



## RESEARCH ARTICLE

# Release of pro-inflammatory cytokines TNF- $\alpha$ , IFN- $\gamma$ and IL-6 by *Burkholderia pseudomallei*-stimulated peripheral blood mononucleocytes of acute myeloid leukemia patients

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### ABSTRACT

Acute myeloid leukemia (AML) is a malignant disease progressed from abnormal production of immature myeloid cells, which is often associated with concurrent infections after diagnosis. It was widely established that infections are the major contributors to mortality in this group due to the prevalence of neutropenia. Gram-negative *Burkholderia pseudomallei* is the causative agent of melioidosis. This disease had been reported in several neutropenic cancer patients undergoing chemotherapy resulting in severe clinical presentations and high mortalities which is in need of critical attention. Studies show that cytokines are important mediators of melioidosis progression and low neutrophil counts are associated with progression of its severity. However, to date, there are no reports on cytokine production in neutropenic cancer patients who are prone to melioidosis. Hence, here we assessed the cytokine production in neutropenic AML patients by introducing *B. pseudomallei* to their peripheral blood mononuclear cell (PBMC) culture *in vitro*. We observed that inflammatory response related cytokines namely TNF- $\alpha$ , IFN- $\gamma$  IL-6 and IL-10 were highly circulated in infected PBMCs suggesting that these cytokines may play important roles in the progression of severity in melioidosis infected neutropenic patients.

**Keywords:** Acute myeloid leukemia; neutropenia; melioidosis; cytokines; *Burkholderia pseudomallei*.

### INTRODUCTION

Leukemia is a clonal malignancy of the myeloid or lymphoid progenitors in the bone marrow. Acute myeloid leukemia (AML) progressed from somatic mutations in myeloid lineage precursors coupled with transcriptomic dysregulation of bone-marrow infiltration that lead to the production of immature myeloid cells (blasts) and disruption in normal haematopoiesis. AML is diagnosed by high blast counts in the blood whereby presentation of 20% or more blasts are determined as AML. The susceptibility of AML patients to life-threatening infectious complications is highly acknowledged as it is the major cause of morbidity and mortality in this group (Klastersky *et al.*, 2000). Both the Gram-negative and Gram-positive bacteria had been seen in acute leukemia cases, and the presentations are more severe in patients with lower neutrophil counts (neutropenia) and impaired granulocyte functions (Hansen *et al.*, 2019). Additionally, accumulation of immature myeloid cells can alter the antigen-specific T-cell response (Almand *et al.*, 2001).

Senescence and exhaustion of T-cell, natural killer (NK) and  $\gamma\delta$  T-cell function are the vital features of immune dysfunction in AML. Thus, AML patients are recognized as immunocompromised and often presented with concurrent infections (Ersvaer *et al.*, 2007).

The importance of innate immune system onset of pathogenic infections, particularly in the regulation of inflammatory cytokines and activation of the adaptive immune system, is well established. For instance, in normal hematopoietic system, pathogenic recognition receptors and pro-inflammatory cytokines induce emergency myelopoiesis during infections, which may consequently promote differentiation of myeloid progenitors and lead to enhanced immune cell productions (Chiba *et al.*, 2018). Poor immune cell productions that result from impaired myelopoiesis plus alteration of hematopoietic system by cytotoxic chemotherapy are the major contributions of neutropenia in AML. Absolute neutrophil counts (ANC) less than 1500 cells/mm<sup>3</sup> is defined as mild neutropenia, while ANC less than 1000 cells/mm<sup>3</sup> is considered moderate and ANC less than 500 cells/mm<sup>3</sup> is

categorized as severe neutropenia (Lustberg, 2012). Cytokine is secreted by a broad-range of immune cells during infections. Neutrophil is one of the vital components in host survival associated with pathogenic clearance via production and amplification of cytokine responses (Tecchio et al., 2014). Cytokine binds to specific receptors of the targeted cells and ultimately induces cellular changes such as inflammation and pain. In AML, cytokines IL-6, IL-8 and TNF-alpha were reported to be elevated during infection and were greatly linked with progression of septic shock (Chaudhary et al., 2012).

