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# Glycogen synthase kinase-3β inhibition improved survivability of mice infected with *Burkholderia pseudomallei*

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**Abstract.** The disease melioidosis, caused by the soil bacteria *Burkholderia pseudomallei*, often manifests as acute septicemia with high fatality. Glycogen synthase kinase-3β (GSK3β) plays a key role during the inflammatory response induced by bacteria. We used a murine model of acute melioidosis to investigate the effects of LiCl, a GSK3 inhibitor on experimental animal survivability as well as TNF-α, IL-1β, IFN-γ, IL-10 and IL-1Ra cytokine levels in blood, lung, liver and spleen of *B. pseudomallei*-infected mice. Our results showed that administration of 100 µg/g LiCl improved survivability of mice infected with 5 X $LD_{50}$ of *B. pseudomallei*. Bacterial counts in spleen, liver and lungs of infected mice administered with LiCl were lower than non-treated controls. Our data also revealed that GSK3β is phosphorylated in the spleen, liver and lung of animals infected with *B. pseudomallei*. However in infected animals administered with LiCl, higher levels of pGSK3 were detected in the organs. Levels of pro-inflammatory cytokines (TNF-α, IL-1β and IFN-γ) and anti-inflammatory cytokines (IL-10 and IL-1Ra) in sera and organs tested were elevated significantly following *B. pseudomallei* infection. With GSK3β inhibition, pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β) were significantly decreased in all the samples tested whilst the levels of anti-inflammatory cytokines, IL-10 (spleen and lung) and IL-1Ra (spleen, liver and sera) were further elevated. This study represents the first report implicating GSK3β in the modulation of cytokine production during *B. pseudomallei* infection thus reiterating the important role of GSK3β in the inflammatory response caused by bacterial pathogens.

## INTRODUCTION

The innate immune response to bacterial infection is primarily dependent on the ability of the host to recognize the pathogen or pathogen products. Activation of a family of trans-membrane receptors known as Toll-like receptors (TLRs) (Medzhitov *et al*., 1997, 1998; Yang *et al*., 1998) present on host cells, triggers a network of intra-cellular signaling pathways involved in the inflammatory response which plays a prominent role in host defense against the early phase of bacterial invasion (Kawai & Akira, 2007; Kumar *et al*., 2009). However, it has been increasingly recognized that benefits of this inflammatory response depends on the ability of the host to regulate the nature and magnitude of the diverse cytokines released in the process (Beurel *et al*., 2010).

*Burkholderia pseudomallei*, a Gram-negative soil bacteria, is the causative agent of melioidosis in humans and animals (White, 2003). During infection, different components of the pathogen are recognized by specific TLRs and other related trans-membrane molecules expressed on host cells. Hence, specific proteins including CD14, MD-2, TLR1, TLR2, TLR3, TLR4, TLR5, TLR8 and TLR 10 are up-regulated in patients with melioidosis (Wiersinga *et al*., 2007). In the absence of TLR2, West *et al*. (2008)

demonstrated that heat-killed *B. pseudomallei*, bacterial lipopolysaccharides (LPS) and lipid A are all capable of triggering production of cytokines from macrophages. Furthermore, Novem *et al.* (2009) observed differential stimulation of cytokines *in vitro* including TNF-α, IL-6 and IL-10 by non-pathogenic *B. thailandensis* and pathogenic *B. pseudomallei*. These results suggest the possible role of inflammatory cytokines in the manifestation of melioidosis.

Glycogen synthase kinase-3 (GSK3), a serine/threonine kinase, was originally identified as one of several protein kinases capable of phosphorylating skeletal muscle glycogen synthase (Embi *et al.*, 1980). This enzyme has since been recognized as a multi-tasking kinase capable of participating in a throng of cellular processes (Dugo *et al.*, 2006; Jope *et al.*, 2007). Dysfunction of GSK3 results in a host of common pathological conditions including diabetes, Alzheimer's disease and cancer (Jope & Johnson, 2004; Jope *et al.*, 2007). Glycogen synthase kinase-3 has two isoforms GSK3α and GSK3β. More recently, GSK3β has been recognized in the regulation of many components of the innate and adaptive immune systems and has also been hailed as the control switch for the regulation of inflammatory response, particularly to attain a favorable balance between assortments of pro- and anti-inflammatory cytokines (Cortés-Vieyra *et al.*, 2012). Given the increasing evidence on the importance of inflammatory cytokines in melioidosis and the widely-accepted notion about the central function of GSK3 in the regulation of inflammatory response, we used a murine model to investigate the role of GSK3 in an acute *B. pseudomallei* infection.

## MATERIALS AND METHODS

### Mice

Male BALB/c mice were supplied by the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia. The 6-8 week old mice used for the study were accommodated in Individual Ventilation Cages (IVC) at the Infection Studies Laboratory located in the Animal House Complex, Universiti Kebangsaan Malaysia (UKM) and fed *ad libitum*. The project was approved by the UKM Animal Ethics Committee (UKMAEC) (reference number FST/2010/MOHAMMED/17-MARCH/293-MARCH-2010-DECEMBER-2012).

### Bacteria

Glycerol stock of *B. pseudomallei* strain D286 was obtained from the Pathogen Laboratory at the Faculty of Science and Technology, UKM; a kind gift from Prof. Dr. Sheila Nathan. In this study, the bacteria was grown in Brain Heart Infusion Broth and cultured on Ashdown agar supplemented with gentamicin.

### Animal infection studies (Acute Melioidosis Mouse Model)

Male BALB/c mice (n=10) were injected intraperitoneally with 5 X $LD_{50}$ (24 854 CFU) or 10 X $LD_{50}$ (49 708 CFU) *B. pseudomallei* suspended in 100 µL PBS. In the experiments to evaluate the effects of LiCl on animal survivability, *B. pseudomallei* – infected mice were administered with 50, 100 or 200 µg/g LiCl intraperitoneally one hour before or after infection. For the control group, infected mice were administered with 0.9% NaCl instead. Survivability of animals was monitored over a 14-day period post-inoculation of bacteria.

### Bacterial load

A group of mice (n=3) was infected with 5 X $LD_{50}$ (24 854 CFU) *B. pseudomallei* and subsequently treated with 100 µg/g LiCl at one hour post-infection. The control group consisted of mice infected with *B. pseudomallei* only. Three mice from each group (n=3) were euthanized at days 0, 1, 2, 3 and 4 post-infection. In another experiment, infected mice (n=4) were administered with LiCl as describe in the animal infection studies above. Organ samples were taken from surviving mice at day 14 post-infection after animals were euthanized. Samples were processed as described by Leakey *et al.* (1998). Briefly, spleen, liver and lung were homogenized in 10 mL PBS containing 0.14 M NaCl, 2.7 µM KCl, 0.01 M $Na_2HPO_4$, 1.7 µM $KH_2PO_4$, pH7.4. The homogenate was then

serially diluted with PBS and spotted onto Ashdown agar. Total bacteria in samples were determined as CFU/mL.

