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The anti-malarial chloroquine modulated cytokine levels and increased animal survivability via Akt-mediated inhibition of GSK3β in *Burkholderia pseudomallei*-infected mice

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Abstract. Melioidosis is a common cause of fatal community-acquired septicaemia and pneumonia in endemic regions even with appropriate antibiotic treatments. The involvement of inflammatory cytokines in the manifestation of melioidosis is well-documented. Anti-bacterial and anti-inflammatory therapies may prove more efficacious against melioidosis rather than just anti-bacterial therapy alone. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway has a central role in regulating the host inflammatory response; and glycogen synthase kinase-3β (GSK3β), a downstream effector molecule within this axis, plays a pivotal role in regulating the production of pro- and anti-inflammatory cytokines. The anti-malarial drug, chloroquine is a novel activator of Akt, and can elicit inhibition of GSK3β via PI3K/Akt signalling. LiCl, a GSK3 inhibitor is reported to increase survivability and modulate cytokine production in *B. pseudomallei*-infected mice. Here we determined the effects of chloroquine administration on animal survivability, cytokine levels and phosphorylation states of GSK3β (Ser9), Akt (Ser473) and NF-κB p65 (Ser536) in a murine model of acute melioidosis infection. Administration of 50 mg/kg b w chloroquine improved survivability (mean 67.0 ± 6.3%) of mice infected with 3 X LD_{50} *B. pseudomallei* compared to controls. Bacterial loads in spleen, liver, lung and blood of infected mice administered with chloroquine were significantly lower than controls. Western blot analysis revealed that the intensities of pAkt (Ser473) and pGSK3β (Ser9) in liver samples of mice administered with chloroquine were significantly ($P<0.05$) higher (2.3- and 4.4-fold respectively) compared to controls. On the other hand, chloroquine treatment signicantly decreased ($P<0.05$) phosphorylation of NF-κB p65 (Ser536) by 0.7-fold compared to control. Chloroquine administration also resulted in significantly reduced levels of pro-inflammatory cytokines (TNF-α, IFN-γ, IL-1β and IL-18) but increased levels of anti-inflammatory cytokines (IL-4 and IL-10) in sera and liver of *B. pseudomallei*-infected mice. Findings from this study demonstrate that the increased survivability of *B. pseudomallei*-infected mice after chloroquine administration is at least in part due to its cytokine-modulating effects elicited via Akt-mediated inhibition of GSK3β that resulted in inhibition of NF-κB activation. This study represents laboratory evidence of the use of chloroquine for cytokine modulation and a plausible effective adjunctive therapeutic for *B. pseudomallei* infection.

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis, a tropical infectious disease associated with mortality due to sepsis associated with overwhelming cytokine production. Death can also occur among patients receiving antibiotic treatments especially those with predisposing conditions such as diabetes mellitus or renal disease (Thong & Arul, 2016). Limiting inflammatory damage during bacterial infections may be an effective therapeutic strategy for melioidosis. *B. pseudomallei* is able to thrive intracellularly due its ability to dysregulate host immune response

signalling pathways. The molecular strategies employed by *B. pseudomallei* to modulate these pathways and evade intracellular killing is however still not well understood (Aschenbroich *et al.*, 2016). Toll-like receptors (TLRs), TLR2 and TLR4 are two important receptors activated by *B. pseudomallei* (West *et al.*, 2008) based on studies using a murine model of melioidosis. Disturbance in the TLR4-TIRAP/Mal pathway leads to sepsis and pneumonia (Wilson *et al.*, 2016). In acute melioidosis, over production of pro-inflammatory cytokines (such as TNF-α, IFN-γ, IL-6 and IL-18) (Lazar Adler *et al.*, 2009) is a major cause of septic shock (Panomket, 2011). TLRs also initiate the activation of phosphoinositide 3-kinase (PI3K) pathway, which is responsible for the regulation of the inflammatory responses during infection. Wang *et al.* (2011) reported that the PI3K/Akt pathway which also depends on the recruitment of MyD88 adaptor protein (Troutman *et al.*, 2012) is one of the main pathways regulating the host inflammatory responses. Activation of this pathway results in phosphorylation and consequent inhibition of GSK3β (Kitagishi *et al.*, 2014).

GSK3 is a serine/threonine protein kinase originally identified by Embi *et al.* (1980) as a key enzyme in the regulation of glycogen metabolism. Since its identification, GSK3 is now known to be a key regulator in many cellular processes, including embryo development, metabolic homeostasis, cell survival, neuronal growth, differentiation and inflammation (Beurel *et al.*, 2015). Dysregulation of GSK3β is implicated in many diseases such as type 2 diabetes, cancer, Alzheimer's disease and sepsis during infection. Inhibition of GSK3β is a potential intervention to treat these diseases. In higher eukaryotes, GSK3 consists of two isoforms, GSK3α and GSK3β, which are highly homologous in their catalytic domains but show remarkable differences in their terminal regions. GSKα and GSK3β are constitutively active under basal condition and become inactive when Ser21 or Ser9 are phosphorylated respectively. GSK3β is involved in the regulation of transcription factors including NF-κB, AP-1, CREB and β-catenin (Cormier & Woodgett, 2017) in cytokine production during inflammation. Since, GSK3β has a pivotal role in the inflammatory responses caused by bacterial pathogens (Cortés-Vieyra *et al.*, 2012), GSK3β inhibitors may be exploited as therapeutics to control infectious diseases (Cheng *et al.*, 2009). Inhibition of GSK3β has been shown to inhibit inflammation in many Gram-negative bacterial infections (Wang *et al.*, 2014) including *B. pseudomallei* (Tay *et al.*, 2012).

