probes to microarray. Expression profiles were compared between A549 cells incubated for 2 hours with the bacteria and normalized with those that were briefly exposed for 1 minute. In each experiment, total RNA was used as a template for synthesizing cRNA probes and hybridized to a microarray containing 18,400 transcripts comprised of more than 22,000 probe sets. The result from the microarray experiment demonstrated a high degree of reproducibility with a correlation coefficient of more than 0.9. A statistical value of fluorescence intensity of each hybridization signal was determined ($p < 0.005$), resulting in 2.54% of the total genes displaying a > 2 fold difference in transcript levels during a 2 hour-exposure of A549 cells to *B. pseudomallei* compared to *B. thailandensis*. The remaining genes were expressed at marginally increased levels, marginally decreased levels, and at no change when compared to control transcripts (Fig. 1A).

In comparison to *B. thailandensis*-infected cells, Fig. 1B shows the genes with higher and lower

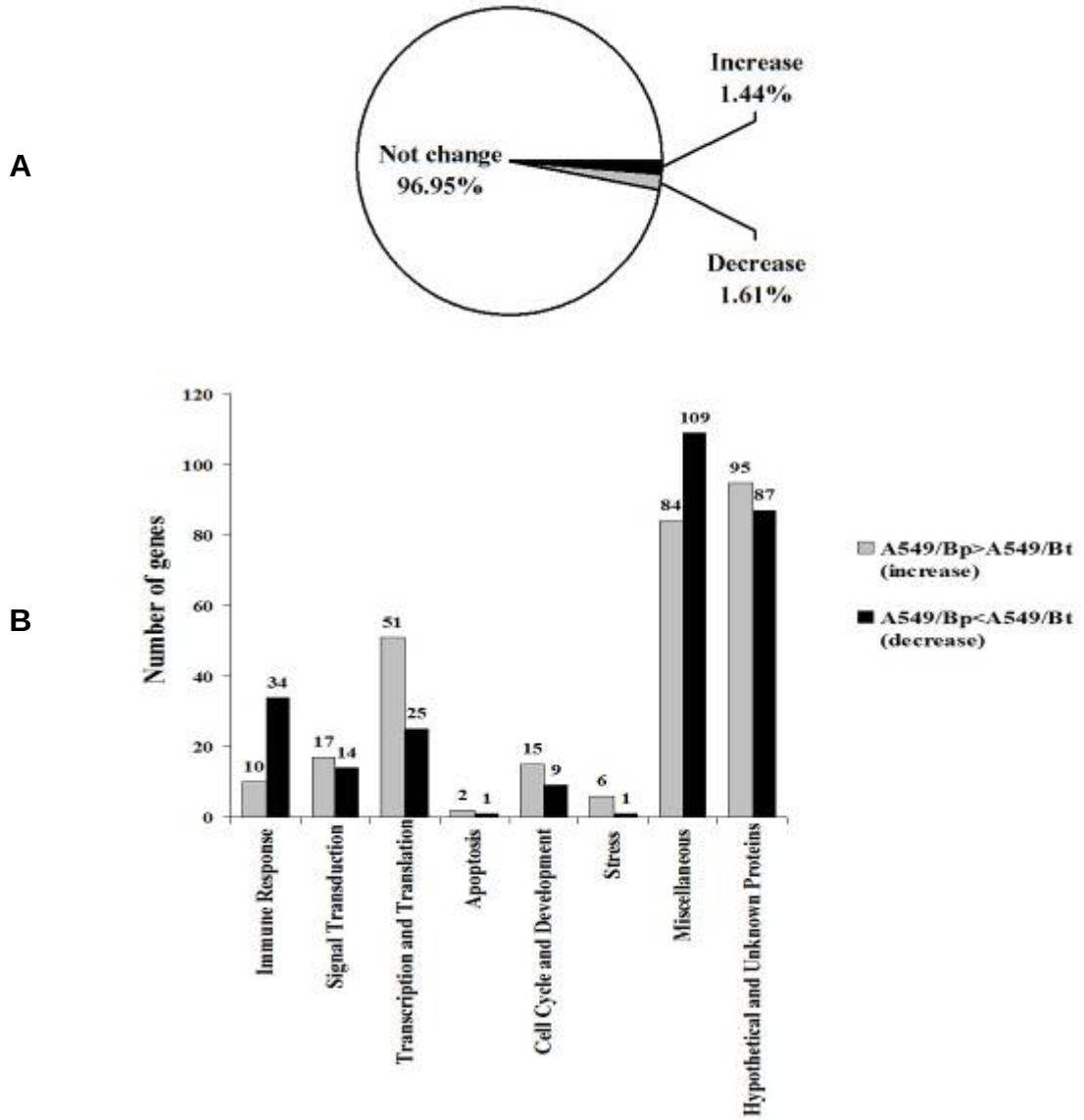

Fig. 1 Analysis of microarray data. (A) Profiles of gene expression divided according to the expression levels in *B. pseudomallei*- and *B. thailandensis*-infected A549 cells. "Increase" represents higher levels of gene expression in *B. pseudomallei*-infected cells and "decrease" represents higher levels of gene expression in *B. thailandensis*-infected cells. A statistical value