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Inhibition of GSK3 Attenuates the Intracellular Multiplication of *Burkholderia pseudomallei* and Modulates the Inflammatory Response in Infected Macrophages and A549 Epithelial Lung Cells

(Perencatan GSK3 Mengurangkan Penggandaan *B. pseudomallei* Intrasel dan Memodulasi Respons Inflamasi dalam Makrofaj dan Sel Peparu Epitelium A549 Terinfeksi)

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ABSTRACT

Burkholderia pseudomallei*, the causative agent of melioidosis, is an intracellular pathogen capable of invading and multiplying in both phagocytic and non-phagocytic cells. Infection results in an inflammatory response involving production of both pro- and anti-inflammatory cytokines. The cellular mechanism regulating this response, believed to play an important role in the pathogenesis of meliodoisis, is not fully understood. In recent years, glycogen synthase kinase-3 (GSK3) has been shown to assume a pivotal role in regulating production of these cytokines. Bacterial infection of host cells activates Toll-like receptors (TLRs) and results in the phosphorylation of GSK3β through activation of the phosphoinositide 3-kinase (PI3K) pathway. In this study, we investigated the effects of GSK3 inhibition in regulating* B. pseudomallei*-induced inflammatory response in macrophages and A549 epithelial lung cells. Our results showed that infection of cells with B. pseudomallei resulted in the increase of anti-inflammatory cytokine, IL-10 and pro-inflammatory cytokine, TNF-α. Pre-treatment of infected cells with GSK3 inhibitors caused further increase in the level of IL-10 but a significant decrease in TNF-α. These changes corresponded with the detection of phosphorylated GSK3β in infected cells treated with LiCl; suggesting that modulation of inflammatory response in* B. pseudomallei *infection involves phosphorylation of GSK3β (Ser 9). This could explain our observations from the invasion assays that pre-treatment of* B. pseudomallei*-infected cells with GSK3 inhibitors resulted in decreased intracellular replication of bacteria within macrophages and A549 epithelial lung cells. In summary, our results demonstrate a regulatory function of GSK3 in the modulation of cytokine levels during* B. pseudomallei *infection.*

Keywords: Burkholderia pseudomallei; *glycogen synthase kinase-3; inflammation; macrophage; melioidosis*

ABSTRAK

Burkholderia pseudomallei*, patogen penyebab melioidosis merupakan bakteria intrasel yang mampu menginfeksi dan mengganda dalam sel fagosit serta sel bukan fagosit. Infeksi mengakibatkan respons inflamasi yang melibatkan penghasilan sitokin pro- dan anti-inflamasi. Mekanisme pengawalan respons tersebut yang dipercayai memainkan peranan penting dalam patogenesis melioidosis masih belum difahami sepenuhnya. Glikogen sintase kinase-3 (GSK3) kini diketahui mempunyai peranan utama dalam pengawalan penghasilan sitokin pro- dan anti-inflamasi. Infeksi sel hos oleh bakteria mengaktifkan reseptor* Toll-like *(TLR) dan mencetuskan pemfosfatan GSK3β melalui pengaktifan tapak jalan fosfoinositid-3-kinase (PI3K). Kami mengkaji kesan perencatan GSK3 dalam pengawalan respons inflamasi yang diaruh oleh* B. pseudomallei dalam *makrofaj dan sel epitelium peparu A549. Hasil yang kami peroleh menunjukkan peningkatan penghasilan sitokin anti-inflamasi, IL-10 dan sitokin pro-inflamasi, TNF-α dalam sel diinfeksi* B. pseudomallei. *Pra-perlakuan sel terinfeksi dengan perencat GSK3 menyebabkan aras sitokin IL-10 meningkat dengan lebih tinggi tetapi penghasilan TNF-α berkurangan secara signifikan. Perubahan aras sitokin IL-10 dan TNF-α berpadanan dengan pengesanan GSK3β terfosfat dalam sel diinfeksi yang diberi perlakuan LiCl. Ini mencadangkan bahawa modulasi respons inflamasi semasa infeksi* B. pseudomallei *melibatkan pemfosfatan GSK3β (Ser 9). Ini juga menjelaskan cerapan daripada asai penaklukan yang menunjukkan pra-perlakuan* perencat *GSK3 menyebabkan pengurangan penggandaan bakteria intrasel dalam makrofaj dan sel epitelium peparu A549 yang diinfeksi* B. pseudomallei. *Secara keseluruhan hasil kami menunjukkan GSK3 terlibat dalam modulasi aras sitokin semasa sel diinfeksi* B. pseudomallei.

Kata kunci: Burkholderia pseudomallei; *glikogen sintase kinase-3; inflamasi; makrofaj; melioidosis*

INTRODUCTION

Burkholderia pseudomallei, a free-living Gram-negative bacterium is the causative agent for the disease melioidosis in humans. The disease is a significant cause of morbidity and mortality whenever it strikes. *B. pseudomallei* is known to secrete various extracellular products; such as exotoxin,

which have been implicated in the pathogenesis of this disease (Ismail et al. 1987). Because of its high virulence, *B. pseudomallei* is currently considered as a category B bio-terrorism agent. Commonly encountered in the tropical environment, especially soil, mud and surface water, it can cause varying manifestations of the disease; ranging from asymptomatic form to fulminating deadly form (White 2003; Wiersinga et al. 2006). It is notorious in infecting people with high risk conditions such as diabetics and other immuno-compromised hosts (Valvano et al. 2005).