**Western Blot**

Organ samples were collected at 2 and 3 hours post-infection. Protein extraction was carried out as described by Wang & Zhu (2003). Organs were homogenized in 1:1 (w/v) extraction buffer containing 9.1 mmol/L $NaH_2PO_4$, 1.7 mmol/L $Na_2HPO_4$, 150 mmol/L NaCl, pH 7.4, 1% IgepalCA-630, 0.5% sodium deoxycholate and 0.1% SDS supplemented with protease inhibitors (1 mM PMSF and 50 µg/mL leupeptin) and phosphatase inhibitors (1 mM $Na_3VO_4$ and 1 mM NaF) followed by incubation on ice for 40 minutes. The samples were then centrifuged at 20 000 g for 30 minutes, 4°C. Aliquots of supernatant were obtained for determination of protein content (Bradford, 1976). The remaining supernatant was diluted with 1:1 (w/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 on 12% resolving gel (Laemmli, 1970). Protein was electro-transferred onto nitrocellulose membranes and probed with specific primary antibodies against total GSK3β (Cell Signaling) or phosphorylated GSK3β (Ser9) (Santa Cruz) followed by HRP-conjugated IgG as a secondary antibody. Detection of immuno-reactive proteins was carried out using ECL western blotting detection reagents (Pierce, USA). β-actin was used as loading control.

**Cytokine assay**

Mice were divided into four groups (n=5) comprising of Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 100 µg/g LiCl (1 hour post-infection); Group III: 100 µg/g LiCl administration only; Group IV: Normal. At day 1 post-infection, mice were euthanized and organs (spleen, liver and lung) excised. Blood was also collected by cardiac puncture and immediately processed to obtain sera. Organs were processed as described by Phelan *et al.* (2002). Briefly, the organs were homogenized in a buffer containing a protease-inhibitor combination (1 mmol/L PMSF, 1 µg/mL pepstatin A and 1 µg/mL leupeptin in phosphate buffered saline solution, pH 7.2), 0.05% sodium azide and 0.5% Triton X-100 in the ratio of 100 mg tissue per mL. The homogenates were then subjected to three rounds of freeze-thaw cycles and incubated at 4°C for one hour. The final homogenate was centrifuged at 12 000 g for 30 minutes. The resultant supernatants and sera were used for cytokine determination, specifically for TNF-α, IFN-γ, IL-1β, IL-10 and IL-1Ra, using cytokine ELISA kits (Ebiosciences, USA).

**Statistical analysis**

Percent survival between animal groups and cytokine assay data were analyzed using GraphPad Prism 5 software. Log rank test was used to evaluate statistical significance for the survival experiments whilst t-test was performed on the cytokine data. Bacterial load was evaluated by Microsoft Office Excel 2007 using t-test at a P value of $<0.05$.

## RESULTS

**LiCl conferred survival advantage to acute *B. pseudomallei*-infected mice**

BALB/c mice infected with a dose of 10 X $LD_{50}$ *B. pseudomallei* (49 708 CFU) or 5 X $LD_{50}$ *B. pseudomallei* (24 854 CFU), succumbed to infection between 2 to 5 days post-infection (Figures 1 and 2). Figure 1 showed that there was no significant difference ($P>0.05$) in mortality rate of infected (10 X $LD_{50}$ *B. pseudomallei*) animals administered with 50 µg/g LiCl compared with *B. pseudomallei*-infected mice (control group). However at higher doses of 100 or 200 µg/g, LiCl administration resulted in improved survivability (30%) of infected animals for both the doses. The results on the rate of animal mortality in *B. pseudomallei*-infected mice showed that acute melioidosis infection was successfully established in the mouse model. This is on the basis that all the animals died between 2-5 days (Leakey *et al.*, 1998) when either of the two doses were employed to infect the animals. In subsequent animal infection studies, we opted to use the lower dose of 5 X $LD_{50}$ in our protocol in

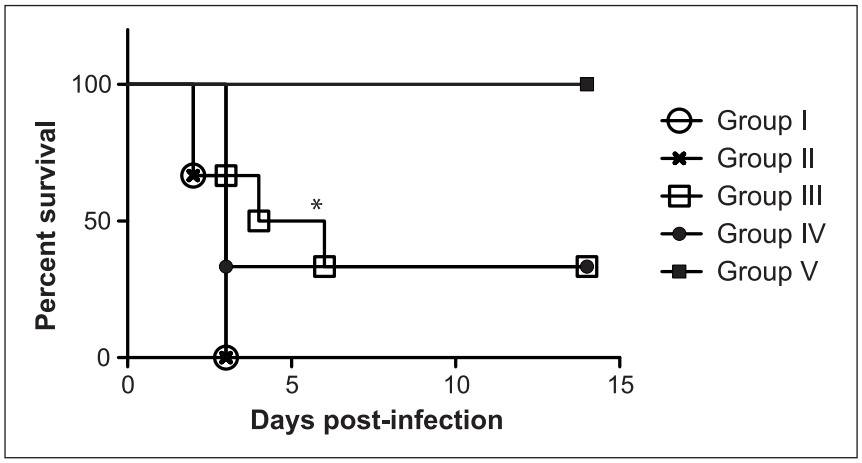



Figure 1. Kaplan-Meier survival curve of mice administered with 10 X 10-day $LD_{50}$ *B. pseudomallei* (49 708 CFU) with and without GSK3 inhibitor (LiCl) treatment at one hour post-infection. Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 50 µg/g LiCl; Group III: *B. pseudomallei* infection + 100 µg/g LiCl; Group IV: *B. pseudomallei* infection + 200 µg/g LiCl; Group V: Normal. Significant difference between tested group and control infected group was evaluated at $P < 0.05$ (*)

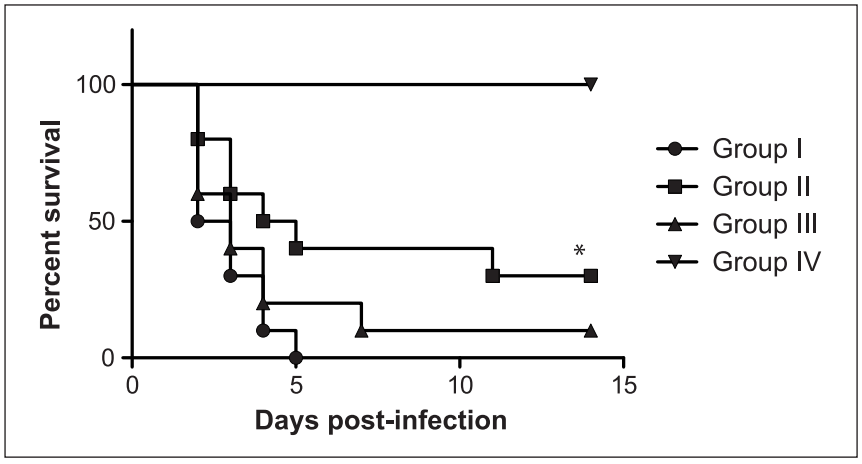



Figure 2. Kaplan-Meier survival curve of mice administered with 5 X 10-day $LD_{50}$ *B. pseudomallei* (24 854 CFU) with and without GSK3 inhibitor (LiCl) treatment at one hour post-infection. Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 100 µg/g LiCl; Group III: *B. pseudomallei* infection + 200 µg/g LiCl; Group IV: Normal. Significant difference between tested group and control infected group was evaluated at $P < 0.05$ (*)

