Abstract

Project Code : BRG5980004

Project Title : Role of NOD-like receptors (NLRs) in *Burkholderia pseudomallei*-infected human alveolar lung epithelial cell line A549

Investigator : Prof. Pongsak Utaisincharoen

E-mail Address : pongsak.uta@mahidol.ac.th

Project Period : 15 June 2016-14 June 2019

Melioidosis is a high mortality rate infectious disease and a major cause of community-acquired sepsis in Southeast Asia and Northern Australia. The pathogenic intracellular bacterium *Burkholderia pseudomallei* is the causative agent of this disease. Inhalation is the one of the major routes of *B. pseudomallei* infection, emphasizing the lungs as the important target organ. After entering into host cells, the bacteria can escape into the cytoplasm and be detected by the host cell cytosolic sensors which trigger the inflammasome pathways through the NOD-like receptors (NLRs), adaptor protein, and inflammatory caspases assembly. Although the canonical inflammasome (NLRP3/ASC and NLRC4) and non-canonical inflammasome (murine caspase-11) have been extensively investigated in murine model of *B. pseudomallei* infection, their role in human, particularly in lung epithelial cells, are neglected and still unknown. In this study, we aimed to investigate the involvement of inflammasome both the canonical (NLRP3/ASC and NLRC4) and non-canonical (human caspase-4) pathways in human lung epithelial A549 cells during *B. pseudomallei* infection. Even though the canonical inflammasome components (NLRP3, ASC, and NLRC4) are expressed and upregulated at both transcriptional and translational levels in *B. pseudomallei* infected human lung epithelial cells, the mature IL-1β and significant pyroptosis level were undetectable in the supernatant. Moreover, depletion of the aforementioned molecules did not alter the intracellular *B. pseudomallei* growth. In contrast to the canonical inflammasome, the human caspase-4 non-canonical inflammasome is importance for the restriction of the intracellular *B. pseudomallei* growth, suggesting the contribution of inflammatory caspase-4 non-canonical inflammasome pathway in promoting the killing of cytosolic bacteria in human lung epithelial cells.

Keywords : A549, *Burkholderia pseudomallei*, caspase-4, NLRs

บทคัดย่อ

รหัสโครงการ: BRG5980004

ชื่อโครงการ: บทบาทของตัวรับสัญญาณ NLRs ในเซลล์มะเร็งเยื่อบุปอดของมนุษย์ที่ติดเชื้อเบอร์โคลเดอเรีย สูโดมาลิไอ

ชื่อนักวิจัย และสถาบัน : ศ.ดร.พงศ์ศักดิ์ อุทัยสินธุเจริญ คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

อีเมล์: pongsak.uta@mahidol.ac.th

ระยะเวลาโครงการ: 15 มิถุนายน 2559 – 14 มิถุนายน 2562

โรคเมลิออยโดสิสจัดเป็นโรคติดเชื้อชนิดหนึ่งที่มีอัตราการตายสูง ซึ่งนำไปสู่การเกิดภาวะโลหิตเป็นพิษจากเหตุติดเชื้อ (sepsis) โรคชนิดนี้พบมากบริเวณเอเชียตะวันออกเฉียงใต้และตอนเหนือของประเทศออสเตรเลีย สาเหตุของโรคเกิดจากการติดเชื้อแบคทีเรียแกรมลบที่ชื่อว่า เบอร์โคลเดอเรีย สูโดมาลลิไอ เชื้อชนิดนี้สามารถติดต่อผ่านสู่ร่างกายมนุษย์โดยผ่านทางการหายใจจึงเป็นการเน้นย้ำว่าปอดน่าจะเป็นอวัยวะเป้าหมายสำคัญ เมื่อมีการติดเชื้อชนิดนนี้เข้าสู่ร่างกาย เชื้อชนิดนี้สามารถที่จะหลบหลีกไปยังบริเวณไซโตพลาสซึมและถูกตรวจจับโดย cytosolic sensers ของโฮสต์ซึ่งกระตุ้นให้เกิด inflammasome pathways โดยอาศัย NLR, adapter protein และ inflammatory caspases ในการจับกลุ่มกันกระตุ้นให้เกิดการส่งผ่านสัญญาณระบบ inflammasome สามารถแบ่งออกเป็น canonical ประกอบด้วย NLRP3/ASC และ NLRC4 และ non-canonical ได้แก่ murine caspase-11/human caspase-4 ซึ่งระบบ inflammasome ทั้งสองระบบดังกล่าวมีการศึกษากันอย่างกว้างขวางในเชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอย โดยทำการทดลองในหนู แต่อย่างไรก็ตามการศึกษาถึงบทบาทและกลไกของระบบ inflammasome ของเชื้อชนิดนี้ในมนุษย์โดยเฉพาะในเซลล์มะเร็งปอดนั้นยังไม่มีรายงาน การศึกษาในครั้งนี้ผู้วิจัยจึงมีเป้าหมายในการที่จะศึกษาบทบาทของระบบ inflammasome ในเซลล์มะเร็งปอดของมนุษย์ชนิด A549 ที่ถูกติดเชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ

จากผลการทดลองพบว่าถึงแม้จะพบการแสดงออกของยีนและโปรตีนในระบบ canonical inflammasome ในเซลล์มะเร็งปอดของมนุษย์ที่ติดเชื้อเบอร์โคลเดอเรีย สูโดมาลิไอ แต่กลับไม่พบการหลั่งของสารน้ำชนิด IL-1β และการเกิด pyroptosis อย่างไรก็ตามเมื่อทำการยับยั้งการแสดงออกของโมเลกุลในระบบ canonical inflammasome (NLRP3/ASC และ NLRC4) ก็ไม่พบความแตกต่างของการเจริญเติบโตของแบคทีเรียที่อยู่ภายในเซลล์ ในทางตรงกันข้ามเมื่อศึกษาระบบ non-canonical inflammasome กลับพบว่า human caspase-4 มีบทบาทสำคัญในการควบคุมการเจริญเติบโตของเชื้อแบคทีเรียภายในเซลล์ ซึ่งจากผลการทดลองอาจกล่าวได้ว่าระบบ non-canonocal inflammasome (caspase-4) มีบทบาทสำคัญในการเพิ่มความสามารถในการทำลายเชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอในเซลล์มะเร็งปอด

คำหลัก : A549, *Burkholderia pseudomallei*, caspase-4, NLRs

Executive summary

Burkholderia pseudomallei is a gram-negative intracellular bacterium that is the causative agent of melioidosis, a serious life-threatening disease in Southeast Asia and Northern Australia. *B. pseudomallei* is naturally found in moist soil and pooled surface water. Although skin inoculation and contaminated water ingestion are generally addressed as the major routes of infection, numerous studies also suggest inhalation as the primary mode of infection during the extreme weather period (1). Melioidosis has a wide range and nonspecific clinical manifestations, which can delay the diagnosis and treatment of the disease. The lung is the commonly affected organ since half of the melioidosis patients suffer from respiratory infection symptoms including pneumonia, pulmonary abscess, and pleuritis (2). With broad range antibiotic resistance and no available vaccine, the understanding of the underlying mechanism of disease particularly in term of host-microbe interaction should be further elucidated (3).

PRRs are expressed in phagocytic innate immune cells such as macrophages and dendritic cells, as well as in non-phagocytic cells like epithelial cells, endothelial cells, and fibroblasts (4). Unlike other PRRs, some NLRs are able to form large multimeric complexes called "inflammasome" (5). The activation of inflammasome results in caspase-1 processing leading to the release of mature pro-inflammatory cytokines IL-1 β and IL-18 and distinct type of inflammatory cell death called pyroptosis. This caspase-1 mediated inflammasome activation is defined as the canonical inflammasome pathway. The well-characterized canonical inflammasomes are the NLRP3/ASC-inflammasome and NLRC4-inflammasome (6). However, many recent studies suggested that non-canonical inflammasome pathway can induce other inflammatory caspases, caspase-11 (in mouse) and caspase-4/-5 (in human), resulting in pyroptosis cell death during intracellular bacterial infections (7, 8).

B. pseudomallei was reported to invade and survive intracellularly in both phagocytic and non-phagocytic cells (9-11). After an escape from endocytic vesicle, *B. pseudomallei* remains in the cytosolic compartment, which can provide the resource for replication. However, the host cell cytosolic sensors are also triggered leading to inflammasome activation in both canonical and non-canonical fashion. Both human and murine *B. pseudomallei* infected macrophages exhibit rapid IL-1 β /-18 secretion followed by cell death which are regulated by caspase-1 (12). Further studies in the murine system identified the non-redundant roles of the well-known canonical inflammasomes during *B. pseudomallei* infection. NLRP3/ASC-inflammasome controls IL-1 β and IL-18 maturation and secretion whereas NLRC4-inflammasome is responsible for pyroptosis (13). Notably, *in vivo* study suggested the harmful effect of IL-1 β though their ability to recruit non-pyroptotic neutrophils leading to severe lung damage in mouse melioidosis model. In contrast, IL-18 was shown to be associated with IFN-γ production in infected mice implying the defensive role in *B. pseudomallei* infection (13). For the non-canonical inflammasome pathway, casp11$^{-/-}$ mice are highly susceptible to *B. pseudomallei* infection and show low survival rate indicating the important role of the non-canonical inflammasome in host protection (7). The function of inflammasome pathways in *B. pseudomallei* infection has been extensively studied only in mouse macrophage. The role of these inflammasome pathways in frontline barrier such as the lung epithelial cells, which are the first encountering cells during *B. pseudomallei* infection, are still unknown. In the present study, we examined the inflammasome activation pattern in both NLRP3/ASC, NLRC4-canonical inflammasomes as well as in caspase-4 non-canonical inflammasome during *B.*

pseudomallei infection in human lung epithelial A549 cells. The consequences of inflammasome activation were measured though IL-1β secretion, pyroptosis cell death, and intracellular survival of *B. pseudomallei*. Additionally, the recent reports demonstrated the role of NLRC3, NLRC5 and NLRP12 that act as an immune regulator in bacterial infection (14). The results showed that *B. pseudomallei*-infected lung epithelial cells was able to stimulate the expression of these negative regulators, suggesting that NLRC3, NLRC5 and NLRP12 may involve in the intracellular survival of *B. pseudomallei* infection.