The lipopolysaccharide (LPS) of *B. pseudomallei* has been demonstrated to be weak stimulators of macrophages (Arjcharoen et al. 2007; Ho et al. 1997; Matsuura et al. 1996). Compared with *B. thailandensis*, a close relative that rarely cause disease, *B. pseudomallei* produce lower concentrations of inflammatory cytokines TNF-α, IL-6 and IL-10 from both stimulated human and murine macrophages *in vitro* (Novem et al. 2009). Offering to explain the difference in the virulence of these two closely-related bacteria, Novem et al. (2009) suggest the more efficient stimulation of cytokine release by non-pathogenic *B. thailandensis* which makes them more susceptible to killing by the host innate immune system. On the other hand, the pathogenic *B. pseudomallei* are able to evade killing by macrophages through their strategic lowering of the cytokine concentration. To achieve this, their LPS are structurally modified to act as weak macrophage activators. All these suggest the role of inflammatory cytokines in the pathogenesis of *B. pseudomallei*.

In *Francisella tularensis* infection, the manifestation of sepsis has been associated with an over-production of pro-inflammatory cytokines including IL-6, IL-12 and TNF-alpha (Cole et al. 2008). Further, it has been shown that LPS is capable of activating the phosphatidylinositol 3-kinase (PI3K) pathway which plays a central role in regulating host inflammatory response (Wang et al. 2010). LPS has also been shown to induce phosphorylation of glycogen synthase kinase 3 (GSK3), suggesting the role of this enzyme in innate immune response (Ohtani et al. 2008). Among the effector molecules of the PI3K pathway, the serine/threonine kinase, GSK3 has been identified as central in regulating the production of pro- and anti-inflammatory cytokines. This enzyme exists in two isoforms; GSK3α (molecular weight 51 kDa) and GSK3β (molecular weight 47 kDa).

Although GSK3 was first identified as one of the key enzymes involved in glycogen metabolism (Cohen & Frame 2001; Embi et al. 1980), it was subsequently shown to be involved in a variety of cellular processes including membrane-to-nucleus signaling, gene transcription, translation, cytoskeletal organization, cell cycle progression and cell apoptosis (Dugo et al. 2006; Jope et al. 2007). Our present study seeks to investigate the role of GSK3 in the intracellular survival of *B. pseudomallei* in macrophages and lung epithelial cells that may relate to host regulatory control of cytokine production.

MATERIALS AND METHODS

BACTERIAL STRAIN AND CULTURE

B. pseudomallei strain D286 was a kind gift from Prof. Sheila Nathan (Pathogen Laboratory, School of Biosciences & Biotechnology, Faculty of Science and Technology, The National University of Malaysia). A single colony with a dry, wrinkled appearance was grown overnight in brain heart infusion broth (BHIB) at 37°C. The culture was sub-cultured at 1:50 in BHIB and grown to mid-log phase. Absorbance readings were measured at 600 nm using UV-spectrophotometer and the colony forming unit (CFU) estimated from a pre-calibrated standard curve.

CELL CULTURE

Human monocyte-like U937 cell line (ATCC No. CRL-1593.2) was purchased from American Type Culture Collection. Cells were maintained in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 25 mM HEPES (complete medium). Prior to experiments, cells were induced to differentiate into macrophages using a total of 1×10^5 U937 cells seeded into a 24-well tissue culture plate and incubated with 10 ng/mL phorbol myristate acetate (PMA) at 37°C in 5% CO_2 for 48 h. After induction, the medium was removed and monolayer cells were washed with phosphate-buffered saline (PBS) to remove traces of PMA.

Cryo-preserved stock of human lung carcinoma cell culture A549 (ATCC No. CCL-185) was kindly provided by Prof. Sheila Nathan. Culture was maintained in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 4500 mg/L D-glucose, 2 mM L-glutamine and 25 mM HEPES buffer (37°C, 5% CO_2).

PHARMACOLOGICAL INHIBITORS

GSK3 inhibitors, lithium chloride (LiCl) and SB216763 were purchased from Sigma. SB216763 was reconstituted in dimethyl sulfoxide and stored at -20°C until used.

INVASION ASSAY

U937 cells (1×10^5) were seeded into 24-well cell culture plates and induced with PMA as described above. A suspension of A549 epithelial cells in serum-free DMEM was seeded into 12-well plates at a density of 1×10^5 cells/mL. Cells were pre-treated with LiCl (1, 5, 10 and 20 mM) or SB216763 (2.5, 5, 10 and 20 μM) for 1 h (5% CO_2 at 37°C) prior to incubation with *B. pseudomallei*.

Bacterial invasion assays were performed according to the method of Elsinghorst (1994). Bacterial culture was prepared using *B. pseudomallei* D286 culture at mid-log phase which was pelleted by centrifugation at 14000 rpm for 1 min, washed with PBS and resuspended in cell culture medium. The bacterial suspension was then added to the cells (in the presence of GSK3 inhibitors) 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). The monolayers were washed with PBS and 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. All invasion assays were performed in triplicates.

WESTERN BLOT

At the various time intervals (0, 30, 60 and 120 min) after incubation with kanamycin, the cells were lysed in buffer containing 50 mM Tris HCl, 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). Equivalent amounts (40 µg) of proteins were separated through 12% polyacrylamide gels 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 GSK3β or pGSK3β (Ser9). Blots were then incubated with HRP-conjugated anti-rabbit IgG (Promega, USA). Detection of immune-reactive bands was carried out using ECL western blot detection reagents (Thermo Scientific, USA).

CYTOKINE ANALYSIS

The levels of IL-10, IL-12 and TNF-α in the supernatants of cell culture infected with *B. pseudomallei* (0, 30, 60 and 120 min post-infection) and pre-treated with GSK3 inhibitors was determined using instructions provided with the enzyme-linked immunosorbent assay kits (IL-10/IL-12/TNF-α ELISA kit, eBioscience) employed.