establishing acute melioidosis infection. When 100 µg/g LiCl was administered to *B. pseudomallei*-infected mice (5 X $LD_{50}$), the results obtained showed improved survivability (30%) of animals (Figure 2). When we repeated the survival experiment, the results obtained showed that animals administered with 100 µg/g LiCl post-infection showed improved survivability (60%) compared with non-treated control (Figure 3). However *B. pseudomallei*-infected mice administered with the higher dose of 200 µg/g LiCl post-infection did not show any significant difference in

survivability compared with the control infected group. When 100 µg/g LiCl was administered one hour prior to infection instead of one hour post-infection, survivability was only 30% higher than the control-infected animals (Figure 3). This showed that post-infection administration of LiCl was more effective in improving survivability of *B. pseudomallei*-infected mice. Further tests showed that post-infection administration of 100 µg/g LiCl resulted in significant ($P<0.05$) better survivability (20%) in *B. pseudomallei* infected animals (Figures 4a and 4b). All the above findings from mice infection studies showed that administration of the GSK3 inhibitor, LiCl conferred survival advantage to *B. pseudomallei*-infected mice.

**LiCl reduced bacterial counts in organs of acute *B. pseudomallei*-infected mice**

We further determined the proliferation of *B. pseudomallei* in target organs affected by the bacteria after infection. The bacterial load in selected organs of infected mice were assessed in both LiCl-treated and non-treated animals. Figure 5 showed that bacterial counts in organs excised from acute *B. pseudomallei*-infected mice rapidly increased; until day 3 post-infection for spleen and liver, and until day 2 for lung. In LiCl-treated mice, the bacterial counts in organs also increased upon infection but the counts were significantly lower compared to organs from non-treated mice. In another infection study, similar bacterial load (Table 1) was found in organs of LiCl-administered mice that were still surviving on day 14 post-infection (Figure 6).

**LiCl increased phosphorylation of GSK3β (Ser9) in organs from *B. pseudomallei*-infected mice**

In an effort to investigate whether the observed effects on LiCl on survival and bacterial load in organs of *B. pseudomallei*-infected mice were related to GSK3β activity, we subsequently compared the levels of phosphorylated GSK3β (Ser9) in organs obtained from LiCl-treated and non-treated *B. pseudomallei*-infected animals using western analysis. All organ samples (spleen, liver and lung) excised from LiCl-treated mice showed higher levels of GSK3β phosphorylation (Ser9) compared to samples from *B. pseudomallei*-infected mice (Figure 7).

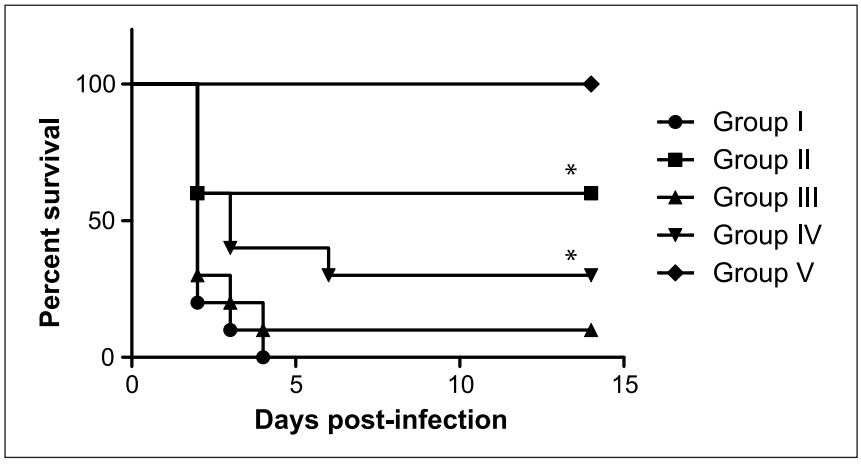



Figure 3. Kaplan-Meier survival curve of mice administered with 5 X 10-day $LD_{50}$ *B. pseudomallei* (24 854 CFU) with and without GSK3 inhibitor (LiCl) treatment at one hour pre- or post-infection. Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 100 µg/g LiCl (1 hour post-infection); Group III: *B. pseudomallei* infection + 200 µg/g LiCl (1 hour post-infection); Group IV: *B. pseudomallei* infection + 100 µg/g LiCl (1 hour pre-infection); Group V: Normal. Significant difference between tested group and control infected group was evaluated at $P < 0.05$ (*)

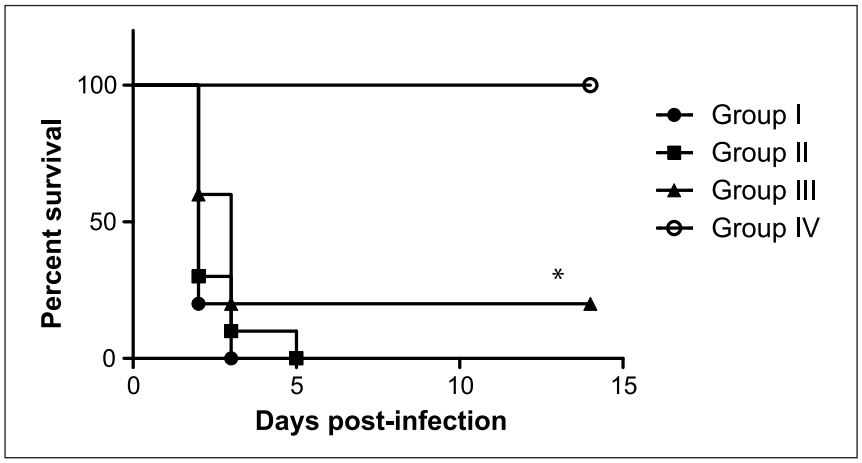



Figure 4a. Kaplan-Meier survival curve of mice administered with 5 X 10-day $LD_{50}$ *B. pseudomallei* (24 854 CFU) with and without GSK3 inhibitor (LiCl) treatment at one hour pre- or post-infection. Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 100 µg/g LiCl (1 hour pre-infection); Group III: *B. pseudomallei* infection + 100 µg/g LiCl (1 hour post-infection); Group IV: Normal. Significant difference between tested group and control infected group was evaluated at $P < 0.05$ (*)

