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GSK3β inhibition confers survival advantage in *Burkholderia pseudomallei*-infected hyperglycaemic mice by regulating inflammatory response

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Abstract. *Burkholderia pseudomallei*, the etiologic agent of melioidosis is a common cause of sepsis mainly in diabetic individuals in South East Asia. Glycogen synthase kinase-3β (GSK3β) plays a pivotal role in modulating inflammatory balance in Gram-negative bacterial infections. In this study, we demonstrate that inhibition of GSK3β significantly improved survival of hyperglycaemic mice acutely infected with *B. pseudomallei*. With GSK3β inhibition, we found significant modulation between pro- (IL-12, TNF-alpha) and anti-inflammatory (IL-10) serum cytokines which may have contributed to bacterial clearance in multiple organs of *B. pseudomallei*-infected hyperglycaemic mice. Concurrently, an increase in phosphorylation of GSK3β at Ser-9 was observed in the liver of *B. pseudomallei*-infected hyperglycaemic mice. Likewise, *B. pseudomallei*-infected non-hyperglycaemic mice upon GSK3β inhibition showed similar trends of bacterial clearance and modulation of serum cytokines; however, the effect of enhanced survival was less substantial than in infected hyperglycaemic mice. Taken together, we demonstrate that inhibition of GSK3β confers survival advantage of hyperglycaemic mice infected with *B. pseudomallei* and offers a potential therapeutic strategy for the treatment of diabetic patients with melioidosis.

INTRODUCTION

B. pseudomallei is a soil saprophyte and is the etiologic agent of melioidosis, an endemic disease in Southeast Asia and Northern Australia. Melioidosis is a major cause for sepsis-related deaths (30-50%) in northern Thailand (Currie *et al.*, 2010; Limmathurotsakul *et al.*, 2010; Wiersinga *et al.*, 2006); with 50% of melioidosis patients comprising individuals with type 2 diabetes. Diabetic individuals with symptoms of acute melioidosis rapidly succumb to infection within 24 hours, even before receiving antibiotic treatment (Currie, 2015).

Several studies have identified the factors of susceptibility of diabetic host to melioidosis (Chanchamroen *et al.*, 2009; Hodgson *et al.*, 2011; Morris *et al.*, 2012; Hodgson *et al.*, 2014). Although delayed activation of the innate immune system is a factor associated with susceptibility of diabetic host to melioidosis (Chin *et al.*, 2012), further research into the dysregulated immune mechanisms contributing to the impaired host-pathogen interactions is vital. Therefore, a more thorough understanding of diabetic host susceptibility mechanism is crucial in targeting the host immunomodulatory system to reduce sepsis due to excessive inflammation during acute melioidosis.

Although the production of inflammatory cytokines plays an important role in early host protection against pathogenic invaders, the inability to modulate the type, magnitude

and duration of the host inflammatory response often affects the host, as observed in chronic inflammatory diseases (Henriksen, 2010). A balance between pro- and anti-inflammatory cytokine production which is mediated by signalling pathways involved in innate immunity is crucial in order to avoid septic shock-related deaths due to uncontrolled inflammation (Cortés-Vieyra *et al.*, 2012; Gan, 2005). Uncontrolled inflammation allows *B. pseudomallei* to survive in the host cell for a long period of time; hence the response of innate immune system is very important to overcome infection (Gan, 2005).

The PI3K/Akt signalling pathway, in which glycogen synthase kinase-3 (GSK3) is a downstream component, is often associated with control of the production of inflammatory cytokines (Martin *et al.*, 2005). GSK3 was initially identified more than three decades ago as a kinase involved in insulin signalling (Embi *et al.*, 1980; Henriksen, 2010). The kinase is now known to be also involved in various other cellular processes including the regulation of host inflammatory response during pathogenic infection (Beurel *et al.*, 2010). Inhibition of GSK3β has been reported to control the excessive inflammatory response and protect the host from death during *Francisella tularensis* infection (Zhang *et al.*, 2009) besides regulating inflammatory response towards Sendai virus, *Shigella flexneri*, *Streptococcus agalactiae*, *Staphylococcus aureus* and *Mycobacterium bovis* infections (Pendaries *et al.*, 2006; Chan *et al.*, 2009; Oviedo-Boyso *et al.*, 2011; Chang *et al.*, 2013; Lutay *et al.*, 2014).

