

Potential of repurposing chloroquine as an adjunct therapy for melioidosis based on a murine model of *Burkholderia pseudomallei* infection

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Abstract. *Burkholderia pseudomallei* is the etiologic agent of melioidosis, a major cause of community-acquired pneumonia and sepsis in the endemic areas. The overall mortality of patients with severe melioidosis remains high due to severe sepsis attributed to overwhelming inflammatory cytokine response in spite of recommended antibiotic therapy. It is crucial that therapeutic approaches beyond just effective antibiotic treatment such as adjunct therapy be considered to mitigate the dysregulated inflammatory signaling and augment host defenses. In an acute *B. pseudomallei* infection model, we have previously demonstrated that treatment with anti-malarial drug, chloroquine, modulated inflammatory cytokine levels and increased animal survivability via Akt-mediated inhibition of glycogen synthase kinase-3 β (GSK3 β). GSK3 β is a downstream effector molecule within the phosphatidylinositol 3-kinase (PI3K)/Akt axis which plays a pivotal role in regulating the production of pro- and anti-inflammatory cytokines. Here we evaluate the effect of chloroquine treatment in combination with a sub-therapeutic dose of the antibiotic doxycycline on animal survivability, cytokine levels and phosphorylation states of GSK3 β (Ser9) in a murine model of acute melioidosis infection to investigate whether chloroquine could be used as an adjunct therapy along with doxycycline for the treatment of melioidosis. Our findings revealed that 50 mg/kg b.w. chloroquine treatment together with a dose of 20 mg/kg b.w. doxycycline improved survivability (100%) of mice infected with 3 X LD₅₀ of *B. pseudomallei* and significantly ($P < 0.05$) lowered the bacterial loads in spleen, liver and blood compared to controls. *B. pseudomallei*-infected mice subjected to adjunct treatment with chloroquine and doxycycline significantly ($P < 0.05$) reduced serum levels of pro-inflammatory cytokines (TNF- α , IFN- γ and IL-6) but increased levels of anti-inflammatory cytokines (IL-4 and IL-10). Western blot analysis demonstrated that the intensities of pGSK3 β (Ser9) in liver samples from mice treated with chloroquine and doxycycline combination were significantly ($P < 0.05$) higher suggesting that the adjunct treatment resulted in significant inhibition of GSK3 β . Taken together the bacteriostatic action of doxycycline coupled with the cytokine-modulating effect of chloroquine gave full protection to *B. pseudomallei*-infected mice and involved inhibition of GSK3 β . Findings from the present study using *B. pseudomallei*-infected BALB/c mice suggest that chloroquine is a plausible candidate for repurposing as adjunct therapy to treat acute *B. pseudomallei* infection.

INTRODUCTION

Melioidosis, an infectious disease caused by the Gram-negative bacillus bacteria, *Burkholderia pseudomallei* is a significant public health concern in endemic regions since it is a major cause of community-acquired pneumonia and sepsis (Wiersinga

et al., 2018). Although this disease is endemic in Southeast Asia and Northern Australia (Limmathurotsakul *et al.*, 2016), melioidosis is now recognized as an emerging neglected tropical infectious disease in many other parts of the world (Molyneux *et al.*, 2017). The high mortality rate in melioidosis in spite of appropriate

antibiotic therapy is attributed to severe sepsis as a result of overwhelming pro-inflammatory cytokine production in the host to eradicate *B. pseudomallei* infection (Kessler *et al.*, 2017). Novel host-directed therapeutics need to be developed to mitigate this dysregulated cytokine production at the early stage of infection and improve bacterial clearance (Chiang *et al.*, 2018). Wilson *et al.* (2016) has reported that post-treatment with a cyclooxygenase-2 (COX-2) inhibitor, tolfenamic acid together with a sub-therapeutic dose of ceftazidime increased the efficacy of the latter for melioidosis treatment in BALB/c mice demonstrating that modulation of the immune system is a potential therapeutic approach for *B. pseudomallei* infection. Hence, an adjunct treatment regime involving the combined use of anti-bacterial agents (antibiotics) with anti-inflammatory (immunomodulatory) agents may be more effective in overcoming septic shock than treatments relying solely on antibiotics.

We have recently demonstrated that treatment with the anti-malarial drug, chloroquine which increased survivability of *B. pseudomallei*-infected mice, was attributed to its cytokine-modulating effects mediated via inhibition of GSK3 β activity. This serine/threonine protein kinase, was initially described as a key enzyme involved in glycogen metabolism (Embi *et al.*, 1980) but is now also known to regulate a wide array of cellular processes. The kinase is implicated in several diseases including bipolar disorder, diabetes mellitus, Alzheimer's disease, inflammation, and cancer. Inhibition of GSK3 β is thus a plausible approach for the treatment of these diseases and many inhibitors of GSK3 β are being evaluated for this purpose (Xu *et al.*, 2018). GSK3 β plays an important role in the host response to pathogenic infections (Wang *et al.*, 2011) and is a point of convergence for the host inflammatory response (Wang *et al.*, 2011). Phosphorylation and consequent inhibition of GSK3 β have been shown to attenuate inflammation in many Gram-negative bacterial infections (Wang *et al.*, 2014) including *B. pseudomallei* (Tay *et al.*,