Research Methodology

Cell line and culture condition

Human alveolar lung epithelial cell line (A549) was obtained from American Type of Culture Collection (ATCC). The cells were cultured in Ham' F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% L-glutamine at 37°C in 5% CO_2 humidified incubator. The culture medium was changed every day and the cell monolayer was subcultured every two days by trypsinization.

Bacterial strains

B. pseudomallei parental wild-type strain (1026b) was used in this study. In some experiment, lipopolysaccharide (LPS) mutant (SRM117) that lacks the O-antigenic polysaccharide moiety was used for comparison (15). Bacteria were cultured in Luria-Bertani (LB) at 37°C with agitation at 150 rpm. Overnight cultures were washed twice with phosphate-buffered saline (PBS) and adjusted to a desired concentration by measuring the optical density at 650 nm and the colony-forming unit (CFU) was calculated from the precalibrated standard curve.

Infection of human alveolar lung epithelial cell line A549

An overnight culture of human alveolar lung epithelial cell line (A549) (1 × 10^6 cells) in a 6-well plate was co-cultured with the bacteria at a multiplicity of infection (MOI) of 10:1 for 2 hrs. To remove extracellular bacteria, the cells were washed 2 times with 1 ml of PBS and residual bacteria killed by incubating in RPMI containing 250 μg/ml kanamycin for 2 hr. The infection was allowed to continue in the medium containing 20 μg/ml of kanamycin until the end of experiment. The medium for culturing the LPS mutant was supplemented with tetracycline at a concentration of 50 μg/ml to prevent possible reversion to the wild type.

Depletion of target proteins in lung epithelial cells (A549)

The canonical and non-canonical inflammasome components were depleted with specific small interfering RNAs. The negative control siRNA, NLRP3, NLRC4, ASC, and CASP-4 were transfected into A549 cells (1.5 × 10^5 cells) 48 hours prior *B. pseudomallei* infection using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's protocol. The expression of the target protein was determined by Western blotting. For NLRP3 and NLRC4 siRNA were purchased from Dharmacon (Lafayette, CO) as the ON-TARGET plus siRNAs in SMARTpool ASC siRNA (siRNA ID: HSS1470) and CASP-4 siRNA (siRNA ID: HSS141457) were purchased from Invitrogen. AllStar Negative Control siRNA (Qiagen, Hilden, Germany) was used as the control.

Western Blotting

The cells were lysed in lysis buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany). The non-specific binding sites on the membrane were blocked with 5% blocking solution (Roche Diagnostics) for 1 hr. before proteins are allowed to react with specific primary antibodies including NLRP3 (D4D8T), NLRC4 (D5Y8E), TMS1 (E1E3I), Caspase-1 and Caspase-4 (Cell Signaling Technology, Danvers, MA) at 4°C overnight. The membrane was washed 3 times with 0.1% PBST and incubated with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL) for 1 hr. at room temperature. The chemiluminescence substrate (Roche Diagnostics) was added and proteins were detected by enhanced chemiluminescence and then exposed to high-performance chemiluminescence film. (Amersham Biosciences)

Quantification of intracellular bacteria

To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed. At the times preferred, the infected cells were washed three times with PBS, then intracellular bacteria were liberated by lysing the infected-A549 with 0.1% Triton X-100 and the released bacteria were cultured on tryptic soy agar. The number of intracellular bacteria, expressed as CFU, was determined by bacterial colony counting after incubating at 37^{o}C for 48 hr.

Determination of mRNA levels

Expression of mRNA levels in living *B. pseudomallei*-treated A549 was determined by RT-PCR. Total RNA was first extracted from treated cells according to the manufacturer’s instruction and used for cDNA synthesis using the avian myeloblastosis virus (AMV) reverse transcription enzyme (Promega, Madison, USA). PCR reaction was performed using specific primer for the target genes (Sequence in Table1). The amplified products were then electrophorese using 1.5% agarose gel and visualize under an ultraviolet lamp.

Gene	Primer	Sequence (5'⟶ 3')
ASC	Forward	GCA-CTT-TAT-AGA-CCA-GCA-CCG
	Reverse	AAG-AGC-TTC-CGC-ATC-TTG-CT
CASP1	Forward	CCC-TGG-TGT-GGT-GTG-GTT-TA
	Reverse	TTC-ACT-TCC-TGC-CCA-CAG-AC
CASP4	Forward	ACA-AAG-TTC-GGG-TCA-TGG-CA
	Reverse	GCT-GAC-TCC-ATA-TCC-CTG-GC
IL-1β	Forward	CAA-CCT-CTT-CGA-GGC-ACA-AGG-C
	Reverse	CTG-CCT-GAA-GCC-CTT-GCT-GT
NLRC3	Forward	CCG-GGA-GAA-CTG-TGG-ACA-AC
	Reverse	AGC-TTC-TCG-TGG-GTG-TTG-AG
NLRC5	Forward	TAC-CTG-AGC-CCT-GAA-TCG-GA
	Reverse	GGT-CTG-AGA-GGC-CCA-GTC-TA
NLRP12	Forward	TTT-GCT-CAT-CAC-CAC-ACG-GC
	Reverse	TCA-GAC-TCA-GCA-GGT-AGA-GC
NLRP3	Forward	TCG-GGA-GGT-GAG-CCT-TGT-GA
	Reverse	ACA-TGC-CGA-GGA-TGG-TCC-AG
NLRC4	Forward	TTG-CCC-TGT-GTG-ACC-TTG-CC
	Reverse	AAG-GGC-TCG-GCT-ATT-GTC-CT
GAPDH	Forward	ATG-GGG-AAG-GTG-AAG-GTC-G
	Reverse	GGG-GTC-ATT-GAT-GGC-AAC-A

ELISA

Human alveolar lung epithelial cell line (A549) was infected with live *B. pseudomallei* at a MOI of 10 for 2 hr before 250 μg/ml of kanamycin was added. The supernatants were collected for cytokine detection at the indicated time point. For cell lysate collection, cells were lysed with 0.1% Triton X-100. The culture supernatants and cell lysate were filtrated with 0.25 μm Acrodisc syringe filter and measured for IL-1β and IL-18 using a specific immunoassay for human IL-1β (BD

Biosciences, San Diego, USA) and IL-18 (R&D Systems, Minnesota, USA) ELISA kit according to the manufacturer's instructions. The absorbance was read at 450 nm.

Caspase activity measurement assay

Human alveolar lung epithelial cell line (A549) was infected with live *B. pseudomallei* at a MOI of 10:1 for 2 hr before 250 μg/ml of kanamycin was added. At the indicate time point, cytosolic content will be collected from infected human epithelial cell line pellet. Cytosolic content were measured caspase-1 activity with caspase-1 colorimetric assay kit (BioVision, California, USA) according to the manufacturer's instruction. Samples were read with microtiter plate reader at 405 nm.

Cytotoxicity assay

To quantify the extent of pyroptosis after bacterial infection, release of lactate dehydrogenase (LDH) in cell culture supernatants was determined. The A549 cells monolayers were infected with *B. pseudomallei* at MOI of 10:1 for 12 hours at 37°C in 5% CO2. At the indicate time point, cell culture and supernatant were collected, and LDH activity was detected by using the CytoTox 96® non-radioactive cytotoxicity assay (Promega, California, USA) according to the manufacturer's instructions. The LDH measurement was recorded absorbance at 490 nm.

Statistical analysis

If not specify otherwise, all experiments in this project will be conducted at least three times. Experimental values will be expressed as means ± the standard error of mean (SEM). Statistical significance of this mean will be assessed by Student's t-test. The values of $p<0.05$, $p<0.01$, and $P<0.001$ was indicated as *, **, and ***, respectively. The $p<0.05$ was considered to be statistically significant and highly significant.

Results

The wild type *B. pseudomallei* was able to survive in human alveolar lung epithelial cell line while LPS mutant *B. pseudomallei* was eliminated.

In order to investigate the ability to survive of *B. pseudomallei* during inflammasome activation in human alveolar lung epithelial cell line A549, intracellular growth profile of infected cells was observed. The A549 cells were infected with either wild type *B. pseudomallei* (1026b) or LPS mutant (SRM117) with MOI of 10:1 as described in research methodology. At the indicated time points, intracellular survival of bacteria was determined by bacteria colony counting on tryptic soy agar. The bacteria internalization was determined at 2 hours post infection. As shown in Figure 1, the wild type infected lung epithelium cells showed slightly lower number of bacteria internalization comparing with LPS mutant infected cells. However, the intracellular survival growth in either wild type or LPS mutant *B. pseudomallei* were dramatically dropped in 4 hours post infection. At the later time point, LPS mutant *B. pseudomallei* showed lower rate of survival comparing to wild type bacteria. Morphology change was able to detect only in wild type infected cells. Cell fusion with bacteria protrusion was clearly observed at 12 hours post infection (Data not shown).

Kinetic gene expression of IL-1β in wild type and LPS mutant *B. pseudomallei* infected human alveolar lung epithelial cell line.

