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Regulatory role of GSK3β in the activation of NF-κB and modulation of cytokine levels in *Burkholderia pseudomallei*-infected PBMC isolated from streptozotocin-induced diabetic animals

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Abstract. Increased susceptibility of diabetics to melioidosis, a disease caused by the *Burkholderia pseudomallei* bacterium is believed to be attributed to dysfunction of the innate immune system. However, the underlying mechanism of the innate susceptibility is not well-understood. Glycogen synthase kinase-3β (GSK3β) plays an important role in the innate inflammatory response caused by bacterial pathogens. The present study was conducted to investigate the effects of GSK3β inhibition by LiCl on levels of pro- and anti-inflammatory cytokines; and the activity of transcription factor NF-κB in *B. pseudomallei*-infected peripheral blood mononuclear cells (PBMC) derived from diabetic-induced and normal Sprague Dawley rats. In addition, the effects of LiCl on intracellular bacterial counts were also investigated. Infection of PBMC from diabetic and normal rats with *B. pseudomallei* resulted in elevated levels of cytokines (TNF-α, IL-12 and IL-10) and phosphorylation of NF-κB in both cell types. Intracellular bacterial counts decreased with time in both cell types during infection. However bacterial clearance was less prominent in diabetic PBMC. *Burkholderia pseudomallei* infection also caused inactivation (Ser9 phosphorylation) of GSK3β in normal PBMC, an effect absent in infected diabetic PBMC. Inhibition of GSK3β by LiCl lowered the levels of pro-inflammatory cytokines (TNF-α and IL-12) in both normal and diabetic PBMC. Similarly, phosphorylated NF-κB (pNF-κB) levels in both cell types were decreased with LiCl treatment. Also, LiCl was able to significantly decrease the intracellular bacterial count in normal as well as diabetic PBMC. Interestingly, the levels of anti-inflammatory cytokine IL-10 in both normal and diabetic PBMC were further elevated with GSK3β inhibition. More importantly, GSK3β in infected diabetic PBMC was inactivated as in their non-diabetic counterparts upon LiCl treatment. Taken together, our results suggest that inhibition of dysregulated GSK3β in diabetic PBMC resulted in the inactivation of NF-κB and modulation of inflammatory cytokine levels. This is evidence that dysregulation of GSK3β is a contributing factor in the molecular basis of innate dysfunction and susceptibility of diabetic host to melioidosis infection.

INTRODUCTION

Burkholderia pseudomallei is the causative organism of melioidosis, a septicaemic disease which is an important cause of septic shock-related deaths (White 2003). Diabetes is an important pre-disposing factor of melioidosis especially in its endemic areas such as South East Asia and Northern Australia (Suputtamongkol *et al.*, 1999; Cheng & Currie 2005).

Previous studies have all shown that susceptibility of diabetics to melioidosis is due to dysfunction of the innate immune system. In one such study it was suggested that the basis of this susceptibility is associated with impaired early cytokine response which led to delayed activation of

the innate immune response in diabetic hosts (Chin *et al.*, 2012). In another study, it was reported that functional deficiencies in diabetic immune cells resulted in failure of these cells to recognise and kill bacteria (Hodgson *et al.*, 2013). In addition, it was also found that lowered intracellular glutathione concentration in diabetic PBMC caused poor bacterial killing and modulation of inflammatory cytokine levels (Tan *et al.*, 2012). Although these findings reiterate that dysfunction of host innate immune system is the reason for the susceptibility of diabetic host to melioidosis, the underlying molecular mechanism for the innate susceptibility is not fully understood.

The host innate immune system is triggered as an initial response to bacterial infection. Pathogen recognition receptors such as Toll-like receptors present on the surface of host cells mediate inflammatory cytokine production stimulated by pathogens through effects on signaling pathways, particularly the PI3K-Akt pathway (Schabbauer *et al.*, 2004). Glycogen synthase kinase-3β (GSK3β) is a downstream component in the PI3K-Akt pathway with a pivotal role in the regulation of inflammatory cytokine production (Cortés-Vieyra *et al.*, 2012). The kinase is known to modulate the balance between pro- and anti-inflammatory cytokine levels in host cells thus suggesting that enzyme inhibition may diminish inflammation (Beurel *et al.*, 2010).