There is a need to develop a melioidosis treatment protocol that can lessen the underlying host-mediated immunopathology of the disease to address the high mortality. At this juncture, it is noteworthy that the anti-malarial drug, chloroquine is also a novel activator of Akt, a critical component of PI3K/Akt signalling thus capable of phosphorylating and consequent inhibition of GSK3β (Halaby *et al.*, 2013). The use of established drugs such as chloroquine to treat diseases other than that it is already used for i.e. repurposing available drugs, is an innovative strategy in the development of new therapeutics. The tolerability, low cost and unique biochemical properties of chloroquine have led to many efforts to evaluate repurposing of this drug for various diseases (Njaria *et al.*, 2015). The potential use of chloroquine as a non-steroidal anti-inflammatory drug for rheumatoid arthritis and multiple sclerosis has been evaluated (Thomé *et al.*, 2013). On-going research initiatives for repurposing chloroquine include studies in various types of cancers (Kimura *et al.*, 2013) and diabetes (Halaby *et al.*, 2013). Chloroquine has been reported to exhibit anti-inflammatory properties and able to protect mice from LPS challenges via a mechanism involving reduction of pro-inflammatory cytokine levels (Hong *et al.*, 2004).

Based on our notion that GSK3 is a plausible target for cytokine modulation in *B. pseudomallei* infection, chloroquine is therefore a feasible candidate to be evaluated for its effect on Akt and GSK3 phosphorylation during *B. pseudomallei* infection. The present investigation aims to evaluate the effects of chloroquine administration on

animal survivability, cytokine levels and phosphorylation (Ser9) of GSK3β in a murine model of acute melioidosis infection. The goal of our study is to determine whether inhibition of GSK3β is a possible therapeutic strategy for treating melioidosis.

MATERIALS AND METHODS

Mice

BALB/c mice (6-8 weeks old) were obtained from the Animal House Complex at the National University of Malaysia (UKM), Malaysia. The animals were housed in Individual Ventilation Cages (IVC) with a bedding of wood, fed on a diet of pellets (rat chow, Barastoc brand from Ridley, Australia) and distilled water ad libitum; and subjected to a 12 hours light/dark cycle. Use of animals in this experiment was approved by and conducted in accordance with the guidelines of UKM Animal Ethics Committee (UKMAEC) (FST/2015/EMBI/ 29-SEPT/701-SEPT-2015-MAC-2017).

Bacterial strain and culture

The virulent D286 strain of *B. pseudomallei* originally isolated from a patient at the Kuala Lumpur Hospital was a kind gift from Prof. Dr. Sheila Nathan, Pathogen Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, UKM. The bacteria were grown in Brain Heart Infusion Broth (BHIB) (Pronadisa Hispanlab, South Africa) at 37°C overnight. The culture was then diluted and absorbance readings were measured at 600 nm (UV-spectrophotometer) to determine the concentration of bacterial culture (CFU/mL) using Mc Farland's standard. The cells were centrifuged at 10,000 g, resuspended in BHIB containing 20% glycerol and frozen immediately in 0.2-mL aliquots at a concentration of 10^9 CFU/mL at -80°C for later use in the infection studies (Hoppe *et al.*, 1999).

Animal studies (Acute Melioidosis Infection)

The 50% lethal dose (LD_{50}) for *B. pseudomallei* was determined according to the method of (Reed & Muench, 1938). Male BALB/c mice (n=9) were injected intraperitoneally (i.p.) with 3 X 10 LD_{50} (300 000 CFU) of *B. pseudomallei* in 200 µL of phosphate-buffered saline (PBS). To evaluate the effects of chloroquine administration on animal survivability, *B. pseudomallei*-infected mice were administered (i.p.) with 10, 25, 50 or 75 mg/kg body weight (b.w.) chloroquine at one hour after infection. For non-treated infected control, *B. pseudomallei*-infected animals were injected (i.p.) with 0.9% sodium chloride (NaCl). Survivability of animals were monitored over a 14-day period post-infection.

Bacterial load

For the effect of chloroquine treatment on bacterial burden (in blood and organs), a group of mice (n=3) was infected with 3 X 10 LD_{50} (300 000 CFU) *B. pseudomallei* and subsequently administered with 50 mg/kg b.w. chloroquine at one hour after infection. The control group consisted of mice infected with *B. pseudomallei* only. Three mice from each group (n=3) were euthanised by carbon dioxide inhalation to obtain blood, liver, spleen and lung samples at day 1, 2, 3 and 4 post-infection. Cardiac puncture was used for collection of blood samples and organs were aseptically removed for further use. Blood and organ samples were also obtained randomly from surviving mice at day 14 post-infection. All samples were processed as described by Leakey *et al.* (1998). Briefly, all test organs (liver, spleen and lung) were homogenised in 10 mL PBS containing 0.14 M NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4, 1.7 mM KH_2PO_4, pH 7.4. The blood and organ homogenates were then serially-diluted with PBS and spotted onto Ashdown agar. Total bacteria in samples were determined as CFU/mL.