Gram-negative, soil-dwelling *Burkholderia pseudomallei* is the causative agent of melioidosis. This disease is endemic in South East Asia and Northern Australia in which the occurrence is more likely to take place during monsoonal season, when the rainwater drive this soil residing etiological agent out to the surface (Gassiep et al., 2020). Global incidences of melioidosis is 165 000 and blamed for 89,000 fatalities worldwide. Melioidosis mortality rate remains high as up to 40% and estimated to be severely underreported in 45 countries (Limmathurotsakul et al., 2016). Primary clinical diagnosis of melioidosis is pneumonia, presented in most acute infections as well as septicaemia, coagulopathy, and shock. In chronic infections, abscesses, suppurative lymphadenitis, skin infection, septic arthritis/osteomyelitis, and liver infection are often presented (Zueter et al., 2016). The clinical presentations can be seen to be more grievous in immunocompromised individuals and linked to higher mortality. Melioidosis had been reported in acquired immunodeficiency syndrome (AIDS),  $\beta$ -Thalassemia, several cancers and neutropenic-induced chemotherapy patients with ovarian cancer, breast cancer, and leukemias (Lin et al., 1980; Currie et al., 2010; Mukhopadhyay et al., 2010; Meumann et al., 2012; Kingsley et al., 2016; Sukauichai & Pattarowas, 2020). Although rare, melioidosis in acute leukemias has been reported in a few cases. Symptoms ranging from fever, cellulitis, septicaemia and patchy pneumonitis are presented and often end with deaths (Tanphaichitra, 1989; Kingsley et al., 2016; Sridhar et al., 2016).

In normal individuals, neutrophil kills up to 90% of intracellular *B. pseudomallei* (Chanchamroen et al., 2009; Saengmuang et al., 2014). The paramount role of neutrophil during infections is well known as it exerts wide range source of diverse chemokines, pro-inflammatory cytokines, and growth factors (Tecchio et al., 2014). Inflammatory responses particularly, cytokine productions onset of infection in melioidosis yields initial protection to hosts. However, dysregulation of cytokine-mediated immune response can trigger lavish inflammation and fatal outcome. Notably IFN- $\gamma$ , IL-18, IL-12, and other cytokines including IL-15, TNF- $\alpha$  and IL-6 favour disease severity (Lauw et al., 1999; Simpson et al., 2000). On contrary, anti-inflammatory cytokine IL-10 supports bacterial killing (Kessler et al., 2017).

Since cytokine and neutrophil productions observed to be impactful in host response during melioidosis infection, it is believed that these responses are severely impaired in neutropenic patients, that is often seen with displeasing outcomes. Therefore, in the present study, we extracted peripheral blood mononuclear cell (PBMC) from severe neutropenic AML patients (ANC < 500 cells/mm<sup>3</sup>) and introduced them with *B. pseudomallei* *in vitro*. Cytokines production following infection with three different strains of *B. pseudomallei* was observed. Additionally, we also measured the expression of cyclooxygenase-2 (COX-2), which is reported as one of the cytokines mediators that is highly produced during inflammation (Ramsay et al., 2003). We

believe that this study may lead to better understanding of cytokine production in neutropenic AML patients during infection with bacteria that are primarily targeted by neutrophils.

## MATERIALS AND METHODS

### Human subjects

Neutropenic AML patients (n=5) were recruited for this investigation. All human related experiments were carried out in accordance with the guidelines and regulations and under examination by the Medical Ethics Committee (MEC) of Universiti Malaya Medical Centre (UMMC), Kuala Lumpur, Malaysia (ref. no. 2019514-7415), and also followed the guidelines of the International Conference on Harmonization Guidelines and the Declaration of Helsinki. All five patients underwent chemotherapy and were confirmed for neutropenia with ANC less than 500 cells/mm<sup>3</sup>. Patients with existing fungal, bacterial and viral infections were excluded. Common bacterial infection in neutropenic individuals such as infection of *Escherichia coli*, *Klebsiella* species, and *Pseudomonas aeruginosa* were also included in exclusion criteria.