of fluorescent intensity of each hybridization signal was determined ($p < 0.005$). (B) Functional based classification of genes with different expression profiles between *B. pseudomallei*-infected and *B. thailandensis*-infected A549 cells.

expression levels in *B. pseudomallei*-infected cells categorized by their molecular functions, such as those involved in immune response or apoptosis. Twelve genes displayed higher levels of expression in *B. pseudomallei*- compared to *B. thailandensis*-infected cells and 35 genes showed lower levels of expression. Genes involved in apoptosis and immune response, such as adhesion molecules and proinflammatory cytokines were selected for further confirmation by RT-PCR and real-time PCR These included an anti-apoptotic gene, *bcl*2, genes that play roles in cell adhesion, such as E-selectin and intercellular adhesion molecule-1 (ICAM-1), cytokine genes such as interleukin-11 (IL-11), and cytokine

regulatory genes such as interferon regulatory factor-1 (IRF-1).

Confirmation of gene expression by RT-PCR and real-time RT-PCR

RT-PCR was performed to confirm the expression level of the selected genes, which were expressed with distinct levels between *B. pseudomallei*- and *B. thailandensis*-infected A549 cells from microarray analysis. There were 4 genes that gave the same expression pattern as in the microarray. These genes are Bcl2, ICAM-1, IRF-1, and LIF (Fig. 2A). The expression of *bcl2* was higher in *B.*