GSK3β which was first identified for its role in the regulation of glycogen metabolism (Embi *et al.*, 1980) is now recognised to be also involved in the regulation of host immune response (Beurel 2011). This kinase is regulated primarily through phospho-inhibition of a serine residue via the PI3K/Akt signaling pathway and/or Wnt signaling pathway (Jope & Johnson 2004). Inhibition of GSK3β decreased TLR-induced production of inflammatory cytokines by inhibition of the transcriptional activity of NF-κB (Martin *et al.*, 2005; Beurel 2011). Its crucial involvement in regulating activity of NF-κB indicates the possible regulatory role of GSK3β in inflammation (Wang *et al.*, 2011; Cortés-Vieyra *et al.*, 2012). In an animal model of sepsis, treatment with GSK3β inhibitors was shown to suppress NF-κB-dependent pro-inflammatory cytokine expression and offer protection from organ injury and endotoxic shock (Woodgett & Ohashi 2005; Dugo *et al.*, 2005). GSK3β has also been shown to regulate NF-κB and TNF-α production during infection of macrophages with Group A *Streptococcus* (Lin 2013).

GSK3β inhibition has been shown to affect the balance of the inflammatory cytokine levels in LPS-induced cirrhotic PBMC (Coant *et al.*, 2011). Inhibition of GSK3β by Lithium chloride (LiCl) significantly reduced production of pro-inflammatory cytokines IL-6, IL-12p40 and TNF-α; but increased production of anti-inflammatory cytokine IL-10 in *Francisella tularensis* infection of macrophages (Zhang *et al.*, 2009). The findings suggest that GSK3β balances pro- and anti-inflammatory cytokine levels. In our own laboratory, we showed that LiCl improved survivability of *B. pseudomallei*-infected mice and attenuated multiplication of the bacteria in phagocytic and non-phagocytic human cells (Tay *et al.*, 2012; Maniam *et al.*, 2013).

As discussed above, diabetics are more susceptible to *B. pseudomallei* infection, but the underlying mechanism of the innate dysfunction is not well-understood. The present study aimed to investigate the effects of GSK3β inhibition on *B. pseudomallei*-infected diabetic immune cells to assess whether GSK3β is implicated in the susceptibility. We pre-incubated PBMC derived from diabetic rats with LiCl, a well-established inhibitor of GSK3β, and determined cytokine levels, activity of NF-κB, intracellular bacterial count and GSK3β phosphorylation status during *B. pseudomallei* infection.

MATERIALS AND METHODS

Bacteria

A *B. pseudomallei* D286 clinical isolate previously characterised based on biochemical tests as well as by 16S rRNA sequencing (Lee *et al.*, 2007) was obtained from the Pathogen Laboratory, School of Biosciences and Biotechnology, Faculty of

Science and Technology, Universiti Kebangsaan Malaysia. Bacteria were grown in Brain Heart Infusion (BHI) broth overnight at 37°C.

In cell infection studies, a single colony with a dry, wrinkled appearance was grown overnight in BHI broth (BHIB) at 37°C. The culture was sub-cultured at 1:50 in the same broth and grown to mid-log phase. Absorbance readings were measured at 600 nm, and the colony forming unit (CFU) estimated from a pre-calibrated standard curve.

Animals

Male Sprague Dawley rats (6-7 weeks old) were obtained from and accommodated at the Animal House Complex, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Rats were caged with a bedding of wood shavings, subjected to a 12-hour light/dark cycle and fed a diet of commercial pellets and water *ad libitum*. All animal experimentations were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (reference number FST/2012/NOOR/21-NOV./465-DEC.-2012-DEC.-2014).

Streptozotocin-induced diabetes in animals

To induce diabetes in experimental animals, rats received intravenous (i.v) injections of streptozotocin (STZ) (Sigma, USA) dissolved in sodium citrate buffer (pH 4.5) at a dose of 45 mg/kg (Szkudelski 2001). Non-diabetic control animals received i.v injections of sodium citrate buffer only. On day 4 after STZ injection, peripheral blood was drawn from the lateral vein and blood glucose levels assessed using Accu-Chek Active Blood Glucose Monitor (Roche Diagnostics, Germany). Rats with blood glucose levels $\geq$13 mmol/L were considered diabetic (Cai *et al.*, 2002). Approximately 90% of experimental animals successfully developed diabetes on day four after STZ injection. Rats remained diabetic for 3-5 days prior to euthanisation and withdrawal of peripheral blood for PBMC isolation.

Isolation and culture of rat PBMC

Rat peripheral venous blood was obtained by cardiac puncture and collected into EDTA-treated, sterile, pyrogen-free tubes (BD Diagnostics, USA) (Parasuraman *et al.*, 2010). PBMC was isolated using Histopaque 1083 (Sigma, USA) following the protocol provided by the manufacturer. Briefly, whole blood was carefully layered onto the Histopaque 1083 surface and centrifuged at 400xg for exactly 30 min at room temperature. After centrifugation, the upper layer to within 2-3 mm of the opaque interface containing the mononuclear cells was carefully aspirated with a pasteur pipet and discarded. The opaque interface containing mononuclear cells was carefully transferred into a clean 15 mL conical centrifuge tube. Ten mL of isotonic phosphate buffered saline (PBS) was added into the cells and centrifuged at 250xg for 10 min. The resultant cell pellet was resuspended in 5 mL of isotonic PBS and centrifuged at 250xg for 10 min. Cells were then washed three times to remove any remaining Histopaque 1083 from the mononuclear cells.