### Isolation of peripheral blood mononuclear cells

PBMCs isolation was performed as previously described (Barathan et al., 2015). Briefly, nine ml of patient's whole blood was taken in BD Vacutainer® heparin lithium tubes (BD Biosciences, Franklin Lakes, NJ, USA). Separations of PBMCs were carried out by density-gradient centrifugation on Ficoll-Paque™ (Amersham Pharmacia, Piscataway, NJ, USA). Later, extracted cells were re-suspended in freezing media consisted of 10% DMSO and 90% fetal bovine serum (FBS). PBMCs were cryopreserved until further use in any assay.

PBMCs were cultured in RPMI-1640 media with additional supplementation of HEPES buffer (25 mM), penicillin (100 U/ml), streptomycin (100  $\mu$ g/ml), sodium pyruvate (1 mM), gentamicin (5  $\mu$ g/ml), L-glutamine (2 mM), sodium pyruvate (1 mM) (all purchased from Life Technologies, Victoria, Australia), and 10% of FBS (Gibco) and incubated at 37°C in a 5% CO<sub>2</sub> cell culture incubator.

### Bacterial strains and infection

Three bacterial strains were used: two isolates of *B. pseudomallei* clinical isolates obtained from University Malaya Medical Center (UMMC), OB (WT, INSDC: APLK0000000.1) and OS (SCV, INSDC: APLL0000000.1) and K9 (*B. pseudomallei* K96243) isolated from a melioidosis patient in Thailand and is commonly used as a reference strain. Characterization of all strains were performed using analytical profile index API 20NE (BioMrieux) test and PCR assay specific for *B. pseudomallei* (Menon et al., 2020). All of them were cultured in Luria-Bertani (LB) broth in a shaking incubator (37°C at 200 rpm). On the following day, all cultured tubes were adjusted to OD 600 nm using phosphate-buffered saline (PBS). Number of viable cells were determined by serial dilution and plating on nutrient agar. Colony forming units (c.f.u.) ml<sup>-1</sup> for all strains were calculated upon 24 hours of growth at 37°C.

Briefly, PMBCs were plated in six-well plates (1 $\times$ 10<sup>6</sup> cells) and incubated for at least 16 hours. Later, live bacteria were introduced at multiplicity of infection (MOI) of 10:1 and further incubated for 6 hours. The PBMCs and supernatant were harvested and stored separately at -80°C for further analysis.

### Cytometric Bead Array (CBA)

Cytokines were determined using Human Th1/Th2/Th17 CBA kit (BD Biosciences, San Jose, California). Upon thawing,

PBMCs were diluted in assay diluent (1:2 v/v) and CBA was carried out according to manufacturer's instructions. In brief, fifty microliters of supernatant were mixed with specific cytokines antibodies (IL-2, IL-4, IL-6, IL-10, TNF- $\alpha$ , IFN- $\gamma$  and IL-17A) with the presence of phycoerythrin-conjugated detection antibody. Then, samples were incubated for 3 hours in the dark before washing with wash buffer, followed by centrifugation at 200g for 5 minutes. Later, the cell pellets were re-suspended in 300  $\mu$ l wash buffer and measured using the BD FACS Canto II Flow Cytometer and analyzed by FACS Array™ Software (BD Bioscience).

#### COX-2 Assay

Total COX-2 was determined using Human/Mouse Total COX-2 Immunoassay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instruction. In brief, bacteria treated PBMCs were centrifuged (500 g x 3 minutes, 4°C) and fixed with 8% formaldehyde. Fixed PBMCs were introduced with primary antibody mixture and incubated for 16 hours at 4°C. Later, cells were stained and read using fluorescence plate reader with excitation at 540 nm and emission at 600 nm.

#### Statistical analysis

Analysis of variance was performed using GraphPad Prism software version 8. Differences were considered statistically significant at a P value of <0.05.

## RESULTS

In this study, phytohemagglutinin (PHA) stimulated PBMCs were used as positive control. Three different strains of *B. pseudomallei* used were OB, OS and K9. OS is a small colony variant (SCV) which is associated with persistent infection and antibiotic resistance whereas OB is its wild-type (See et al., 2017). K9 was isolated from a melioidosis patient and had been widely used in experimental *B. pseudomallei*. Healthy individual cytokines were referred from unpublished data by Barathan et al. and presented as green dotted baseline in Figure 1.