2012) since GSK3 β is known to be able to modulate other key players in inflammation such as NF- κ B (Medunjanin *et al.*, 2016). Based on these observations, we hypothesize that chloroquine, in combination with one of the currently-used antibiotics is a potential candidate to be repurposed as adjunct therapy for the treatment of melioidosis. Chloroquine is a novel activator of Akt and can elicit inhibition of GSK3 β via PI3K/Akt signaling (Halaby *et al.*, 2013). Chloroquine has been reported to exhibit anti-inflammatory properties and is able to protect mice from lipopolysaccharide (LPS) challenges via a mechanism involving reduction of pro-inflammatory cytokine levels (Hong *et al.*, 2004). In addition, chloroquine was shown to block TNF- α and IL-6 synthesis in LPS-stimulated mouse macrophages and human monocytes (Jang *et al.*, 2006). Chloroquine has also been reported to inhibit high-mobility group box 1 protein (HMGB1) inflammatory signaling and to protect mice from lethal sepsis (Yang *et al.*, 2013).

Initiatives for repurposing chloroquine include studies in various types of cancers (Cuffi *et al.*, 2013; Kimura *et al.*, 2013) and in diabetes (Asamoah *et al.*, 1990; Halaby *et al.*, 2013). In the case of melioidosis, repositioning existing drugs such as chloroquine in combination with currently-employed antibiotics such as doxycycline for melioidosis is a rational strategy to mitigate sepsis in *B. pseudomallei* infections. Doxycycline is an effective antibiotic in post-exposure prophylaxis of melioidosis (Estes *et al.*, 2010). Several studies have shown that doxycycline treatments reduced mortality in animal trials (Sivalingam *et al.*, 2008; Wang *et al.*, 2012). Sivalingam *et al.* (2008) demonstrated that 100% of *B. pseudomallei*-infected mice survived when doxycycline was administered at the time of challenge. Four Malaysian strains of *B. pseudomallei* tested were reported susceptible to doxycycline (Ahmad *et al.*, 2013). Another study with *B. pseudomallei* showed low rates of resistance to doxycycline when tested against 50 strains (2%) (Thibault *et al.*, 2004). Similarly Di Caprio *et al.* (2015)

reported that doxycycline was effective at preventing death from exposure to aerosolized *B. pseudomallei*.

In the present study we evaluated the effect of chloroquine adjunct treatment together with a sub-therapeutic dose of doxycycline, using a murine model of acute melioidosis infection. Although doxycycline is not the antibiotic employed in current clinical regime for treatment of the acute phase of melioidosis, in this study we have selected this bacteriostatic agent to prove a point that any increase in bacterial clearance in the adjunct treatment is likely due to enhance action of the immune cells. Animal survivability, cytokine levels and phosphorylation states of liver GSK3 β (Ser9) were assessed to investigate whether chloroquine can serve as an adjunct therapy (and repurposed) for the treatment of melioidosis in a mouse model of acute *B. pseudomallei* infection.

MATERIAL AND METHODS

Bacterial culture and animals

A stock of *B. pseudomallei* strain D286 was obtained from Prof. Dr. Sheila Nathan, Pathogen Laboratory, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). The bacterial culture was prepared according to Hoppe *et al.* (1999) and frozen back in 0.2-mL aliquots in Brain Heart Infusion Broth (BHIB) containing 20% glycerol at a concentration of 10⁹ CFU/mL at -80°C for later use in the infection studies. Male BALB/c mice approximately 6-8 weeks old were purchased from Takriff Bestari Sdn. Bhd. Kuala Lumpur, Malaysia. Animal use protocols were approved and conducted according to the guidelines from the UKM Animal Ethics Committee (UKMAEC) (FST/2015/EMBI/29-SEPT/701-SEPT-2015-MAC-2017).

Animal studies

Male BALB/c mice (n=9 per group) were injected intraperitoneally (i.p.) with 3 X 10 LD₅₀ (3 X 10⁵ CFU) (bacterial CFUs were confirmed by plating the inoculum on

Ashdown agar) of *B. pseudomallei* in 200 μ L of phosphate-buffered saline (PBS), where the 50% lethal dose (LD₅₀) for *B. pseudomallei* (based on our previous study (Ganesan *et al.*, 2018) was quantitated using the method of. To determine a suitable dose of doxycycline in the infection studies based on Gelhaus *et al.* (2013) and on comparable human treatment dose for melioidosis (Chaowagul *et al.*, 2005), 10, 20 and 40 mg/kg b.w. were injected intraperitoneally (i.p.) into *B. pseudomallei*-infected mice to ascertain the sub-optimal dose of doxycycline that can elicit ~50% survival of the animals. To evaluate the effects of adjunct treatment with chloroquine on animal survivability, infection experiments were conducted using a single intraperitoneal administration of chloroquine [50 mg/kg body weight (b.w.)] (the optimum dose based on our previous study (Ganesan *et al.*, 2018). Based on the parameters obtained from the above experiments, infection studies (using 3 X 10⁵ CFU *B. pseudomallei*) were then carried out as follows: chloroquine (50 mg/kg b.w.) was administered at one hour post-infection while sub-optimal dose of doxycycline (20 mg/kg b.w.) was injected at six hours post-infection initially and every 12 hours thereafter with the last at day 14 post-infection. The infection study continued until day 28 post-infection. Another two groups of infected mice were treated with either chloroquine (50 mg/kg b.w.) or doxycycline (20 mg/kg b.w.) alone. For non-treated infected control, *B. pseudomallei*-infected animals were injected (i.p.) with 0.9% sodium chloride (NaCl). Survivability of animals were monitored for 28 days. The infection experiment was repeated.