After the final wash, the number of cells was enumerated and cells resuspended in an appropriate volume of cell culture medium. PBMC derived from normal rats (normal PBMC) was cultured (37°C, 5% CO2) using complete RPMI medium; whereas, PBMC derived from diabetic rats (diabetic PBMC) was cultured (37°C, 5% CO2) in complete RPMI supplemented with 5g/L D-glucose to maintain hyperglycaemic condition in diabetic PBMC.

***In vitro* infection of PBMC**

PBMC (1 x 10^6 cells) were seeded into 24-well cell culture plates. Cells were pre-treated with LiCl (2.5, 5, 10 and 20 mM) for 1 h (5% CO2 at 37°C) prior to incubation with *B. pseudomallei* at an MOI of 10:1 (Pongcharoen *et al.*, 2008).

Bacterial invasion assays were performed as described previously (Elsinghorst 1994). *Burkholderia pseudomallei* D286 culture at mid-log phase pelleted by centrifugation at 10 000 x g for

1 min, washed with PBS, and resuspended in cell culture medium. The bacterial suspension was then added to the cells at MOI of 10:1. After 2 h incubation in 5% CO_2 at 37°C, the monolayers were washed with PBS, and further incubated for 2 h in culture medium containing kanamycin (250 µg/mL) to kill extracellular bacteria. The monolayers were then washed three times with PBS. Final wash with PBS was spotted onto Ashdown agar in order to quantify any remaining extracellular bacteria. Cells were lysed with 0.1% Triton X-100 at various time points during incubation with kanamycin (time zero was taken 15 min after incubation in antibiotic-containing medium) and intracellular bacteria quantified by drop-plating of the serially-diluted lysate on Ashdown agar plates. Quantification of intracellular bacteria was done by subtracting the number of extracellular bacteria determined in the last wash with PBS from the total intracellular bacterial count. Experiments were done twice with triplicates in each experiment.

Western blot analysis

At various time intervals (15, 30, 60 and 120 min) after incubation of PBMC with kanamycin, the cells were lysed in buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, phosphatase and protease inhibitors (1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.5 mM PMSF, 1 µg/mL aprotinin, 5 µg/mL leupeptin and 1 mM NaF) followed by incubation on ice for 20 min. The samples were then centrifuged at 20 000 xg for 30 min at 4°C and supernatant collected.

Aliquots of supernatant (50 µL) were used for determination of protein content (Bradford 1976). The remaining sample (450 µL) was diluted with 1:1 (v/v) sample buffer containing 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, β-mercaptoethanol and 0.5% bromophenol blue for protein separation by SDS-PAGE. Equivalent amounts (30 µg) of protein samples were separated through 12% polyacrylamide gels (Laemmli 1970) and then electro-transferred onto nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare, USA). The membrane was blocked with 3% BSA for 1 h before overnight incubation with rabbit polyclonal antibody to pGSK3β (Ser9), GSK3β, (1000x dilution) or β-actin (3000x dilution) (Cell Signaling Technology, USA). Blots were then incubated with HRP-conjugated anti-rabbit IgG (10 000x dilution) (Promega, USA). Detection of immuno-reactive bands was carried out using ECL western blot detection reagents (Thermo Scientific, USA).

Cytokine assay

Levels of IL-10, IL-12 and TNF-α in supernatants of rat PBMC culture (120 min post-infection) infected with *B. pseudomallei* were determined using methods provided together with rat enzyme-linked immuno-sorbent assay kits (IL-10/IL-12/TNF-α ELISA kit, eBioscience, USA) employed.

Nuclear factor-κB phosphorylation assay

A fluorimetric cell-based assay was employed to determine the phosphorylation status of NF-κB (Ser536-p65). Phosphorylation at Ser536 of p65 subunit is an indication of activation of NF-κB, and is involved in transcriptional activity, nuclear translocation and protein stability (Lewander *et al.*, 2012). A total of 100 µL containing ~1 x 10^4 PBMC was seeded into each well of a black clear flat-bottom 96-well plate pre-treated with 20 mM LiCl followed by incubation with *B. pseudomallei* as described above. Cell-based assay was carried out according to the protocol provided with the EnzyFluo™ NF-κB phosphorylation assay kit (BioAssay Systems, USA). Plates were then read at $\lambda_{ex/em}$= 530/585 for phosphorylated NF-κB (pNF-κB) and at $\lambda_{ex/em}$= 360/450 for total protein. Results were expressed as absorbance of pNF-κB/absorbance of total protein.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad, San Diego, CA, USA). Data of cytokine analysis were compared by two-way repeated measures ANOVA. All other data were compared by unpaired Student's t-tests. Comparisons were considered to be significant at $P < 0.05$. Data were expressed as mean ± standard error (SE).