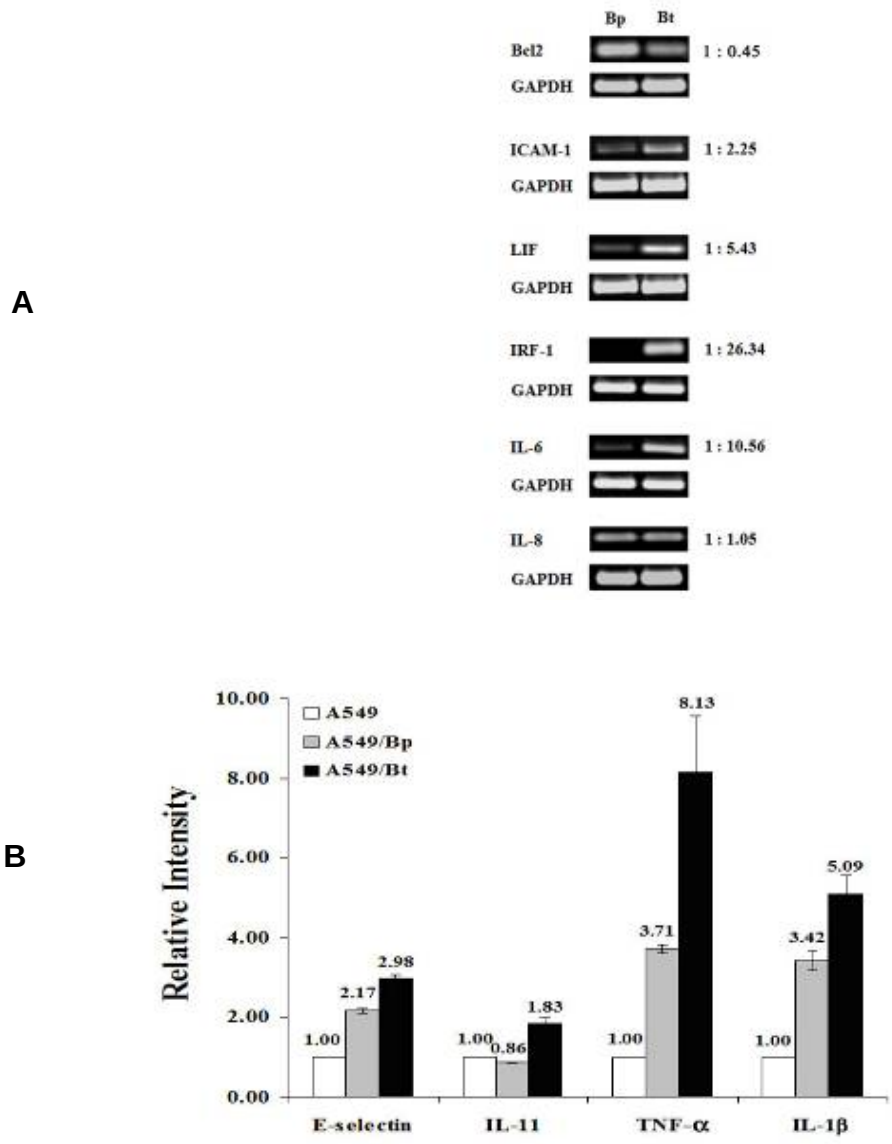

Fig. 2 Confirmation of microarray results by RT-PCR and real-time RT-PCR. (A) RT-PCR analysis of the selected genes expressed in A549 cells after 2 hours of *B. pseudomalllei* (Bp) infection compared to *B. thailandensis* (Bt) infection. The relative ratios of target gene levels were normalized to the house-keeping gene GAPDH. The results are from a representative of 2 reproducible independent experiments. (B) Real-time RT-PCR analysis of gene expression of A549 cells after 2 hours of *B. pseudomallei* infection (A549/Bp) compared to *B. thailandensis* infection (A549/Bt). The relative intensity of target genes was normalized to the house-keeping gene, GAPDH. The cycle threshold of uninfected A549 cells was used as a calibrator for each gene. The results represent mean ± SD from 2 independent experiments, each performed in duplicate.

pseudomallei-infected A549 cells; the proportion of gene expression in *B. pseudomallei*-infected cells compared to *B. thailandensis*-infected cells was 1:0.45. Conversely, IRF-1 gene showed lower expression level in *B. pseudomallei*-infected A549 cells, and the relative intensity ratio between *B. pseudomallei* and *B. thailandensis*-infected cells was 1:26.34. The levels of both ICAM-1 and LIF gene expression in *B. pseudomallei*-infected cells were 2-fold lower than in *B. thailandensis*-infected cells. The relative intensity ratio of IL-6 gene expression in *B. pseudomallei* and *B. thailandensis*-infected cell was 1:10.56. Moreover, there was no difference in IL-8 expression level between *B. pseudomallei*- and *B. thailandensis*-infected A549 cells.

The expression levels of E-selectin, IL-11, TNF-α, and IL-1β genes were further quantitated by real-time PCR (Fig. 2B). The cycle thresholds of the genes expressed by *B. pseudomallei*- and *B. thailandensis*-infected A549 cells were compared to those expressed by uninfected cells after subtracting with GAPDH. The data from this experiment matched the results of the microarray and RT-PCR experiments. The calculated expression ratio of the relative intensity of E-selectin in *B. pseudomallei*- to *B. thailandensis*-infected A549 cells was 1:1.5. Using this technique, the values of IL-11 gene expression were also in accordance with the result from the microarray. The ratio of expression for *B. pseudomallei* and *B. thailandensis* was 1:2.0. TNF-α was also analyzed because there were several TNF-α superfamily members and TNF-α -induced proteins that showed different levels of gene expression in the microarray analysis. The expression level of TNF-α in *B. pseudomallei*-infected A549 cells was 2.2 fold lower than that seen in the *B. thailandensis*. Another proinflammatory cytokine, IL-1β, was also subjected to RT-PCR analysis the expression ratio between *B. pseudomallei*-and *B. thailandensis*-infected cells was found to be 1:1.5.