Recently, we reported that inhibition of GSK3β not only decreased the number of intracellular bacterial load, but also modulated the levels of inflammatory cytokines in peripheral blood mononuclear cells (PBMC) derived from hyperglycaemic animals by lowering the activity of NFκβ (Maniam *et al.*, 2015). We also showed that inhibition of GSK3β by lithium chloride (LiCl) was able to improve survival of *B. pseudomallei*-infected non-diabetic BALB/c mice by balancing the levels of pro- and anti-inflammatory cytokines (Tay *et al.*, 2012). These studies are now extended to evaluate the effects of GSK3β inhibition on susceptibility of hyperglycaemic mice to *B. pseudomallei* infection. We show here that GSK3β inhibition using LiCl, a potent GSK3β inhibitor in *B. pseudomallei*-infected hyperglycaemic mice significantly reduced bacterial load in multiple organs by modulating cytokines, thus improving survival.

MATERIALS AND METHODS

Bacteria culture

B. pseudomallei D286 strain (a kind gift from Prof. Sheila Nathan, School of Biosciences and Biotechnology, Universiti Kebangsaan Malaysia) was used for all experiments. Bacteria were cultured and maintained as previously described (Chin *et al.*, 2012; Tay *et al.*, 2012).

Animals

BALB/c mice were used throughout the study because the course of infection in BALB/c mice was similar to that which occurs in acute human infection (Leakey *et al.*, 1998). Male BALB/c mice (5-6 weeks old) were obtained from the Institute of Medical Research, Malaysia. Mice were housed in individually ventilated cages at the Infection Studies Laboratory, Animal House Complex, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. The project was approved by the UKM Animal Ethics Committee (reference number FST/2012/NOOR/21-NOV./465-DEC.2012-DEC.-2014).

Streptozotocin-induced hyperglycaemia in animals

To induce hyperglycaemia in experimental animals, BALB/c mice were injected with 45 mg/kg body weight (b.w) streptozotocin (STZ) intraperitoneally (IP) for six consecutive days (Leiter, 1982). Non-hyperglycaemic mice received placebo IP injection of 0.1 M sodium citrate buffer (pH 4.5). On day 10 after the first STZ injection, peripheral blood was obtained from lateral

vein and glucose levels determined using an Accu-chek Active Blood Glucose Monitor (Roche Diagnostics, Germany). Mice with glucose levels exceeding 13 mmol/L were considered hyperglycaemic (Williams *et al.*, 2011). Mice remained hyperglycaemic for 3-5 days before inoculation with bacteria.

Animal infection studies

Mice were inoculated through IP with 4X LD_{50} (7.2 x 10^4 CFU) of *B. pseudomallei*. For the purpose of GSK3β inhibition studies, animals were administered with 100 mg/kg b.w LiCl through IP at one hour post-infection (Zhang *et al.*, 2009; Tay *et al.*, 2012). Survival of BALB/c mice was monitored daily for a duration of ten days post-infection (Liu *et al.*, 2002; Barnes *et al.*, 2008).

Bacterial load determination

Mice were euthanised at days 1, 2, 3 and 4 post-infection to obtain liver, spleen and lung from each mouse. Samples were processed as described previously (Leakey *et al.*, 1998).

Cytokine analysis

Peripheral blood was collected from BALB/c mice at 24 hours post-infection and processed immediately to obtain sera as described previously (Zhou *et al.*, 2010). Levels of IL-10, IL-12 and TNF-α were determined using mouse enzyme-linked immunosorbent assay kits as per manufacturer instructions (eBioscience, USA).

Western blotting

Liver samples were collected at 24 hours post-infection. Liver tissue lysates were subjected to 12.5% SDS-PAGE and electro-transferred to nitrocellulose membrane as described previously (Yeh *et al.*, 1997). The membrane was then probed with specific primary antibodies against total GSK3β or phosphorylated GSK3β (Ser9) (Cell Signalling Technology, USA) followed by HRP-conjugated IgG as a secondary antibody and visualised by an ECL kit (Pierce, USA). β-actin was used as a loading control. Densitometric analysis of immunoreactive bands was conducted using BIO-1D software (Vilber-Lourmat, Germany).