Bacterial load

To evaluate the effect of adjunct treatment on bacterial burden in liver, spleen and blood, a similar *in vivo* infection experiment as above was undertaken with separate groups of mice (n=3). The control group consisted of mice infected with *B. pseudomallei* only. Mice were euthanized by carbon dioxide inhalation and liver and spleen removed aseptically at day 3, 7, 14 and 21 post-infection. Cardiac puncture was used for

collection of blood samples. Blood and organ samples were also obtained randomly from surviving mice at day 28 post-infection (to observe any reduction of bacterial loads). All samples were processed as described by Leakey *et al.* (1998) for bacterial load determination.

Western blot analysis

Mice liver were collected at 24 hours post-infection on the basis that most of cytokines (IFN- γ , TNF- α , IL-1 β , IL-4 and IL-10) were reported to be expressed as early as 24 hours post-infection in melioidosis infection studies using BALB/c mice (Breitbach *et al.*, 2009; Hodgson *et al.*, 2013; Ulett *et al.*, 2000). Protein extraction was carried out as described by Wang & Zhu (2003). Liver samples were homogenized in 1:1 (w/v) extraction buffer containing 9.1 mmol/L NaH₂PO₄, 1.7 mmol/L Na₂HPO₄, 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 μ g/mL leupeptin and 100 μ g/mL aprotinin) and phosphatase inhibitors (1 mM Na₃VO₄, 1 mM NaF and 100 mM EDTA). The homogenates were centrifuged at 20 000 g for 30 min at 4°C. Protein concentration in the supernatants were determined by Bradford method. 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). Proteins were then electro-transferred onto nitrocellulose membranes and probed and incubated with primary monoclonal antibodies against total GSK3 β or phosphorylated GSK3 β (Ser9) (Cell Signaling, USA). This was followed by secondary antibody incubation with HRP-conjugated IgG (Cell Signaling, USA). β -actin was used as loading control. Detection of immuno-reactive proteins was carried out using Western Lightning™ Chemiluminescence Reagent Plus (MA, USA).

Cytokine assay

Mice were divided into two groups (n=6) comprising of Group I: *B. pseudomallei* infection only; Group II: *B. pseudomallei* infection + 50 mg/kg b.w. chloroquine + 20 mg/kg doxycycline. Blood samples were collected at 24 hours post-infection (similar to that for Western analysis) by cardiac puncture during carbon dioxide anesthesia 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-6, IL-10 and IL-4) using enzyme-linked immunosorbent assay kits (Qiagen, Germany).

Statistical analysis

Log-rank test was conducted for Kaplan-Meier survival analysis whilst t-test was performed on the bacterial load using Prism 6.0 software (Graphpad). Cytokine analysis data were expressed as mean \pm SEM based on samples obtained from six mice per treatment. Western blot analysis data obtained were expressed as mean \pm SEM based on duplicate densitometric analysis (calculated based on two separate experiments). P value of < 0.05 between groups was considered statistically significant.

RESULTS

Chloroquine treatment with a sub-therapeutic dose of doxycycline gave full protection in a mouse model of acute *B. pseudomallei* infection

BALB/c mice were challenged (i.p.) with a local clinical isolate of *B. pseudomallei* (D286). The LD₅₀ for *B. pseudomallei* D286 was determined (via tests on animals which kill 50% of the test population) to be 1 X 10⁵ CFU. Mice infected with a dose exceeding 10⁵ CFU *B. pseudomallei* have been reported to develop paresis of both hind legs, piloerection and appear lethargic leading to paralysis before death from the effect of infection (Feezor *et al.*, 2003). Figure 1

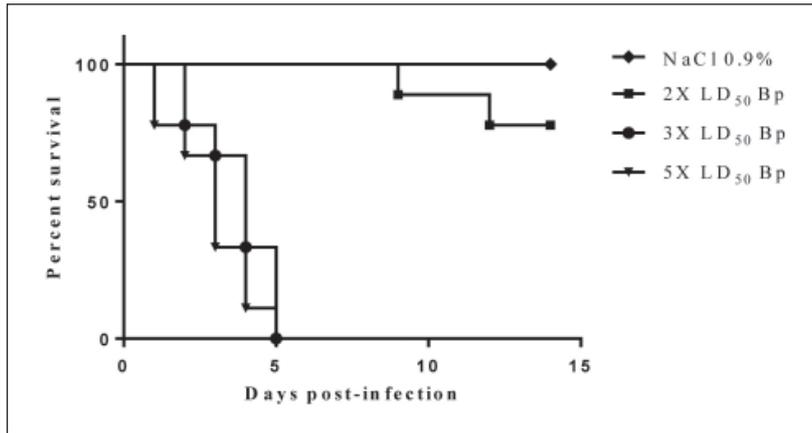


Figure 1. Kaplan-Meier survival curve of BALB/c mice (n=9 per group) administered with 2X, 3X and 5X LD₅₀ of *B. pseudomallei*. The survivability of mice observed for 14 days as compared to control treated with NaCl 0.9%.