Secretion of Interleukin-8 by epithelial cells in the presence of *B. pseudomallei* and *B. thailandensis* and the characteristic of bacterial cell adhesion

Data from the microarray and PCR confirmation showed lower expression of E-selectin and ICAM-1 in *B. pseudomallei*-infected lung epithelium compared to that of cells infected with *B. thailandensis*, suggesting that there might be a reduction in PMN recruitment to the site of infection. We therefore measured the levels of IL-8 secretion in supernatant collected from the cultures of epithelial cells infected of *B. pseudomallei* and *B. thailandensis*. Fig. 3A demonstrates that there was no significant difference between IL-8 secretion in both cultures during 15 h of co-cultivation and the levels of IL-8 secretion were comparable to those of TNF-α-induced A549 cells.

Additionally, the capacity for PMNs to adhere to epithelial cells was determined. Lung epithelial cells showed similar levels of PMN adhesion upon infection by both *B. pseudomallei* and *B. thailandensis*, (Fig. 3B). The adhesion capacity of both bacteria-infected A549 cells was not significantly different from the positive control, TNF-α treated A549 cells. PMN showed less than 40% binding capacity for untreated A549 cells.

Induction of apoptosis by *B. pseudomallei* and *B. thailandensis*

The anti-apoptotic gene, *bcl2*, was expressed at a higher level in *B. pseudomallei*-infected A549 cells compared to *B. thailandensis*-infected cells. Therefore, we further investigated the degree of apoptosis of lung epithelial cells after infection with these bacteria. It was found that both *Burkholderia* species did not induce DNA ladder formation in the infected A549 cells (data not shown). Staining with anti-Annexin V-FITC and propidium iodide was also performed to confirm the results of DNA fragmentation analysis. The results presented in Figs. 4A and 4B demonstrate that both *B. pseudomallei* and *B. thailandensis* could not induce apoptosis of the infected cells even though the incubation time was extended from 12 hours to 24 hours.

DISCUSSION

Although *B. pseudomallei* is considerably different from its avirulent counterpart *B. thailandensis* in many respects, information about how these differences might have impact on host cell responses are still limited. Clearly, these two species differ in their pathogenic capacity.[9,12,15] The A549 human lung epithelial cell line is known to be a highly susceptible host cell to *B. pseudomallei* infec-

tion[9] and has been used as a host cell model to study the infection caused by several other intracellular bacteria infections. This is one reason that led us to use this cell line in our microarray study. DNA microarray technology allows simultaneous comparison of mRNA levels of several thousand genes, thus enabling the evaluation of a wide pattern of gene expression.[16-18] In the present study, the GeneChip Human U133A microarray set from Affymetrix, which represents approximately 22,000 transcribed genes, was used to identify potential target genes for *B. pseudomallei* and *B. thailandensis*. After exposure of A549 cells to bacteria for 2 hours, many genes, particularly those involved in anti-apoptosis, cytokine production, and immune cell recruitment, were found to be expressed at different levels. Real-time reverse transcription PCR and/or reverse transcription PCR were used to validate the microarray results. In this study, we demonstrated for the first time the differences in gene expression profiles between *B. pseudomallei*- and *B. thailandensis*-infected A549 cells, especially those involved in inflamma-