Western blot analysis

Liver organs were collected from experimental mice at 2 hours post-infection. The 2 hours samples were analysed because our previous study revealed that phosphorylation of GSK3β was detected in spleen, liver and

lungs, as early as two hours after infection with *B. pseudomallei* (Tay *et al.*, 2012). Liver organs at 24 hours post-infection were also analysed because levels of cytokines (IFN-γ, TNF-α, IL-1β, IL-6 and IL-10) were observed in BALB/c mice 24 hours post-infection (Hodgson *et al.*, 2013; Ulett *et al.*, 2000). Protein extraction was carried out as described by Wang & Zhu (2003). Liver samples were homogenised 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, 0.1% SDS supplemented with protease inhibitors (1 mM PMSF, 50 µg/mL leupeptin and 100 µg/mL aprotinin) and phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF and 100 mM EDTA) followed by incubation on ice for 40 minutes. The homogenates were centrifuged at 20 000 g for 30 min at 4ºC. Protein concentration in the supernatant was determined by Bradford method (Bradford, 1976). Protein samples were diluted with 1: 1 (w/v) sample buffer consisting of 0.06 M Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.25% bromophenol blue. Protein separation was then conducted using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% resolving polyacrylamide gels (Laemmli, 1970). Separated proteins were then electro-transferred onto nitrocellulose membranes (Amersham hybond-ECL, GE Healthcare, USA) and blocked with 3% BSA in Tris buffered saline with Tween (TBST). Membranes were probed and incubated for 18 h at 4°C with primary monoclonal antibodies, anti-GSK3β, anti-phosphoSer9-GSK3β, anti-Akt, anti-phosphoSer473-Akt, anti-NF-κB, or anti-phosphoSer536-NF-κB (Cell Signaling, USA) followed by incubation with HRP-conjugated IgG (Cell Signaling, USA) as a secondary antibody for 2 hours at room temperature. β-actin was used as loading control. Detection of immuno-reactive proteins was carried out using SuperSignal® West Pico chemiluminescent HRP substrate (Pierce, USA). Band area intensity was quantified using a densitometer (Vilbert Lourmat 302, France).

Cytokine assay

Mice were divided into four groups (n=6) comprising of Group I: Normal; Group II: 50 mg/kg b.w. chloroquine administration only; Group III: *B. pseudomallei* infection only; Group IV: *B. pseudomallei* infection + 50 mg/kg b.w. chloroquine (1 hour post-infection); At day 1 post-infection, blood samples were collected by cardiac puncture during carbon dioxide anaesthesia and immediately processed to obtain sera (Phelan *et al.*, 2002). Whole blood samples were allowed to clot for 30 min at 37°C and centrifuged at 2,000 g for 15 minutes at 4°C. The sera collected were then used for cytokine determination (specifically for TNF-α, IFN-γ, IL-1β, IL-18, IL-10 and IL-4) using enzyme-linked immunosorbent assay kits (Qiagen, Germany).

Statistical analysis

Data obtained from the study were examined statistically using Prism 6.0 software (Graphpad). Log-rank test was conducted for Kaplan-Meier survival analysis whilst t-test was performed on the bacterial load and cytokine data. Western blot analysis data obtained were expressed as mean ± SD based on duplicate densitometric analysis of one representative blot. Cytokine analysis data were expressed as mean ± SEM based on samples obtained from 6 mice per treatment. P value of < 0.05 between groups was considered statistically significant.

RESULTS

Chloroquine increased survivability of acute *B. pseudomallei*-infected mice

BALB/c mice were challenged (i.p) with a local clinical isolate of *B. pseudomallei* (D286). The 10-day LD_{50} for *B. pseudomallei* D286 was determined to be 1 X 10^5 CFU. The mice infected with a dosage of $>10^5$ CFU *B. pseudomallei* developed paresis of both hind legs, piloerection and appeared lethargic leading to paralysis before death from the effect of infection (Feezor *et al.*, 2003). Figure 1 showed that 3 X LD_{50} (300 000 CFU) is a good dose to use in

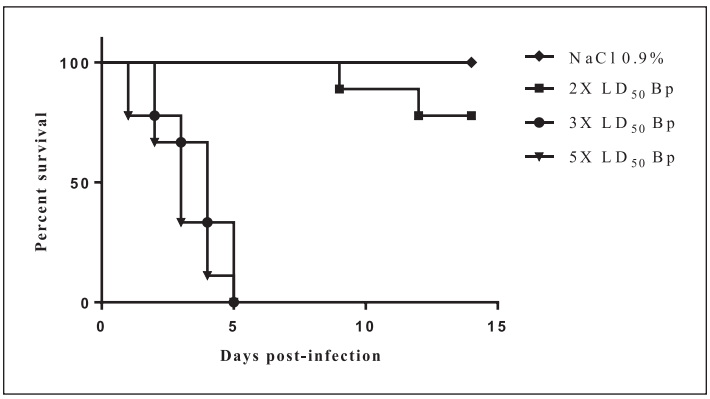

Figure 1. Kaplan-Meier survival curve of BALB/c mice (n=9) administered with 2 X, 3X and 5X LD_{50} of *B. pseudomallei* for 14 days observation period as compared to control treated with NaCl 0.9%.