RESULTS

MULTIPLICATION OF *B. pseudomallei* IN CELLS

B. pseudomallei is a facultative intracellular bacterium which can survive and multiply in both phagocytic and non-phagocytic cells (Utaisincharoen et al. 2004). *B. pseudomallei* is able to invade non-phagocytic cells including HeLa, CHO, A549 and Vero (Kespichayawattana et al. 2004). Cells were infected with *B. pseudomallei* at an MOI of 10:1 in order to assess the viability and multiplication of bacteria within U937 macrophages and A549 epithelial cells. Evaluation of the initial colony forming unit (CFU) of bacteria which successfully invaded U937 macrophages showed that 2.8% of the original inoculum entered macrophages. This is comparable with the data reported in J774A.1 and RAW264.7 macrophages infected by *B. pseudomallei* isolates (Gong et al. 2011; Wand et al. 2011). The number of intracellular *B. pseudomallei* increased up until 120 min after kanamycin treatment (3.39×10^5 CFU/mL) signifying multiplication of *B. pseudomallei* within the phagocytic cells during this period (Figure 1). In the case of the human respiratory epithelial cell line A549, the invasive capacity of *B. pseudomallei* was reported to be significantly greater than that of *B. thailandensis* (Kespichayawattana et al. 2004). The number of bacteria that invaded epithelial cells was 3-4% of the bacterial inoculum employed. An exponential increase of up to 2.8×10^7 CFU/mL of intracellular bacteria was observed in epithelial cells 2 h post-infection (Figure 1). Studies by Phewkliang et al. (2010) showed that percentage internalisation of *B. pseudomallei* into human epithelial cell line was 1.2% for *B. pseudomallei* 1026b and 3.6% for *B. pseudomallei* SR1015. The invasion data obtained here with *B. pseudomallei* D286 showed percentage invasion obtained was comparable with that established by other researchers and reiterate various reports that *B. pseudomallei* can invade and replicate intracellularly within eukaryotic cells.

MULTIPLICATION OF *B. pseudomallei* IN CELLS IN THE PRESENCE OF GSK3 INHIBITORS

To evaluate how GSK3 inhibitors affected replication of *B. pseudomallei* in cells, bacterial CFUs in phagocytic and non-phagocytic cells pre-exposed to various concentrations of LiCl or SB216763 were determined at 0, 60 and 120 min post-kanamycin treatment. Neither LiCl nor SB216763 affected the viability of *B. pseudomallei*, macrophages or epithelial cells at the tested concentrations.

Pre-treatment with LiCl (Figure 2) or SB216763 (Figure 3) had no effects on internalisation of *B. pseudomallei* in macrophages and epithelial cells based on the CFU/mL of bacteria in treated and non-treated cells at 0 min post-infection.

In macrophages pre-treated with 10 mM and 20 mM LiCl (Figure 2), the number of intracellular bacteria at 60 min post-infection was only 33 and 37%, respectively, compared with the control. Similarly, the number of intracellular bacteria at 120 min post-infection was lowered to 10 and 20 mM LiCl pre-treatment to 40 and 39%, respectively. Pre-treatment of macrophages with the GSK3 inhibitor SB216763 also significantly lessened the multiplication of bacteria within macrophages to about 50% at 60 min post-infection for both 10 and 20 µM SB216763 (Figure 3). At 120 min post-infection, the multiplication of intracellular bacteria was attenuated to 30, 65 and 47%, respectively, in macrophages pre-treated with SB216763 at concentrations of 5, 10 and 20 µM. Taken together, this showed that pre-treatment with GSK3 inhibitors (LiCl or SB216763) suppressed multiplication of intracellular bacteria in macrophages even though both inhibitors did not have any effect on bacterial invasion. In epithelial cells similarly pre-treated with either LiCl or SB216763, attenuation of intracellular bacterial multiplication was also observed within the concentrations tested at both 60 and 120 min post-infection time points.

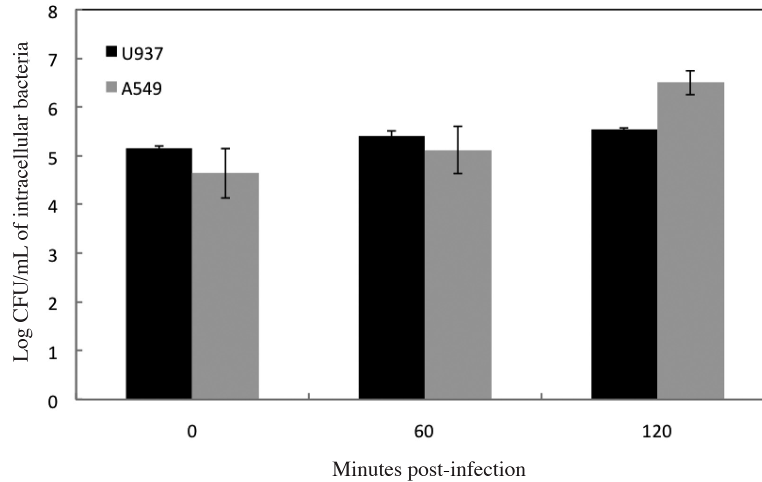


FIGURE 1. Intracellular CFU of *B. pseudomallei* D286 in U937 macrophages and epithelial cells. Cells were incubated with *B. pseudomallei* at an MOI of 10:1 for 2 h at 37°C in 5% CO_2 to allow bacterial entry. Cells were washed and further incubated in medium containing kanamycin (250 µg/mL) for up to 120 min. The bacterial count was enumerated at various time points post-infection by cell lysis and plating onto Ashdown agar. One representative experiment out of two independent experiments is shown. Each experiment was done in triplicates

