CCL20 Production in Mouse Monocyte Cell Lines Infected with *Burkholderia pseudomallei*

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ABSTRACT

The aim of this study was to determine the ability of live, heat-killed (HK) and paraformaldehyde (PP) killed *B. pseudomallei* 1026b to stimulate CCL20 in RAW 264.7 cell lines compared to a non virulent *B. thailandensis* UE5 and other bacteria. We demonstrated here that only live but not killed bacteria could induce CCL20 expression. The effect of PP and HK in the induction of CCL20 was similar. As *B. thailandensis* UE5 could invade cell lines at 30 times less than *B. pseudomallei* 1026b, we then used live *B. thailandensis* UE5 at MOI 300:1 compared to *B. pseudomallei* 1026b at MOI 10:1 in the stimulation of CCL20. The results demonstrated that *B. thailandensis* UE5 could induce higher amount of CCL20 in RAW 264.7 cells. This indirectly indicated that the induction of CCL20 is depending on the number of intracellular bacteria rather than the bacteria outside. To find out the potential molecule(s) that stimulate CCL20, LPS, capsule or flagellin mutants of *B. pseudomallei* 1026b were investigated. The results indicated that flagellin mutant stimulated lower CCL20 levels compared to wild type and other mutants. The finding suggests that the flagellin might involve in the release of CCL20 in RAW 264.7 cells.

Keywords: CCL20, *Burkholderia pseudomallei, RAW 264.7 cells*

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Introduction

Burkholderia pseudomallei is a Gram-negative intracellular bacterium that can be transmitted by cutaneous, ingested or airborne route. *B. pseudomallei* 1026b can survive in surface water of pH ranging from 2-9 (Finkelstein et al., 2000). It causes melioidosis, that can be present both acute and chronic forms (Anandan et al., 2010). In Northeastern Thailand, approximately 20% of community-acquired bacterial infection caused by melioidosis. Septicaemic melioidosis is the most fatal with mortality rate approximately 40% (Boonsawat et al., 1990;Cheng, Currie, 2005;Intarak et al., 2014). This bacterium can replicate and survive within macrophages for many months or years (Jones et al., 1996;Pruksachartvuthi et al., 1990;Utaisincharoen et al., 2004). It can also induce a cell-to-cell fusion and leading to multinucleated giant cell (MNGC) formation in the tissues of patients with melioidosis and cell cultures (Suparak et al., 2005;Utaisincharoen et al., 2004). Cells in immune response including monocytes and macrophages play role in early response to kill the bacteria (Goodyear et al., 2010;Hoebe et al., 2004;Pegoraro et al., 2014;Rowland et al., 2006). Infection of pulmonary epithelial cells or human lung epithelial cells with *B. pseudomallei* 1026b lead to the expression of proinflammatory cytokines for example gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-8 and CC chemokine ligand 20 (CCL20) (Barnes et al., 2008;Lauw et al., 2000;Santanirand et al., 1999;Sim et al., 2009).

The CCL20 or macrophage inflammatory protein-3α (MIP-3α) only binds to CCR6 that expressed in lymphocytes (Homey et al., 2000;Schutyser et al., 2003) in lung and tissues (Nakayama et al., 2001). CCL20 plays an important role in the control of bacterial infections (Fahy et al., 2004) and represents a link between innate and adaptive immune responses (Rivero-Lezcano et al., 2010). CCL20 play an important role in the antimicrobial activity and in the antibacterial response against a broader range of bacteria particularly against Gram-negative bacteria for example *S. enteritidis* (Fahy et al., 2004) and *H. pylori* (Nandi et al., 2014). However, the mechanism and knowledge of CCL20 stimulation by *B. pseudomallei* 1026b is limited.

In this study, we therefore investigated the effects of *B. pseudomallei* 1026b on the production of CCL20 in mouse monocyte cell lines (RAW 264.7)

Objectives

To study the ability of live, heat-killed (HK) and paraformaldehyde (PP) killed *B. pseudomallei* 1026b to stimulate CCL20 in RAW 264.7 cell lines compared to other bacterial strains. The tentative molecules that stimulated CCL20 were investigated using several mutants.

Materials and Methods

Cell line preparations

The mouse monocyte cell lines (RAW 264.7) were obtained from American Type Culture Collection (ATCC). Cell lines were cultured in RPMI-1640 medium (Gibco, Invitrogen Corporation, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Invitrogen Corporation, USA), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco Invitrogen Corporation, USA) in humidified incubator containing 5% CO_2 at 37°C.

Table 1 Bacterial strains and cell lines used in this study.