Statistical analysis

Statistical analysis was performed using GraphPad Prism v6.0. Significant differences between experimental groups were determined using log-rank test and Kaplan-Meier survival curves were plotted to indicate surviving animals at each time point. Data from cytokine analysis was analysed using two-way ANOVA measures. All other data were compared using Student's t-test. Comparisons were significant at $P < 0.05$.

RESULTS

GSK3β inhibition significantly conferred survival advantage in *B. pseudomallei*-infected hyperglycaemic mice

The BALB/c mice used in this study are well-known animal models frequently used to study acute infections; hence innately susceptible to *B. pseudomallei* infection. BALB/c mice are reported to succumb from disease within 96 hours after *B. pseudomallei* infection (Leakey *et al.*, 1998). In the present study, the median survival of hyperglycaemic and non-hyperglycaemic BALB/c mice was 2 and 2.5 days respectively after *B. pseudomallei* infection. The insignificant differences of survival between the infected hyperglycaemic and non-hyperglycaemic mice from this study were an expected condition since the BALB/c mice is an innately susceptible model to *B. pseudomallei* infection; therefore hyperglycaemic state has no effect on survival.

We have previously shown that GSK3β inhibition significantly improved survival of *B. pseudomallei*-infected non-hyperglycaemic mice (Tay *et al.*, 2012). In order to compare the selectivity of GSK3β inhibition on survival of *B. pseudomallei*-infected hyperglycaemic and non-hyperglycaemic mice, we treated cohorts of mice using a selective GSK3β inhibitor LiCl at one hour post-infection. Lithium, a medication for bipolar disorder is a direct or indirect inhibitor of GSK3 and widely used in multiple disease settings (Freland and Beaulieu, 2012). We found that administration of GSK3β inhibitor LiCl did not alter glucose

levels in both hyperglycaemic and non-hyperglycaemic mice (Fig. 1A). However, GSK3β inhibition significantly improved survival of hyperglycaemic (median survival 9.5 days) as well as non-hyperglycaemic infected mice (median survival 5 days) compared to infected mice without treatment (Fig. 1B-C). Likewise, GSK3β inhibition-mediated improved survival was significantly more pronounced in infected hyperglycaemic mice compared to infected non-hyperglycaemic mice by day five, where almost 70% of infected non-hyperglycaemic mice were dead compared to 100% survival in infected hyperglycaemic mice ($P < 0.001$). Thus, our data strongly suggest the involvement of GSK3β in the susceptibility of hyperglycaemic host to *B. pseudomallei* infection.

GSK3β inhibition enhanced bacterial clearance in organs of *B. pseudomallei*-infected mice

Previously, we have shown that inhibition of GSK3β enhances bacterial clearance in PBMC of hyperglycaemic rat (Maniam *et al.*, 2015) which is in parallel with the improved survival of *B. pseudomallei* infected hyperglycaemic mice in this study. In order to further examine the effects of GSK3β inhibition in host antibacterial defence, we harvested multiple organs from hyperglycaemic and non-hyperglycaemic mice on days 1, 2, 3 and 4 post-infection. We found an increasing trend in bacterial load from day 1 to day 4 post-infection in multiple organs (Fig. 2A-C), demonstrating the ability of *B. pseudomallei* to invade and replicate in both hyperglycaemic and non-hyperglycaemic mice.

All organs of hyperglycaemic mice examined showed significantly more CFU count than organs of non-hyperglycaemic mice on day 1 post-infection (Fig. 2), which confirmed acute septicaemic melioidosis was successfully established in hyperglycaemic animals (Chin *et al.*, 2010). As observed from the survival plot in Figure 1, 70% of hyperglycaemic mice succumbed to infection within 2 days post-infection.

