Reduced Interleukin-17 Expression of *Burkholderia pseudomallei*-Infected Peripheral Blood Mononuclear Cells of Diabetic Patients

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**SUMMARY** *Burkholderia pseudomallei* is the causative agent of melioidosis. One of the main risk factors for *B. pseudomallei* infection in endemic areas is diabetes mellitus. The present study investigated IL-17 mRNA and protein expression by peripheral blood mononuclear cells in response to *B. pseudomallei* infection in 10 diabetic patients in comparison to 10 healthy blood donors. The IL-17 expression in diabetic patients was significantly lower (*p*<0.05) than in the controls. However, IL-23 mRNA expression of the 2 groups was comparable. The present findings suggest that melioidosis affects T cell IL-17 production and that patients with diabetes mellitus have a defective IL-17 production in response to this type of infection.

*Burkholderia pseudomallei*, a Gram-negative bacillus found in soil and water, is the causative agent for melioidosis which is endemic in Southeast Asian countries and Northern Australia.¹ While *B. pseudomallei* is highly virulent in man and animals, other members of this family of bacteria, like *Burkholderia thailandensis* are avirulent.

The human immunopathogenesis of melioidosis is not completely understood. There are reports on increased levels of a number of proinflammatory cytokines and chemokines, suggesting that these mediators may be involved in the pathogenesis of melioidosis.¹ One of those proinflammatory cytokines produced by a subset of T cells distinct from T helper-type 1 or –type 2 cells² and involved in the host defense against bacterial infections³ is IL-17.

It is well documented that in diabetes mellitus immune cells, such as macrophages and neutrophils are impaired, possibly leading to an increased susceptibility to infections including those caused by Gram-negative bacteria.⁴ It has been shown that insulin levels are associated with bacteremic melioidosis.⁵ To date, the status of IL-17 expression associated with *B. pseudomallei* infection and diabetes mellitus has not been established. Thus the present study was conceived to investigate the possible involvement of IL-17 expression in the pathogenesis of melioidosis.

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melioidosis, particularly in diabetes mellitus which is the most common risk factor for this disease. The expression of IL-17 mRNA and protein in peripheral blood mononuclear cells (PBMC) stimulated with virulent *B. pseudomallei* and avirulent *B. thailandensis* was assessed in diabetic patients and compared to that of healthy individuals. In addition, IL-23 gene expression was investigated for its involvement in IL-17 expression.

**MATERIALS AND METHODS**

**Subjects**

Subjects included in this study were 10 diabetic patients attending the Out-Patient Department of Naresuan University Hospital as part of their follow-up routine and 10 healthy blood donors (aged 20-36 years) from the Blood Bank Centre, Naresuan University. The diagnosis of diabetes was established by recording fasting blood sugar levels ≥ 125 mg/dl two times. Nine patients were diagnosed with non-insulin dependent (type 2) diabetes mellitus and one patient with insulin-dependent (type 1) diabetes mellitus. Eight of the 10 diabetic patients had an elevated HbA1c of more than 5.7%. This study was approved by the Local Ethical Committee of Naresuan University (permission number 4602040009) and written informed consents were obtained from all diabetic patients involved.

**Bacterial strains**

The *B. pseudomallei* and *B. thailandensis* cultures used in this study were kindly provided by V. Wuthiekanun (Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand). *B. pseudomallei* 392a was isolated from a Thai patient with septicemic melioidosis. *B. thailandensis* 264E was cultured from soil collected from the northeastern part of Thailand known to be endemic for melioidosis. The identification of these bacteria was carried out by colonial morphology and biochemical tests. The bacteria were cultured in tryptase soy broth at 37°C, harvested at a mid-log phase, and then washed three times in PBS. Bacterial counts were then checked spectrophotometrically and verified by viable count analysis. Prior to use, the bacterial suspension was diluted to achieve the desired concentration in sterile PBS.