During intracellular bacterial infection, inflammasome complex, which consists of cytosolic innate immune sensors, an adaptor protein, and caspase-1, is likely to be activated. One of the consequences of caspase-1 activation is proteolytic maturation and secretion of IL-1β. IL-1β considers as the powerful pro-inflammatory cytokine that can further induce defense

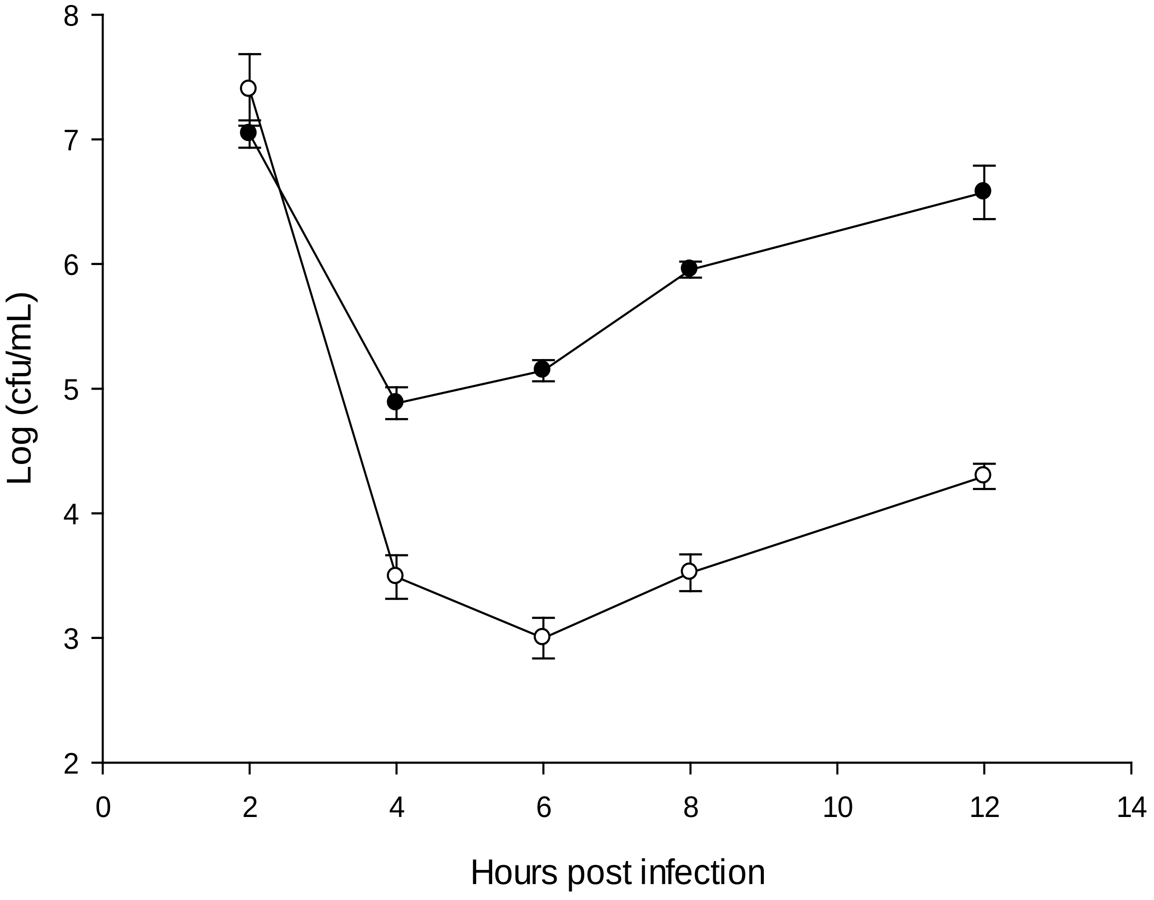


Figure 1. Intracellular bacterial growth profile in wild type *B.pseudomallei* or LPS mutant *B.pseudomallei* infected human alveolar epithelial cell. Wild type or LPS-mutant *B.pseudomallei* was applied to human alveolar epithelial cell line, A549, (1×10^6 cells) at MOI of 10 for 2 hours. The intracellular bacterial growth at desired time points was determined by bacterial colony counting in tryptic soy agar plate. Data represent the means and standard errors of 3 independent experiments.

against infection. Therefore, the pattern of IL-1β expression during *B. pseudomallei* infection in A549 was investigated. The *il-1β* expression profile was observed by using RT-PCR. The result showed that expression of *il-1β* was inducible after *B. pseudomallei* infection in both strain. However, the expression pattern of wild type and LPS-mutant infected A549 cells was slightly different. During wild type infection, *il-1β* expression was observed at 6 hours post infection and gradually increased to the highest level at 8 hours post infection. In contrast to LPS mutant infection, *il-1β* gene expression was able to detect at the earlier time point, 4 hours post infection (Figure 2). The result suggested that the inflammasome activation was potentially triggered during *B. pseudomallei* infection in either wild type or LPS mutant strain. The *gapdh* expression was using as internal control in the experiment.

The inflammasome cytokines profiling in wild type or LPS mutant *B.pseudomallei* infection in alveolar epithelial cell line.

The remarkable result of inflammasome activation is the production of powerful proinflammatory cytokine such as IL-1β and their family, IL-18. In order to understand the pattern of this mechanism, IL-1β and IL-18 production in translational level were observed during either wild type or LPS mutant *B. pseudomallei* infection in human alveolar cell line. The supernatants of infected cells were collect to detect for accumulated IL-1β and IL-18 by using ELISA. Notably, either IL-1β or IL-18 production was unable to detect in cell supernatants in any time point (Figure 3 and 4). Since the upregulation of il-1β was detected in transcription level (Figure 2), cytosolic content of *B. pseudomallei* infected cells were further collected. IL-1β level in cell lysates was also determined using ELISA. The result revealed that IL-1β was produced intracellularly in both wild type and LPS mutant *B. pseudomallei* infected cells.

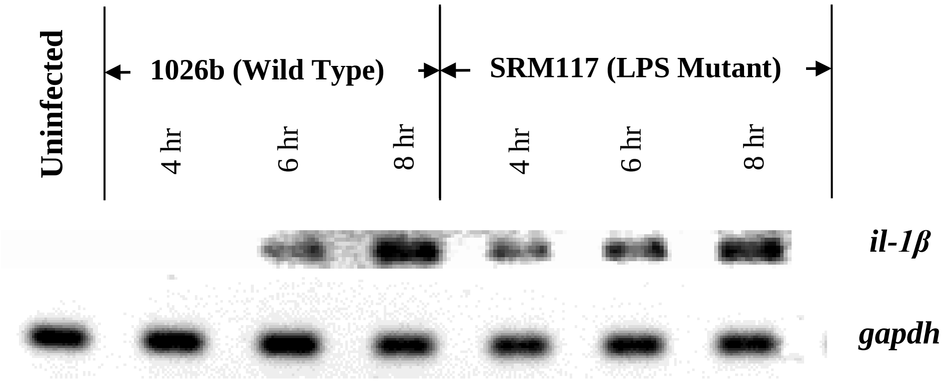


Figure 2. Kinetics *il-1β* gene expression in A549 during wild type and LPS mutant *B.pseudomallei* infection. A549 cells (1×10^6 cells) were infected with wild type or LPS-mutant *B. pseudomallei* at MOI of 10. Cell pellets were collected and lysed at indicated time. Gene expressions were determined by RT-PCR.

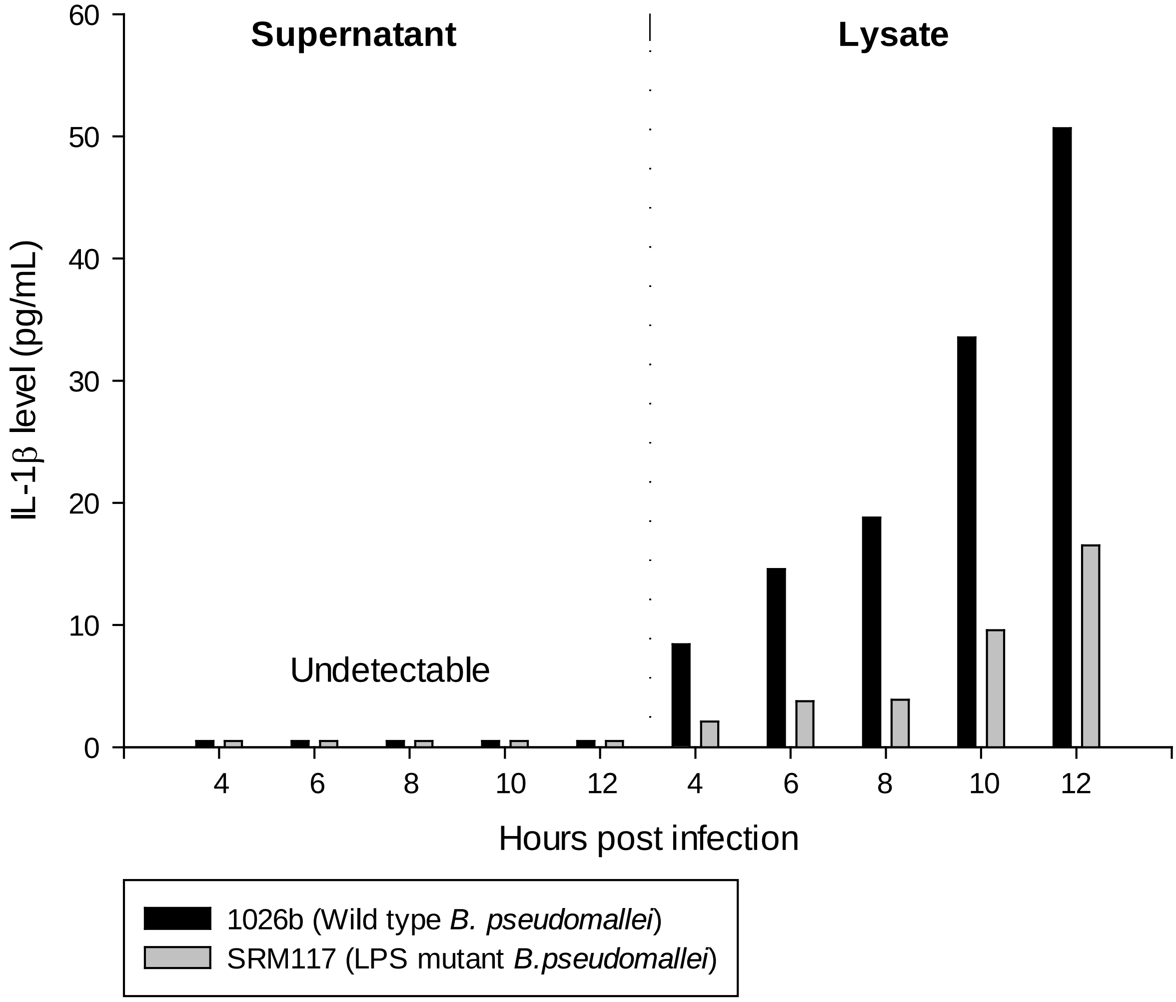


Figure 3. Profiling of secreted and intracellular IL-1β production in wild type *B.pseudomallei* or LPS mutant *B.pseudomallei* infected human alveolar epithelial cell. Wild type or LPS-mutant *B.pseudomallei* was applied to human alveolar epithelial cell line, A549, ($1{\times}10^6$ cells) at MOI of 10 for 2 hours. At the indicated time point, supernatants and cell lysate were collected for cytokine detection at the indicated time point using ELISA.