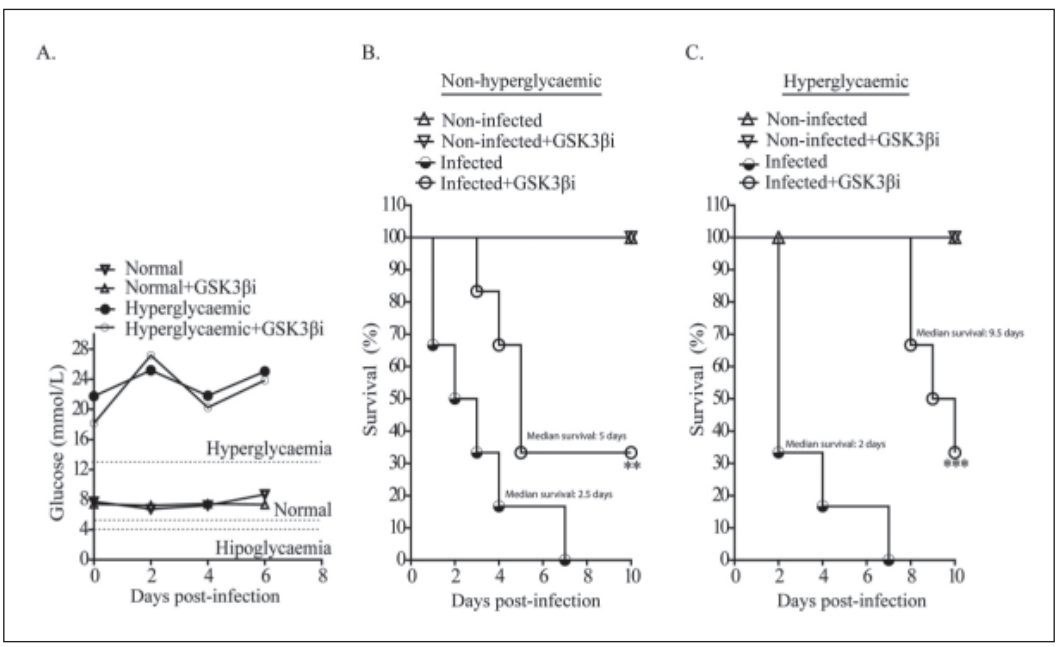

Figure 1. GSK3β inhibition prolonged survival of *B. pseudomallei*-infected hyperglycaemic mice.
A. Blood glucose levels of non-infected hyperglycaemic and non-hyperglycaemic mice in the presence or absence of GSK3β inhibitor LiCl. **B-C**. Kaplan-Meier survival analysis of non-hyperglycaemic (B) and hyperglycaemic (C) mice (n=7) infected with 4X 10-day LD_{50} *B. pseudomallei* with and without GSK3β inhibitor LiCl (100 mg/kg b.w.) treatment at one hour post-infection. Data shown is a representative of three independent experiments. **denotes significant value at $P < 0.01$ and *** $P < 0.001$.