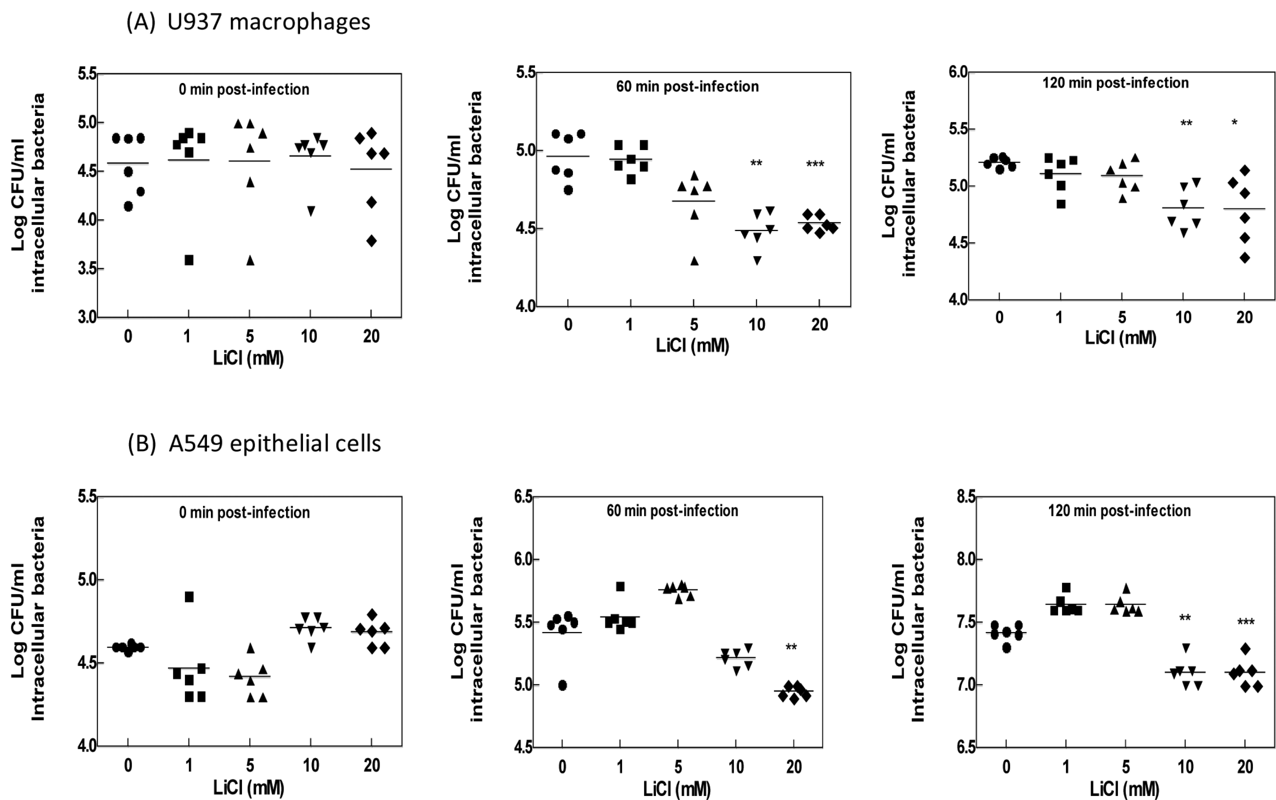


FIGURE 2. Intracellular CFU of *B. pseudomallei* in U937 macrophages and A549 epithelial cells pre-treated with LiCl. Cells were pre-incubated with LiCl or vehicle (control) for 1 h prior to infection with *B. pseudomallei* at an MOI of 10 bacteria per cell. Cells were washed and further incubated in medium containing kanamycin (250 µg/mL) for 0 h, 1 h and 2 h (time zero was taken 15 min after incubation with antibiotic-containing medium). Cells were then lysed with 0.1% Triton X-100 and intracellular bacteria were quantified by serial dilution plating of the lysate. Results shown are combined from two independent sets of experiments. Each experiment was done in triplicates. Statistical analysis was performed using a Student's t-test (*$p< 0.05$; **$p<0.01$; ***$p<0.001$)