RESULTS

GSK3β inhibition modulated inflammatory cytokine levels in diabetic and normal PBMC

Burkholderia pseudomallei infection increased the levels of TNF-α by 10- and 6-times respectively in normal and diabetic PBMC upon infection (Figure 1A). The level of TNF-α in infected diabetic PBMC is 1.4 times higher than in normal PBMC. The level of IL-12 significantly ($P < 0.05$) increased in diabetic and normal PBMC by 1.4-times respectively (Figure 1B) upon infection. Levels of anti-inflammatory cytokine IL-10 also showed increases by 3.4- and 2.6- times upon *B. pseudomallei* infection in normal and diabetic PBMC respectively compared to their non-infected controls (Figure 1C). This shows that *B. pseudomallei* infection increased levels of TNF-α, IL-12 and IL-10 in normal as well as diabetic PBMC compared to non-infected cells.

Pre-treatment of cells with LiCl decreased the levels of TNF-α (by 3.1- and 2.2-times respectively) and IL-12 (by 1.1- and 1.4-times respectively) in normal and diabetic PBMC (Figure 1A) compared to non-infected cells. Level of IL-10 was further increased (by 2- and 1.7-times) respectively in normal and diabetic cells pre-treated with LiCl compared to their respective infected controls. Level of IL-10 in LiCl-treated *B. pseudomallei*-infected normal PBMC is higher by 1.3-times compared to diabetic PBMC. This shows that inhibition of GSK3β modulates levels of pro- and anti-inflammatory cytokines in diabetic and normal PBMC infected with *B. pseudomallei*.

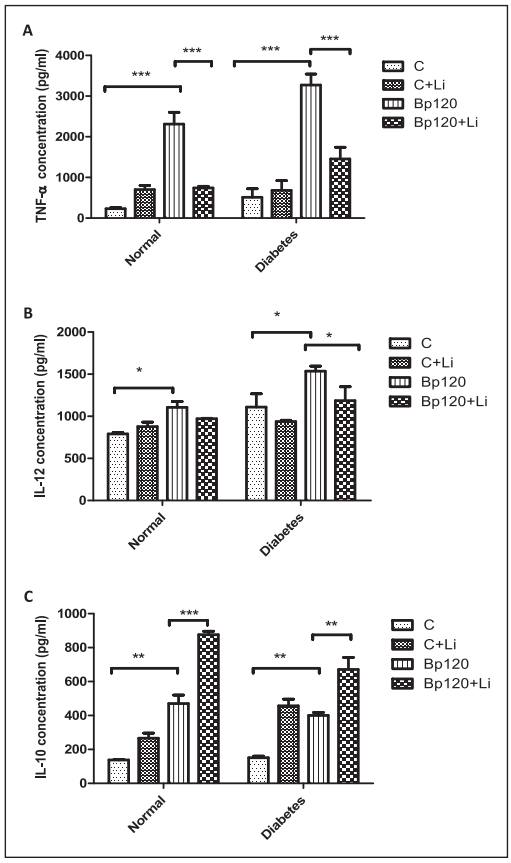


Figure 1. Effects of LiCl on the levels of pro-inflammatory cytokines TNF-α (A) and IL-12 (B), and anti-inflammatory cytokine IL-10 (C) in normal and diabetic PBMC upon *B. pseudomallei* infection. C: control non-infected; C+Li: control non-infected + 20 mMLiCl; Bp120: *B. pseudomallei*-infection at 120 min post-infection; Bp120+Li: *B. pseudomallei*-infection at 120 min post-infection + 20 mMLiCl. Data represent means and standard errors of two separate experiments, each carried out in triplicates.

*denotes significance of $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

GSK3β inhibition suppressed *B. pseudomallei*-induced activation of NF-κB

NF-κB is an important transcription factor that regulates many diverse cellular processes, including cytokine-mediated inflammation. Phosphorylation of NF-κB at p65/RelA (Ser 536) causes activation and translocation of NF-κB into the nucleus and directed production of pro-inflammatory cytokines such as TNF-α and IL-12 (Park *et al.*, 2011).

We first analysed the level of pNF-κB in both normal and diabetic PBMC at different time points following *B. pseudomallei* infection. The level of pNF-κB in diabetic PBMC started to rise at 30 min (2-times higher compared to non-infected) post-infection and a maximal level of pNF-κB was observed at 120 min post-infection (3-times higher compared to non-infected). In normal PBMC, a maximal level of pNF-κB was obtained at

120 min post-infection with 3.4-times higher level compared to non-infected control (Figure 2). These results indicate that *B. pseudomallei* infection increased the activation of NF-κB through phosphorylation in both normal and diabetic cells.