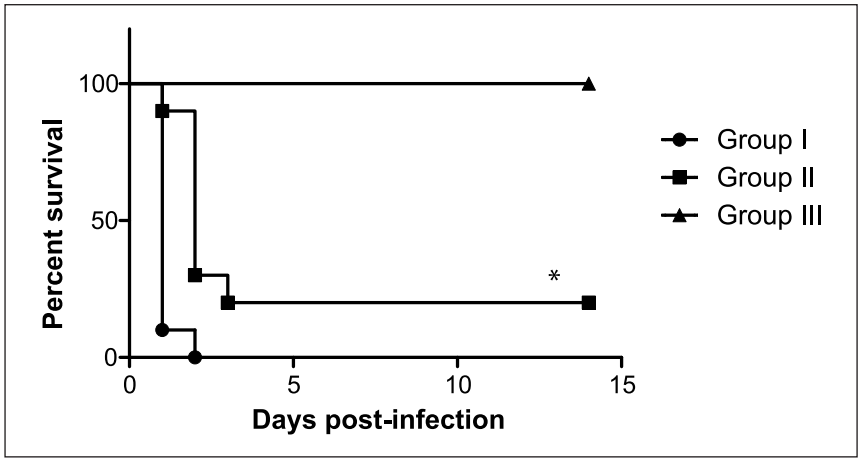



Figure 4b. Kaplan-Meier survival curve of mice administered with 5 X 10-day $LD_{50}$ *B. pseudomallei* (24 854 CFU) with and without LiCl treatment at one hour post-infection. Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 100 µg/g LiCl (1 hour post-infection); Group III: Normal. Significant difference between tested group and control infected group was evaluated at $P < 0.05$ (*)

Table 1. Bp load of surviving LiCl-treated infected mice at day 14 post-infection (n=4). Data is from triplicate analysis with mean ± SE. The corresponding survival curve is shown in Figure 6

| | Spleen | Liver | Lung |
|---|---|---|---|
| Log (CFU/mL) | 7.147 ± 0.735 | 6.767 ± 0.359 | 4.574 ± 0.246 |

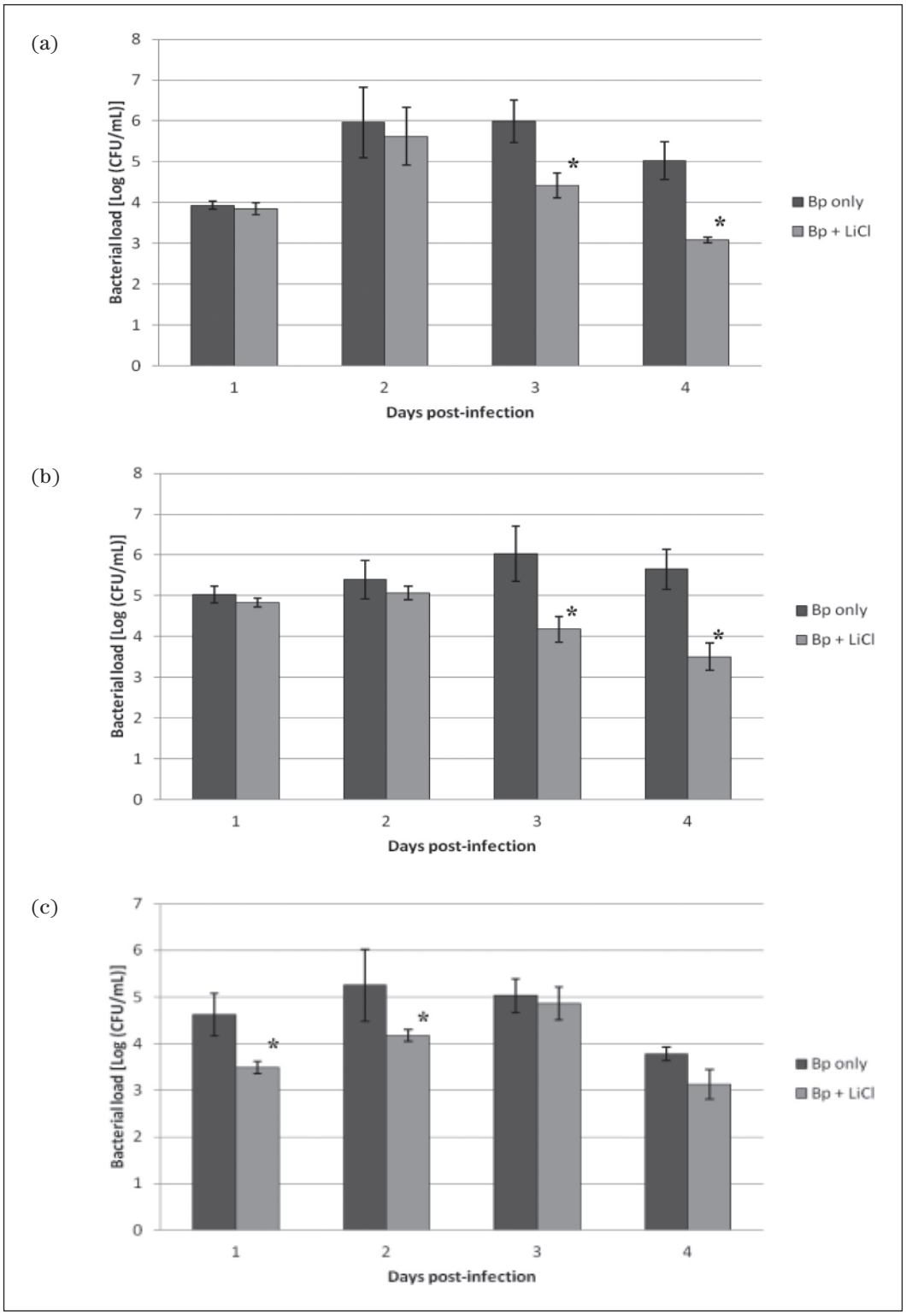



Figure 5. Bacterial load day 1 to day 4 post-infection in (a) spleen (b) liver and (c) lung of mice administered with 5 X 10-day $LD_{50}$ *B. pseudomallei* (24 854 CFU) with or without GSK inhibitor (LiCl) treatment at one hour post-infection. Significant difference between treated group and control infected group was evaluated at $P < 0.05$ (*)

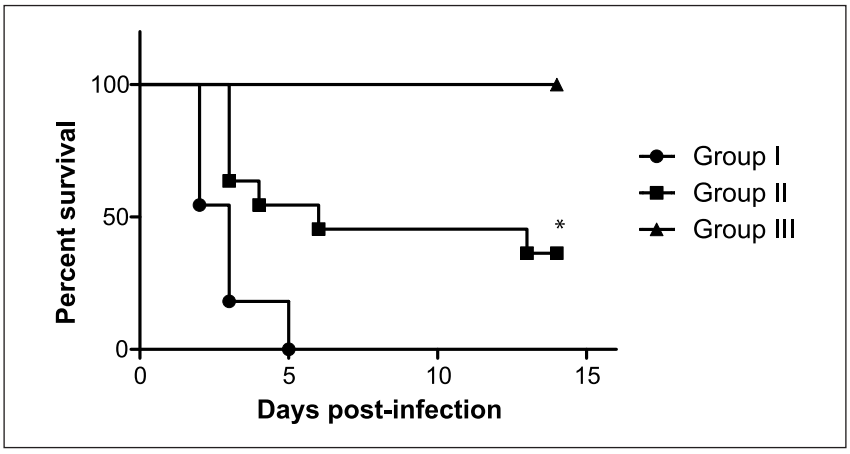



Figure 6. Kaplan-Meier survival curve of mice administered with 5 X 10-day $LD_{50}$ *B. pseudomallei* (24 854 CFU) with and without GSK3 inhibitor (LiCl) treatment at one hour post-infection. Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 100 µg/g LiCl (1 hour post-infection); Group III: Normal. Significant difference between tested group and control infected group was evaluated at $P < 0.05$ (*)