CBA assay revealed that inflammatory cytokines TNF- $\alpha$ , IL-10, IFN- $\gamma$  and IL-6 are elevated in all strains, contrarily IL-2, IL-4 and IL-17A are less expressed (Figure 1). K9 shows significantly higher expression of IL-6, followed by OB and OS. Similarly, IL-6 also is the highest cytokine expressed in PHA. Meanwhile, in OB and OS, TNF- $\alpha$  is the highest to be expressed. Compared to healthy individual baseline, OS and K9 displayed elevation of IL-6. IL-10 is also elevated in OB and K9. Moreover, TNF-alpha and IFN-gamma are highly elevated in all strains. Interestingly, suppression of IL-17A was detected in all strains compared to healthy individuals. On the other hand, COX-2 assay revealed that that no significant difference can be observed between strains even though K9 exhibited slightly higher COX-2 reading compared to OB and OS (Figure 2).

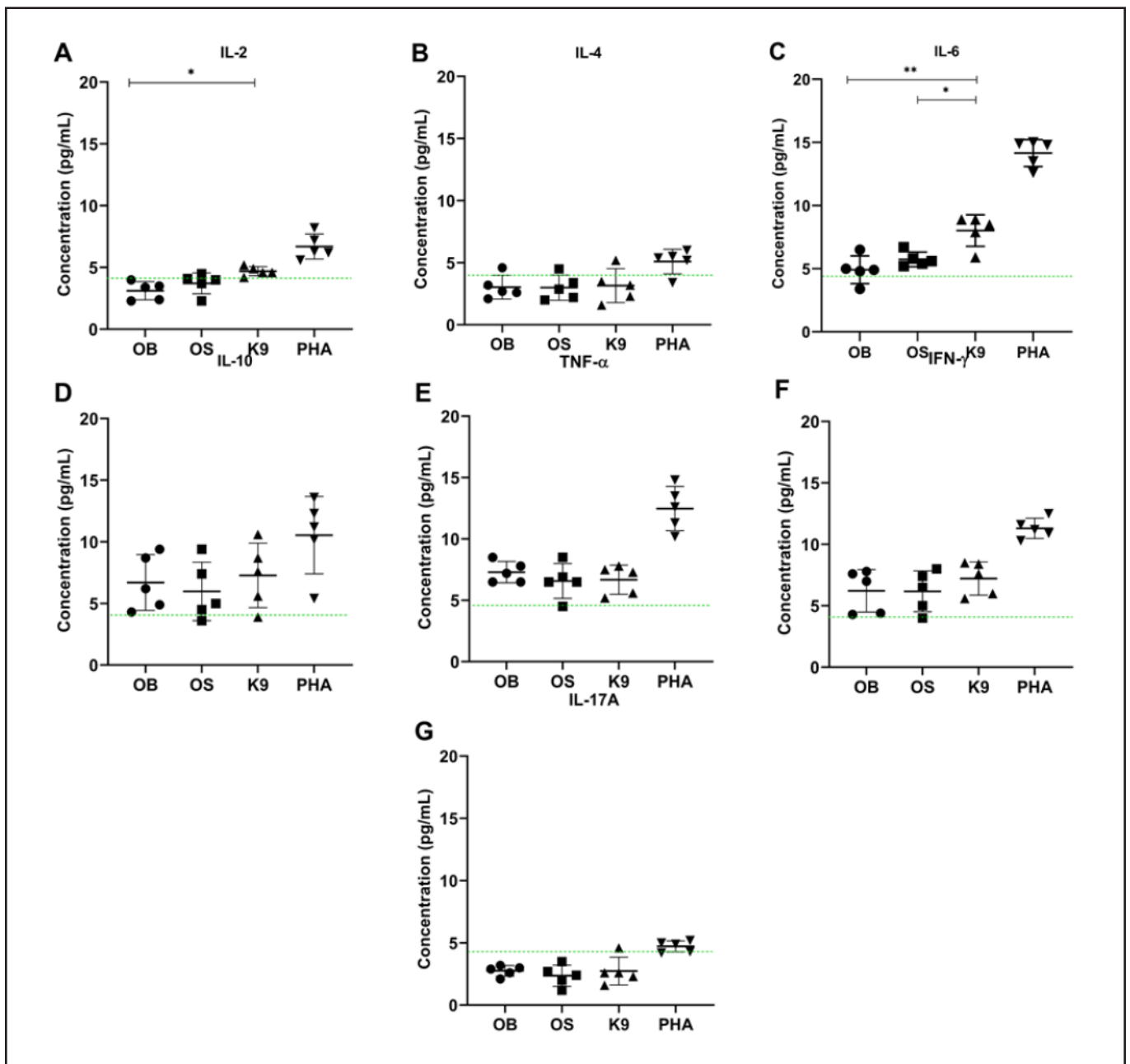
## DISCUSSION

It was deeply recognized that bacterial infections in individuals with neutropenia exerted poor outcomes particularly severe clinical manifestations and high mortalities. Although *E. coli*, *Klebsiella pneumoniae* and *P. aeruginosa* remained the most familiar pathogens in neutropenic individuals, *B. pseudomallei* infections have been reported in several cases (Mukhopadhyay et al., 2010; Sukauichai & Pattarowas, 2020). Melioidosis in neutropenic acute leukemias also have been reported and showed fast disease progression. Previous literature showed that

cytokine productions were responsible for immunity as well as severity during *B. pseudomallei* infection. We speculate that severity in melioidosis infected neutropenic patients is mediated by circulation of pro-inflammatory cytokines. To the best of our knowledge, we are the first to attempt investigation of cytokines productions in neutropenic AML patients upon infection with *B. pseudomallei*.