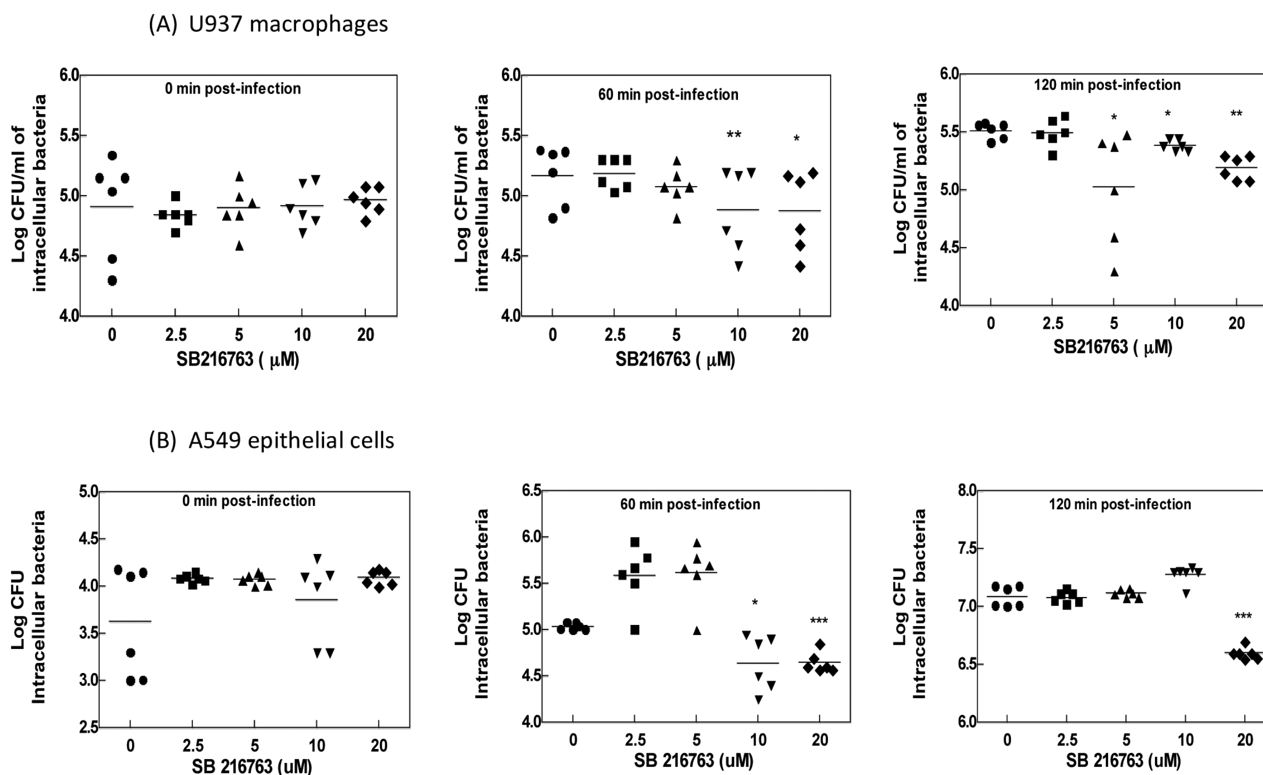


FIGURE 3. Intracellular CFU of *B. pseudomallei* in U937 macrophages and A549 epithelial cells pre-treated with SB216763. Cells were pre-incubated with SB216763 or vehicle (control) for 1 h prior to infection with *B. pseudomallei* at an MOI of 10 bacteria per cell. Cells were washed and further incubated in medium containing kanamycin (250 μg/mL) for 0 h, 1 h and 2 h (time zero was taken 15 min after incubation with antibiotic-containing medium). Cells were then lysed with 0.1% Triton X-100 and intracellular bacteria were quantified by serial dilution plating of the lysate. Results shown are combined from two independent sets of experiments. Each experiment was done in triplicates. Statistical analysis was performed using a Student's t-test (*p< 0.05; **p<0.01; ***p<0.001)

In summary, our results from the invasion assays showed that at the concentrations tested, pre-treatment of cells with GSK3 inhibitors (LiCl and SB216763) attenuated multiplication of the intracellular *B. pseudomallei* in macrophages and in A549 epithelial lung cells.

PHOSPHORYLATION OF GSK3B IN *B. pseudomallei* -INFECTED CELLS

The phosphorylation state of GSKβ in cells was determined 2 h post-infection by western blotting. Phosphorylated GSK3β (Ser 9) was not observed in non-infected cells (Figure 4). However, in *B. pseudomallei*-infected cells, phosphorylated GSK3 (Ser 9) was detected in macrophages and A549 epithelial cells as early as 15 min and persisted through 120 min post-infection. The intensity of the phosphorylated GSK3 (Ser 9) band was more pronounced in cells that were pre-treated with GSK3.

CYTOKINE PRODUCTION IN *B. pseudomallei*-INFECTED CELLS IN THE PRESENCE OF GSK3 INHIBITORS

Pathogenic infection is commonly associated with inflammatory cytokine production in the host. In lieu of this, levels of anti-inflammatory cytokine, IL-10; and pro-inflammatory cytokines, TNF-α and IL-12 secreted by phagocytic and non-phagocytic cell lines in response to *B. pseudomallei* infection were determined using cytokine-specific ELISA kits (eBiosciences, USA). *B. pseudomallei* infection gradually increased the levels of IL-10 and TNF-α in macrophages up to 17- and 13-times, respectively, at 2 h post-infection compared with non-infected control macrophages (Figure 5). *B. pseudomallei*-infected epithelial cells also showed increased in IL-10 and TNF-α levels (up to 1.8- and 5.2-times, respectively). However, no significant changes in IL-12 were detected during early infection of *B. pseudomallei* in both macrophages and epithelial cells. This is because while other cytokines involved in innate immune response are detected early during bacterial invasion, IL-12 was produced relatively later in the course of infection (Miettinen et al. 1998).

Pre-treatment of macrophages with either LiCl or SB216763 increased the level of IL-10 cytokine by about 50- and 44-times, respectively, compared with non-infected control macrophages (Figure 6). TNF-α cytokine level was 7.6- and 5.6-times more with pre-treatment of infected macrophages with either LiCl or SB216763, respectively, as compared with non-infected control macrophages. This change in TNF-α level however, was significantly lower compared with infected cells. Pre-treatment of macrophages with GSK3 inhibitors had no effects on IL-12 level.