**Isolation of peripheral blood mononuclear cells and bacterial stimulation**

Twenty milliliters of heparinized (20 U/ml) venous whole blood was obtained from each healthy donor and diabetic patient. The PBMC were isolated by a standard density gradient centrifugation using Lymphoprep (Axis- Shield Poc AS, Oslo, Norway) containing sodium metrizoate (9.6% w/v) and polysaccharide (5.6% w/v). The PBMC obtained were resuspended in RPMI-1640 (Sigma, MO, USA) containing 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml), and 2 mM L-glutamine (all reagents were from Sigma) and referred to as complete medium. The PBMC were counted and adjusted to 1×10^6 cells/ml. Bacterial stimulation was performed by first plating the PBMC at 1×10^5 cells/well in 96-well tissue culture plates in a final volume of 200 μl and then co-culturing with either live *B. pseudomallei* or live *B. thailandensis* at an MOI ratio of 10:1, each set carried out in triplicate. In addition, 10 μg/ml phytohemagglutinin (PHA; Sigma) was added to the positive control wells and the complete medium was used as negative controls. The plates were then incubated at 37°C for 72 hours in a 5% CO2 humified atmosphere. At the end of the culture period, the culture supernatants were harvested and kept frozen at −70°C until used for the IL-17 ELISA assay. The PBMC were used for IL-17 mRNA analysis by real-time PCR.

In order to study the kinetics of the ability of the bacteria to induce IL-17 protein expression, initial experiments were performed by stimulating the PBMC from 10 healthy donors (not the same donors as in the above experiment) with live *B. pseudomallei* or live *B. thailandensis* at an MOI ratio of 10:1 compared to unstimulated PBMC in similar culture conditions as above but during a 3- to 24-hour period.

**Total RNA extraction and cDNA synthesis**

Total RNA was extracted from the PBMC cultures using the Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The RNA was quantified and cDNAs were synthesized using the Superscript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Briefly, 0.5 μg RNA tem-
plates were mixed with 0.5 μg/ml Random Hexamers (Applied Biosystems, Foster, USA) and 1 mM dNTPs, incubated at 65°C for 5 minutes and chilled on ice for 1 minute. Forty units RNaseOUT™, 200 U Superscript™ III RT, 10x RT buffer, 0.1 M DTT, and 25 mM MgCl₂ were added according to the supplier’s instructions. The reaction mixture was incubated at 25°C for 10 minutes, followed by 50°C for 50 minutes. The reaction was terminated by heating at 85°C for 5 minutes and chilling on ice. Finally, 1 μl of RNase H was added and incubated at 37°C for 20 minutes. The synthesized cDNA was used directly as template in the PCR.

Real-time PCR and determination of the relative expression levels of IL-17

The reactions were performed using a GeneAmp 5700 Sequence Detection System (Applied Biosystem, Foster City, CA, USA). All reagents used in real-time PCR were from Applied Biosystem. Expression of IL-17 mRNA was determined using Pre-Developed Taqman®Assay Reagents for IL-17. The 18S ribosomal RNA assay kit was used as an internal control. The PCR reactions were the same for IL-17 and 18S rRNA. For each run, the cDNA was mixed with 1.25 μl of 20x IL-17 Primers and Probe, 12.5 μl of 2x Taqman®Universal PCR Master Mix and the volume was adjusted to 25 μl with RNase-free water. PCR reactions were loaded into MicroAmp optical 96-well reaction plates and run using the conditions recommended by the manufacturer, i.e., 1 cycle at 50°C for 2 minutes, 1 cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 s and 60°C for 1 minute.

Results of the real-time PCR were presented as Ct values, where Ct was defined as the threshold cycle of the PCR at which the amplified product was first detected. ΔCt was the difference in Ct values of the IL-17 gene and the 18S rRNA, to normalize for the difference in the amounts of total nucleic acid added to each reaction mixture. Then, the ΔCt for each experimental sample (stimulated PBMC) was subtracted from the ΔCt of the calibrator (unstimulated PBMC). This difference was designated as ΔΔCt. The relative level of IL-17 mRNA expression was reported as $2^{-\Delta\Delta C_{t}}$.