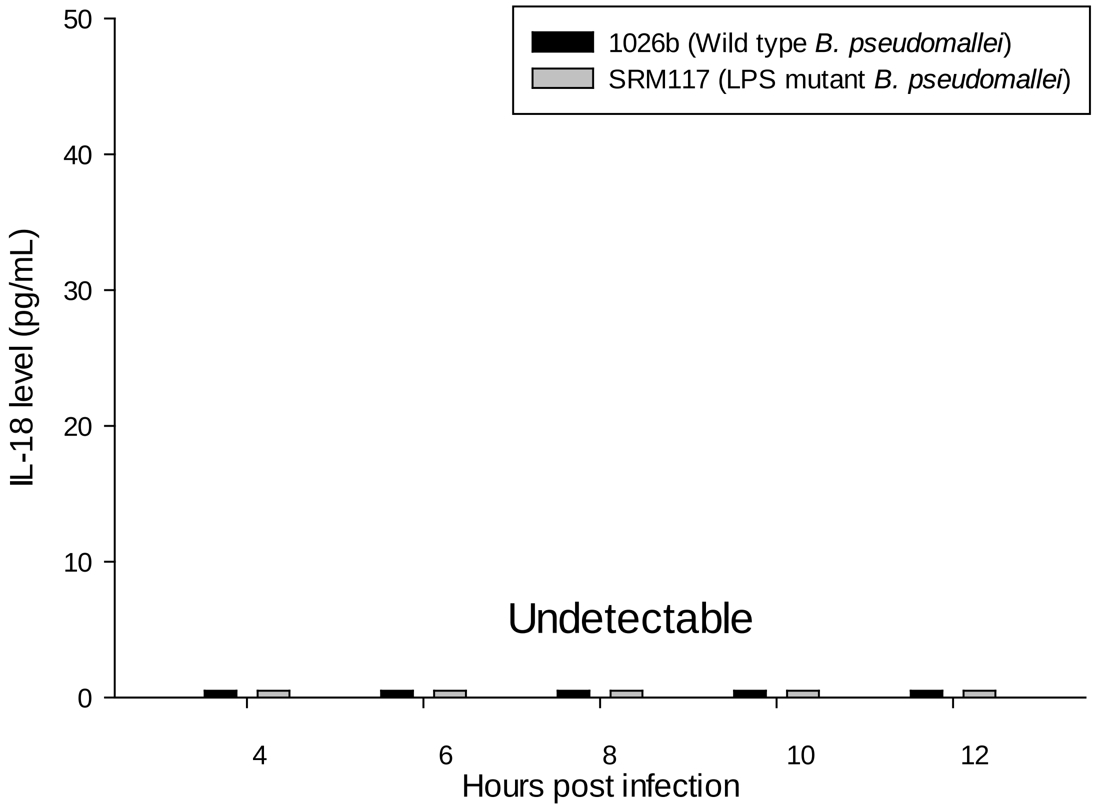


Figure 4. IL-18 production in wild type *B. pseudomallei* or LPS mutant *B. pseudomallei* infected human alveolar epithelial cell supernatant was undetectable. Wild type or LPS-mutant *B. pseudomallei* was applied to human alveolar epithelial cell line, A549, (1×10^6 cells) at MOI of 10 for 2 hours. At the indicated time point, supernatants were collected for IL-18 detection at the indicated time point using ELISA.

Moreover, wild type *B. pseudomallei* was able to induce higher level of intracellular IL-1β production than LPS mutant strain (Figure 3). The LPS mutant *B. pseudomallei* infected lung epithelial cells established the higher level of pyroptosis comparing to wild type infected cells.

Another outcome of inflammasome activation was a unique form of cell death called pyroptosis. Characteristics of pyroptosis are cell swelling and lysis that are the result from permeabilization of plasma membrane causing by caspase-1 activation. Lactate dehydrogenase (LDH) is the enzyme that is normally found intracellularly. Therefore, the releasing of LDH in cell culture supernatants could infer the pyroptosis pattern during wild type and LPS mutant *B. pseudomallei* infected cells. The result demonstrated that the LPS mutant infected cells showed the stronger pyroptosis level than the wild type infected cells after 8 hours post infection (Figure 5).

The inhibition of caspases activity did not alter the intracellular growth pattern of *B. pseudomallei* in infected human alveolar lung epithelial cell line.

In the recent study suggested that pyroptosis could effectively cut replication cycle and re-expose premature intracellular bacteria to extracellular milieu. In order to understand the role of pyroptosis in intracellular bacterial growth in alveolar lung epithelial cells during *B. pseudomallei* infection, twenty μg/ml of Z-VAD-FMK, a pan-caspase inhibitor, was applied 1 hour prior to infection of wild-type (1026b) or LPS mutant (SRM117) strain to irreversibly stop all caspases activity. After 8 hours of infection, the intracellular survival of bacteria was measured. In LPS mutant infected A549, the intracellular growth at 8 hours post infection was dramatically lower than the wild-type strain (Figure 5). The result was consistent to the survival pattern in Figure 1. The results inferred the possible involvement of pyroptosis level and bacterial clearance since the LPS mutant infected cells also showed the higher LDH release comparing to wild type infection (Figure 5). However, the pan-caspases inhibitor treated cells did not alter the clearance pattern resulting in similar intracellular survival level of bacteria either in wild type or in LPS mutant strain (Figure 6). The result suggested the unrelated function of controlling intracellular growth of caspases during *B. pseudomallei* infection in human lung epithelial cells.

The caspase-1 activity was unable to detect via colorimetric assay kit in *B. pseudomallei* infected human alveolar lung epithelial cell line.

In order to understand the key member of inflammasome complex, caspase-1 activation pattern in *B. pseudomallei* infected lung epithelial cells was quantitated by using caspase-1 colorimetric assay kit. However, colorimetric assay was unable to detect the activation of caspase-1 (Figure 7). The other caspase-1 activity detection need to be further examined.

The wild type *B. pseudomallei* delayed caspase-1 expression and activation to the late time-point in infected human lung epithelial cells.

Our recent report demonstrated that wild-type *B. pseudomallei* had ability to survive and multiply intracellularly within human lung epithelial cells. Inflammasome activation is considered as one of the important defensive mechanisms in encountering the intracellular bacteria. In order to investigate whether wild-type *B. pseudomallei* manipulated the inflammasome activation in human lung epithelial cells, gene and protein expression and maturation profiles of caspase-1 in canonical inflammasome activation pathway was examined.

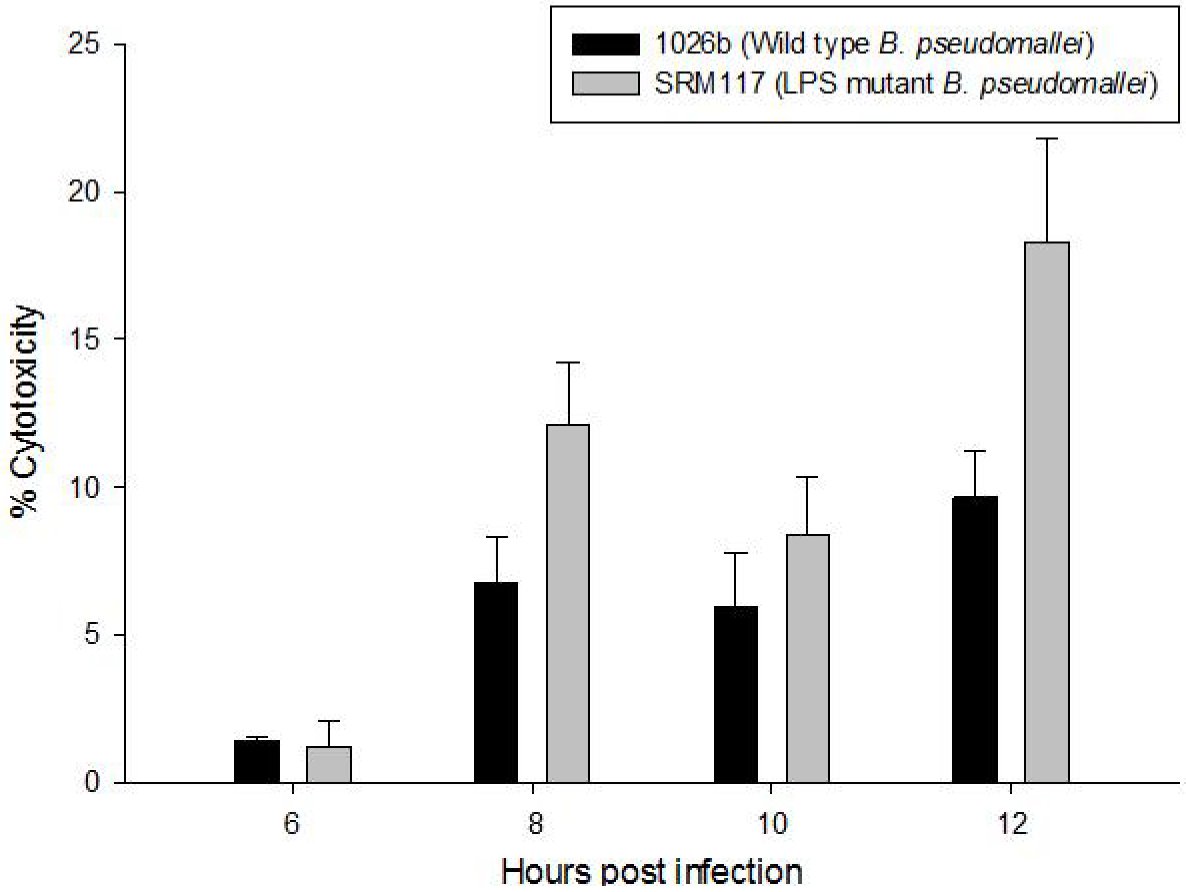


Figure 5. Pyroptosis level in wild type *B. pseudomallei* or LPS mutant *B. pseudomallei* infected human alveolar epithelial cell. Wild type or LPS-mutant *B. pseudomallei* was applied to human alveolar epithelial cell line, A549, (1×10^6 cells) at MOI of 10 for 2 hours. At the indicated time point, supernatants were collected and determined for LDH release at the indicated time point by using cytotoxicity assay. Data represent the means and standard errors of 3 independent experiments.