Similar observations in cytokine levels were detected in epithelial cells pre-incubated with GSK3

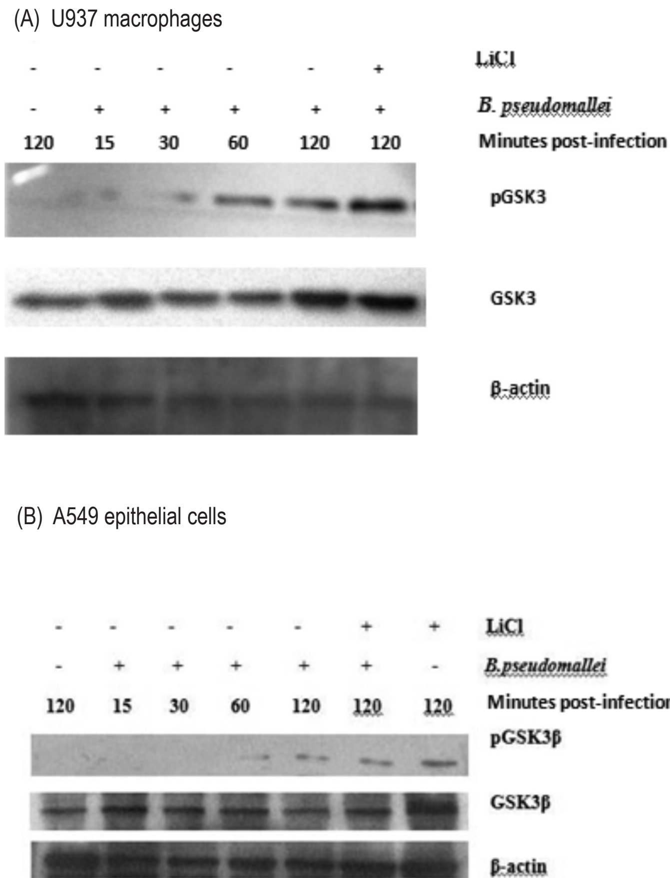
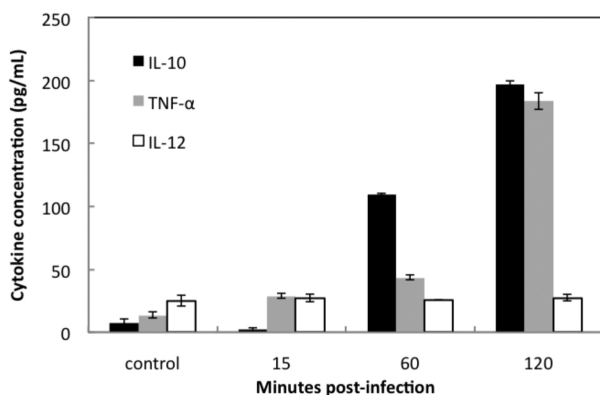


FIGURE 4. Serine 9 phosphorylation of GSK3β in *B. pseudomallei*-infected macrophages and epithelial cells in the presence or absence of LiCl. The cells were pre-incubated with LiCl or vehicle for 1 h before infection with *B. pseudomallei* at MOI of 10:1. Cells were washed and further incubated in medium containing kanamycin (250 μg/mL). At 15, 30, 60 and 120 min post-infection, cells were lysed and phosphorylation of GSK3β was determined by immunoblotting

(A) U937 macrophages



(B) A549 epithelial cells

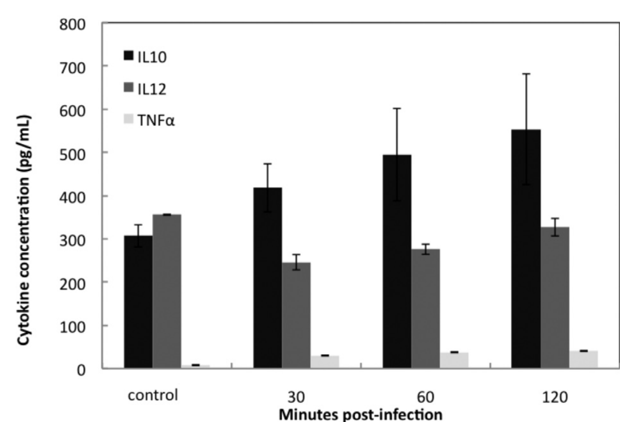
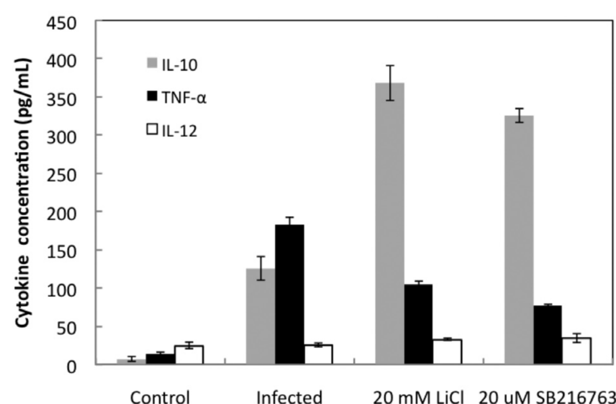


FIGURE 5. Levels of cytokines in macrophages and epithelial cells during infection with *B. pseudomallei*. Cells were infected with *B. pseudomallei* at MOI of 10 bacteria per cell. Cells were washed and further incubated in medium containing kanamycin (250 μg/mL) for 15, 30, 60 and 120 min and the levels of IL-10, TNF-α and IL-12 in the culture supernatants were analysed by ELISA. Control cells consisted of uninfected, untreated normal cells

(A) U937 macrophages



(B) A549 epithelial cells

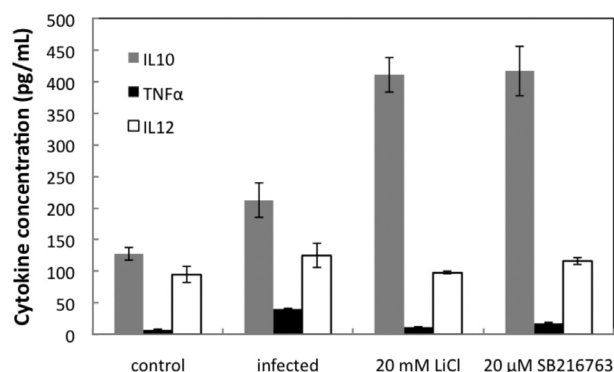


FIGURE 6. Levels of cytokines in macrophages and epithelial cells in the presence or absence of GSK3 inhibitors during infection of *B. pseudomallei*. Cells were pre-incubated with LiCl, SB216763 or vehicle for 1 h before infection with *B. pseudomallei* at MOI of 10:1. Cells were washed and further incubated in medium containing kanamycin (250 µg/mL) for 2 h and the levels of IL-10, TNF-α and IL-12 in the culture supernatants were analysed by ELISA. Control cells consisted of uninfected, untreated normal cells