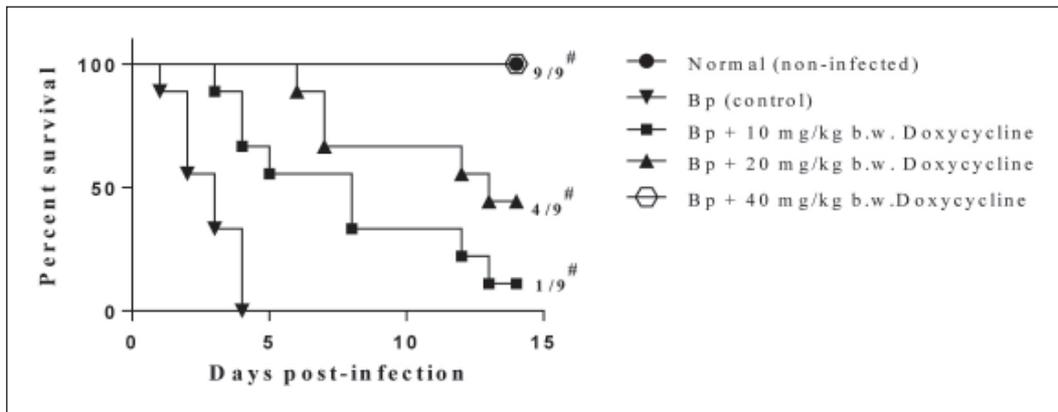


Figure 2. Kaplan-Meier survival curve for determination of sub-optimal dose of doxycycline in *B. pseudomallei*-infected mice. Survival of BALB/c mice (n=9 per group) administered intraperitoneally with 3 X 10⁵ CFU (3 X LD₅₀) *B. pseudomallei* D286 with 10, 20 and 40 mg/kg b.w. doxycycline. Non-treated infected (Bp + 0.9% NaCl) mice were used as control and survivability monitored for 14 days. # denotes number of surviving animals at day 14 post-infection.

shows that 3 X LD₅₀ (3 X 10⁵ CFU) was a suitable dose to use in establishing acute melioidosis infection; all mice infected with 3 X LD₅₀ bacteria died within day 2-5 post-infection indicating acute infection by *B. pseudomallei* (Leakey *et al.*, 1998). To determine the suitable dose of doxycycline for use in subsequent infection studies, animal survivability experiments using three different doses (10, 20 and 40 mg/kg b.w.) of doxycycline were conducted to ascertain optimal and sub-optimal doses. Results obtained (Figure 2) showed that treatment

with 20 mg/kg b.w. doxycycline resulted in nearly 50% animal survivability compared to 100% survivability at a dose of 40 mg/kg b.w. whilst the dose of 10 mg/kg b.w. resulted in only 11% survivability. Thus, the sub-optimal dose of 20 mg/kg b.w. was employed in subsequent adjunct experiments.

Results obtained from the adjunct studies showed that mice treated with a sub-therapeutic dose of doxycycline (20 mg/kg b.w.) showed 44% survivability (Figure 3a). Infected animals treated with 50 mg/kg b.w. chloroquine alone showed 56% survivability.