Measurement of IL-23 mRNA expression by the PBMC

Total RNA was extracted, the RNA was quantified and the cDNAs were synthesized as above for the study of IL-17 mRNA expression. The expression of the IL-23 gene was performed. Briefly, in the total volume of 50 μl, the reactions consisted of 1x PCR buffer, 0.2 μM dNTP, 2mM MgCl₂ (Invitrogen), 1.08 μM of forward primer 5'-CAGCAGC-TCTCTCGGAAT-3', 1.08 μM of reverse primer 5'-ACAACCATCTTCACTGGATACG-3', 0.125 U Taq DNA polymerase (Promega, Madison, USA), and 1 μl cDNA template. Distilled water was used as negative control. The cycling conditions were as follows; 1 cycle at 95°C for 5 minutes, followed by 40 cycles at 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute with a final extension at 72°C for 7 minutes. The amplified products were analyzed by a 1% agarose gel electrophoresis. PCR using primers for β-actin was used as positive control. The amounts of IL-23 mRNA expression were then analyzed using a gel documentation system (GelDoc 2000: Bio-Rad, Hercules, CA, USA) with the Quantity One program (GelDoc 2000: Bio-Rad). Measurement of IL-17 in the PBMC cultured supernatants by ELISA

The IL-17 protein secreted by the cultured PBMC was measured in the supernatants using a commercial IL-17 Duoset ELISA Development System (R&D System, Minneapolis, MN, USA) according to the manufacturer’s instructions. The absorbance was read at 450 nm using the Packard SpectraCount® Microplate Photometer (PerkinElmer Life Sciences, IL, USA).

Statistical analysis

Comparisons between the two groups for mRNA and protein expression were made with the Student’s t-test using Microsoft Excel software. P values of less than 0.05 were considered significant.

RESULTS

Kinetics of IL-17 protein expression in PBMC from healthy donors

This initial study was performed to confirm the ability of B. pseudomallei and B. thailandensis to
induce the expression of IL-17 by PBMC of healthy donors (n = 10) at 3, 6, 18, and 24 hours (Fig. 1). A significant increase in IL-17 expression was noted with <i>B. thailandensis</i> (p < 0.05) but not with <i>B. pseudomallei</i> as compared to unstimulated PBMC at 18 and 24 hours. It was unexpected to observe this significantly higher expression of IL-17 in PBMC stimulated with <i>B. thailandensis</i> compared to those stimulated with <i>B. pseudomallei</i> at 18 (p < 0.05) and 24 hours (p < 0.01) (Fig. 1).

**IL-17 mRNA and protein expression**

At 72 hours, both <i>B. pseudomallei</i> and <i>B. thailandensis</i> induced a significantly increased expression of IL-17 mRNA and protein compared to the unstimulated PBMC of each group. Unstimulated PBMC data from healthy donors and diabetic patients were similar, both exhibiting low levels of IL-17 mRNA (Mean Ct ± SEM, 35.83 ± 1.02 vs. 38.30 ± 1.02) and protein (Mean pg/ml ± SEM, 25.32 ± 2.90 vs. 22.64 ± 2.40) expression. It is of interest to note that in both normal and diabetic groups, <i>B. thailandensis</i> induced higher levels of IL-17 mRNA and protein expression than <i>B. pseudomallei</i>. However, this difference was only statistically significant for protein levels (p < 0.001). The levels of stimulation by these two bacterial species were noticeably lower than those obtained by PHA, the positive control.

When comparing the IL-17 expressions of the healthy and diabetic groups, the results clearly demonstrated that the PBMC from diabetic patients stimulated with <i>B. pseudomallei</i> and <i>B. thailandensis</i> expressed significantly lower IL-17 mRNA and protein levels than those from healthy donors (Fig. 2A, 2B). Stimulation with PHA also induced an IL-17 mRNA expression that appeared to be depressed in diabetic patients when compared to that of healthy controls.

**IL-23 mRNA expression**

IL-23 mRNA was significantly increased in PBMC stimulated with PHA (p < 0.05) compared to unstimulated controls (Fig. 3). Infection with both <i>B. pseudomallei</i> and <i>B. thailandensis</i> caused a significantly reduced expression of IL-23 mRNA (p < 0.05) compared to unstimulated controls (Fig. 3). However, there were no differences when comparing IL-23 mRNA expression either between diabetic and normal PBMCs stimulated with PHA or between diabetic and normal PBMCs stimulated with the two bacteria (Table 1, Fig. 3).
DISCUSSION