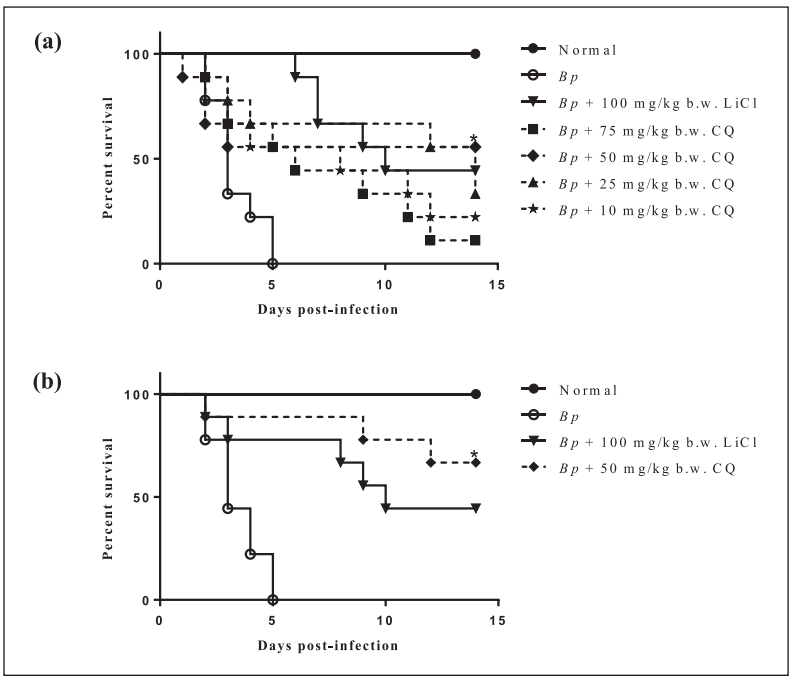

Figure 2. Representative Kaplan-Meier survival curve of BALB/c mice (n= 9 per group) administered with 3 X 10^5 CFU (3 X LD_{50}) of *B. pseudomallei* D286 intraperitoneally. (a) Infected mice with or without chloroquine treatment (10, 25, 50 and 75 mg/kg b.w.) at 1 hour post-infection (n=9). (b) Infected mice with or without chloroquine treatment (best dose of 50 mg/kg b.w.) at 1 hour post-infection (n=9). Non-treated (non-infected) mice were used as control. Significant difference between tested groups and control infected group was evaluated at $p<0.05$ (*).

establishing acute melioidosis infection; all mice infected with 3 X LD_{50} bacteria died within day 2-5 post-infection indicating establishment of acute infection by *B. pseudomallei* (Leakey *et al.*, 1998). Infected mice administered with 10, 25, 50 and 75 mg/kg b.w chloroquine resulted in improved survivability by 22%, 33%, 56% and 11% respectively compared to non-treated infected control ($P<0.05$) (Figure 2a). The

survivability of mice were significantly increased in a dose-dependent manner and were seen in all treated groups except at the highest dose (75 mg/kg b.w.) possibly because of slight toxicity. A repeat experiment with a selected dose of 50 mg/kg b.w. chloroquine based on highest survivability showed an improved survival of 67% compared to non-treated infected control ($P<0.05$) (Figure 2b). The findings indicate that post-infection administration of 50 mg/kg b.w. chloroquine resulted in significant ($P<0.05$) improvement of survivability in *B. pseudomallei*-infected animals.

Chloroquine reduced *B. pseudomallei* bacterial burden in organs and blood in *B. pseudomallei*-infected mice

The bacterial loads in organs and blood of infected mice were determined in chloroquine-treated and non-treated mice. High bacterial counts (or bacterial loads) of *B. pseudomallei* (up to > 10^8 CFU) were detected by day 4 post-infection in both liver and spleen of non-treated mice. Both of these organs have been reported to be more prone to melioidosis infection (Laopaiboon *et al.*, 2009). Increase in the bacterial counts were also observed in lung and blood of non-treated infected mice. Significant decrease in bacterial loads were seen in blood, liver, spleen and lung of *B. pseudomallei*-infected mice administered with 50 mg/kg b.w. chloroquine from day 1 until day 4 post-infection. Drastically reduced bacterial counts were also observed on day 14 post-infection in mice which remained alive throught out the animal study. However, chloroquine did not entirely clear the total numbers of bacteria on day 14 post-infection. The findings suggest that the improved survivability conferred by chloroquine administration was not due to the reduction in bacterial load (Figure 3).

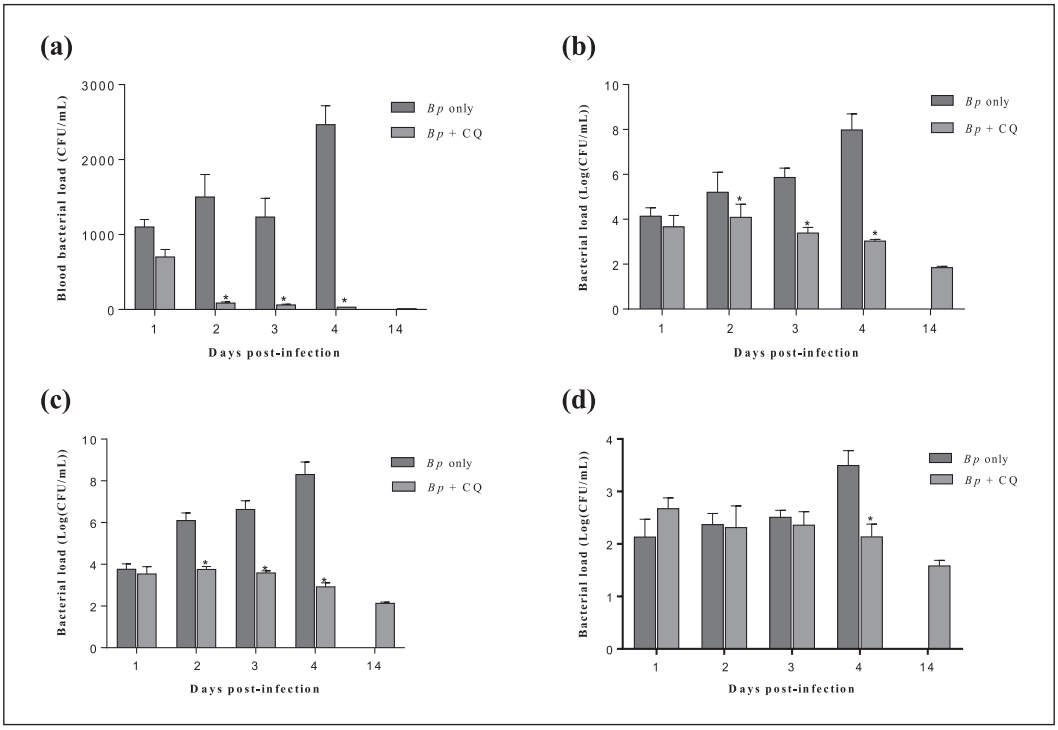

Figure 3. Bacterial loads in the (a) Blood (b) Liver (c) Spleen (d) Lung of BALB/c mice (n=3 per group) at day 1, 2, 3, 4 and 14 administered with 3 X LD_{50} (300 000 CFU) of *B. pseudomallei* with or without chloroquine treatment at 1 hour post-infection. The data for control mice at day 14 are not represented as all the mice died within 5 days post-infection. Significant difference between treated groups and control infected groups was evaluated at $P < 0.05$ (*).