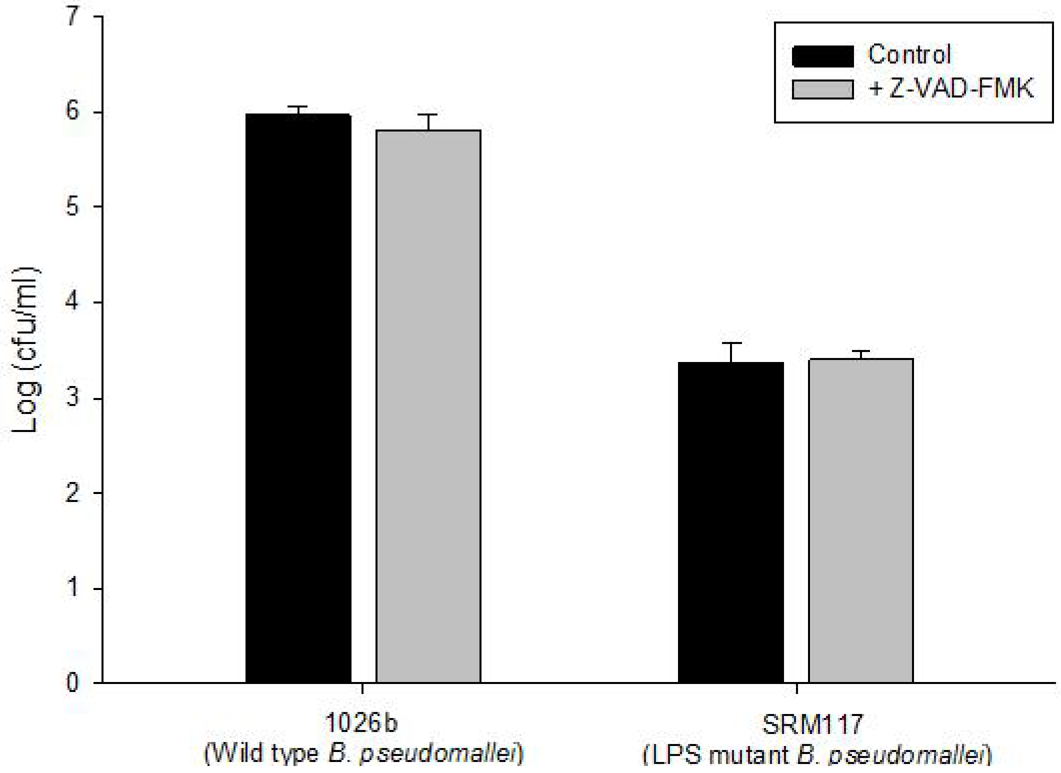


Figure 6. Caspases activation does not interfere the intracellular growth of *B.pseudomallei* in infected human lung epithelial cells. Human lung epithelial cell line (1×106 cells) was pre-treated with Z-VAD-FMK (20 µg/ml) 1 hour prior to infection. Wild type or LPS-mutant *B. pseudomallei* was applied to the cell culture at MOI of 10 for 2 hours following by kanamycin protection assay. The number of intracellular bacteria was determined at 8 hours post infection. Data represent the means and standard errors of 3 independent experiments.

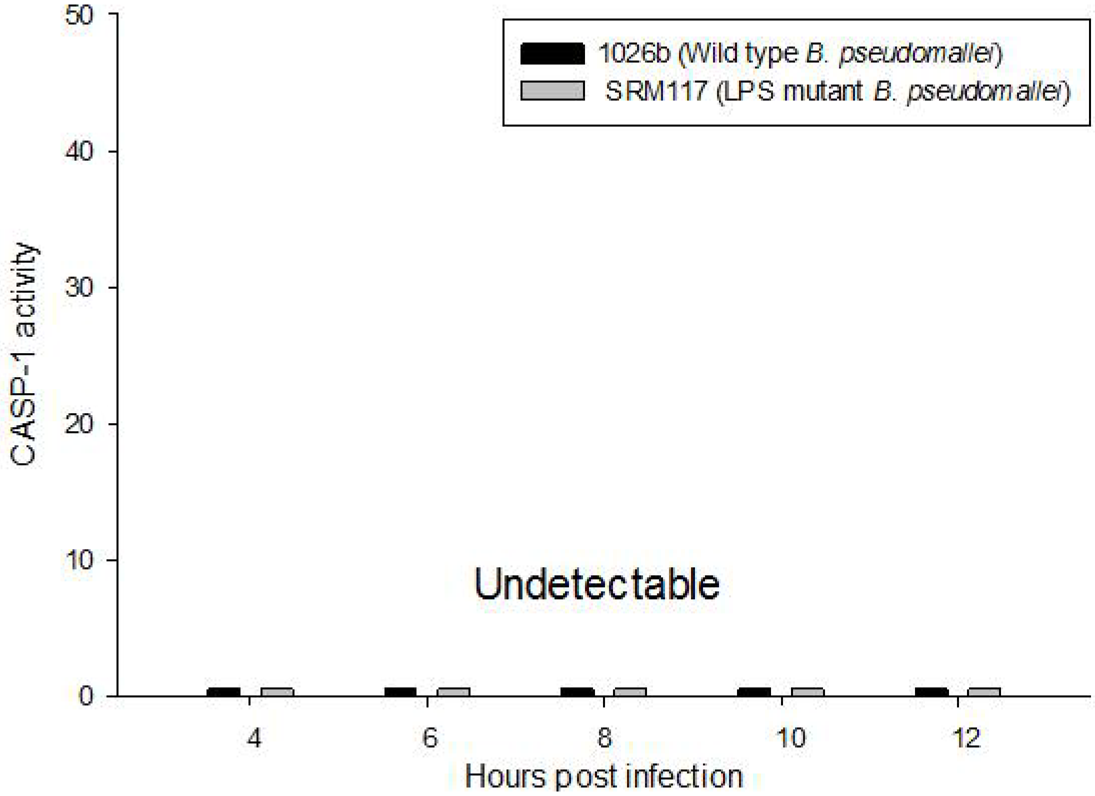
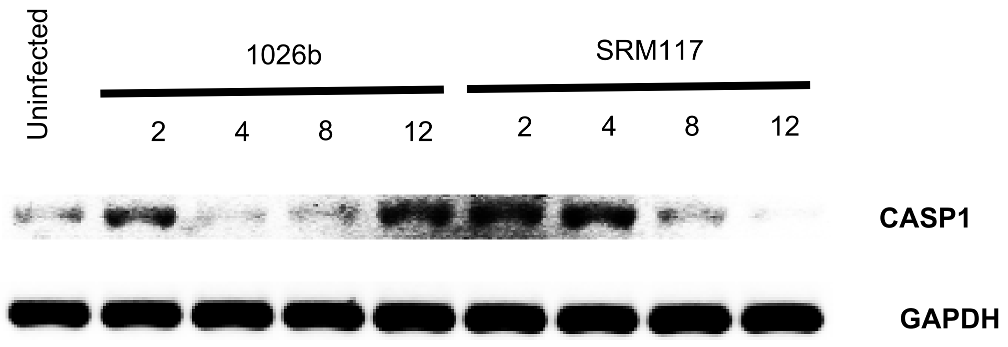


Figure 7. Caspase-1 activity was unable to detect in *B. pseudomallei* infected A549 via caspase activity measurement assay in wild type *B. pseudomallei* or LPS mutant *B. pseudomallei* infected human alveolar epithelial cell. Wild type or LPS-mutant *B. pseudomallei* was applied to human alveolar epithelial cell line, A549, (1×10^6 cells) at MOI of 10 for 2 hours. At the indicated time point, cytosolic contents were detected by caspase activity measurement assay.

The results demonstrated that the caspase-1 gene expression was upregulated in wild-type *B. pseudomallei* infected human lung epithelial cells (Figure 8A). The upregulation was firstly observed in the early time-point; 2 hours post infection (p.i.), and then sharply dropped in 4 hours pi before re-upregulating and reaching its maximum at 12 hours p.i.. The caspase-1 maturation pattern was further examined using Western blotting. The caspase-1 is the proteolytic cleavage enzyme that is produced in inactive form. The proteolytic maturation of caspase-1 can be indicated by detection of either 20kDa (p20) or 10kDa (p10) caspase-1 subunit. In consistent with gene expression, the caspase-1 maturation in wild type infected human lung epithelial cells was clearly observed at 12 hours p.i. (Figure 8B).

To further investigate the correlation between caspase-1 maturation pattern and intracellular bacterial growth pattern, the non-virulent lipopolysaccharide (LPS)-mutated *B. pseudomallei* strain (SRM117) was used in the experiment for comparison. The LPS-mutated *B. pseudomallei* is lacking in O-antigenic polysaccharide moiety resulting in less aggressive than wild-type *B. pseudomallei* 1026b strain. In contrast to wild-type *B. pseudomallei*, our group had been demonstrated that LPS-mutated *B. pseudomallei* was unable to survive and multiply intracellularly even though the LPS-mutated *B. pseudomallei* established the higher bacterial internalization than wild-type 1026b strain. Unlike wild-type strain infection, caspase-1 gene expression dramatically upregulated in 2 hours p.i. then slowly decline and diminished at 12 hours p.i (Figure 8A). For caspase-1 maturation, the active form (p20) was also triggered at earlier time point as 4 hours p.i. and remained until 12 hours p.i. (Figure 8B). The results indicated that wild-type *B. pseudomallei* was able to detain caspase-1 expression and maturation in infected human lung epithelial cells.