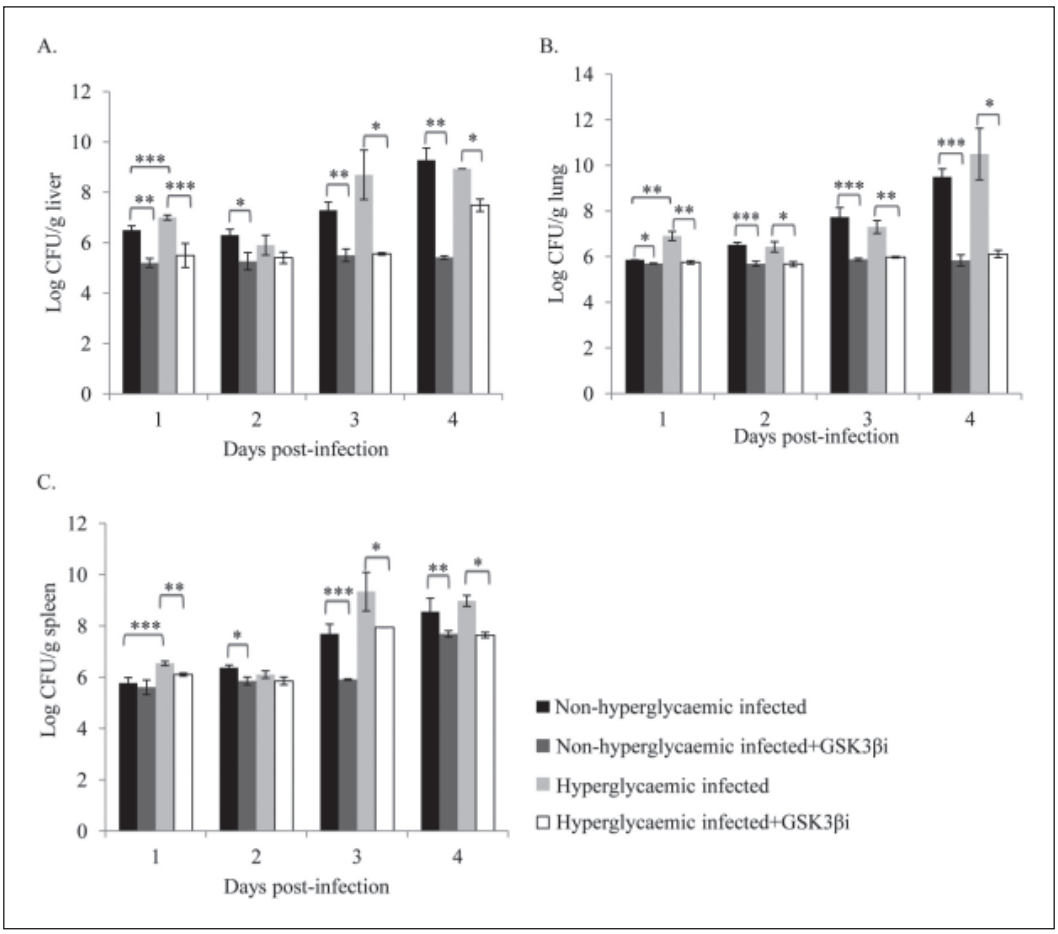

Figure 2. Inhibition of GSK3β decreased bacterial loads in organs of *B. pseudomallei*-infected hyperglycaemic and non-hyperglycaemic BALB/c mice.
Mice (n=3) were infected with *B. pseudomallei* and intraperitoneally injected with LiCl (100 mg/kg) at 1 hour post-infection. Mice were euthanised on Day 1, 2, 3 and 4 and liver (A), lung (B) and spleen (C) were excised aseptically to determine bacterial loads by serial dilution plating on Ashdown agar. Data represents one of three independent experiments. * denotes significant value at $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Therefore, the significantly higher bacterial count in organs of hyperglycaemic mice may be an indication of the animals succumbing to infection quicker than the non-hyperglycaemic mice with the acute phase of infection.

Interestingly, GSK3β inhibition significantly decreased bacterial load throughout day 1 to day 4 post-infection in all organs of hyperglycaemic and non-hyperglycaemic infected mice (Fig. 2), suggesting the mechanism of protection mediated via GSK3β inhibition may be partly due to increased bacterial clearance in the organs of infected animals. However, we were not able to correlate the bacterial clearance with pronounced increased survival of LiCl treated hyperglycaemic mice as compared to that in non-hyperglycaemic mice since the cohorts exhibited similar clearance profiles.

GSK3β inhibition modulated levels of cytokines in *B. pseudomallei*-infected hyperglycaemic mice

Poor control of the innate immune response during early *B. pseudomallei* infection is one of the reasons for increased susceptibility of hyperglycaemic host to melioidosis (Morris *et al.*, 2012). We therefore determined the

levels of pro- (TNF-α and IL-12) and anti-inflammatory (IL-10) cytokines in the serum of infected mice. Hyperglycaemia was associated with an increase in the basal level of serum TNF-α ($P < 0.01$) and IL-12 ($P < 0.05$) cytokines than that in non-hyperglycaemic mice (Fig. 3), a typical pro-inflammatory characteristic of diabetes and this was significantly increased following *B. pseudomallei* infection ($P < 0.001$; Fig. 3). Consistent with our *in vitro* findings in rat PBMC (Maniam *et al.*, 2015), inhibition of GSK3β significantly lowered the levels of both pro-inflammatory cytokines, TNF-α and IL-12 in hyperglycaemic and non-hyperglycaemic mice (Fig. 3). Moreover, pronounced IL-12 reduction was more significant in the infected hyperglycaemic mice compared to the infected non-hyperglycaemic mice. In spite of this, we also found a significant increase in the level of anti-inflammatory cytokine IL-10 following GSK3β inhibition, further suggesting a mechanism of inflammatory control during *B. pseudomallei* infection. Collectively, our data strongly suggest that GSK3β is able to modulate pro- and anti-inflammatory cytokines in both hyperglycaemic and non-hyperglycaemic *B. pseudomallei*-infected mice, conferring protection against melioidosis related death.