inhibitors. Pre-treatment of epithelial cells with LiCl or SB216763 resulted in further increase in levels of IL-10 (3.2- and 3.3-times, respectively). TNF-α cytokine level was 1.4- and 2.3-times more with the pre-treatment of infected macrophages with either LiCl or SB216763, respectively, as compared with non-infected control epithelial cells (Figure 6). This change in TNF-α level is significantly lower compared with the infected cells. However, no significant changes in IL-12 was detected during infection or with pre-treatment of A549 cells with GSK3 inhibitors.

DISCUSSION

During infection by Gram-negative bacteria, the host innate immune response is triggered by lipopolysaccharide (LPS), the major outer membrane component of such bacteria, resulting in the production of both pro- and anti-inflammatory cytokines needed to curb invasion (Elson et al. 2007). LPS, however, may also result in over-stimulation of cytokine release leading to sepsis primarily responsible for high mortality in acute melioidosis. Results from the cytokine level studies in the present study supports the notion that proper balance of pro- and anti-inflammatory cytokines is important for successful invasion of the host by *B. pseudomallei*.

Host recognition of *B. pseudomallei* involves transmembrane receptors, specifically TLR2 and TLR4 (Hii et al. 2008; West et al. 2008; Wiersinga et al. 2007). The underlying cellular mechanisms which regulate the inflammatory cytokine response following TLR stimulation is believed to be mediated through GSK3.

Activation of the PI3K pathway by TLR2 agonists gave differential effects on the production of anti-inflammatory IL-10 and the pro-inflammatory IL-12 cytokines (Fukao et al. 2002). Inhibition of the PI3K activity was reported to reduce IL-10 production but increase IL-12 levels. This is however not a universal phenomenon for bacterial infection of host cells.

Our investigations revealed that *B. pseudomallei* infection caused an increase in the release of anti-inflammatory cytokines IL-10 and TNF-α in macrophages. Inhibition of GSK3 activity further increased IL-10 production. This observation suggests a beneficial role for GSK3 inactivation in ensuring the ability of the host to control an overproduction of cytokines during *B. pseudomallei* infection. IL-10 has been reported to be capable of preventing cytokine overproduction in response to both microbial infections and cancer development (Chan et al. 2009). Studies on the role of GSK3 activity using other pathogens also appear to concur with our observations. GSK3 inhibition protected mice from lethal infection with *Francisella tularensis* by increasing production of anti-inflammatory cytokines and reducing pro-inflammatory cytokines (Zhang et al. 2009). For enhanced survival in the host, *Mycobacterium* infection resulted in inhibition of host GSK3 activity that led to increased IL-10 production (Chan et al. 2009). Specific inhibition of GSK3 in BCG-infected human macrophage cells resulted in increased production of IL-10 which was also a potent suppressor of IFN-γ production (Chan et al. 2009). These investigations suggest that GSK3 acts as a negative regulator of anti-inflammatory cytokine IL-10 production in response to bacterial invasion.

Our present study showed that inhibition of GSK3 led to significant reduction of TNF-α secretion by macrophages and epithelial cells. Similar observations have been documented for *Staphylococcus aureus* and *F. tularensis* infections upon GSK3 inhibition (Cheng et al. 2009; Zhang et al. 2009). The pathological role of TNF-α have attracted much attention in recent years. Exogenous and endogenous factors from bacteria, viruses and parasites are known to stimulate production of TNF-α and other cytokines. Lipopolysaccharide from bacterial cell walls is

an especially potent stimulus for TNF-α synthesis (Tracey & Cerami 1994).

Hence, TNF-α can bring about beneficial effects for the host in inflammation and in protective immune responses against a variety of infectious pathogens. On the other hand, depending on the levels produced and released during infection, TNF-α can also exert host-damaging effects (Pfeffer 2003). In melioidosis, it is tempting to speculate a scenario depicting the role of TNF-α during local infection or inflammation. Produced by macrophages, low levels of TNF-α could act to enhance immune responsiveness. It can stimulate blood-vessel growth, increase energy mobilization, induce the release of other cytokines and promote wound-healing (Dalmas et al. 2012). But when overwhelming infection occurs, as in the case of acute melioidosis, large quantities of TNF-α could reach the circulation and cause septicemic shock. If a persisting infection develops and TNF-α is chronically secreted, it could result in death. Future studies will undoubtedly advance our understanding of these effects of TNF-α and that of the other cytokines. The development of novel therapies for inflammation and septic shock in melioidosis may be based on such advances.

Our results, however, painted a different picture of prototypical pro-inflammatory cytokine IL-12. Infection with *B. pseudomallei* failed to show a significant increase in the production of pro-inflammatory cytokine IL-12 during early stage of infection. Inhibition of GSK3 also failed to exert any impact on the secretion of IL-12. We offer the following possible explanation for this unexpected observation in our study. While other cytokines involved in innate immune response are detected early during bacterial invasion, IL-12 was produced relatively later in the course of infection (Miettinen et al. 1998). Indeed it has been demonstrated by others that LPS-stimulated human macrophages showed significant increase in IL-12 production after 24 h (Coant et al. 2011; Miettinen et al. 1998; Zhang et al. 2009). It is possible that in our study, IL-12 production was not pronounced because this specific inflammatory cytokine did not play a significant role in the early response of infection by *B. pseudomallei*. IL-12, however, may be crucial in the adaptive immune response. It is known to be primarily secreted by the peripheral lymphocytes after infection, particularly by B-cells and to a lesser extent by T-cells (Morris et al. 1994). Here, in the acquired immune response, IL-12 is involved probably in the selection of host immunoglobulin isotypes (Morris et al. 1994). For instance, at picomolar concentrations, IL-12 has been shown to markedly inhibit the synthesis of IgE by peripheral blood mononuclear cells (Brunda 1994).