To investigate the effects of GSK3β inhibition on *B. pseudomallei*-mediated activation of NF-κB, PBMC derived from normal and diabetic rats were pre-treated with LiCl for 1 h before infection. Inhibition with GSK3β resulted in a significant ($P < 0.05$) decrease in the levels of pNF-κB in both diabetic and normal PBMC by 3-times and 2-times respectively compared to non-treated infected cells. These results indicate that GSK3β regulates *B. pseudomallei*-induced cytokine production by affecting NF-κB activity/phosphorylation.

Impaired bacterial clearance in diabetic PBMC than normal PBMC

The number of intracellular bacteria in infected PBMC was determined using the protocol previously described in order to assess the internalisation and intracellular bacterial clearance in diabetic and normal PBMC (Elsinghorst 1994). To assess the number of internalised bacteria in PBMC, cells were lysed at 15 min after incubation with kanamycin and internalised bacteria spotted onto Ashdown agar selective for *B. pseudomallei*. Incubation time point of 15 min was chosen as invasion time point on the basis that 15 min was the minimum time required to kill all extracellular bacteria with 250 µg/mL kanamycin (data not shown).

Internalised bacterial number in diabetic PBMC was 4.5 x 10^6 CFU/mL, compared to 3.3 x 10^6 CFU/mL in normal PBMC (Figure 3). The number of internalised bacteria in normal PBMC did not differ significantly ($P > 0.05$) from that in diabetic PBMC which means that both diabetic and normal PBMC were able to uptake bacteria. A previous study showed that bone marrow-derived dendritic cells (BMDC) isolated from chronic diabetic patients had lower number of internalised bacteria compared to non-diabetic BMDC (Williams *et al.*, 2011). Peritoneal-elicited macrophages (PEM)

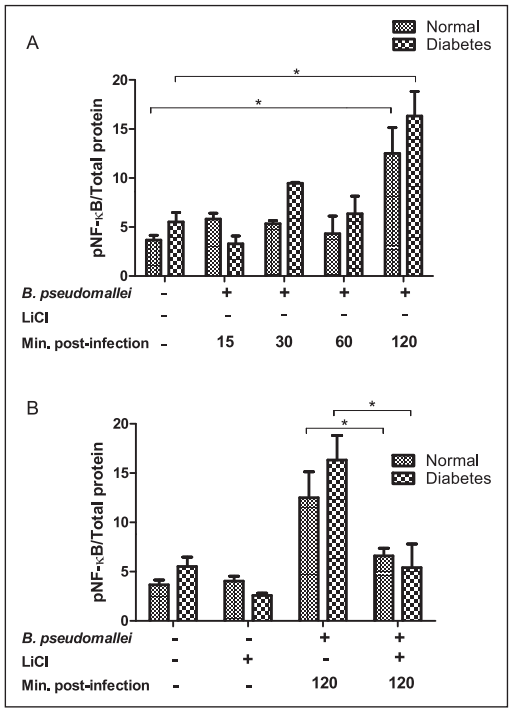


Figure 2. Levels of pNF-κB in normal and diabetic PBMC infected with *B. pseudomallei* in the absence (A) or presence (B) of LiCl. Data represent means and standard errors of two separate experiments, each carried out in triplicates.
*denotes significance of $P < 0.05$.

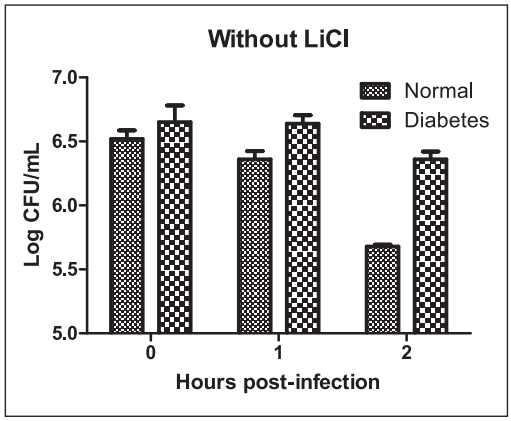


Figure 3. Number of intracellular *B. pseudomallei* (log CFU/mL) in normal and diabetic PBMC during invasion (0 h post-infection), 1h and 2h post-infection. Data represent means and standard errors of two separate experiments, each carried out in triplicates.
*denotes significance of $P < 0.05$.

isolated from diabetic patients did not show significant difference in internalisation of bacteria compared to PEM from non-diabetic patients (Williams *et al.*, 2011). Internalisation of *B. pseudomallei* into cells thus relies on immune cell types (i.e. PBMC, PEM, BMDC, etc.). Our study showed no difference between internalisation of *B. pseudomallei* into PBMC isolated from diabetic or normal rats.