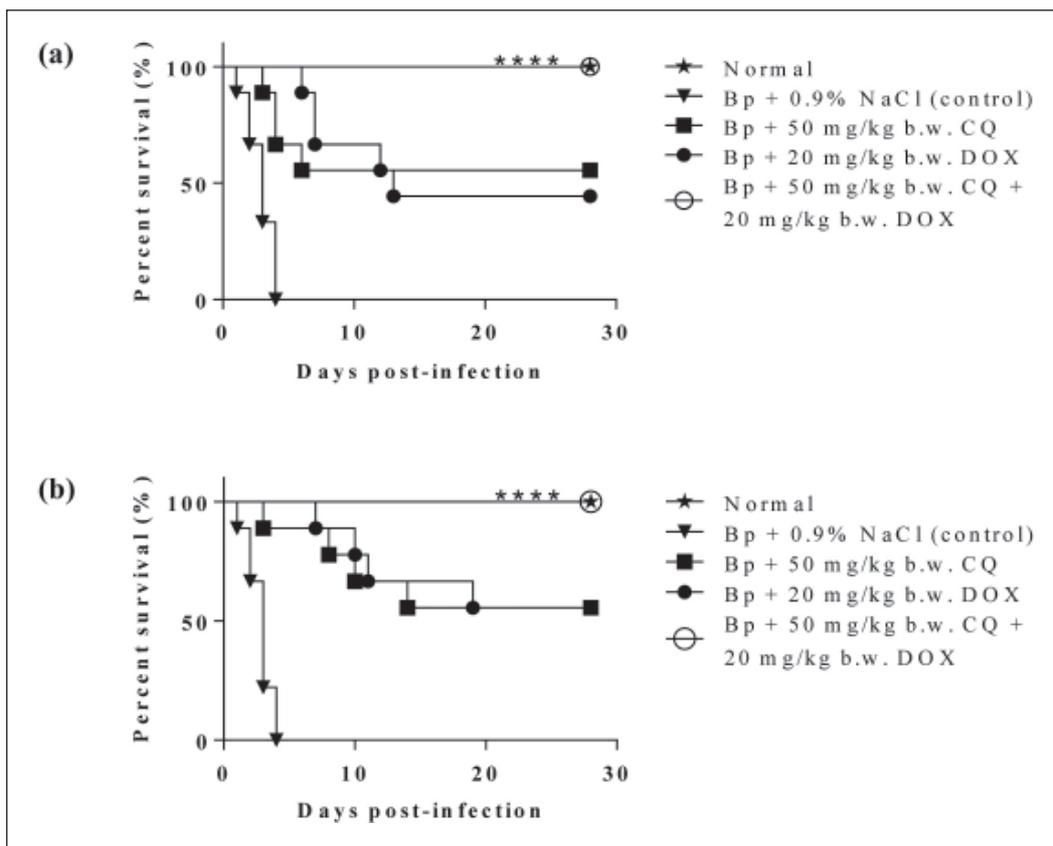


Figure 3. Kaplan-Meier survival curve of BALB/c mice (n=9 per group) administered intraperitoneally with 3×10^5 CFU ($3 \times LD_{50}$) of *B. pseudomallei* D286 with or without treatments. (a) Data represent infected mice treated with chloroquine alone (Bp + 50 mg/kg b.w. CQ), doxycycline alone (Bp + 20 mg/kg b.w. DOX) and adjunct treatment (Bp + 50 mg/kg b.w. CQ + 20 mg/kg b.w. DOX) (b) Repeat experiment. Non-treated infected (Bp + 0.9% NaCl) mice were used as control and survivability was monitored for 28 days. Significant difference between tested and control groups was evaluated at via Mantel-Cox test **** $p < 0.0001$.

To assess if immune modulation can potentiate the efficacy of antibiotic treatment of acutely-infected animals, experimental mice were given adjunct treatment with 50 mg/kg b.w. chloroquine (one hour post-infection) followed by 20 mg/kg b.w. doxycycline (six hour post-infection and consequently every 12 hours till day 14 post-infection). This adjunct treatment resulted in 100% survivability of infected animals monitored at day 28 post-infection (Figure 3a). A repeat experiment (Figure 3b) for the combination treatment also yielded 100% survivability compared to the control group (*B. pseudomallei*-infected mice without treatment). Mice which received adjunct

treatment also did not show any adverse symptoms of toxicity (ruffled hair, lethargy and hunched posture) and appeared as active as the animals in the normal (non-infected) group.

Adjunct treatment (chloroquine and doxycycline) reduced *B. pseudomallei* bacterial burden in organs and blood of *B. pseudomallei*-infected mice significantly

The bacterial loads in liver, spleen and blood of infected mice were determined in adjunct-treated and non-treated mice at 3, 14, 21 and 28 days post-infection. High bacterial counts of *B. pseudomallei* ($\sim 10^5$ to $\sim 10^6$ CFU) were