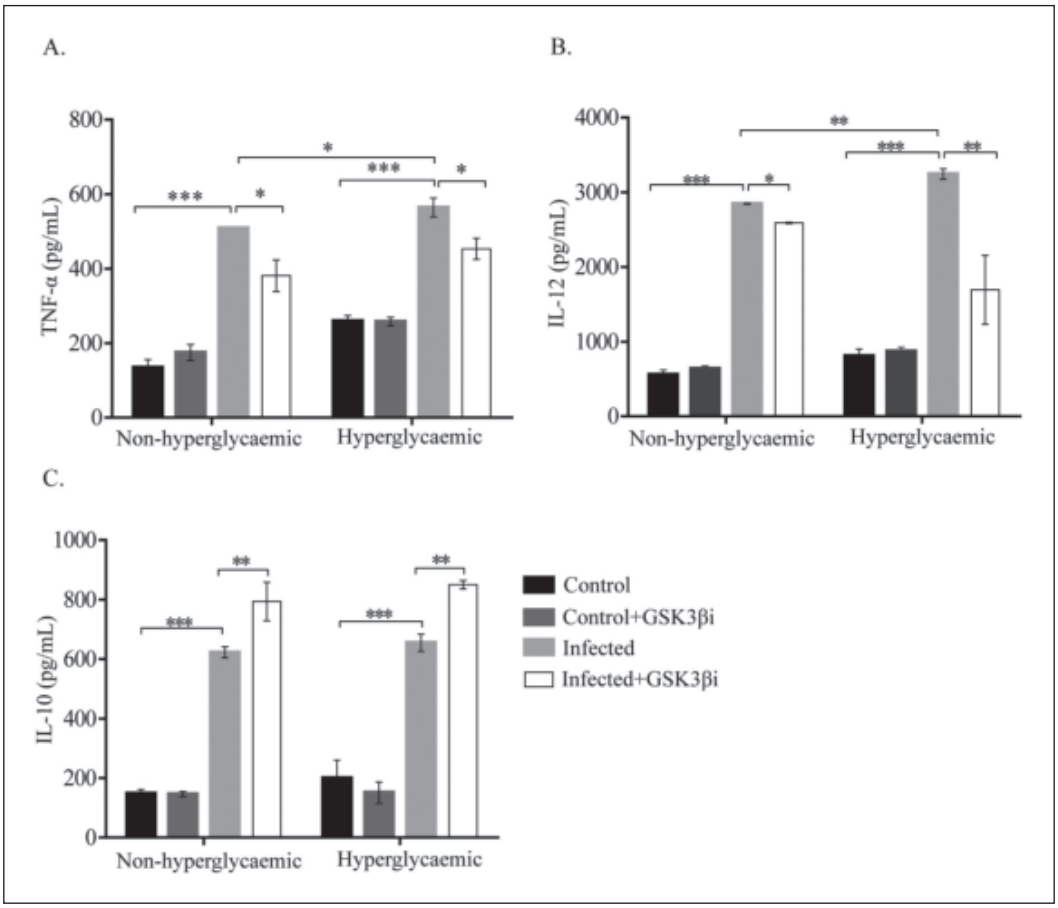

Figure 3. GSK3β inhibition modulated inflammatory cytokine levels in *B. pseudomallei* infected hyperglycaemic and non-hyperglycaemic mice.

Mice (n=3) were infected with *B. pseudomallei* (7.2×10^4 CFU) and administered with LiCl (100 mg/kg b.w.) thereafter. Mice were euthanised 24 hours post-infection and blood was withdrawn to obtain serum for TNF-α (A), IL-12 (B) and IL-10 (C) cytokine analysis. Data represents one of three independent experiments. * denotes significant value at $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

LiCl enhanced phosphorylation of GSK3β at Ser-9 in liver of *B. pseudomallei*-infected mice

Phosphorylation at Ser-9 residue of GSK3β marks the reduced activity of the enzyme (Jope and Johnson, 2004). To investigate whether the observed effects of LiCl were due to its modulation of GSK3β activity, we analysed protein lysates from liver of LiCl-treated and non-treated *B. pseudomallei*-infected animals to determine pGSK3β Ser-9 levels. *B. pseudomallei* infection significantly decreased GSK3β activity in both hyperglycaemic and non-hyperglycaemic mice (Fig. 4). Moreover, livers of LiCl-treated infected hyperglycaemic mice showed higher pGSK3β at Ser-9. LiCl treatment in both infected hyperglycaemic and non-hyperglycaemic mice displayed a similar profile of pGSK3β. The increase in pGSK3β levels upon LiCl treatment in hyperglycaemic and non-hyperglycaemic mice corresponds to the increased survival of mice (Figure 1) and suggests that phosphoinactivation of GSK3β during *B. pseudomallei* infection offers protection to the host by promoting bacterial clearance in organs and modulating the inflammatory response.