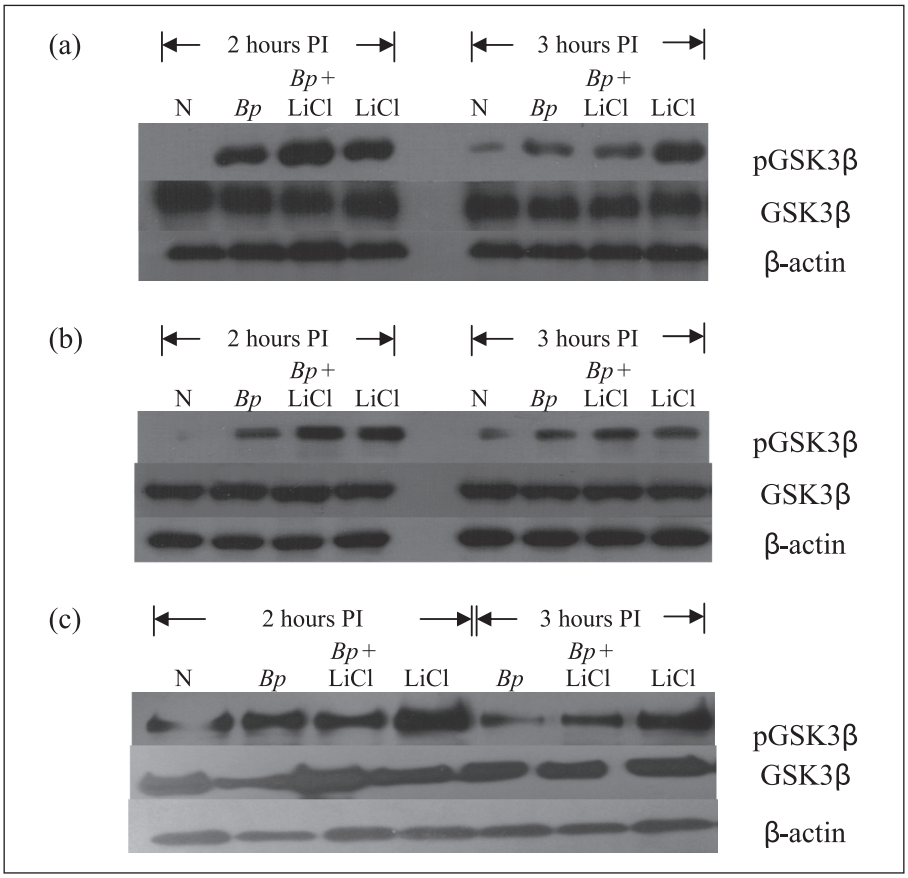



Figure 7. GSK3β, pGSK3β (Ser9) and β-actin of normal (N), infected (*Bp*), LiCl-treated infected (*Bp*+LiCl) and LiCl-treated uninfected (LiCl) mouse organs (a) spleen (b) liver and (c) lung at three different time points (2 and 3 hours post-infection PI)

**Effects of LiCl administration on the levels of pro- and anti-inflammatory cytokines in *B. pseudomallei*-infected mouse organs**

To determine the effects of LiCl administration on the cytokine levels during acute *B. pseudomallei* infection, we carried out experiments to determine the levels of pro-inflammatory cytokines (TNF-α, IFN-γ and IL-1β) and anti-inflammatory cytokines (IL-10 and IL-1Ra) in organs and sera of infected mice with or without LiCl treatment (Figure 8).

*Burkholderia pseudomallei* infection elevated the levels of TNF-α, IFN-γ and IL-1β by up to 1.92-15.78 fold compared to non-infected controls in all samples analyzed (Figures 8a, b and c). These elevated levels of pro-inflammatory cytokines were however, reduced ($P<0.05$) with LiCl treatment (Figures 8a, b and c) thus possibly explaining the improved survivability of *B. pseudomallei*-infected mice administered with LiCl described above (Figures 1, 2, 3 and 4). Mortality in mice acutely infected with *B. pseudomallei* has been shown to be associated with overwhelming levels of pro-inflammatory cytokines.

The effect of GSK3 inhibition (by LiCl) on anti-inflammatory cytokines measured in this study was however not as clear-cut as that obtained for pro-inflammatory cytokines. The levels of IL-10 were up to 2.22-2.76 folds higher in all samples obtained from infected animals (except for lung) as compared to that from non-infected controls (Figure 8d). The level of this anti-inflammatory cytokine in lung was similar to control ($P>0.05$). IL-10 levels were however, raised with LiCl administration into the animals; but only in spleen and lung samples (Figure 8d).

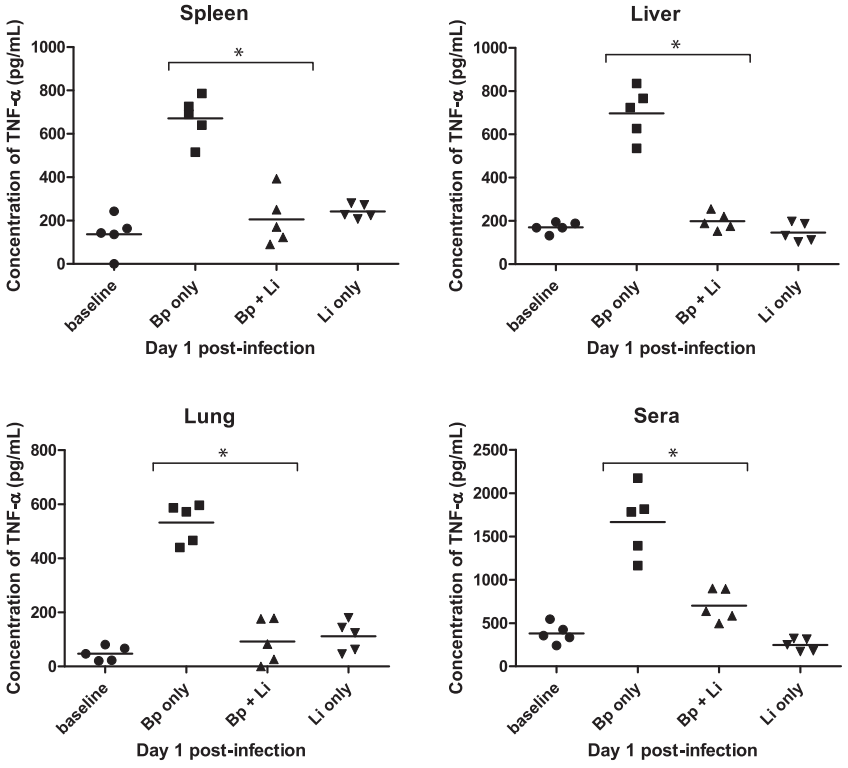
The levels of another anti-inflammatory cytokine measured in this experiment, IL-1 receptor antagonist (IL-1Ra), were also raised with *B. pseudomallei* infection; this time in all samples tested. Further elevations of IL-1Ra were observed in spleen, liver and sera samples upon LiCl administrations. In contrast, administration of the GSK3 inhibitor, LiCl into infected animals decreased lung IL-1Ra. Further increase of anti-inflammatory cytokine levels as a result of LiCl administration could possibly be a contributing factor in attenuating the harmful effects of pro-inflammatory cytokines; consequently the increase in the survivability observed in LiCl-treated *B. pseudomallei*-infected mice.