A) RT-PCR



B) Western Blotting

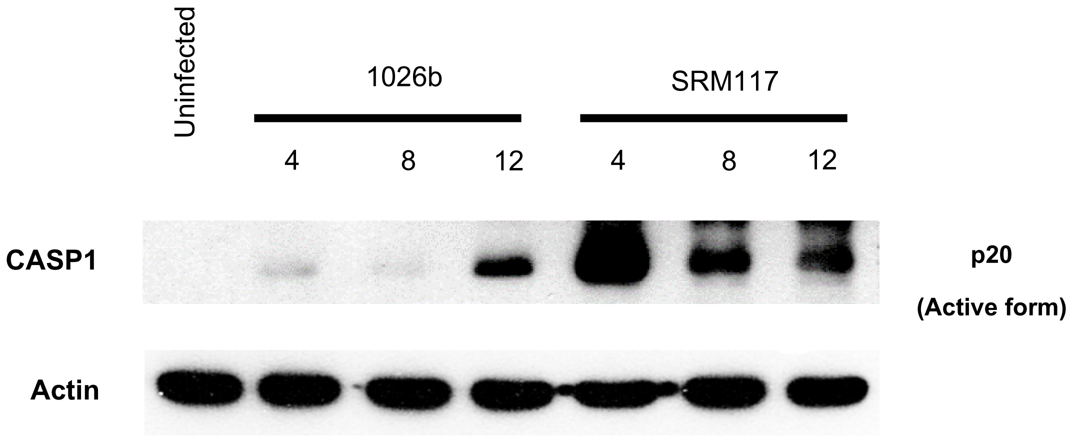
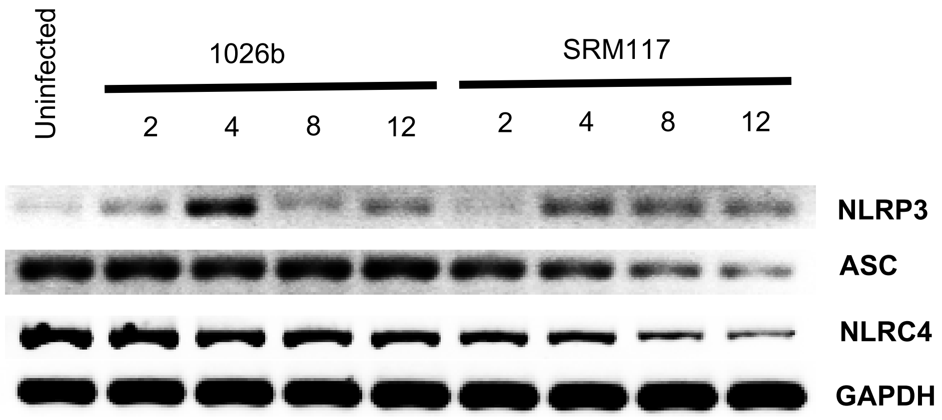


Figure 8. The wild type *B. pseudomallei* managed to delay caspase-1 gene expression and proteolytic maturation in infected human lung epithelial cells. A549 cells were infected with wild-type or LPS-mutated *B. pseudomallei* for 2 hours at MOI of 10. The cell lysates were collected at indicated time point. (A) Caspase-1 gene expression was assessed by RT-PCR. GAPDH was the internal control of RT-PCR. (B) The active form of caspase-1 was showed at 20kDa. Actin was the internal control of Western blotting. Each experiment was conducted three times minimum.

Kinetic gene expression of canonical inflammasome components (NLRP3, NLRC4 and ASC) in wild type and LPS mutant *B. pseudomallei* infected human lung epithelial cells.

Since activation of caspase-1 is regulated by muticomplex protein called 'inflammasome'. The canonical inflammasome complex consists of cytosolic sensors, NOD-like receptors (NLRs), adaptor molecule, ASC, and inflammatory caspase-1. Among NLRs inflammasome, NLRP3/ASC and NLRC4 inflammasome complexes were the extensive studied and were reported to be activated and contribute to defensive role during many intracellular bacterial infection including *B. pseudomallei* (13). Whether the delay of caspase-1 activation was in consequence from upstream molecules of canonical inflammasome complex, NLRP3/ASC and NLRC4 gene and protein profiles in *B.pseudomallei* infected human lung epithelial cells were validated. NLRP3 is the well-characterized NLR that can be activated by various stimuli either from pathogen or damage tissue. In order to interact with caspase-1, NLRP3 requires ASC adaptor protein to complete the complex resulting in caspase-1 recruitment and activation. During *B. pseudomallei* infection, NLRP3 gene expression was upregulated (Figure 9A). In the same manner as caspase-1, the delayed upregulation of NLRP3 protein expression was observed in wild-type *B. pseudomallei* infected cells comparing to LPS mutant (Figure 9B). For ASC adaptor protein, both gene and protein expression of infected cells were comparable during wild-type *B. pseudomallei* infection (Figure 9A and 9B). Another important caspase-1 inducing inflammasome was NLRC4 inflammasome. NLRC4 gene and protein expression remained in constant level during wild-type *B. pseudomallei* infection whereas NLRC4 protein expression was completely abolished in LPS mutant infected cells (Figure 9A and 9B). The GAPDH and actin expression was using as internal control for RT-PCR and Western blotting,

A. RT-PCR



B. Western Blotting

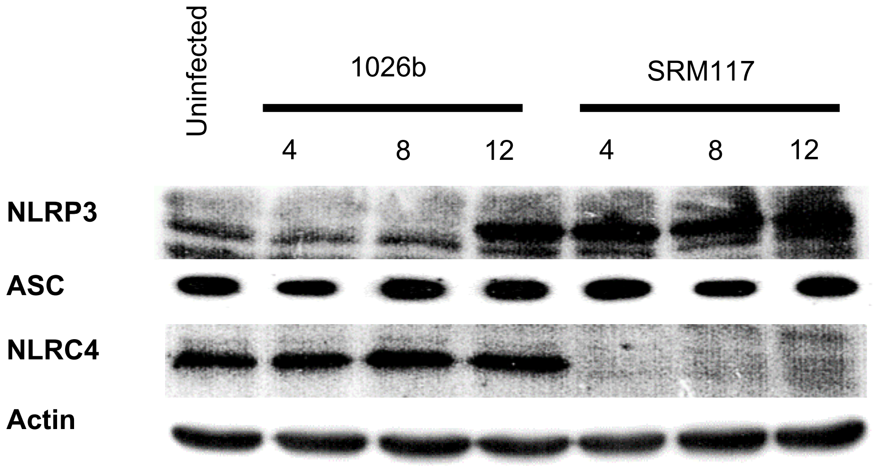


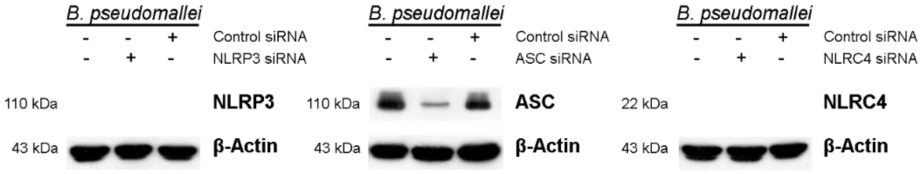
Figure 9. Kinetics expression profiles of canonical inflammasome components in human lung epithelial cell line (A549) during wild-type and LPS-mutated *B. pseudomallei* infection. A549 cells were infected with wild-type or LPS-mutated *B. pseudomallei* for 2 hours at MOI of 10. The cell lysates were collected at indicated time point. The canonical inflammasome machinery gene and protein expression (NLRP3, ASC, and NLRC4) were assessed by RT-PCR (A) and Western blotting (B), respectively. GAPDH was the internal control of RT-PCR while Actin was the internal control of Western blotting. Each experiment was conducted three times minimum.

respectively. Altogether, the canonical inflammasome components kinetic profiles emphasized NLRP3/ASC inflammasome complex in regulation of caspase-1 maturation in human lung epithelial cells during *B. pseudomallei* infection.

The canonical inflammasome activation pathway did not alter the intracellular growth of *B.pseudomallei* and pyroptosis level in infected human lung epithelial cells.

We further assessed the involvement of canonical inflammasome activation in lung epithelial cells during *B. pseudomallei* infection. Each of inflammasome signaling molecules was depleted in order to determine their role in intracellular bacterial restriction and pyroptosis cell death. Pyroptosis is the another reported consequence that occur following inflammasome activation leading to the rapid formation of plasma membrane pores disrupting the balance between extracellular environment and cytosolic compartment resulting in osmotic cell lysis (16). The lung epithelial A549 were transfected with specific small interfering RNA (siRNA) 48 hours prior to *B. pseudomallei* infection. The depleting efficiency of each inflammasome molecules was confirmed through Western Blotting (Figure 10A). After 12 hours of wild-type *B. pseudomallei* infection, cell supernatant and cell lysates were collected to determine LDH releasing level and intracellular survival, respectively. Neither intracellular bacterial survival nor pyroptosis level was altered in canonical inflammasome machinery depleted lung epithelial cells following *B. pseudomallei* infection (Figure 10B and 11). Although NLRC4 depleted cells showed the significantly lower pyroptosis level than negative siRNA treated cells indicating the role in mediating pyroptosis cell death, the contribution was not sufficient to alter intracellular *B. pseudomallei* growth. Collectively, the data suggested that both well-known canonical inflammasome complex (NLRP3/ASC and NLRC4) did not affect intracellular *B. pseudomallei*

A. Western Blotting



B. Intracellular bacterial growth

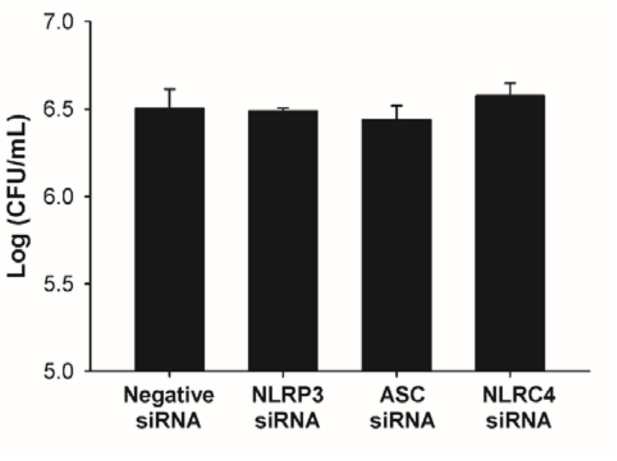


Figure 10. Canonical inflammasome machinery, NLRP3, ASC adaptor protein and NLRC4, did not involve in controlling intracellular growth of *B. pseudomallei* in infected human lung epithelial cells. The human lung epithelial cells were transfected with specific small interfering RNA (siRNA) of each inflammasome machinery including NLRP3, ASC, and NLRC4 for 48 hours before *B. pseudomallei* infection. At MOI of 10, *B. pseudomallei* was incubated with inflammasome components silencing A549 cells for 2 hours before performing kanamycin protection assay. The infected cell lysates were collected at the 12 hours post infection. The depleting efficiency was confirmed by Western blotting (A). The Triton X-100 treated cell lysates were quantitated the number of intracellular bacteria using pour plate method. The number of intracellular bacteria was also determined at 12 hours post infection by bacterial colony counting (B). Each experiment was conducted three times minimum. The samples were measured from three independent experiments and showed as mean ± SEM. The Student's t-test was applied to analyze the data statistically. The significant values of $p<0.05$, $p<0.01$, and $P<0.001$ was indicated as *, **, and ***, respectively.