DISCUSSION

Diabetes mellitus is widely known as a major predisposing factor for melioidosis (Currie, 2015), where poor glycaemia control in diabetic patients results in failure of intracellular bacteria elimination and increased susceptibility to melioidosis. Ineffective antimicrobial and phagocytic activities previously reported in diabetic

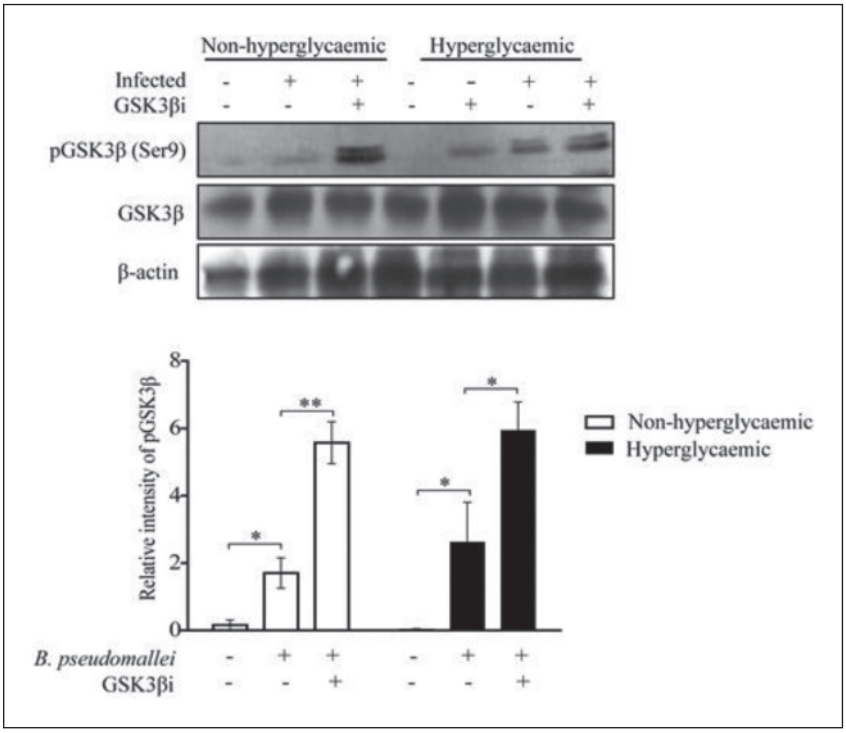

Figure 4. Phosphorylation profile of GSK3β (Ser9) (A) and relative intensity level of pGSK3β (Ser9) as determined by densitometeric analysis (B) of liver proteins from hyperglycaemic and non-hyperglycaemic *B. pseudomallei*-infected mice with and without LiCl treatment.

Mice (n=3) were infected with *B. pseudomallei* and administered with LiCl one hour post-infection. Mice were euthanised at 24 hours post-infection and liver were excised aseptically to proceed with protein analysis. Data represents mean densitometer values of triplicates (* denotes significant value at $P < 0.05$ and ** $P < 0.01$).

hosts could also be contributing to the uncontrollable bacterial dissemination and development of the melioidosis (Hodgson *et al.*, 2011).

Over the past decade, it has been shown that the co-morbidity of melioidosis and diabetes are associated with impairment of host inflammatory response, in which uncontrolled levels of inflammatory cytokines may cause severe sepsis and death in melioidosis (Hotchkiss and Karl, 2003; Netea *et al.*, 2003). The importance of GSK3β in controlling the host innate immune system has been demonstrated in a number of infections including *B. pseudomallei* (Chan *et al.*, 2009; Zhang *et al.*, 2009; Oviedo-Boyso *et al.*, 2011; Tay *et al.*, 2012; Chang *et al.*, 2013; Lutay *et al.*, 2014). Previously, we have shown that the inhibition of GSK3β confers survival advantage to non-hyperglycaemic *B. pseudomallei*-infected animals and macrophages through modulation of inflammatory responses (Tay *et al.*, 2012). Furthermore, *B. pseudomallei* requires GSK3β dependent NFκB activation to replicate and survive within hyperglycaemic PBMCs (Maniam *et al.*, 2015).