Types	References/Sources
Bacterial strains	
B. pseudomallei 1026b (wild type)	Clinical isolated from blood patient with septicemic melioidosis
B. pseudomallei 1026b MM35 (flagellin mutant)	(DeShazer et al., 1997)
B. pseudomallei 1026b SR1015 (capsule mutant)	(Reckseidler et al., 2001)
B. pseudomallei 1026b SRM117 (LPS mutant)	(DeShazer et al., 1998)
B. thailandensis UE5	Environmental isolated from Thailand
E. coli ATCC25922	American Type Culture Collection (ATCC)
Salmonella group B	Clinical specimens from patients (KhonKaen Hospital, Thailand)
Cell lines	
RAW 264.7 (Mouse monocyte cell lines)	American Type Culture Collection (ATCC)

Bacterial strain preparations

Bacterial strains (Table 1) were used in this study. The bacteria were grown on LB agar at 37°C for 48 h, before a single colony of each strain was replicated in 3 ml LB broth for 16-18 h in 37°C with shaking at 200 rpm. The overnight culture were adjusted optical density 600 nm (OD_{600}) to 0.1 before inoculated into 20 ml LB broth in an Erlenmeyer flask of 1% inoculums size and incubated until reach mid-log phase. The number of bacteria (CFU/ml) was quantitated by colony count.

Infection of RAW 264.7 cells with bacterial strains.

The RAW 264.7 cells ($5x10^5$ cells/well) were seeded in 24-well plate and incubated at 37°C, 5% CO_2 overnight. On the day of infection, cells lines were co-cultured with live bacteria, heat-killed or paraformaldehyde killed bacteria at MOI 10:1 or 300:1. After 1 h post infection, extracellular bacteria were removed by washing three times with PBS. Then after the infected cells were added with RPMI complete medium containing 250 µg/ml kanamycin for 2 h to kill extracellular bacteria incubated for further in RPMI complete medium containing 20 µg/ml kanamycin until indicated times. At 2, 6 and 12 h post infections. Viability of RAW 264.7 cells in response to *B. pseudomallei* 1026b (wild type) and *B. pseudomallei* MM35 (flagellin mutant) were examined and counting by using a hem cytometer at 6 and 12 h post infection.

ELISA assay for detection of mouse CCL20 in culture supernatant.

The culture supernatant was collected at each time point and the CCL20 levels were determined using CCL20/MIP-3αELISA assay kit (DuoSet ELISA development system; R&D systems, USA). The capture antibodies were coated in a 96-well micro plate and incubated overnight at room

temperature, then wash three times with PBS containing 0.05% tween 20 blocked with reagent dilulent (PBS/ 1%BSA) for 1 h. The plates were washed three times with PBS and then sample or standards were added and incubated for 2 h at room temperature. After washing three times with PBS, the detection antibody were added and incubated for 2 h at room temperature. The streptavidin-HRP conjugated at the dilution of 1:200 were added and incubated for 20 min at room temperature. Then unbound conjugates were removed by wash with PBS and added substrate solution was added for 20 min. Finally, the stop solution were added and the OD of each well were determined immediately, using a microplate reader set to 450 nm (TECAN/Sunrise Basic, Switzerland).

Statistical analysis

Statistical analysis was performed by using one-way ANOVA test. A probability of $P<0.05$ were considered significant. Experimental data were presented as means value ± the standard deviation of the duplicate and representation of two independent experiments.

Results

Live *B. pseudomallei* 1026b stimulated CCL20 in RAW 264.7 cell lines

The CCL20 levels secreted by RAW 264.7 cells were significantly increased when stimulated with live bacteria. At 12 h after infection *B. pseudomallei* 1026b could induce significantly higher amount of CCL20 in RAW 264.7 cells (0.00756±0.00075 pg/10^2 cells) (Figure 1A) whereas heat-killed *B. pseudomallei* 1026b or live and heat-killed *B. thailandensis* UE5, *E. coli* ATCC25922 and *Salmonella* group B induced very low levels of CCL20 (Figure 1B).