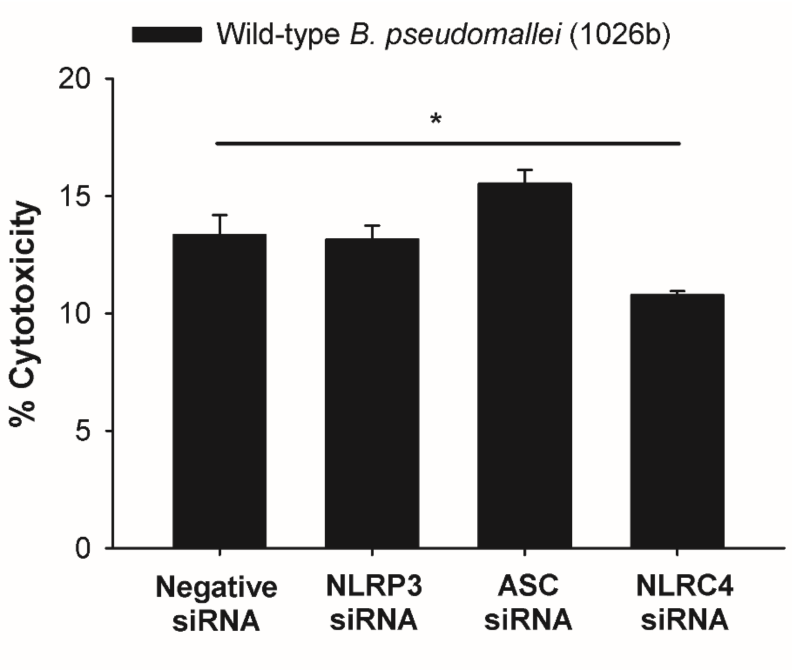


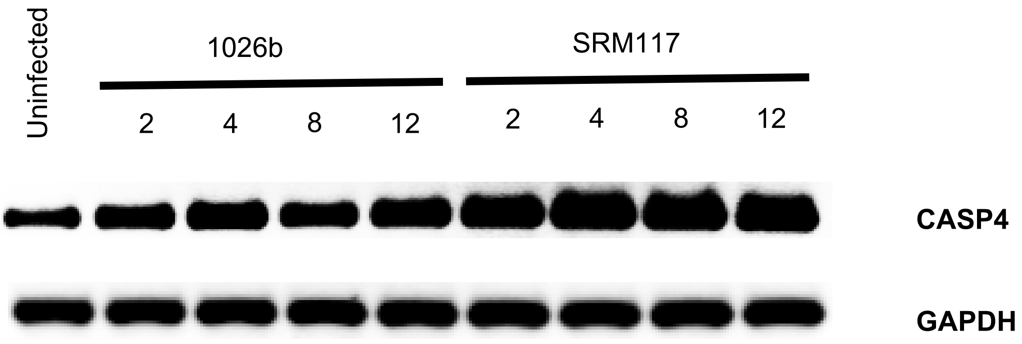
Figure 11. NLRC4 canonical inflammasome complex mediated pyroptosis cell death in response to *B.pseudomallei* infection in human lung epithelial cells. The human lung epithelial cells were transfected with specific small interfering RNA (siRNA) of each inflammasome machinery including NLRP3, ASC, and NLRC4 for 48 hours before *B. pseudomallei* infection. At MOI of 10, *B. pseudomallei* was incubated with inflammasome components silencing A549 cells for 2 hours before performing kanamycin protection assay. The LDH release in cell culture supernatants was determined using cytotoxicity assay to indicate the pyroptosis level. Each experiment was conducted three times minimum. The samples were measured from three independent experiments and showed as mean ± SEM. The Student's t-test was applied to analyze the data statistically. The significant values of $p<0.05$, $p<0.01$, and $P<0.001$ was indicated as *, **, and ***, respectively.

restriction and only NLRC4 inflammasome mediated pyroptosis cell death in human lung epithelial cells.

Non-canonical caspase-4 inflammasome activation was also delayed in wild-type *B. pseudomallei* infected human lung epithelial cells.

The non-canonical inflammasome activation was also reported to play role during *B. pseudomallei* infection as Caspase-11$^{-/-}$ mice were suffered from high bacterial burden and high mortality rate (7). In human, the non-canonical inflammasome activation is processed though caspase-4 and caspase-5, the ortholog of murine caspase-11. In this study, we aim to determine the function of the non-canonical caspase-4 inflammasome in human lung epithelial cells during *B. pseudomallei* infection. The caspase-4 gene and protein expression were investigated. The results demonstrated that the non-canonical caspase-4 mRNA level in wild type and LPS mutant infected cells was upregulated and maintained throughout the time of infection (Figure 12A). However, the LPS mutant was able to induce higher level of transcriptional expression than wild-type *B. pseudomallei*. We further performed the western blotting to investigate the cleaved form of caspase-4 (p20) known as the active form indicating the non-canonical inflammasome activation. Interestingly, the delay maturation of caspase-4 in wild-type infected cells was also observed as well (Figure 12B). All together, the data suggested that both inflammasome activation pathways, canonical caspase-1 and non-canonical caspase-4, were hindered in human lung epithelial cells during wild-type *B. pseudomallei* infection.

A. RT-PCR



B. Western Blotting

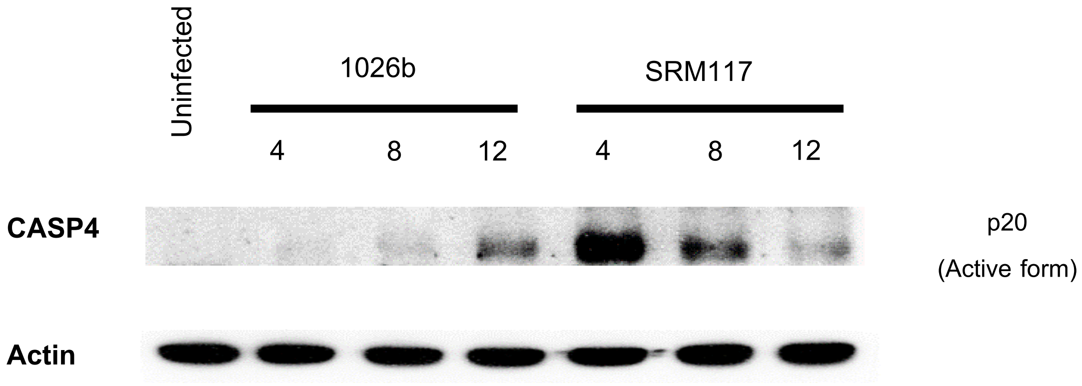


Figure 12. The wild type *B. pseudomallei* also detained non-canonical caspase-4 inflammasome gene expression and proteolytic maturation in infected human lung epithelial cells. A549 cells were infected with wild-type or LPS-mutated *B. pseudomallei* for 2 hours at MOI of 10. The cell lysates were collected at indicated time point. (A) Caspase-4 gene expression was assessed by RT-PCR. GAPDH was the internal control of RT-PCR. (B) The active form of caspase-4 was showed at 20kDa. Actin was the internal control of Western blotting. Each experiment was conducted three times minimum.

Non-canonical caspase-4 inflammasome pathway restricted the intracellular growth of *B. pseudomallei* through pyroptosis independent pathway.

In order to validate the role of non-canonical caspase-4 inflammasome during *B. pseudomallei* infection in human lung epithelial cells, caspase-4 expression was depleted using caspase-4 specific siRNA before *B. pseudomallei* infection. The depleting efficiency was determined via Western Blotting (Figure 13A and 13C). Notably, the infected caspase-4 depleted cells established the higher number of *B. pseudomallei* comparing to the negative siRNA treated cells in both wild type and LPS mutant infection (Figure 13B and 13D). In addition, bacterial internalization was comparable between caspase-4 depleted cells and negative siRNA treated cells highlighting their role in intracellular bacterial restriction not in bacterial internalization (Figure 14). The correlation between non-canonical inflammasome and pyroptosis level is previously reported to contribute in intracellular bacterial restriction in many model including murine lung epithelial cells and human intestinal epithelial cells (8, 17). However, the pyroptosis level of caspase-4 depleted cells and negative siRNA treated cells was comparable during wild-type *B. pseudomallei* infection suggesting the alternative unknown pathway in restriction of intracellular bacterial growth (Figure 15). Taken together, the results solidly pointed out the important role in intracellular bacterial killing of non-canonical caspase-4 inflammasome, not canonical pathway, in *B. pseudomallei* infected lung epithelial cells.