Chloroquine administration resulted in increased phosphorylation of GSK3β (Ser9) in liver of *B. pseudomallei*-infected mice

To further investigate whether increased survivability and bacterial load reduction described above in chloroquine-treated infected mice are related to Akt/GSK3β signaling pathway, we determined the phosphorylation states of both GSK3β (Ser9) and Akt (Ser473) using western analysis. Levels of phosphorylated GSK3β (Ser9) and Akt (Ser473) in liver were determined in chloroquine-treated and non-treated infected mice. Administration of chloroquine (50 mg/kg b.w) significantly increased ($P<0.05$) pAkt (Ser473) in liver by 1.3 fold at 2 hours and 2.3 fold at 24 hours after infection compared to non-treated infected animals. Chloroquine administration in *B. pseudomallei*-infected mice resulted in a significant increase ($P<0.05$) in the levels of pGSK3β (Ser9) in liver by 3.2 fold at 2 hours and 4.4 fold at 24 hours post-infection compared to control. These findings revealed that the activation of Akt led to the inhibition of the host (liver) GSK3β upon treatment with chloroquine during acute melioidosis infection. The effects of chloroquine observed implicates the involvement of GSK3β inhibition mediated through activation of Akt. We also investigated how GSK3β has an impact on the activation of NF-κB p65 (Ser536) in *B. pseudomallei*-infected mice treated with chloroquine. Our results showed that chloroquine treatment significantly decreased ($P<0.05$) phosphorylation of pNF-κB p65 (Ser536) by 0.7-fold (detected at 2 and 24 hours post-infection) compared to control. In this study, we showed that chloroquine treatment decreased phosphorylation of pNF-κB p65 (Ser536) in liver of *B. pseudomallei*-infected mice indicating that chloroquine caused inhibition of NF-κB p65 (Ser536) activation (Figure 4).

Administration of chloroquine modulated the production of pro- and anti-inflammatory cytokines in *B. pseudomallei*-infected mice serum

Next we investigated the modulation of pro- and anti-inflammatory cytokine levels in chloroquine-treated and non-treated animals during melioidosis infection. The levels of pro-inflammatory cytokines TNF-α, IFN-γ, IL-1β and IL-18 were high in sera of *B. pseudomallei*-infected mice and reduced ($P<0.05$) by 2.7, 2.0, 1.8 and 1.5 times the level of control with 50 mg/kg b.w chloroquine treatment. Treatment with chloroquine also increased the levels of anti-inflammatory cytokines IL-4 and IL-10 by 3.4 and 6.9 fold compared to non-treated infected mice. These findings suggest that chloroquine modulated the production of inflammatory cytokines in *B. pseudomallei*-infected animals due to its ability to activate Akt pathway which led to the inhibition of GSK3β. Further increase of anti-inflammatory cytokine levels with administration of chloroquine is a possible contribution in reducing the detrimental effects of pro-inflammatory cytokines; consequently increasing the survivability of *B. pseudomallei*-infected mice (Figure 5).

DISCUSSION

Our findings revealed that chloroquine administration into *B. pseudomallei*-infected mice significantly improved animal survivability. The improvement in survivability seen here with chloroquine administration (67%) is comparable to that observed in our previous animal infection studies (60%) where LiCl, a GSK3 inhibitor was employed (Tay *et al.*, 2012) suggesting that chloroquine is as effective as LiCl in protecting *B. pseudomallei*-infected animals. The effect on animal survivability observed here could not have been attributed to the anti-bacterial action on the part of chloroquine if any. At the dose employed, chloroquine did not show any effect on *B. pseudomallei* growth in culture based on the findings from the present study.

In this study, we observed significantly lowered bacterial counts in spleen, liver, lung and blood of infected animals administered with chloroquine although incomplete clearance was seen in the organs of the surviving mice on day 14 post-infection. Nevertheless, the bacterial loads in the organs of chloroquine-treated *B.*