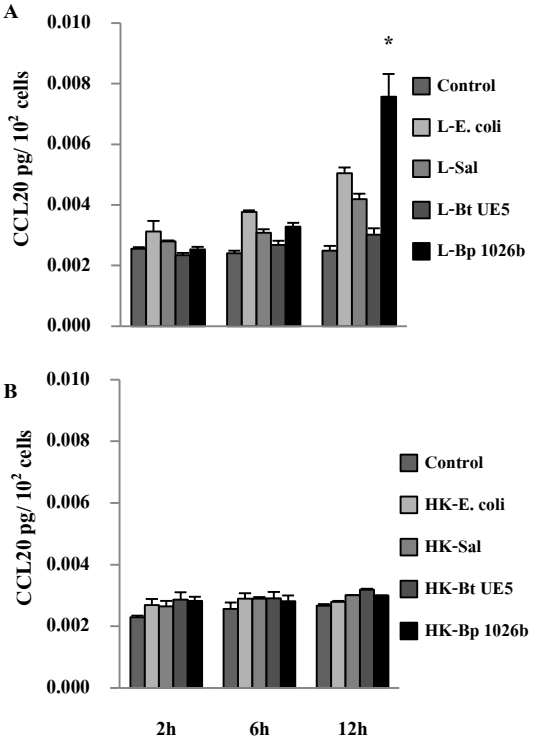


Figure 1 CCL20 secretions in RAW 264.7 cells infected with live (A) and heat-killed bacteria (B) at M0I 10:1. The culture supernatant was collected at 2, 6 and 12 h. Each bar represents means ± SD of CCL20 levels. Values of *$P<0.05$ were considered statistical significantly different from controls.

Heat and paraformaldehyde killed bacteria stimulated low and similar levels of CCL20.

We next assessed the effect of heat-killed (HK) and paraformaldehyde (PP) killed *B. pseudomallei* 1026b on the stimulation of CCL20. Our results showed that HK and PP stimulated similar and low CCL20 levels in RAW 264.7 cells at 6 and 12 h post infection (Figure 2).

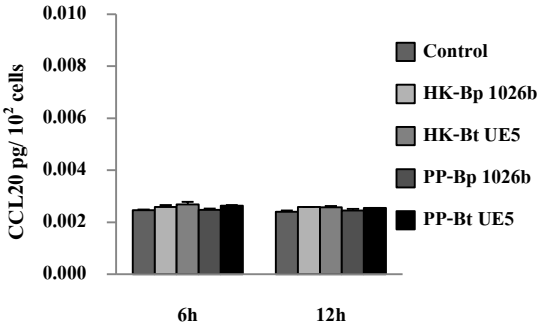


Figure 2 Effect of paraformaldehyde-killed (PP) and heat-killed (HK) bacteria in RAW 264.7 cells production of CCL20. The cells ($5x10^5$ cells/well) were incubated with MOI 10:1 of HK and PP *B. pseudomallei* 1026b and *B. thailandensis* UE5 at 6 and 12 h and assayed for CCL20 chemokine production by CCL20 ELISA. Each bar represents means ± SD of CCL20 levels.

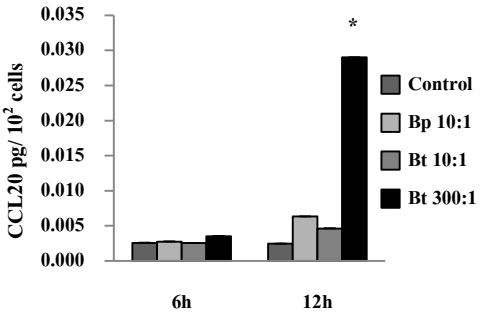


Figure 3 *B. thailandensis* UE5 at MOI 300:1 induces significantly CCL20 levels in RAW 264.7 cells. The cells ($5x10^5$cells/well) were incubated with *B. thailandensis* UE5 at MOI 300:1, *B. pseudomallei* 1026b or *B. thailandensis* UE5 at MOI 10:1 for 6 and 12 h. Each bar represents means ± SD of CCL20 level. Values of $*P<0.05$ were considered statistical significantly different from controls.

Level of CCL20 secretions depended on the number of intracellular bacteria.

Because of live *B. thailandensis* UE5 could invade 30 times less than live *B. pseudomallei* 1026b, in this experiment we then used *B. thailandensis* UE5 at MOI 300:1 compared to *B. pseudomallei* 1026b at MOI 10:1 in the stimulation of CCL20 in RAW 264.7 cells at 6 and 12 h. Our results showed that *B. thailandensis* UE5 at MOI 300:1 could induce higher levels of CCL20 (0.029 pg/10^2 cells) whereas *B. pseudomallei* 1026b at MOI 1:10 gave 0.0063 pg/10^2cells (Figure 3).

The ability of *B. pseudomallei* MM35 (flagellin mutant) infection in RAW 264.7 cells.

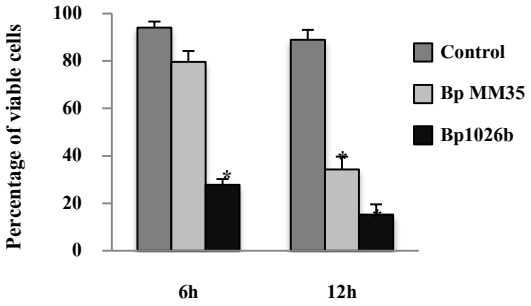
To determine viability of RAW 264.7 cells were infected with *B. pseudomallei* 1026b (wild type) and *B. pseudomallei* MM35 (flagellin mutant). Our result demonstrated that cells infected with flagellin mutants had significant higher viable RAW 264.7 cells than wild type (79.56±4.70 cells/ml compared to 34.31±5.34 cells/ml in wild type) at 6 h post infection (Figure 4).