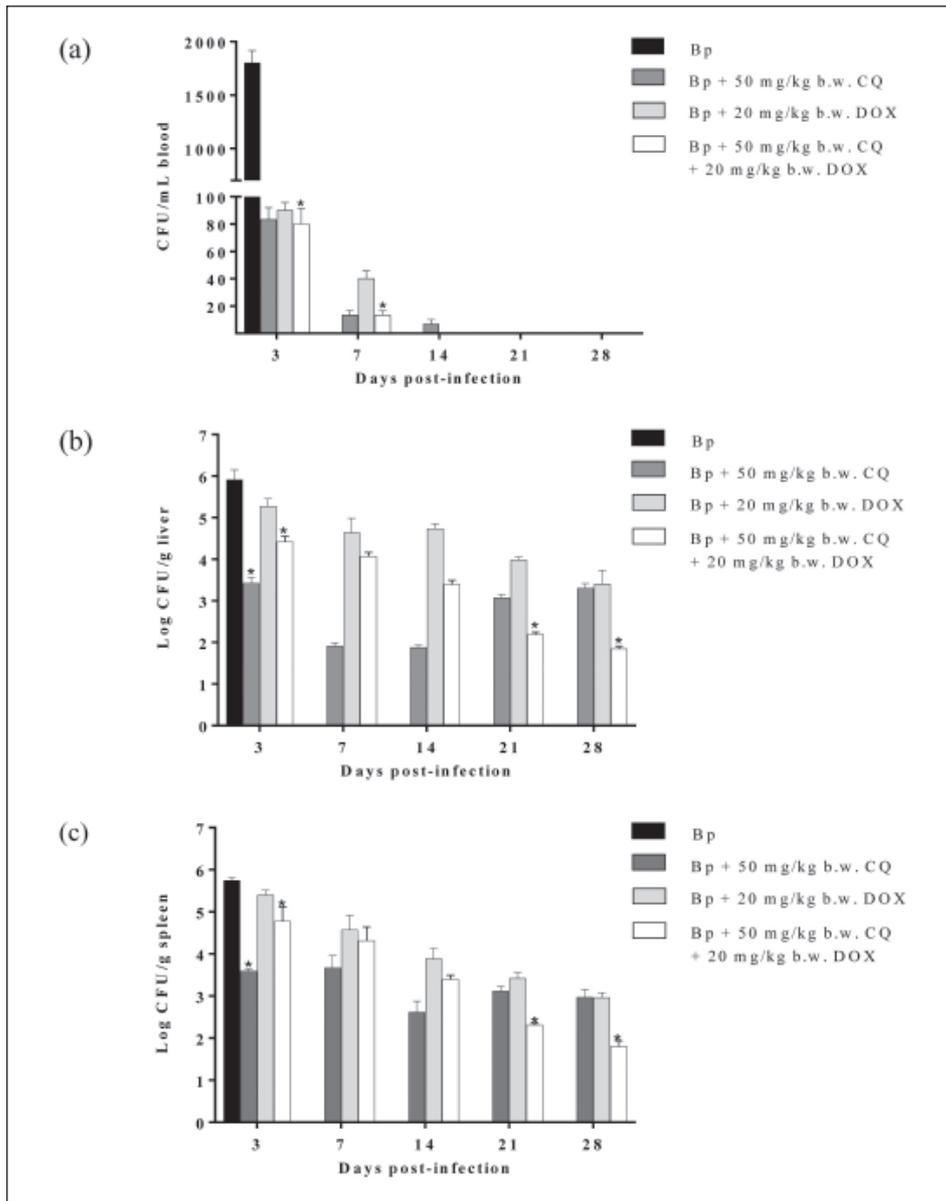


Figure 4. Bacterial loads in the (a) blood (b) liver (c) spleen of BALB/c mice (n=3 per group) at day 3, 7, 14, 21 and 28 post-infection administered with 3 X LD₅₀ (3 X 10⁵ CFU) of *B. pseudomallei* with or without adjunct treatment. The data for control mice on day 7, 14, 21 and 28 post-infection are not represented as all the mice died within 5 days post-infection.

detected by day 3 post-infection in both liver and spleen of non-treated mice (Figures 4b and 4c). Both of these organs are targets in melioidosis infection (Laopaiboon *et al.*, 2011). The bacterial load in blood of the infected group of animals given adjunct treatment continued to decline with time and no bacterial load was observed at day 14, 21

and 28 post-infection (Figure 4a). Significant decrease in bacterial loads were seen in liver and spleen of *B. pseudomallei*-infected mice administered with 50 mg/kg b.w. chloroquine followed with 20 mg/kg b.w. doxycycline at 3, 7 and 21 days post-infection (Figure 4b and 4c). Drastic reduction in liver and spleen bacterial counts were also observed on day

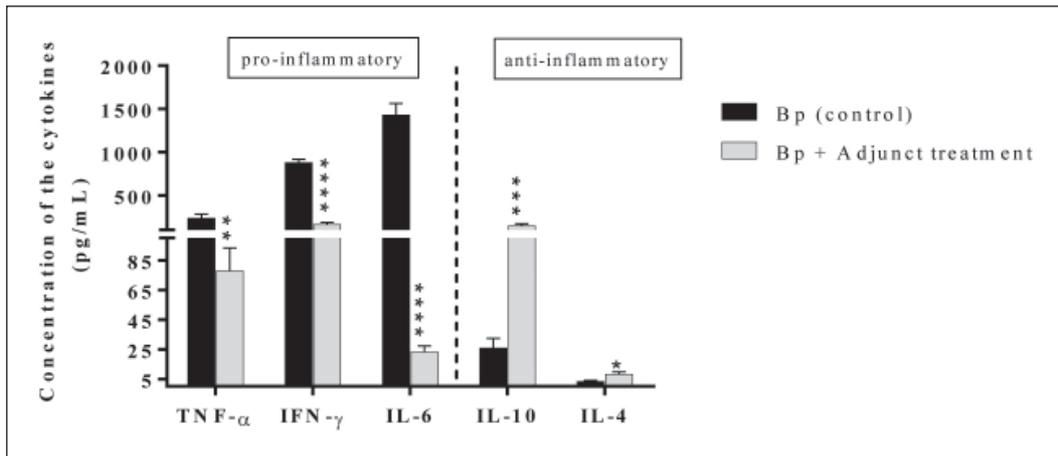


Figure 5. Cytokine levels in serum of *B. pseudomallei*-infected mice in response to adjunct treatment. The levels of pro-inflammatory (TNF- α , IFN- γ and IL-6) and anti-inflammatory (IL-4 and IL-10) cytokines levels in sera of mice (n=6 BALB/c per group) administered with 3×10^5 CFU ($3 \times LD_{50}$) of *B. pseudomallei* with or without adjunct treatment. Data are expressed as mean \pm SEM. Significant difference as compared with control infected group was evaluated at $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).

28 post-infection in mice treated with chloroquine-doxycycline combination. Those treated infected animals also remained alive throughout the animal study (28 days). Adjunct treatment however did not completely clear the total numbers of bacteria in both organs on day 28 post-infection. Thus although the overall bacterial load in the experimental animal organs were not cleared completely with the adjunct treatment, animal survivability increased significantly.

Adjunct treatment (chloroquine and doxycycline) modulated the levels 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 treated and non-treated animals during melioidosis infection. Serum was taken at day one post-infection for analysis because detectable levels of cytokines (IFN- γ , TNF- α , IL-6 and IL-10) were observed in BALB/c mice 24 hours post-infection (Hodgson *et al.*, 2013; Ulett *et al.*, 2000). The levels of pro-inflammatory cytokines TNF- α , IFN- γ and IL-6 in sera of *B. pseudomallei*-infected mice were reduced ($P < 0.05$) to 0.32, 0.20 and 0.02 times with adjunct treatment. Adjunct

treatment also increased the levels of anti-inflammatory cytokines IL-4 and IL-10 to 2.50 and 5.70 times the level in control (non-treated infected mice) (Figure 5). Overall, based on the results obtained, adjunct treatment resulted in significant ($P < 0.05$) reduction of pro-inflammatory cytokines compared with chloroquine treatment only.