The mechanisms of host immune responses in melioidosis are poorly understood. The present study demonstrated that in vitro exposure of PBMC to virulent *B. pseudomallei* as well as avirulent *B. thailandensis* stimulated IL-17 mRNA and protein expression by PBMC. As IL-17 has been shown to be essential in antigen-specific T cell responses, this cytokine should play a significant role in the response to infection with these bacteria. Moreover, in the diabetic patients, of which 8/10 had elevated HbA1c suggesting a poorly controlled disease, levels of both mRNA and protein of IL-17 were lower. In addition, the present study showed that both *B. pseudomallei* and *B. thailandensis* caused reduced IL-23 mRNA expression in the PBMC. Although IL-23 has been thought to promote IL-17 synthesis, recent works have shown that in the presence of other cytokines, namely IFN-γ and IL-4, the expression of IL-17 cannot be enhanced by IL-23. Thus, it appears that the IL-17 expression of PBMC stimulated by *Burkholderia spp* in the present study was not directly associated with IL-23. IL-6 has also been shown to be increased in mice and humans infected with *B. pseudomallei* and this cytokine may be involved in the induction of IL-17 expression. However, several other yet unidentified factors could also be involved in the control of IL-17 expression in melioidosis.

It is possible that patients with high blood glucose levels have impaired cytokine responses, at least in terms of reduced production of IL-17, probably by activated T cells. In Thailand, type 2 diabetes mellitus is more common than type 1 diabetes mellitus and in our centre almost all of the patients are type 2. Although the sample size was small, we obtained consistent results that were confirmed by statistical analysis. This finding supports a previous study that diabetes is associated with a dysregulation of the cytokine production in response to bacterial infection.

It should be noted that in the present study, although the sex ratio of the two groups was similar, the mean age of the diabetic patients (21-67 years) was considerably higher than that of the healthy donors (20-36 years). As aging is known to be associated with a number of immune deviations, it is possible that this factor might have played a role here. However, immune deviation such as T cell proliferation is mostly found in individuals older than 65 years old and aging is also associated with a more inflammatory process involving higher production of proinflammatory cytokines such as TNF-α. In the present study, the diabetic patients were not in that extreme age and the stimulation with PHA gave similar results. It appears unlikely that the age difference was responsible for the results reported.

![Fig. 2](image_url)

Fig. 2 Comparison of IL-17 mRNA (A) and protein (B) expression between healthy (HD, n = 10) and diabetic (DM, n = 10) groups in response to *B. pseudomallei* (Bp) and *B. thailandensis* (Bt) infection as well as PHA. DIabetic patients show a significantly lower expression of IL-17 mRNA and protein. IL-17 mRNA was measured using real-time PCR and the data is presented as a multiple of the unstimulated control of each group. The IL-17 protein was measured by ELISA and the results are presented in pg/ml. Bar and line represent the mean and standard error of the mean respectively. *p < 0.05.
In this study, the amounts of total RNA in both sample groups as determined by spectrophotometric analysis were similar, which reflected comparable initial cell numbers in both groups before RNA extraction was performed. In addition, the real-time PCR technique used in this study compared the relative gene expression of IL-17 and 18s rRNA which is recommended as a standard normalization of the target gene\textsuperscript{19}. For these reasons, it is unlikely that cell death is responsible for the lower IL-17 expression in diabetic patients in the present study. Moreover, the amounts of IL-23 mRNA expression in the healthy and diabetic groups were similar, reflecting a comparable amount of cells producing this cytokine.

Diabetes mellitus is a known risk factor for chronic or severe infection owing to impaired phagocyte function that increases the susceptibility to infection. The present study demonstrated a lower IL-17 expression in PBMC from diabetic patients in response to both \textit{B. pseudomallei} and \textit{B. thailandensis}, which suggests that reduced IL-17 expression in diabetic patients is associated with a defective T cell function. There are no data currently available on the functional impairment of IL-17 expression in diabetic patients and further studies are needed to clarify the role of this cytokine in the host response to infection in hyperglycemic subjects. Whether changes in T cells, which are the major source of IL-17, are implicated in the impaired immunity in diabetic patients is not known. Identification of the T cell impairment associated with this severe life threatening infection in diabetes mellitus may shed light on the host responses to infection as well as the pathogenesis of diseases for which diabetes mellitus is a risk factor.

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