Notably, our study found that pro-inflammatory cytokines TNF- $\alpha$ , IFN- $\gamma$  and IL-6 were highly expressed in neutropenic AML upon exposure to *B. pseudomallei*. These cytokines had been observed to increase severity in melioidosis (Lauw et al., 1999; Simpson et al., 2000). Similarly, these cytokines also had been detected to circulate in type-2 diabetic (T2D) individuals with melioidosis. It is noteworthy that T2D is the most prevalent risk factor associated with vulnerability towards melioidosis. Clinical studies have shown that diabetic individuals exhibited limitation in neutrophil chemotactic, phagocytic and microbicidal activities similar to AML patients (Alba-Loureiro et al., 2007; Bornemann et al., 2020). As neutrophils were identified as the major immune cells involved in intracellular killing of *B. pseudomallei*, neutrophil dysfunctions were believed to be the underlying factor in rapid progression of melioidosis in this group of individuals. In BALB/c mouse models, neutrophils were found to be the major cells containing *B. pseudomallei* antigen (Laws et al., 2011). Additionally, activated neutrophils were observed to be rapidly recruited to the infection site and produced most of the inflammatory cytokines upon *B. pseudomallei* infection in resistant C57BL/6 mice (Easton et al., 2007). Moreover, neutrophils were reported to have the ability to induce autophagy adjacent to phagosomes containing *B. pseudomallei* in a Type III secretion system-dependent manner suggesting killing of *B. pseudomallei* by neutrophils also through macroautophagy (Rinchai et al., 2015).