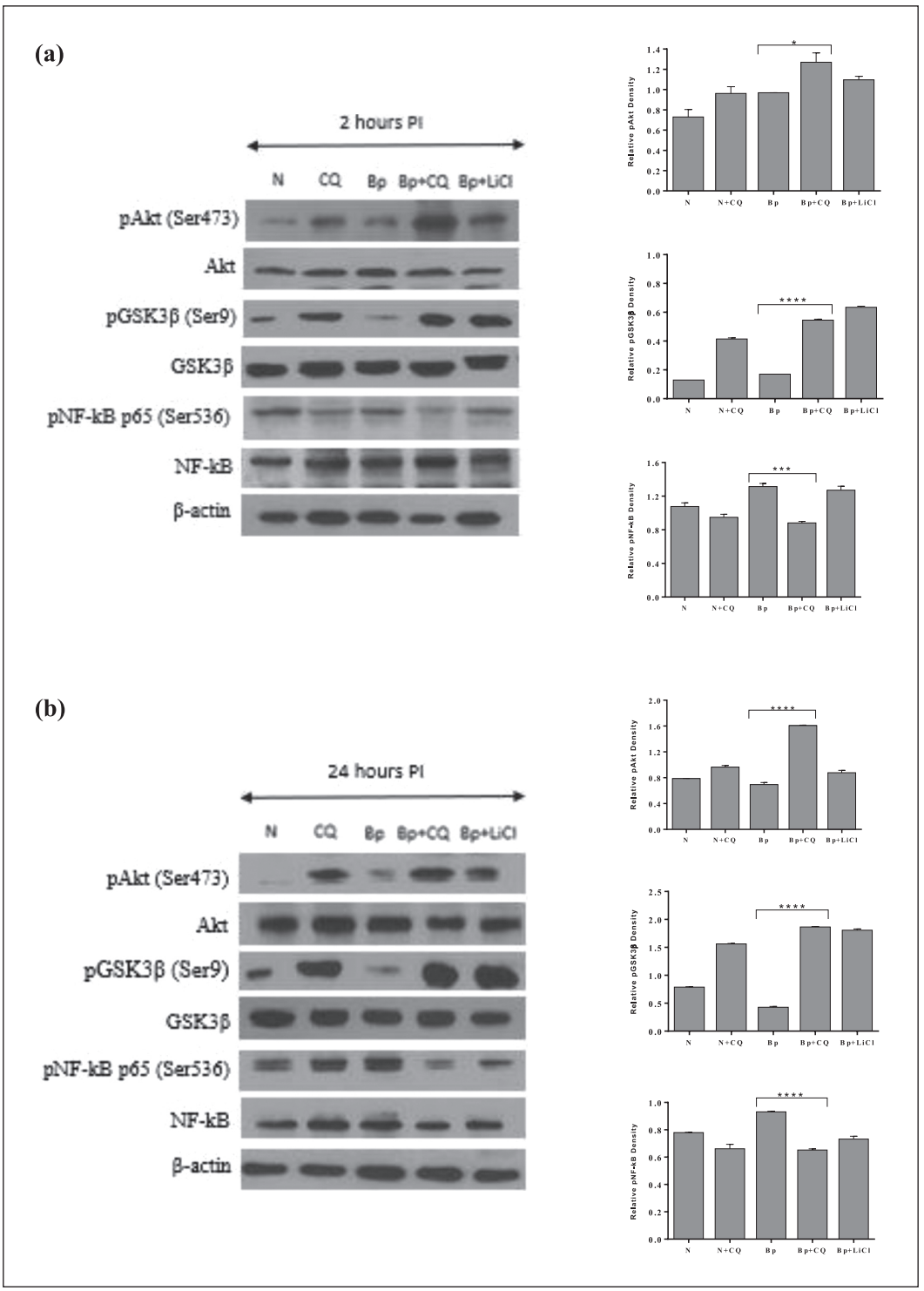

Figure 4. Relative phosphorylation levels of pAkt/Akt, pGSK3β/GSK3β and pNF-kB/ NF-kB in liver of uninfected (N), CQ-treated uninfected (CQ), *B. pseudomallei*-infected (*Bp*), CQ-treated infected (*Bp*+CQ) and LiCl-treated infected (*Bp*+LiCl) mice at 2 and 24 hours post-infection. Data presented as mean ± SD. Significant difference between tested and control groups was evaluated at $p<0.05$(*), $p<0.001$ (***), $p<0.0001$ (****). Representative Western blotting images are shown.