Recent studies showed delayed response of immune system in both *in vivo* and *in vitro* diabetes models during early phase of *B. pseudomallei* infection (Chin *et al.*, 2011; Hodgson *et al.*, 2011). Based on this observation, we examined in the present study the number of intracellular bacteria at 1 and 2 h post-infection to evaluate host response during early stages of infection. At 1 h and 2 h post-infection, the intracellular bacterial count in diabetic PBMC was 4.4 x 10^6 and 2.3 x 10^6 CFU/mL respectively. However, in normal PBMC, the number of intracellular bacteria was relatively lower (2.3 x 10^6 and 0.48 x 10^6 CFU/mL at 1 h and 2 h post-infection respectively). Significant ($P < 0.05$) decrease in intracellular bacterial count with time was observed in normal PBMC. The change in the bacterial number was however not significant in diabetic cells. This difference is likely to be due to impaired phagocytic activity in PBMC of diabetic rats as previously reported (Lecube *et al.*, 2011).

LiCl restored bacterial clearance in diabetic PBMC

In order to determine the effects of cell-permeable GSK3β inhibitor, LiCl, on the intracellular *B. pseudomallei* bacterial number in diabetic and non-diabetic PBMC, cells were pre-incubated with LiCl prior to infection and invasion assays performed as described above.

Pre-treatment of cells with LiCl significantly increased bacterial clearance in normal, as well as diabetic PBMC, as evidenced by a drop in intracellular bacterial count at 1 h and 2 h post-infection (Figure 4).

To ascertain whether LiCl lowered the number of bacteria (in BHIB broth) by direct killing of *B. pseudomallei*, bacteria was incubated with 20 mM LiCl for up to six hours. Viability of *B. pseudomallei* was not affected by LiCl incubation (Supplementary Figure S1). Controls used were *B. pseudomallei* without LiCl. Therefore, the drop in intracellular bacterial numbers in the presence of LiCl involves the host mechanism for killing intracellular bacteria.

GSK3β phosphorylation increased in LiCl pre-treated cells

Phosphorylation of GSK3β was assessed by immunoblotting of PBMC proteins separated by SDS-PAGE using pGSK3β (Ser9) and GSK3β antibodies. As yet, no study has been performed in diabetic immune cells infected with *B. pseudomallei*. Protein samples were pooled from PBMC of three rats due to low signals in proteins extracted from individual animals.

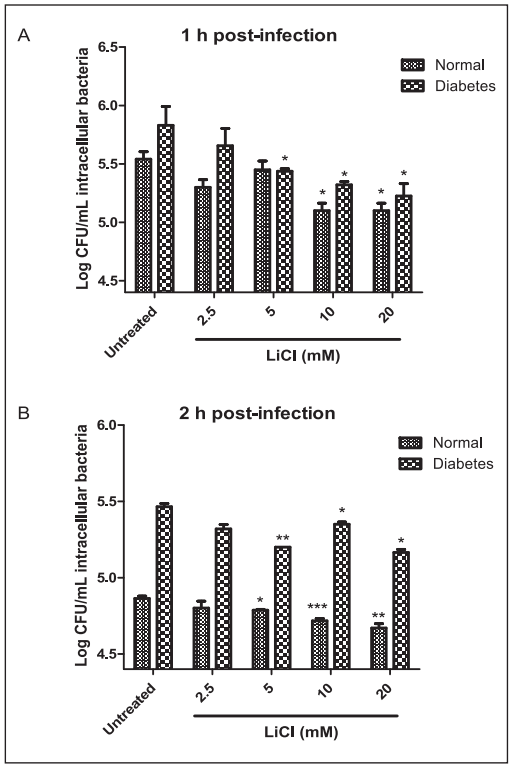


Figure 4. Number of intracellular *B. pseudomallei* (log CFU/ml) in normal and diabetic rat-derived PBMC at 1 h (A) and 2 h (B) post-infection. Data represent means and standard errors of two separate experiments, each carried out in triplicates.

*denotes significance of $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ as compared to untreated from respective groups (normal/diabetic).