## DISCUSSION

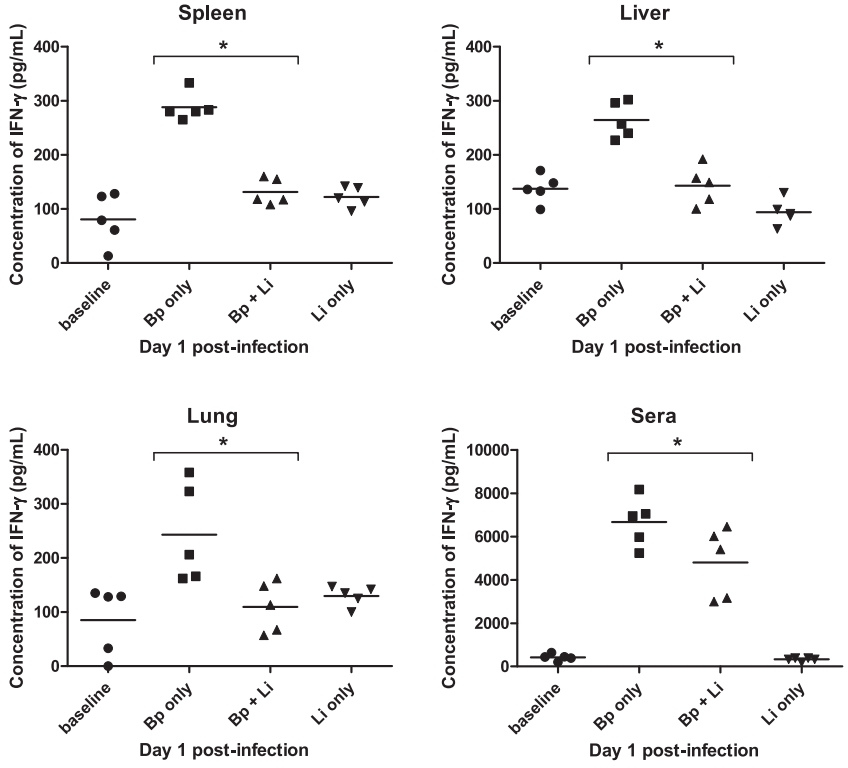
The enzyme GSK3β has been implicated in a number of bacterial infections including infections by *Francisella tularensis* and *Salmonella typhimurium*, through its central role in the regulation of host innate inflammatory response (Duan *et al.*, 2007; Zhang *et al.*, 2009). In *Salmonella* infection, the host GSK3β activity was found to increase resulting in the elevated production of pro-inflammatory cytokine IL-8 (Duan *et al.*, 2007). However, following inhibition of GSK3β by LiCl, IL-8 secretion was reported to be suppressed. In *F. tularensis* infection, inhibition of GSK3β by LiCl significantly reduced production of pro-inflammatory cytokines IL-6, IL-12p40 and TNF-α; but resulted in the increase in anti-inflammatory cytokine IL-10 production (Zhang *et al.*, 2009). The regulatory role of GSK3β in modulating the nature and magnitude of cytokines being secreted appears to give distinct survival advantage to the host under assault by bacterial pathogens. This could explain why *F. tularensis*-infected mice administered with LiCl survived longer than control non-treated animals (Zhang *et al.*, 2009). In another study, mice administered with GSK3 inhibitor and subsequently challenged with *E. coli* lipopolysaccharides were protected from the expected manifestation of endotoxic shock (Ko *et al.*, 2010). The above observations lend more evidence to the notion that the suppressed release of pro-inflammatory cytokines during innate immune response could be beneficial to the host during bacterial infections.

We investigated the effect of GSK3β inhibition and showed improved survivability of mice infected with 5 X $LD_{50}$ of *B. pseudomallei*. This observation was further espoused by significantly lowered bacterial counts in the infected spleen, liver and lungs

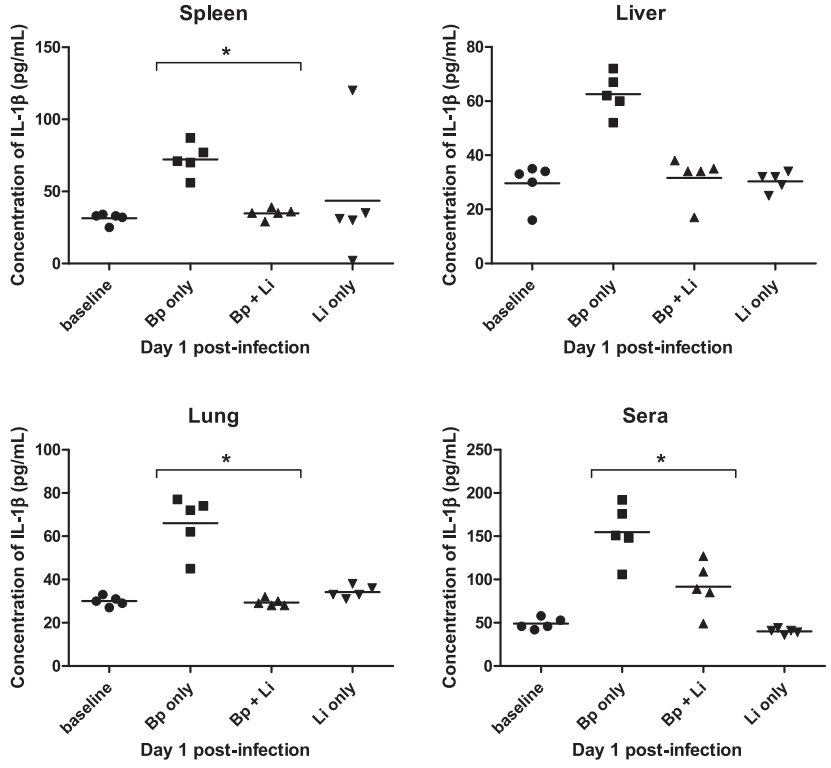
(a) TNF-α


Spleen
Concentration of TNF-α (pg/mL)
1000
800
600
400
200
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
Liver
Concentration of TNF-α (pg/mL)
1000
800
600
400
200
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
Lung
Concentration of TNF-α (pg/mL)
800
600
400
200
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
Sera
Concentration of TNF-α (pg/mL)
2500
2000
1500
1000
500
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection

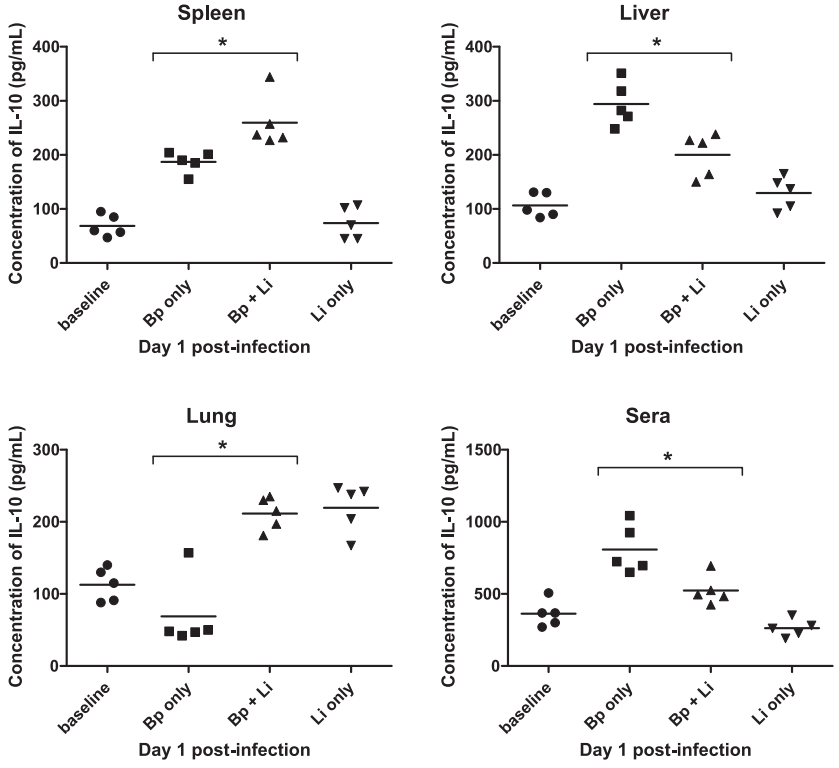
(b) IFN-γ


Spleen
Concentration of IFN-γ (pg/mL)
400
300
200
100
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
Liver
Concentration of IFN-γ (pg/mL)
400
300
200
100
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
Lung
Concentration of IFN-γ (pg/mL)
400
300
200
100
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
Sera
Concentration of IFN-γ (pg/mL)
10000
8000
6000
4000
2000
0
*
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection

(c) IL-1β


Spleen
Liver
Lung
Sera
Concentration of IL-1β (pg/mL)
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
*

(d) IL-10


Spleen
Liver
Lung
Sera
Concentration of IL-10 (pg/mL)
baseline
Bp only
Bp + Li
Li only
Day 1 post-infection
*

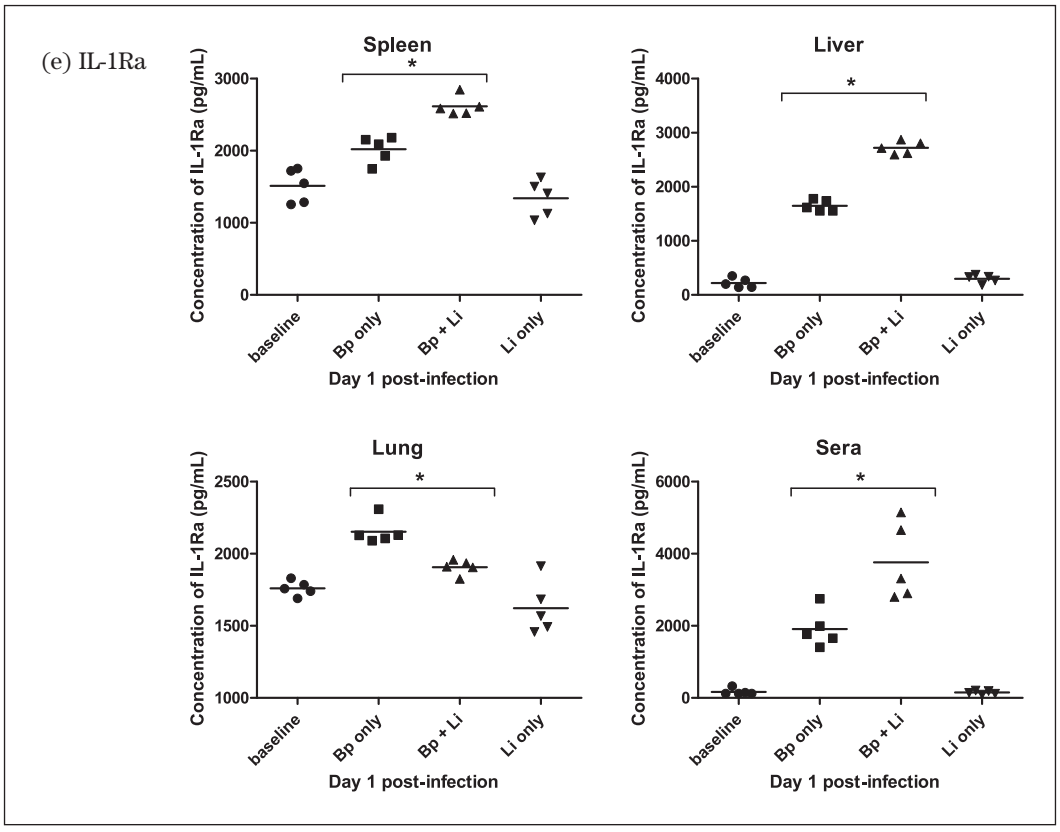



Figure 8. The levels of cytokines (a) TNF-α (b) IFN-γ (c) IL-1β (d) IL-10 and (e) IL-1Ra in mouse organs (spleen, liver and lung) and sera administered with 5 X 10-day $LD_{50}$ *B. pseudomallei* (24 854 CFU) with or without GSK3 inhibitor (100 µg/g LiCl) treatment at one hour post-infection. Group I: Normal (acts as a baseline); Group II: *B. pseudomallei* infection only; Group III: *B. pseudomallei* infection + 100 µg/g LiCl; Group IV: 100 µg/g LiCl only. Significant difference as compared with control infected group was evaluated at $P < 0.05$ (*)

of LiCl-administered mice. *B. pseudomallei* infection was not completely suppressed by LiCl administration of the infected animals as seen from the CFU in the organs of the surviving mice on day 14 post-infection. The improved survivability of the LiCl-treated animals was likely attributed to the inhibition of GSK3β and consequent modulation of the inflammatory response to the infection.