Flagellin is potential molecule stimulated CCL20 in RAW 264.7 cells.

To investigate the molecules that relate to CCL20 stimulation, *B. pseudomallei* 1026b (wild

type) and their mutants were used in this study. Our results were shown in Fig 5. At 6 h *B. pseudomallei* 1026b (wild type) and capsule mutant (SR1015) were significantly induce CCL20 and at 12 h post infection *B. pseudomallei* 1026b (wild type), capsule mutant (SR1015) and LPS mutant (SRM117) were also significantly induce CCL20 secretion in RAW 264.7 cells but not in the case of the flagellin mutant (MM35).

Figure 4 **Percentage of viable RAW 264.7 cells.**



The cells were co-culture with *B. pseudomallei* 1026b and *B. pseudomallei* MM35 (flagellin mutant) at MOI 10:1. Each bar represents means ± SD of two independent experiments. Values of **P*<0.05 were considered statistical significantly different from controls.

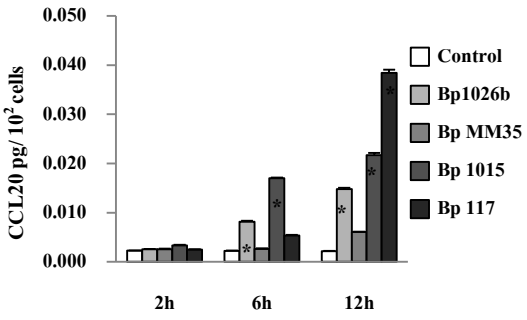


Figure 5 Effect of *B. pseudomallei* 1026b and their mutants to stimulate CCL20 in RAW 264.7 cells. The culture supernatant was collected at 2, 6 and 12 h. Each bar represents means ± SD of CCL20 levels. Values of **P*<0.05 were considered statistical significantly different from controls.

Discussion

In this study, we used RAW 264.7 cells to demonstrate different virulence factor of *B. pseudomallei* 1026b to induce CCL20. The ability of live bacteria compared to heat-killed bacteria suggested that live bacteria could invade and multiple that may contain several antigens or pathogen-associated molecular patterns (PAMP) for cytokine stimulations. The bacterial cell surface of *B. pseudomallei* 1026b such as LPS, capsule, flagellin or TLRs are involve in the activation of innate immune response leading to expression of pro-inflammatory cytokines. In contrast, the heat-killed (HK) or paraformaldehyde-killed (PP) bacteria could not elevate the CCL20 level. This result is similar when a live non-virulent *B. thailandensis* UE5 was used. We also investigated the virulent of both *B. pseudomallei* 1026b and *B. thailandensis* UE5 to confirm this hypothesis as the number of viable RAW 264.7 cells on 12 h after were 48.48±3.94 and 76.73±5.38, respectively (data were not shown). Since the ability of invasion by *B. thailandensis* UE5 was 30 times lower than *B. pseudomallei* 1026B, the CCL20 levels stimulated might be due to the number of intracellular bacteria. We confirm this finding by infecting the cells with avirulent *B. thailandensis* UE5 at MOI 300:1 compared to virulent *B. pseudomallei* 1026b at

MOI 10:1. The results revealed that avirulent *B. thailandensis* UE5 could not only induce CCL20 but its level was higher than virulent *B. pseudomallei* 1026b. The results suggested that expression of CCL20 in RAW 264.7 cells is depending on the number of intracellular bacteria.

Flagellin of *B. pseudomallei* 1026b is one of the important molecules of the virulence factor and involve in colonization and penetration within mucosal layer and adhesion to or invasion of epithelial cells (Duan et al., 2013). In this study, we demonstrated here that flagellin mutants could not stimulate CCL20 in RAW 264.7 cells. This might be due to the inability to adhere or invade the host cells. Our results demonstrated that at 2 and 6 h post infection *B. pseudomallei* 1026b could invade 10 and 28 times higher than *B. pseudomallei* MM35 (flagellin mutant) in RAW 264.7 cells.

Conclusion

Although the role of CCL20 is still controversial in melioidosis, our results demonstrated that live *B. pseudomallei* 1026b could stimulate CCL20 and its flagellin might be tentative molecular pattern that stimulated CCL20 in RAW 264.7 cells. The role of CCL20 and the mechanism of stimulation remain to be further investigation.

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