Adjunct treatment (chloroquine and doxycycline) resulted in increased phosphorylation of GSK3 β (Ser 9) in liver of *B. pseudomallei*-infected mice

To investigate whether the increased survivability and bacterial load reduction in adjunct-treated infected mice described above involves GSK3 activity, we determined the phosphorylation states of liver GSK3 β (Ser9) using western analysis. Densitometric analysis (Figure 6) of the phosphorylated GSK3 β (Ser9) showed a significant increase due to the combination treatment (adjunct treatment i.e. Bp+CQ+DOX) in liver by 5.3 ± 0.7 fold at day one post-infection compared to control group (Bp). In addition, increased levels of pSer9 GSK3 β were detected in animals receiving chloroquine treatment alone (Bp+CQ) by 3.7 ± 0.9 fold and treatment of doxycycline alone (Bp+DOX) by 3.5 ± 1.0 fold compared to control.

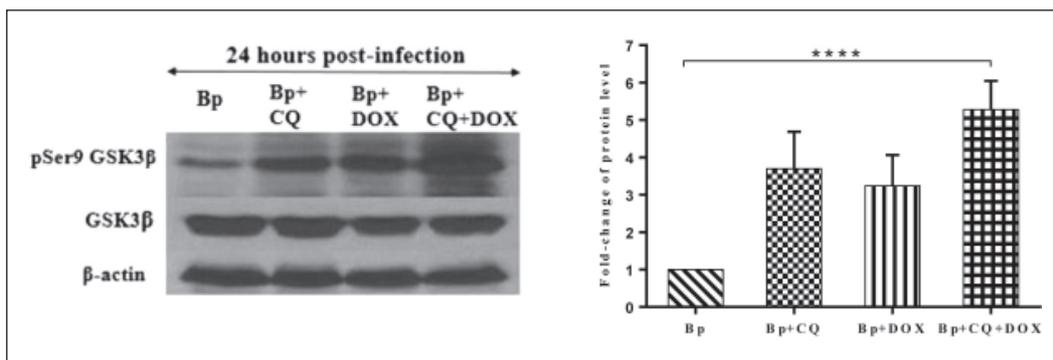


Figure 6. Levels of pGSK3 β (Ser9) significantly increased in liver of *B. pseudomallei*-infected mice in response to adjunct treatment. Western blotting analysis of total GSK3 β protein expression levels were normalized to β -actin and pGSK3 β was normalized to total GSK3 β protein level. Liver organs were obtained from *B. pseudomallei*-infected (Bp), chloroquine-treated infected (Bp+CQ), doxycycline-treated infected (Bp+DOX) and chloroquine-doxycycline treated infected (Bp+CQ+DOX) groups of mice at 24 hours post-infection. Densitometric measurements are represented as mean \pm SEM of treated group compared to non-treated control. Significant difference between tested and control groups was evaluated at $p < 0.0001$ (****). One representative western blotting image is shown here.

These findings imply that doxycycline or chloroquine treatment alone was also able to cause inhibition of liver GSK3 β but to a lower extent.

DISCUSSION

In the endemic areas, melioidosis is associated with high mortality even with antibiotic treatment (Limmathurotsakul *et al.*, 2016) due to severe sepsis as a result of overwhelming inflammatory response. Host-directed therapeutics which may modulate the host inflammatory cytokine response at an early stage of infection may be a feasible strategy to improve bacterial clearance (Chiang *et al.*, 2018). Findings from this study show that adjunct treatment involving a reported cytokine-modulating agent, chloroquine with a sub-therapeutic dose of the antibiotic, doxycycline significantly increased survival outcome and decreased organ bacterial burdens in *B. pseudomallei*-infected mice. Animal survivability with adjunct treatment was 100% compared to chloroquine treatment alone (67%) or treatment with a sub-therapeutic dose of doxycycline (50%). In this study, we observed significantly lowered bacterial counts 28 days post-infection in spleen, liver and blood

of infected animals administered with a combination of chloroquine and doxycycline when compared to those treated with chloroquine or doxycycline alone. The bacterial loads in the organs of chloroquine-treated *B. pseudomallei*-infected animals were significantly lower than the control infected animals suggesting that chloroquine treatment may have boosted the innate defense and consequently improved bacterial clearing resulting in better animal survivability. This protective effect of chloroquine corroborates findings from a study by Hong *et al.* (2004) in which 30 mg/kg b.w. of chloroquine protected mice from lethal challenge by LPS. The lower bacterial load in liver, spleen and blood of *B. pseudomallei*-infected mice administered with a sub-optimal dose of doxycycline is most likely due to its bacteriostatic activity in inhibiting bacterial growth through inhibition of bacterial protein synthesis in 30S ribosomal subunit (Holmes & Charles, 2009). Nevertheless, a therapeutic dose of doxycycline alone was effective at preventing death from exposure to *B. pseudomallei*, corroborating previous reports by Gelhaus *et al.* (2013) and Sivalingam *et al.* (2008) in which 100% of mice survived when doxycycline was administered to *B. pseudomallei*-infected

mice. Doxycycline is active against *B. pseudomallei* and mostly used in the eradication stage of melioidosis therapy, but not in the initial intensive phase (Dance, 2014). It is interesting to evaluate whether doxycycline besides its bacteriostatic property can serve as an immunomodulator in adjunct therapy in combination with antibiotics frequently employed in treatment for the acute phase melioidosis, such as ceftazidime (β -lactams) although Mohamad *et al.* (2018) reported that doxycycline in combination with β -lactams had no synergistic effect when tested *in vitro* and concluded that doxycycline offers no additional benefit to be used in combination with front-line antibiotics in the intensive phase of melioidosis. In light of the findings from our work and various previous reports that doxycycline has anti-inflammatory effects *in vivo*, studies to assess the benefit of combined therapy of doxycycline and first-line antibiotics such as ceftazidime is warranted.