LiCl inhibits the activity of GSK3β by increasing the phosphorylation of Ser9 residue of the kinase (Sinha *et al.*, 2005). This inhibition is thought to occur through direct competition for magnesium ions required for enzyme activity (Ryves & Harwood, 2001); or indirectly by causing the increase in phosphorylation of serine residues (Zhang *et al.*, 2003). Our data revealed that phosphorylation of GSK3β occurred in the three mouse organs analyzed (spleen, liver and lungs), as early as two hours after infection with *B. pseudomallei*. These results showed that LiCl resulted in the inhibition of GSK3β (Sinha *et al.*, 2005) and consequently explain why *B. pseudomallei*-infected mice administered with LiCl survived longer than control non-treated animals. It is widely accepted that GSK3β inhibition is important in modulating the production of pro- and anti-inflammatory cytokines as a result of *B. pseudomallei* infection (Wang *et al.*, 2011).

Interleukin 1β (IL-1β) is one of the major pro-inflammatory cytokines known to be produced in sepsis (Cannon *et al.*, 1990). Excessive acute activation of the IL-1β system contributes to multi-organ failure

caused by sepsis (Cohen, 2002). TNF-α is a pro-inflammatory cytokine (Dinarello 2000) produced during bacterial infection, injury, or other microbial invasions (Kelvin *et al.*, 1994). Excess secretion of TNF-α causes endotoxic shock, an often fatal complication of infection (Wright *et al.*, 1990). This cytokine is known to have profound effects on numerous cell types that, in turn, secrete a variety of inflammatory mediators, creating a complex network of interactions and inflammatory cascades (Dinarello, 1996; Thompson & Lotze, 1999). IFN-γ is an efficient amplificatory cytokine produced by T-lymphocytes in response to IL-12, produced by monocytes/macrophages activated by microbial products (Redl & Schlag, 1999). IFN-γ is able to increase the level of LPS-induced plasma TNF-α and also increase LPS- or TNF-α-induced mortality (Doherty *et al.*, 1992; Bundschuh *et al.*, 1997).

Improved survivability and reduction in pro-inflammatory cytokine levels in *B. pseudomallei*-infected mice with GSK3β inhibition observed in the present study implicate the involvement of this kinase in the modulation of excessive pro-inflammatory response. For example, in LPS-stimulated non-cirrhotic immune cells, constitutively active GSK3β favors production of pro-inflammatory cytokines. On the other hand, GSK3β may promote or inhibit inflammation depending on the types of pathogen and host cells (Cortés-Vieyra *et al.*, 2012). The present study concurs with findings of Zhang *et al.* (2009) which showed that LiCl was able to reduce production of pro-inflammatory cytokines in *F. tularensis*-infected murine macrophages.

IL-10 is an important anti-inflammatory cytokine (Opal & DePalo, 2000) which inhibits nuclear translocation of nuclear factor-κB (NF-κB) following LPS stimulation (Clarke *et al.*, 1998) and promotes degradation of messenger RNA for pro-inflammatory cytokines (Opal *et al.*, 1998). During inflammatory response, IL-10 serves to attenuate the harmful effects of pro-inflammatory cytokines. Thus, physiologically an inadequate production of IL-10 after a systemic injury or infection could well result in detrimental consequences to the host (Opal & DePalo, 2000). IL-1Ra is an anti-inflammatory cytokine which antagonizes the effects of IL-1β and IL-1α (Gary *et al.*, 2009). Early treatment with IL-1Ra reduced mortality from endotoxic shock (Li *et al.*, 1995) and improved survival of animals from *E. coli*-induced septic shock (Fischer *et al.*, 1992). Thus any imbalance of pro- and anti-inflammatory cytokines renders the host more susceptible to disease development.

It is noteworthy that results obtained from the study showed decreased levels of IL-10 in the liver and sera of LiCl-administered infected mice. A plausible explanation for this is that GSK3β may promote or inhibit inflammation depending on the types of pathogen and host cells (Cortés-Vieyra *et al.*, 2012), hence levels of anti-inflammatory cytokines may not necessarily increase in all organs.

At the cellular level, inhibition of GSK3β by LiCl reduces the release of pro-inflammatory cytokines including IL12p40, IL-6, TNF-α and IFN-γ (Martin *et al.*, 2005). Evidences are accumulating on the differential effects of GSK3β inhibition on the secretion of pro- and anti-inflammatory cytokines. For example, in infections involving *S. typhimurium* (Duan *et al.*, 2007), *F. tularensis* (Zhang *et al.*, 2009), and enteroviruses (Wong *et al.*, 2005), production of pro-inflammatory cytokines decreases whilst levels of anti-inflammatory cytokines IL-10 increases with GSK3β inhibition. This shows that the host inflammatory response to microbial infections is complex and may vary according to the invading pathogens and innate response requires the engagement and articulation of a variety of bio-signaling pathways triggered downstream of TLRs.

Under normal circumstances, GSK3β is capable of regulating the production of pro- and anti-inflammatory cytokines without compromising the integrity of the host innate immune response. The balance of this cytokine response is well articulated with respect to the nature and magnitude of different cytokines released. An excessive production of either pro- or anti-inflammatory cytokines could only result in detrimental

instead of beneficial effects to the host. It is also tempting to speculate that death of high risk melioidosis patients with tendencies to develop sepsis and sepsis-induced organ failures may have resulted from such markedly imbalanced cytokine response.

In our attempt to understand the pathogenic mechanisms involved during *B. pseudomallei* invasion, previous studies from our laboratory had probed into the cellular determinants believed 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 *et al.*, 1982). While our previous investigations gave insights into the events occurring during the microbial-host cell interactions before establishment of the disease, the present study focused on the early events of the infection process. Our results on the effects of GSK3β inhibition by LiCl and the increase in survivability of infected mice suggest the following sequence of events during the early phase of infection. 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 phosphorylation and consequent inhibition of GSK3β, resulting in the production of inflammatory cytokines. Our findings showed that IL-10 (spleen, liver and sera) and IL-1Ra (spleen, liver, lung and sera) levels in *B. pseudomallei*-infected mice were elevated during the initial phase of invasion. However, the infected animals succumbed to the infection even though IL-10 could attenuate the harmful effect of pro-inflammatory cytokines. It could be that the production of anti-inflammatory cytokines, IL-10 and IL-1Ra were not high enough to suppress the harmful effects of pro-inflammatory cytokines. This imbalance of pro- and anti-inflammatory cytokines renders the host more susceptible to developing the disease, hence the mortality observed in our murine model for acute melioidosis. In LiCl-administered infected mice, where more pronounced pGSK3 were detected in the organs, the levels of IL-10 (in spleen and lung); and IL-1Ra (in spleen, liver and sera), were however, much higher than in organs of infected animals that were not administered with LiCl. This difference in the level of GSK3 inhibition may be a contributing factor for the improved survivability of animals in this LiCl-administered group.

In conclusion, in the early phase of *B. pseudomallei* infection, the role of GSK3 is crucial in achieving and maintaining a balance of cytokine response. Hence determining and maintaining a balanced profile of pro-inflammatory cytokines in the host is crucial and GSK3 may be a plausible novel target for acute melioidosis infection.

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