In this study, we observed higher levels of serum TNF-α and IL-12 in hyperglycaemic mice than non-hyperglycaemic mice in response to *B. pseudomallei* infection. These results concur with increased pro-inflammatory response (IL-12, IL-8 and CP1) in the blood of *B. pseudomallei*-infected individuals with diabetes type 2 compared to individuals without diabetes (Morris *et al.*, 2012). Furthermore, in a murine model of type 2 diabetes, experimental animals quickly succumbed to septicaemia-related melioidosis due to severe hypoglycaemia and extreme pro-inflammatory response (TNF-α and IL-1β) (Hodgson *et al.*, 2011). A subsequent study conducted by the same group (Hodgson *et al.*, 2013) showed that diet-induced type 2 hyperglycaemic mice are relatively highly susceptible to *B. pseudomallei* infection due to weak immune signalling in mice. On the contrary, Chin *et al.* (2012) reported lower expression of pro-inflammatory cytokines in hyperglycaemic host which resulted in a delayed activation of the innate immune response during *B. pseudomallei* infection. Williams *et al.* (2011) reported that *B. pseudomallei* infection did not alter levels of pro-inflammatory cytokines in immune cells isolated from acute hyperglycaemic mice; however immune cells from chronic hyperglycaemic mice showed decreased levels of inflammatory cytokines (IL-12, IL-18 and IL-10) compared to non-hyperglycaemic immune cells. The inconsistent reports of augmented, attenuated or unchanged cytokine responses to infection in association with diabetes attests that the basis of innate immune response for the synergy seen between diabetes and melioidosis requires further investigation (Hodgson *et al.*, 2015).

Inhibition of GSK3β augmented the level of IL-10 and dampens pro-inflammatory cytokines IL-12 and TNF-α upon *B. pseudomallei* infection in hyperglycaemic mice showing the attainment of a balanced inflammatory response which in turn delayed the death of infected mice. These imply that inhibition of GSK3β potentially rescued hyperglycaemic mice from inflammation and septicaemia caused by high bacterial load and ameliorates the innate immune system to overcome infection. A similar study showed that inhibition of GSK3β lowered the level of TNF-α, which subsequently reduced mortality of BALB/c mice during infection with *Streptococcus* Group A (Chang *et al.*, 2013). In addition, inhibition of GSK3β has also been previously shown to improve survival of mice infected with *F. tularensis* (Zhang *et al.*, 2009) and protects mice from *E. coli* LPS induced endotoxic shock (Ko *et al.*, 2010).

It is clear from our study that the improved survival of host is dependent on inhibited bacterial growth and a well-modulated inflammatory response; thus suggesting the role of GSK3β as a regulatory switch for inflammation in *B. pseudomallei*-infected hyperglycaemic animals. The failure of host to modulate the activity of GSK3β suggests a novel regulatory factor of susceptibility of diabetic host to *B. pseudomallei* infection, thus contributing to impaired innate immune system. In addition, the enhanced survival of *B. pseudomallei*-

infected hyperglycaemic mice as compared to non-hyperglycaemic mice observed in this study may also be attributed to the additional effects of LiCl and GSK3β inhibition on its actions on glucose transport and insulin-related signalling pathway related to diabetes/hyperglycaemia. Therefore, other effects of LiCl apart from those on GSK3β may confound the enhanced survival of hyperglycaemic mice compared to non-hyperglycaemic mice.

In conclusion, we suggest that GSK3β inhibition reduces the susceptibility of hyperglycaemic mice to *B. pseudomallei* infection by modulating the inflammatory response, thus giving protection against *B. pseudomallei* infection. GSK3β inhibitors like LiCl could potentially act as immunomodulators to target the innate immune system in *B. pseudomallei*-infected diabetic host.

Conflict of Interest

The authors disclose no potential conflicts of interest.

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