Our present study also revealed that inhibition of GSK3 activity significantly reduced the intracellular survival of *B. pseudomallei* in macrophages and epithelial cells. This suggests that *B. pseudomallei* requires intact host GSK3 activity for its successful invasion. Such requirement has also been reported in other infection models including *Salmonella typhimurium* (Duan et al. 2007), *Yersinis pestis* (Sauvonnet et al. 2002) and *F. tularensis* (Zhang et al. 2009). Any attempt to provide some explanations for this reduced survival of bacterial pathogens in host cells with dysfunctional GSK3 activity would be speculative at this juncture. Nonetheless, our data suggest two important points: the pro- and anti-inflammatory cytokine network could assume a diversity of biological functions to determine the course of *B. pseudomallei* infection and the regulation of the overlapping and synergistic effects between many different cytokines is critical in the protection against *B. pseudomallei* infection or causing tissue injury and death to infected host cells.

We postulate that the reduced survival of *B. pseudomallei* upon inhibition of GSK3 pathway may relate to the failure of this intercellular pathogen in establishing a replicative niche after its internalization by the host cells. Once ingested, bacterial pathogens may undergo varying pathways to establish a replication niche for themselves. Some pathogens escape from being killed by the lysosomal enzymes in the phagocytic vacuoles by travelling along the 'end-to-end track' formed by host cell microtubules to end up in affable cell compartments such as the endoplasmic reticulum (ER) and cell cytoplasm. Here, they can safely and rapidly replicate to cause detrimental effects to the host cells and result in diseases. Others employ actin-mediated mobility to escape from the vacuoles. The mechanism(s) employed by *B. pseudomallei* to delay lysosomal fusion and avoid killing by lytic enzymes and acidic pH remains unclear.

Many pathogens interfere with the phosphorylation mechanism of the host to survive and cause disease (Kwok et al. 2007). A number of intracellular pathogens interfere with signaling pathway of the phophoinositide (PI) metabolism which is known to determine several crucial events during infection including membrane trafficking, actin rearrangement and cell survival (Duronio 2008; Krauß & Haucke 2007; Toker & Cantley 1997; Weber et al. 2009).

Evidence has been accumulating to suggest the importance of PI metabolism in the internalization of pathogens to form phago-lysosomal vacuoles in the host cells. For instance, PI3K has been shown to play a role in the phagocytosis of many pathogens (Ireton et al. 1996; Lindmo & Stenmark 2006; Toker & Cantley 1997; Weber et al. 2009). The initial contact and subsequent uptake of bacterial pathogens into the host cells is believed to be dependent on PI3K pathway and the ensuing downstream signaling in macrophages (Charpentier et al. 2009; Tachado et al. 2008).

It is thus quite plausible that *B. pseudomallei* has evolved as a pathogen that is dependent on the host cell phosphorylation pathways. It may have targeted and exploited the host phosphorylation system as part of its strategy to establish an environment beneficial for replication. Hence, when the GSK3 activity was inhibited, processes known to be GSK3-regulated and crucial in host-pathogen encounter are disrupted. These processes

include cell motility, microtubule function, cell adhesion and inflammation; all are prerequisites to the ingestion and intracellular survival of the pathogen within the macrophage phagocytic vacuoles.

In our attempt to understand the pathogenic mechanisms involved during invasion by this deadly bacteria, our team had previously probed into the cellular determinants deemed critical in the host defense against *B. pseudomallei*. Studies include the resistance of *B. pseudomallei* to the bactericidal action of normal human serum (Ismail et al. 1988), inhibition of protein and DNA synthesis by the bacterial exotoxin (Mohamed et al. 1989) and rapid killing of the bacteria by human polymorphonuclear leukocytes (Razak & Ismail 1982). While our previous investigations gave insights into the events occurring during the microbial-host cell interactions before the establishment of the disease, our present study focused on the events taking place relatively earlier in the infection process. The attenuation of intracellular multiplication of bacteria as a result of GSK3 inhibition leads us to speculate the following sequence of events taking place during early phase of infection by *B. pseudomallei*. Firstly, the pathogen-associated molecular patterns (PAMPs) of *B. pseudomallei* including LPS and lipid A are recognized by the TLR2 and TLR4 of the host cells (West et al. 2008). This is followed by over-production of pro-inflammatory cytokines if GSK3 is not inhibited. Under attack, this imbalance of pro- and anti-inflammatory cytokines renders the host more susceptible to developing the disease melioidosis.

Conclusion

To our knowledge, our study represents the first attempt to elucidate the role of GSK3 in *B. pseudomallei* infection. The results of our investigation brought to light the possible regulatory role of GSK3 in ensuring a proper balance between anti- and pro-inflammatory cytokine productions that must be met to avoid detrimental effects to the host cells and contribute to the progression of melioidosis. GSK3 is thus crucial in the regulation of innate immune responses during *B.pseudomallei* infection. Our initial study here suggests that GSK3 may be a target for novel therapeutic approaches aimed at managing acute and threatening stages of melioidosis. Inhibitors of GSK3, such as LiCl, for example, may be useful in controlling excessive pro-inflammatory response to *B. pseudomallei* infection in advanced and life-threatening melioidosis.

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