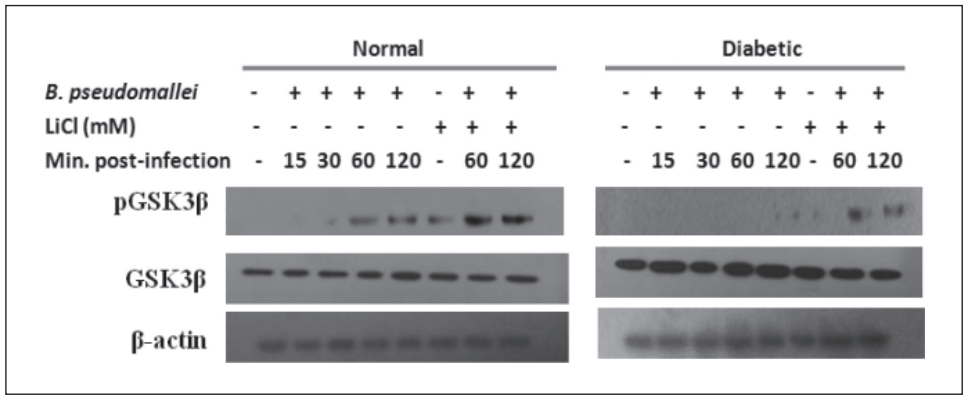
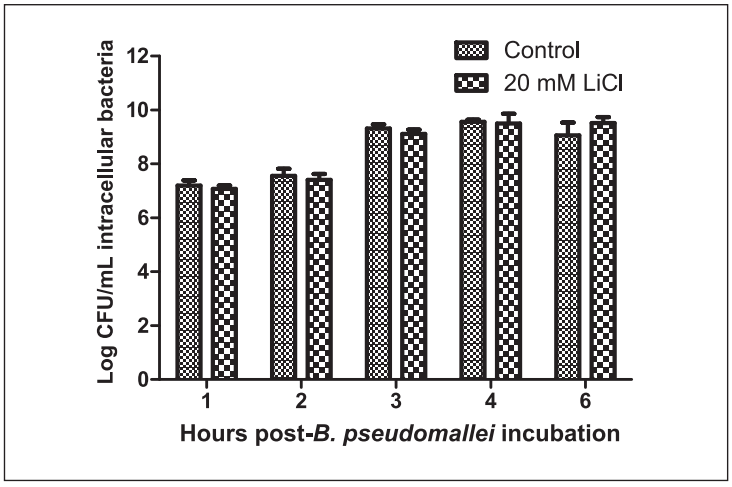


Figure 5. Profile of pGSK3β and total GSK3β of *B. pseudomallei*-infected normal and diabetic PBMC in the presence of LiCl. β-actin was used as a control to ensure equal protein loading.



Supplementary Figure S1. Effects of LiCl on viability of *B. pseudomallei*. *B. pseudomallei* were incubated in 20 mM LiCl for 6 h and number of viable *B. pseudomallei* quantified by serial dilution plating on Ashdown agar. *B. pseudomallei* in medium without the presence of LiCl was used as a control.

Overall, the total GSK3β protein band intensity was higher in diabetic PBMC compared to normal PBMC (Figure 5). In both non-infected diabetic and normal PBMC, there was no significant phosphorylation of GSK3β on Ser9 was detected. pGSK3β band absent in diabetic PBMC was however detected in normal PBMC at 120 min post-infection with *B. pseudomallei*. With LiCl pre-treatment, the phosphorylated protein (pGSK3β) was detected in both normal (increased intensity) and diabetic *B. pseudomallei*-infected PBMC.

DISCUSSION

The present study is an investigation on the early response of *B. pseudomallei* infection in normal and diabetic immune cells. Adherent PBMC, which is enriched with monocytes and macrophages, has previously been used for infection and inflammation studies (Mühl & Dinarello 1997; Chandra *et al.*, 2007; Dewi *et al.*, 2008). In experiments related to susceptibility of melioidosis patients, delayed activation of the innate immune system and impaired

functional responses in macrophages of diabetic host have been reasons proposed for diabetics to be prone to melioidosis (Hodgson *et al.*, 2011; Chin *et al.*, 2012). Pattern recognition receptors in diabetic macrophages failed to recognise and kill *B. pseudomallei* (Hodgson *et al.*, 2011). In addition, uncontrolled and prolonged hyperglycaemia in diabetes disrupts dendritic cell and macrophage function and causes higher resistancy in *B. pseudomallei* infection (Williams *et al.*, 2011). How these abnormalities in diabetic immune cells contribute to their susceptibility to melioidosis has yet to be established.

GSK3 was first identified for its regulatory role in glycogen metabolism (Lawrence & Roach 1997; Embi *et al.*, 1980). The GSK3 family is encoded by two genes, *GSK3α* and *GSK3β*. GSK3β is now known to be involved in the regulation of numerous cellular processes through various signaling pathways. GSK3β regulates several transcription factors, including NF-κB to control inflammation during pathogenic infections (Doble & Woodgett 2003; Burnham *et al.*, 2007). Here, we investigated the involvement of GSK3β in the inflammatory response of diabetic PBMC during *B. pseudomallei* infection.