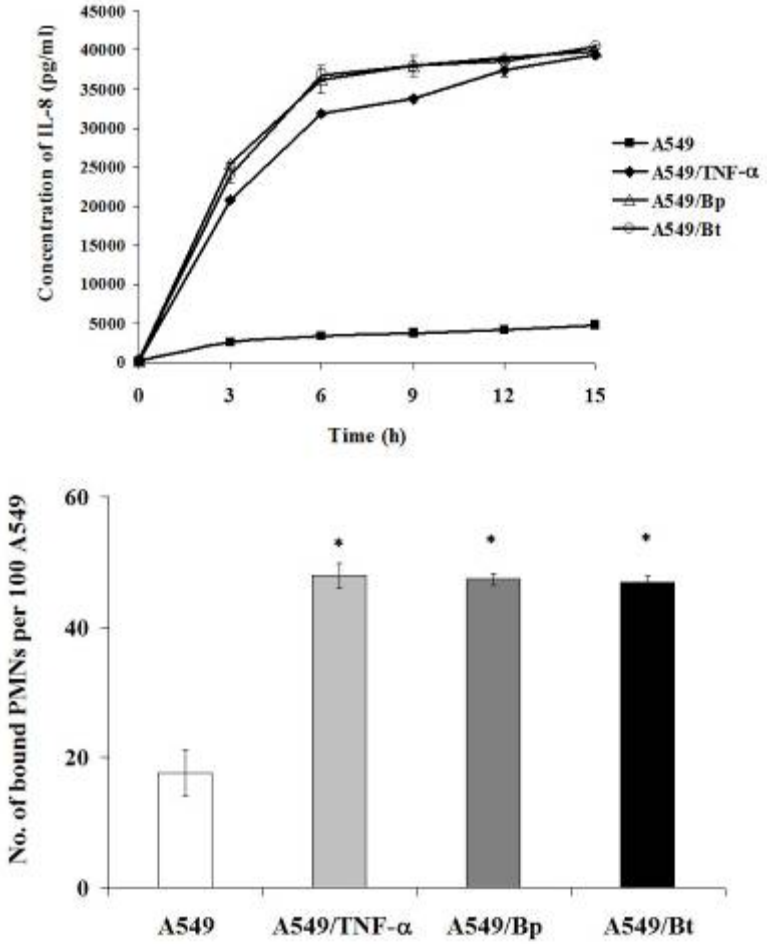

Fig. 3 Ability of *B. pseudomallei*- and *B. thailandensis*-infected A549 cells to recruit PMNs. (A) IL-8 production by infected A549 cells. The cells were infected with either *B. pseudomallei* (A549/Bp) (△) or *B. thailandensis* (A549/Bt) (○) at the MOI of 10. TNF-□ stimulated A549 cells (A549/TNF□) was used as positive control (◆). Uninfected cells were used as negative control (■). At the indicated time interval, the supernatant was collected and analyzed by ELISA. The results represent mean ± SD from 2 independent experiments, each performed in duplicate. (B) Adhesion of PMNs to *B. pseudomallei* or *B. thailandensis* stimulated A549 cells. Unstimulated A549 cells were used as a negative control. TNF-α stimulated A549 cells was used as a positive control. The results represent mean ± SD from two independent experiments, each performed in duplicate.

tion, immune response, cell adhesion, and apoptosis. Shortly after the microarray experiment was initiated and completed, our group reported *B. pseudomallei* to be more efficient in adherence to and invasion of A549 cells than *B. thailandensis*.

Innate immune mechanisms are critical in determining the outcome of infections caused by many bacterial pathogens. The microarray data of the present study showed that *B. pseudomallei* interfered with the transcription of genes involved in the production of proinflammatory cytokines (TNF-α, IL-11, IL-6 and LIF), cytokine regulatory protein (IRF-1), and adhesion molecules (E-selectin and ICAM-1), whereas *B. thailandensis* seemed to enhance the expression of these genes. The inhibition of these genes by *B. pseudomallei* would theoretically result in a reduction of recruitment of innate immune cells to the site of infection, which in turn could influence the degree of host inflammatory response and bacterial elimination. *B. pseudomallei* has been shown to modulate the macrophage bactericidal response in favor of its intracellular survival and persistence in the human host, and this process may be associated with relapse melioidosis.[11,19,20]

Similar to the findings in this study, IRF-1 expression was previously found to be reduced in *B. pseudomallei*-infected cells.[21] The function of IRF-1 was demonstrated in IRF-1-deficient mice, which have reduced $CD8^+$ T cells function, functionally impaired natural killer cells, and dysregulation of IL-12p40 and iNOS induction.[22] In the case of *B. pseudomallei*, our group previously demonstrated that *B. pseudomallei* interferes with the expression of IRF-1, which may in turn be related to its prolonged intracellular survival inside macrophages.[23] In that study, exogenous IFN-γ or IFN-β was added to the macrophage cultures at different time points relative to infection time and it was shown that IFN-γ was able to induce more sustained IRF-1 expression compared to IFN-β.[23] The protective role of IFN-γ against *B. pseudomallei* has been shown previously by others in animal model.[24,25]