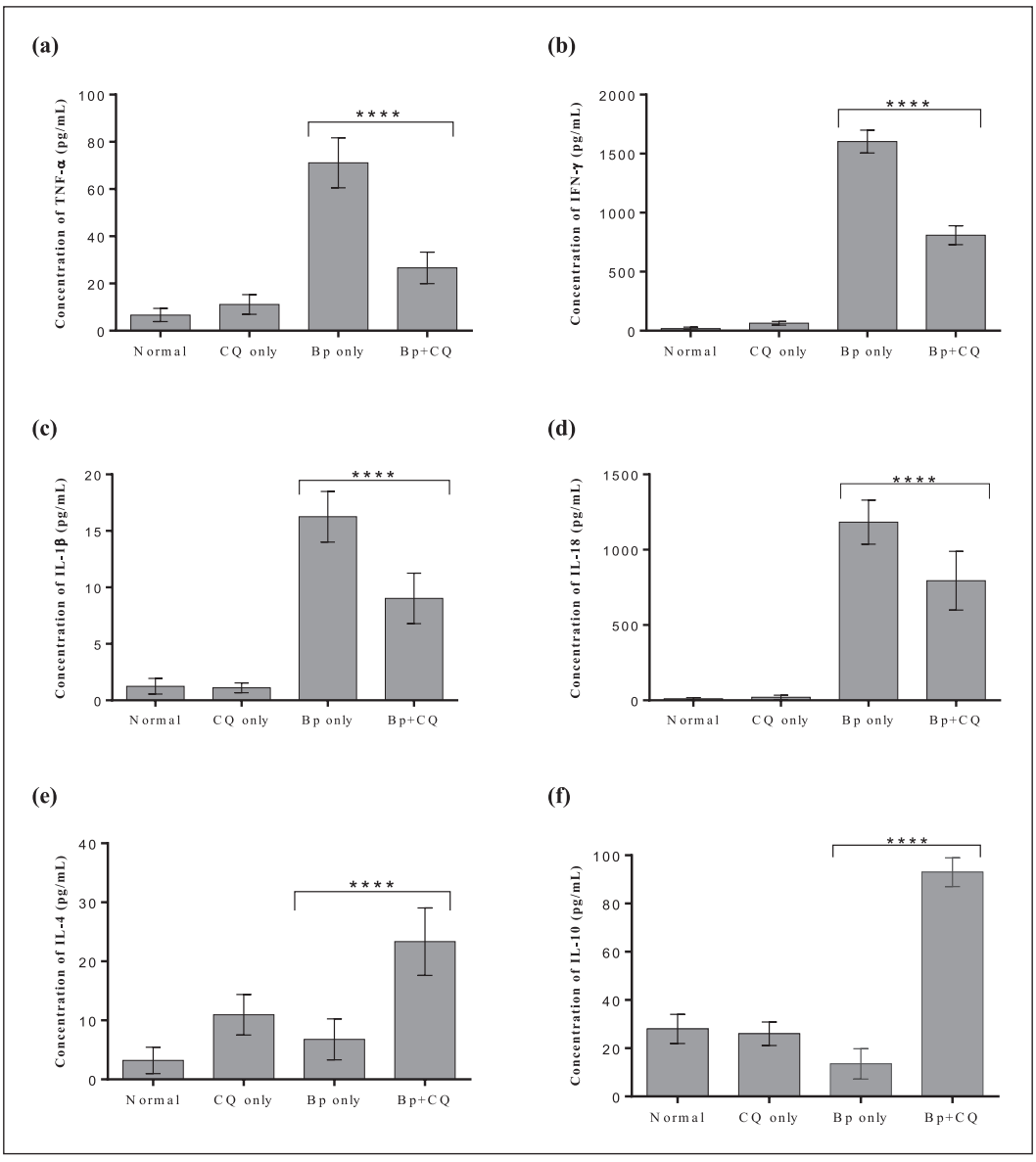

Figure 5. The levels of pro-inflammatory (a) TNF-α (b) IFN-γ (c) IL-1β (d) IL-18 and anti-inflammatory cytokines (e) IL-4 and (f) IL-10 levels in sera of mice administered with 3 X 10^5 CFU (3 X 10-day LD_{50}) of *B. pseudomallei* with or without (50 mg/kg b.w. chloroquine) treatment at 1 hour post-infection. Data are expressed as mean ± SEM; n = 6 BALB/c mice per group. Significant difference as compared with control infected group was evaluated at $P < 0.0001$ (****).

pseudomallei animals were significantly lower than the control infected animals suggesting that chloroquine treatment may have boosted the innate defence resulting in prolonged animal survivability. This protective effect of chloroquine corroborate findings from a study by Hong *et al.* (2004) where it was reported that 30 mg/kg b.w. of chloroquine protected mice from lethal challenge by LPS. Recently, Chen *et al.* (2017) also demonstrated that chloroquine facilitated negative regulation of NLRP3 inflammasome, and conferred protection against lethal endotoxic shock induced with LPS in BALB/c mice. Our present study represents the first report on the effect of

chloroquine on animal survivability in a murine model of acute *B. pseudomallei* infection. While the present study was being carried out, Chua *et al.* (2016) also reported that chloroquine was able to increase survival of *B. pseudomallei*-infected hissing cockroaches by inhibiting intracellular growth and escape from the phagosome and subsequent multinucleated giant cells (MNGC) formation due to its pH alkalisation effect inside the host cells. Chua *et al.* (2016) proposed that there may be multiple mechanisms by which intracellular growth of *B. pseudomallei* and virulence can be affected. However, the mechanism of the multifactorial effects of chloroquine inhibition on cellular pathogenesis of *B. pseudomallei* was not specified. The effect of chloroquine treatment in murine and higher order animal models of *B. pseudomallei* infection has yet to be reported.

Uncontrolled inflammation, as characterised by excessive cytokine release, also contributes to acute and chronic clinical disorders, including sepsis. Inflammation induced by *B. pseudomallei* infection is a major factor in the immuno-pathogenesis of melioidosis thus implicating the important role of inflammatory cytokines in the manifestation of this disease (Wiersinga *et al.*, 2007). Chloroquine as an anti-malarial drug also possesses anti-inflammatory properties (Hong *et al.*, 2004). Since LPS is a major virulence factor of *B. pseudomallei* implicated in the induction of septic shock (Chantratita *et al.*, 2013), we are of the opinion that chloroquine treatment may protect *B. pseudomallei*-infected animals against sepsis. Our results clearly demonstrated that in mice experimentally-infected with *B. pseudomallei*, chloroquine administration resulted in significantly lower sera levels of pro-inflammatory cytokines (IL-1β, IL-18, IFN-γ and TNF-α) whilst anti-inflammatory cytokines (IL-10 and IL-4) were elevated. A recent study by Chen *et al.* (2017) also demonstrated that chloroquine reduced IL-1β and IL-18 in sera, peritoneal fluid, and lung tissues in a mouse model of endotoxic shock. It was suggested that the improved animal survivability was due at least in part to the dampening effect of chloroquine on the release of pro-inflammatory cytokines. Ceballos-Olvera *et al.* (2011) reported that IL-1β and IL-18 are necessary in the immune response toward *B. pseudomallei* infection but over expression of both cytokines may lead to septic melioidosis. In a murine model of *B. pseudomallei* infection, early hyper-production of IFN-γ after two days resulted in death (Ulett *et al.*, 2000). TNF-α is produced during bacterial infection, injury and other microbial invasions (Tracey & Cerami, 1994) but over production of this cytokine leads to endotoxic shock and mortality in sepsis melioidosis (Suputtamongkol *et al.*, 1992). In a recent study, Dunachie *et al.* (2017) showed reduced IL-23 and IL-10 cytokine responses in diabetic individuals, suggesting dysregulated bacterial immunity in acute melioidosis patients. To the best of our knowledge, our findings represent the first report on the relationship between the effect of chloroquine on mice survivability and levels of pro-and anti-inflammatory cytokines in experimentally-induced acute *B. pseudomallei* infection *in vivo*. Thus, we have provided evidence that chloroquine administration into *B. pseudomallei*-infected mice decreased the levels of pro-inflammatory cytokines and increased the levels of anti-inflammatory cytokines in sera and that this cytokine-modulating effect could have contributed to the improved animal survivability in *B. pseudomallei* infected mice. Next, we attempted to provide an explanation of a possible molecular mechanism on the cytokine-modulating effect of chloroquine that may have contributed to the improved animal survivability.