Our western analysis showed that at 24 hours post-infection, higher levels of GSK3 β was phosphorylated (at Ser9) in liver of infected animals which received adjunct treatment compared to that in animals receiving chloroquine or doxycycline treatment alone. It is likely that the higher level of phosphorylated GSK3 seen here is a consequence of the phosphorylation and activation of Akt (Beurel *et al.*, 2015) since GSK3 which remains active in the early phase of infection then can result in the failure of the host to control infection due to dysregulation of inflammatory cytokine production which involve PI3K/Akt signaling. The increased survivability of *B. pseudomallei*-infected mice seen in the present study after adjunct treatment (chloroquine and doxycycline combination) was shown to be due to Akt-mediated inhibition of GSK3 β . As explained in our previous study (Ganesan *et al.*, 2018), reduction of bacterial load in organs and blood of *B. pseudomallei*-infected mice treated with chloroquine alone could be attributed at least in part to its inhibitory effect on GSK3 β as a consequence of Akt activation. It is noteworthy from our animal infection

studies, that treatment with a sub-therapeutic dose of doxycycline on its own resulted in significant phosphorylation of Akt and GSK3 β (Figure 6). This finding suggests that chloroquine (Ganesan *et al.*, 2018) and doxycycline each can cause inhibition of GSK3 β through activation of Akt. It has been previously reported that GSK3 β in the constitutively active state preferentially favors production of pro-inflammatory cytokines (Ohtani *et al.*, 2008). GSK3 β inhibition is crucial in modulating the pro- and anti-inflammatory cytokines as a result of *B. pseudomallei* infection (Wang *et al.*, 2011).

Next, we investigated the involvement of GSK3 β as a regulator in the production of inflammatory cytokines. Pro-inflammatory cytokine production is important in increasing macrophage anti-microbial activity and regulating pathogen growth (Ehlers *et al.*, 1992). In humans infected with melioidosis (Wiersinga *et al.*, 2007) and in BALB/c and C57BL/6 models of the disease, these cytokines (TNF- α , IFN- γ and IL-6) have been reported to be elevated (Ulett *et al.*, 2000). TNF- α is the earliest pro-inflammatory cytokine produced during bacterial infections or other microorganisms and plays an important role in early immune responses such as innate immunity that allows macrophage and neutrophil to travel to infection sites (Coant *et al.*, 2011). *B. pseudomallei* infection can also induce excessive IFN- γ cytokine production within 24 hours after infection and cause acute melioidosis (Santanirand *et al.*, 1999). Our cytokine analysis revealed that the serum levels of pro-inflammatory cytokines, TNF- α , IFN- γ and IL-6 were significantly reduced ($P < 0.05$) in infected mice which received adjunct treatment compared to the control (*B. pseudomallei*-infected mice without treatment).

Recent studies have shown that chloroquine as well as doxycycline possess strong immunomodulatory effects (Bode *et al.*, 2014; Oh *et al.*, 2016). The anti-inflammatory cytokine IL-10 is involved in host defense mechanisms to control inflammatory responses induced by bacterial infections (Zhang *et al.*, 2009). IL-4 (another

anti-inflammatory cytokine) on the other hand, plays a major role in the activation of B cells and T cell polymorphisms and to reduce the production of Th1 and pro-inflammatory cytokines response in acute melioidosis patients (Chin *et al.*, 2010). During acute melioidosis infection, excessive production of pro-inflammatory cytokines greatly reduced IL-10 and IL-4 production in the host (Hodgson *et al.*, 2013) and this may lead to septicemia. In the present study, *B. pseudomallei*-infected mice treated with adjunct treatment (chloroquine and doxycycline) was found to have significantly increased levels of IL-10 and IL-4 than the control group. Improved survivability and reduction in pro-inflammatory cytokine levels in *B. pseudomallei*-infected mice with GSK3 β inhibition observed in the present study implicate the involvement of this kinase in the modulation of excessive pro-inflammatory response. Most importantly, we showed that the adjunct treatment with the sub-therapeutic dosage of antibiotic significantly increased survival outcome and decreased organ bacterial burdens. The improved survivability of the adjunct-treated animals was likely at least in part mediated via inhibition of GSK3 β and consequent modulation of the inflammatory response to the infection.

We have provided evidence to show that that chloroquine is potentially useful for adjunct therapy in melioidosis. To the best of our knowledge the present study is the first report for repurposing of chloroquine as adjunct treatment for melioidosis using mice infection studies. Drugs in clinical use for unrelated conditions, primarily those that can affect the balance of the cytokine response may have benefits in melioidosis (Laws *et al.*, 2019). Previously, we have shown that chloroquine is a potential candidate for repurposing based on its GSK3-inhibitory action.

The present findings support our notion that inhibition of GSK3 β is a plausible strategy to mitigate dysregulation of cytokine balance during acute melioidosis infections (Ganesan *et al.*, 2018; Tay *et al.*, 2012) and that GSK3 β is a relevant molecular target for host-directed adjunct therapy for *B. pseudomallei*

infections in mice. In conclusion, chloroquine is a plausible candidate to be repurposed for treatment of melioidosis. Further studies on adjuvant use of chloroquine with other antibiotics commonly used in melioidosis therapy is needed to confirm that immunomodulation via GSK3 β inhibition and subsequent dampening of pro-inflammatory cytokine response is an effective adjunct therapy strategy for melioidosis to warrant human clinical trials.

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