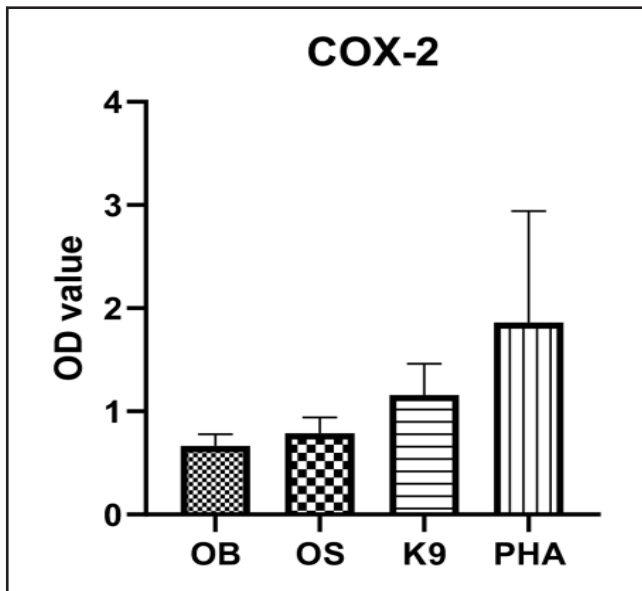
In melioidosis, monocyte-derived IL-10 was found to be beneficial in host response as it improved the killing of *B. pseudomallei* *in vitro* (Kessler et al., 2017). In the present study, we found that IL-10 is highly expressed in infected neutropenic AML PBMCs. In other settings, albeit favoring the host, IL-10 can also alter the proper bacterial clearance by inducing excessive suppression of immune response. In the context of melioidosis, overexpression of IL-10 contributes to suppression of IFN- $\gamma$ , TNF- $\alpha$  and IL-6 and may support pathogenic persistence (Kessler et al., 2017). Experimental mice showed early production of IFN- $\gamma$ , TNF- $\alpha$  and IL-6 are important in the immunopathogenesis of this disease (Ulett et al., 2000). Hence, high production of IL-10 may not be beneficial and as such it is not surprising that IL-10 was found to be highly expressed in melioidosis patients who did not survive rather than survivors (Kaewarpai et al., 2020). Moreover, we discovered that cytokine IL-17A was suppressed in AML PBMCs after infection compared to uninfected healthy individuals. Although the role of IL-17A was not known in melioidosis, healthy individuals showed no changes of IL-17A before and after infection as previously reported (Kaewarpai et al., 2020). Past study also have shown that AML patients did not exhibit defects in levels of IL-17A compared to healthy control (Zhu et al., 2015). Thus, we believe that the unique action of *B. pseudomallei* suppression of IL-17A in immunocompromised patients like AML might be involved in disease severity, as IL-17A was discovered to induce protective immunity. In addition, defect-IL-17A mice also displayed extreme engagement with infection which highlights the role of IL-17A as an important mediator in host defense.



**Figure 1.** Effect of different *B. pseudomallei* strains (OB, OS and K9) and positive control (PHA) on cytokines production after *in vitro* infection of AML PBMCs (MOI 10). The supernatants were obtained after 6 hours of incubation and quantified using BD cytometric Bead Array (CBA). A (IL-2), B (IL-4), C (IL-6), D (IL-10), E (TNF- $\alpha$ ), F (IFN- $\gamma$ ) and G (IL-17A) were plotted in horizontal bar against concentration (pg/ml). Green dotted baseline represent cytokines from healthy individual's PBMCs without AML or bacterial stimulation acquired from Barathan *et al.* (Unpublished data) Results from each strains were compared using analysis of variance; \* $p < 0.05$ , and \*\* $p < 0.01$  indicate statistically significant differences at the indicated p-values.

On the other hand, our study also highlights the differences in cytokines response in different strains of *B. pseudomallei* in infected neutropenic AML patients. We observed similar cytokine production trait in all of the strains except for IL-6. K9 strain shows higher production of IL-6 rather than OB and OS. In contrast with IL-10, the function of IL-6 in melioidosis is also not fully elucidated. However, IL-6 was found to be highly expressed in severe melioidosis and in non survivors suggesting it negatively impacts patients (Kaewarpai *et al.*, 2020). COX-2 assay revealed that there are no significant differences between the strains even though K9 expressed higher OD value. However, it is noted that

COX-2 was also produced in neutropenic AML PBMC. Although their roles are still controversial, COX-2 was seen to be elevated during bacterial and viral infection. In virus, COX-2 facilitates their replication and modulates the inflammatory responses (Steer & Corbett, 2003). In melioidosis, COX-2 inhibitor suppresses *B. pseudomallei* growth in macrophages and exhibited significant protection to mice hosts, indicating COX-2 inhibitor as a potential immunotherapeutic treatment in this disease (Asakrah *et al.*, 2013). However, their mechanism in melioidosis is not well understood and warrants further elucidation.



**Figure 2.** Effect of different *B. pseudomallei* strains (OB, OS and K9) and positive control (PHA) on COX-2 production after *in vitro* infection of AML PBMCs (MOI 10). Error bars indicate standard deviation.

Immunocompromised individuals like neutropenic AML were linked with high mortality in melioidosis and low neutrophils count produced inferior outcomes. Thus, the need for further research on this group of individuals is warranted and clinicians should be aware that this group of individuals need additional medical attention.

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#### Conflict of Interest

Authors declare no conflict of interest.

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