As with other Gram-negative bacterial infections, *B. pseudomallei* infection can cause alterations in proteins involved in various intracellular inflammatory cascades. The PI3K/Akt pathway has been shown to play a central role in regulating the host inflammatory response of which GSK3β, a downstream component is now recognised as a point of convergence for the host inflammatory response. In an animal model of L6 muscle cells, chloroquine has been shown to be a novel activator of Akt, a

critical component of PI3K/Akt signalling, resulting in inhibition of GSK3β (Halaby *et al*., 2013). Recent studies also demonstrated that chloroquine was able to increase phosphorylation of Akt in myotubes (Spears *et al*., 2016).

Our western analysis showed that an upstream mediator of GSK3β, Akt, was also phosphorylated (at Ser473) in liver of chloroquine-treated infected mice. pGSK3β (Ser9) was detected at 2 and 24 hours after infection with *B. pseudomallei*. It is likely that the phosphorylation of GSK3 seen here is a consequence of the phosphorylation and activation of Akt. Our findings also indicate a lowered level of pNF-κB (Ser536). NF-κB is the key transcription factor for the inflammatory and immune gene expression in MyD88-dependent pathways as it is believed to be activated during *B. pseudomallei* infection leading to the translation of many pro-inflammatory cytokines (West *et al*., 2012). The mechanism of chloroquine involves activation of Akt subsequent phosphorylation and inhibition of GSK3β via PI3K/Akt signalling. In melioidosis patients, TLR1, TLR2 and TLR4 were shown to be overly expressed (Wiersinga *et al*., 2007) and the over expression of these receptors during the early phase of bacterial invasion causes the dysregulation of the downstream effector (NF-κB) which leads to the septicaemia with overwhelming bacterial loads in organs and blood. GSK3 which remains active in the early phase of infection then results in the failure of the host to control infection due to dysregulation of inflammatory cytokines which involve PI3K/Akt signalling. In the current study we have shown that treatment with chloroquine attenuated NF-κB p65 activation during *B. pseudomallei* infection. Thus, our findings on the effect of chloroquine on the activation of Akt-mediated inhibition of GSK3β, and inhibition of NF-κB activation suggest a crucial role of GSK3β in the mechanism of modulation of inflammatory cytokines in *B. pseudomallei*-infected mice. It has been previously reported that GSK3β in the constitutively active state preferentially favours production of pro-inflammatory cytokines (Ohtani *et al*., 2008). GSK3β inhibition is meaningful in modulating the pro- and anti-inflammatory cytokines as a result of *B. pseudomallei* infection (Wang *et al*., 2011). *B. pseudomallei* infection studies from our laboratory demonstrated that inhibition of GSK3β *in vitro* and *in vivo* resulted in lowered levels of pro-inflammatory cytokines whilst anti-inflammatory cytokine levels were elevated (Maniam *et al*., 2013; Tay *et al*., 2012). It is noteworthy that recently, D'elia *et al*. (2017) reported that activation of the mitogen-activated protein kinases (MAPKs) p38 and ERK were both significantly altered during both *in vitro* and *in vivo B. pseudomallei* infections. However, they found that the inhibition of MAPKs during *B. pseudomallei* infection did not result in the modulation of the pro-and anti-inflammatory cytokine balance.

Various mechanisms have been proposed to explain how chloroquine influences cytokine production during infection (Weber & Levitz, 2000). Yang *et al*. (2013) reported that the protective effects of chloroquine and inhibition of cytokine release were mediated through inhibition of high-mobility group box 1 (HMGB1) release in macrophages, monocytes, and endothelial cells; chloroquine specifically inhibited HMGB1-induced Iκ-B degradation and NF-κB activation. Chen *et al*. (2017) explained that chloroquine attenuated NF-κB and MAPK activation and prohibited expression of IL-1β, IL-18, and NLRP3 in LPS-treated murine bone marrow-derived macrophages (BMDMs), demonstrating its inhibitory effect on the priming signal of NLRP3 activation. Hong *et al*. (2004) showed that chloroquine reduced the expression of TLR9 and TLR4 mRNA and activation of NF-κB. All these findings including our results point to the fact that the cytokine-modulating effect of chloroquine involves the regulation of NF-κB.

We have therefore provided evidence to support our notion that chloroquine treatment is beneficial to the host inflammatory response and in part contributes to the increased survivability of mice in an acute melioidosis infection. Repurposing chloroquine for potential

adjunctive therapy of melioidosis by combining anti-bacterial (antibiotics) and anti-inflammatory (GSK3β inhibitor) therapies may prove more efficacious than individual therapy. Wilson *et al*. (2016) recently reported immune modulation as an effective adjunctive post-exposure therapeutic for *B. pseudomallei* infection. Post-exposure to a COX-2 inhibitor, tolfenamic acid, a non-steroidal anti-inflammatory drug, with a sub-therapeutic dose of ceftazidime increased anti-bacterial efficacy and this was more effective to treat melioidosis in BALB/c mice (Wilson *et al*., 2016).

Findings from this study demonstrate that the increased survivability of *B. pseudomallei*-infected mice after chloroquine administration is at least in part due to its cytokine-modulating effects elicited via Akt-mediated inhibition of GSK3β that resulted in inhibition of NF-κB activation. This study thus represents laboratory evidence of the use of chloroquine for cytokine modulation and a plausible effective adjunctive therapeutic for *B. pseudomallei* infection. In conclusion, our findings revealed a novel pharmacological effect of chloroquine and its molecular basis in *B. pseudomallei* infection i.e. the anti-malarial chloroquine modulated cytokine levels and increased animal survivability via Akt-mediated inhibition of GSK3β.

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