Besides IFN, the expression of several other

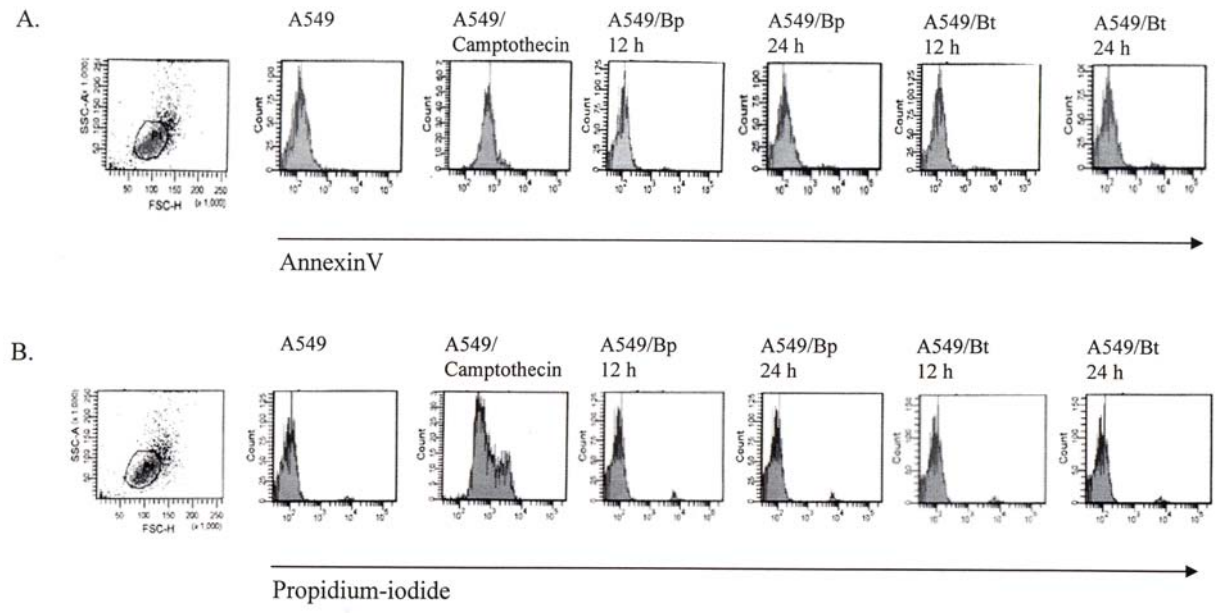

Fig. 4 Detection of apoptosis of infected A549 cells using anti-Annexin V-FITC (A) and propidium iodide (B) staining. Uninfected cells were used as negative control and camptothecin-induced A549 cells were used as positive control. A549 cells were infected with *B. pseudomallei* and *B. thailandensis* for 12 and 24 hours before staining. The results shown are from representative of two independent experiments.

proinflammatory cytokines by infected cells is known to be altered by many other bacteria. Many of these cytokines, like IL-6, LIF, IL-11, oncostatin M (OSM), and ciliary neurotrophic factor (CNTF), use a receptor composed of a common signal transducing component called gp130.[26,27] IL-6 has an essential role in the release of LIF, which is known to be involved in regulating the pulmonary response to inflammation.[28] Neutropenic rats receiving high doses of IL-11 intravenously can survive lethal sepsis caused by *Pseudomonas aeruginosa.*[29] These IL-11-treated rats had prolonged survival time, reduced pathologic changes, and lower systemic levels of bacterial endotoxins and concentrations of *P. aeruginosa* in target tissues.[29] It was shown in mice infected with *E. coli* that IL-6, IL-11, and LIF could readily activate alveolar epithelial STAT3, which in turn promoted neutrophil recruitment and limited the degree of infection and lung injury.[30] The information from our microarray experiments showed that the expression of IL-6, IL-11, and LIF were reduced by *B. pseudomallei*, consistent with the above studies. Therefore, it is tempting to postulate that *B. pseudomallei* manipulate the host response by reducing the production of inflammatory cytokines, which may in turn interfere with PMN recruitment and subsequent elimination of the bacteria.