The present study revealed higher levels of pro-inflammatory cytokines IL-12 and TNF-α in diabetic PBMC than normal PBMC at early infection. In contrast, level of anti-inflammatory cytokine IL-10 was lower in diabetic PBMC than normal PBMC. The hyper-production of TNF-α and defective IL-10 production have been shown to characterise an early 'pro-inflammatory phenotype' of LPS-stimulated immuno-suppressed PBMC. A defect in IL-10 production has previously been shown to be involved in TNF-α hyper-production in LPS-stimulated cirrhotic PBMC (Coant *et al.*, 2011). In addition, accumulation of pro-inflammatory cytokines during *B. pseudomallei* infection causes septicaemia and death of diabetic host due to septic shock.

In the presence of high concentrations of pro-inflammatory cytokines, bacteria may adapt and utilise cytokines to their growth advantage (Kanangat *et al.*, 1999). Infection studies of human U937 monocytes and PBMC with *S. aureus*, *Pseudomonas aeruginosa* and *Acinetobacter sp.* showed that lower concentrations of pro-inflammatory cytokines decreased the intracellular bacterial growth; conversely, at higher concentrations, intracellular growth of bacteria increased significantly (Kanangat *et al.*, 1999).

Our results reiterate previous findings that phagocytic response in PBMC is impaired in diabetes mellitus (Lecube *et al.*, 2011; Lin 2013). In normal PBMC, number of *B. pseudomallei* within PBMC significantly decreased with time. On the contrary, lowering of intracellular bacterial counts in diabetic PBMC was not significant compared to normal PBMC. The latter suggests that bactericidal activity in diabetic PBMC is deficient. Similar decreased capacity to phagocytise and kill bacteria was reported in diabetic macrophage infected with *S. aureus* (Rich & Lee 2005). Impaired phagocytosis and bactericidal functions observed in these macrophages have been linked to cellular abnormality associated with some degree of insulin deficiency (Sima *et al.*, 1988). Phagocytic function of macrophages and expression of adhesion molecules may be altered during hyperglycaemia; thus, interfering with recruitment of immune cells to the inflammatory sites (Sunahara & Martins 2012).

Pathogen recognition by TLR during infection activates the PI3K-Akt pathway which can then lead to phosphorylation and inactivation of GSK3β of host (Nikoulina *et al.*, 2002).On the other hand, our results showed that GSK3β in diabetic PBMC remains active (unphosphorylated at Ser9) upon *B. pseudomallei* infection. This suggests that PI3K-Akt pathway in diabetic PBMC was not stimulated upon infection; thus, GSK3β remained active. It is important to note that the GSK3β in diabetic macrophage is dysregulated than normal macrophage, due to dysfunction of PI3K-Akt pathway involved in insulin signaling (Eldar-Finkelman *et al.*, 1999; Eldar-Finkelman & Kaidanovich 2002). Therefore, it is not unexpected that GSK3β remained active

in diabetic PBMC upon *B. pseudomallei* infection based on the above reasoning.

GSK3β of *B. pseudomallei*-infected cells pre-incubated with LiCl were found to be phosphorylated. As a result of the inhibition of GSK3β, inactivation of NF-κB was observed. Increased level of pNF-κB as observed during infection of normal and diabetic PBMC indicates an active NF-κB which translocates to the nucleus and promotes transcription of genes associated with pro-inflammatory cytokine production (Cortés-Vieyra *et al.*, 2012). These have led to decreased levels of pro-inflammatory cytokines whilst the level of anti-inflammatory cytokine increased. This shows that the inhibition of GSK3 by LiCl modulated the levels of cytokines in *B. pseudomallei*-infected diabetic PBMC. The above observations could in part be the contributing factor for a more efficient bacterial clearance in the presence of LiCl obtained.

It is noteworthy that (Coant *et al.* (2011) also showed that LiCl reversed the overwhelming levels of pro-inflammatory cytokines TNF-α and IL-12 in normal and cirrhotic LPS-induced PBMC. Again, this emphasises the importance of GSK3β in modulating cytokine levels during infection.

In addition, our results are in agreement with previous observations showing that GSK3β positively regulates *F. tularensis* and *E. coli* LPS-induced NF-κB activity and pro-inflammatory cytokines within human monocytic cells (Martin *et al.*, 2005). In *Streptococcus* Group A (GAS) infection, inhibition of GSK3β was shown to reduce NF-κB activation, and subsequent TNF-α production, which indicates that GSK3β acts upstream of NF-κB in GAS-infected macrophages (Lin 2013).

In conclusion, our results suggest that inhibition of dysregulated GSK3β in diabetic PBMC resulted in the inactivation of NF-κB and modulation of inflammatory cytokine levels. This is evidence that dysregulation of GSK3β is a contributing factor in the molecular basis of innate dysfunction and susceptibility of diabetic host to melioidosis infection.

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