The lungs and airways are normally populated by a large number of leukocytes that help defending against infection and play crucial roles in the pathogenesis of a wide variety of bacterial diseases.[31] The recruitment of leukocytes into the lung and airways initially involves selectin- and integrin-mediated events required for recruitment of leukocytes into organs.[32,33] Neutrophil recruitment, in particular, is essential in controlling infections caused by some extracellular gram-negative bacteria, including *P. aeruginosa, Klebsiella pneumoniae,* and *Legionella pneumophila.*[31,34-36] The role of neutrophils in resistance to infection has also been demonstrated in a *B. pseudomallei* mouse model showing that neutrophils from resistant C57BL/6 mice are essential in the control of pulmonary as well as subcutaneous *B. pseudomallei* infections, thus suggesting that its function does not depend on the route of exposure.[37] The observation reported in the present study on differential expression of gene encoding for adhesion molecules, especially E-selectin, in A549 cells infected with *B. pseudomallei* and *B. thailandensis* clearly defined their role in melioidosis. Previous studies have shown chemokine IL-8 production by human lung epithelium infected with *B. pseudomallei* to be somewhat depressed compared with tissue infected with other bacteria.[38] However, in the present study, the quantity of IL-8 was similar in both *B. pseudomallei*- and *B. thailandensis*-infected A549 cells when measuring up to 15 hours of infection. Moreover, the experimental data presented in Figure 3B showed that the binding of PMNs to *B. pseudomallei*- and *B. thailandensis*-infected A549 cells was similar. However, because the number of bacteria inside A549 cells may vary with the number of microbes in the co-culturing step, the quantity of IL-8 secretion may change in parallel with the number of intracellular bacteria, which could in turn influence the magnitude and degree of activation of the recruited PMNs. In our experimental design, using a MOI of 10, the number of intracellular *B. thailandensis* was considerably lower than that of *B. pseudomallei*.

Although *B. pseudomallei* appeared to induce transcription of an anti-apoptotic gene (*bcl2*) in A549 cells with higher efficiency than *B. thailandensis*, their ability to induce apoptosis were similar. This is unexpected, as it was previously reported that *B. pseudomallei* was more effective than *B. thailandensis* in inducing apoptosis in HeLa cells after 18 hours of incubation at a MOI of 50.[39] In the present study, apoptosis was not seen in either *B. pseudomallei*- or *B. thailandensis*-infected A549 cells, even after a 24 hour incubation at a MOI of 10 (Fig. 4A and 4B). It is possible that the very low expression of the *bcl2* gene induced by *B. thailandensis* is sufficient to inhibit apoptosis in A549 cells and these bacteria may manipulate this gene for their own advantage to survive inside A549 cells. This process can therefore extend the period of bacterial survival and allows them to replicate inside the cell. At this point of time, it is still unclear whether the apoptotic cell death in *B. pseudomallei* infection is beneficial or detrimental to the host. Pathogens may either induce or inhibit apoptosis as an immune-evasion mechanism. The inhibition of host cell apoptosis allows for intracellular persistence of these pathogens. A large number of microbial virulence factors have been shown to promote macrophage apoptosis or impair its antibacterial activity.[40] One example is the case of *Shigella flexneri* infection, where the inva-

sion gene (*ipaB*) can directly activate caspase-1, which then induces apoptosis and inflammation.[40] This form of apoptosis usually occurs early after exposure of the macrophages to the pathogens, thus facilitating evasion of immune clearance. Our data are consistent with the interpretation that *B. pseudomallei* can manipulate A549 cells for its own advantage, allowing prolonged survival inside the harmless environmental niche of the non-phagocytic cells.

The data presented in this study clearly show that early phase of the host cell response to infection by *B. pseudomallei* and *B. thailandensis* are not very different. Both bacteria have similar ability to survive inside the host cells, to induce PMN recruitment, and to inhibit host cell apoptosis. Nevertheless, *B. pseudomallei* could manipulate host cell immune response more efficiently through the suppression of proinflammatory cytokine production. Further experimental investigation to dissect the underlying mechanisms for such manipulation may explain why infection by *B. pseudomallei* is more virulent than infection by *B. thailandensis.*

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