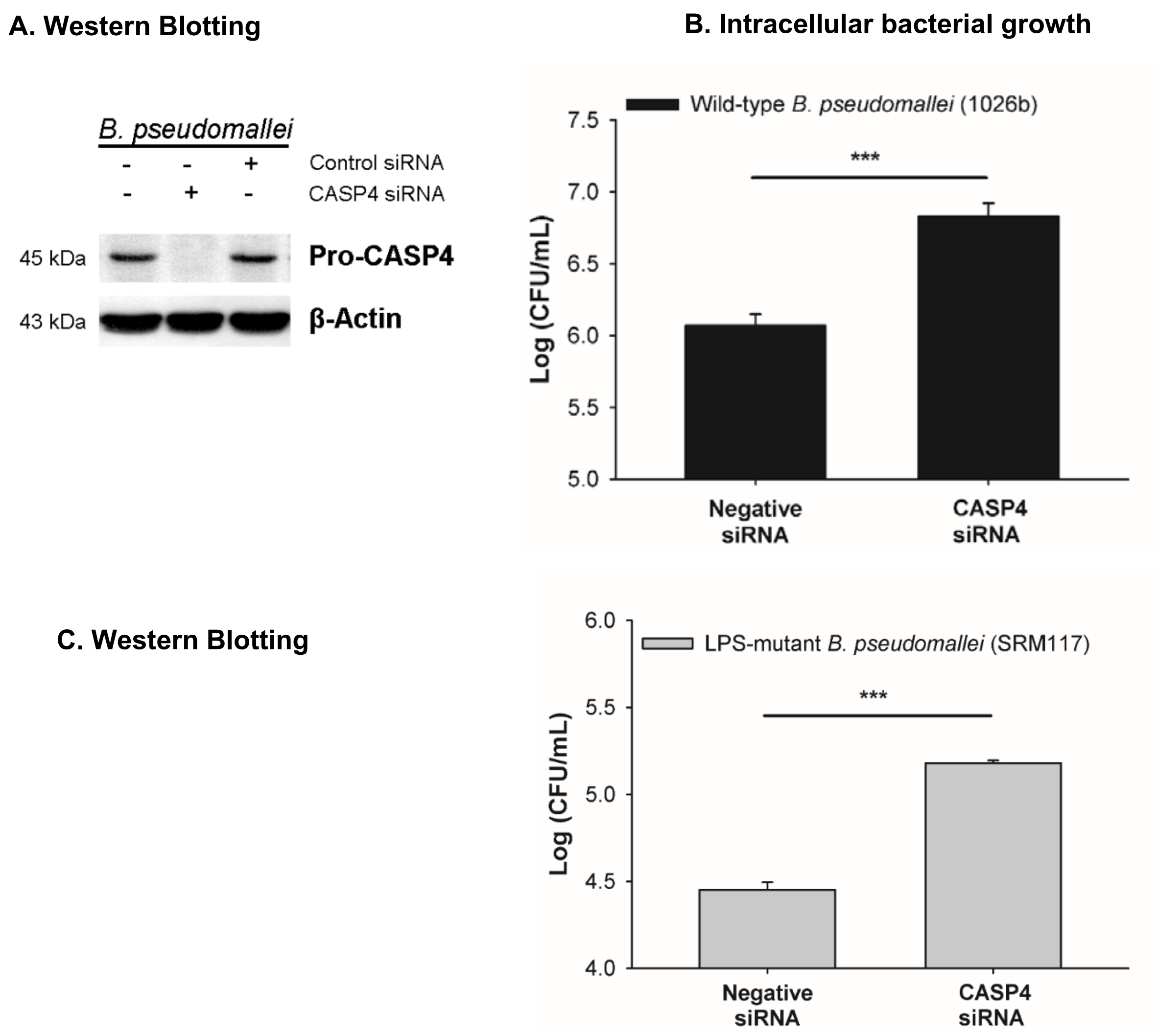


Figure 13. Non-canonical caspase-4 inflammasome was responsible for intracellular growth restriction in *B.pseudomallei* infected human lung epithelial cells. The human lung epithelial cells were transfected with specific small interfering RNA (siRNA) of caspase-4 for 48 hours before wild-type or LPS-mutated *B. pseudomallei* infection. At MOI of 10, *B. pseudomallei* was incubated with inflammasome components silencing A549 cells for 2 hours before performing kanamycin protection assay. The infected cell lysates were collected at the 12 hours post infection. The depleting efficiency was confirmed by Western blotting (A and C). The Triton X-100 treated cell lysates were quantitated the number of intracellular bacteria using pour plate method. The number of intracellular bacteria was also determined at 12 hours post infection by bacterial colony counting (B and D). Each experiment was conducted three times minimum. The samples were measured from three independent experiments and showed as mean ± SEM. The Student's t-test was applied to analyze the data statistically. The significant values of $p<0.05$, $p<0.01$, and $P<0.001$ was indicated as *, **, and ***, respectively.

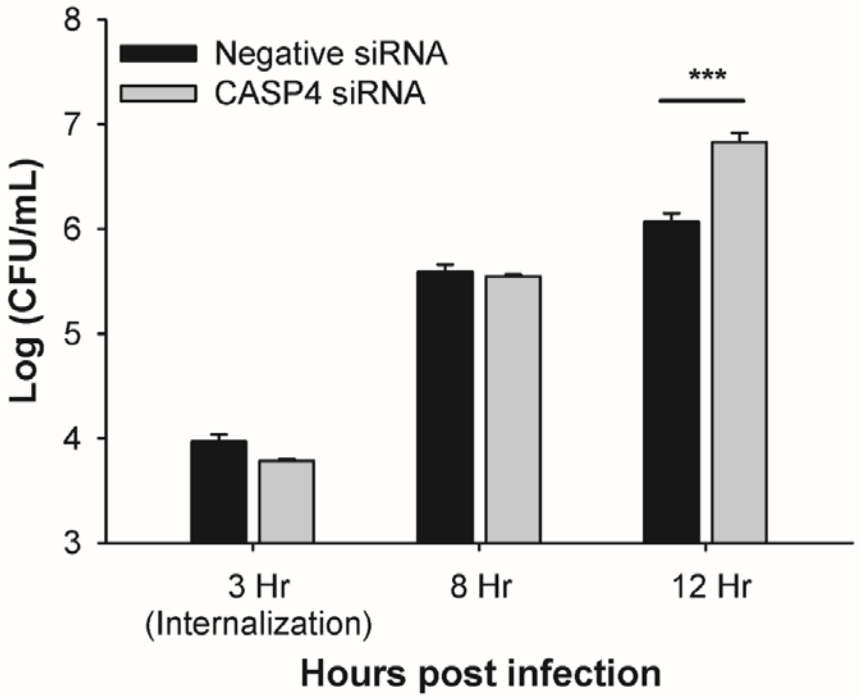


Figure 14. Non-canonical caspase-4 inflammasome did not interfere bacterial internalization and was responsible for bacterial restriction in the late time point. The human lung epithelial cells were transfected with specific small interfering RNA (siRNA) of caspase-4 for 48 hours before *B. pseudomallei* infection. At MOI of 10, *B. pseudomallei* was incubated with inflammasome components silencing A549 cells for 2 hours before performing kanamycin protection assay. The infected cell lysates were collected at the indicayed time points. The Triton X-100 treated cell lysates were quantitated the number of intracellular bacteria using pour plate method. The number of intracellular bacteria was also determined at 12 hours post infection by bacterial colony counting. Each experiment was conducted three times minimum. The samples were measured from three independent experiments and showed as mean ± SEM. The Student's t-test was applied to analyze the data statistically. The significant values of $p<0.05$, $p<0.01$, and $P<0.001$ was indicated as *, **, and ***, respectively.

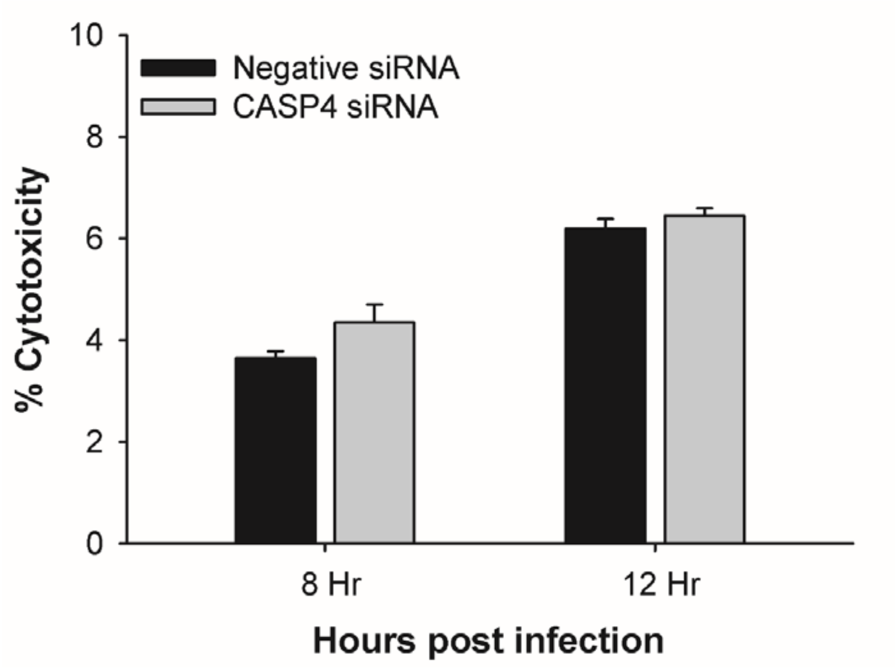


Figure 15. Non-canonical caspase-4 inflammasome control bacterial killing using alternative pathway in response to *B. pseudomallei* infection in human lung epithelial cells.

The human lung epithelial cells were transfected with specific small interfering RNA (siRNA) of caspase-4 for 48 hours before *B. pseudomallei* infection. At MOI of 10, *B. pseudomallei* was incubated with inflammasome components silencing A549 cells for 2 hours before performing kanamycin protection assay. The LDH release in cell culture supernatants was determined using cytotoxicity assay to indicate the pyroptosis level. Each experiment was conducted three times minimum. The samples were measured from three independent experiments and showed as mean ± SEM. The Student's t-test was applied to analyze the data statistically. The significant values of $p<0.05$, $p<0.01$, and $P<0.001$ was indicated as *, **, and ***, respectively.

NLRC3 mRNA expression level was remained unchanged throughout the infection of wild-type *B. pseudomallei* in human lung epithelial cells.

Unlike the typical canonical inflammasome pathways (NLRP3/ASC and NLRC4) that can initiate pro-inflammatory response during gram-negative bacteria infection, NLRC3 is reported as the immune regulator that antagonises inflammatory pathway (18, 19). To investigate the expression of NLRC3 during pathogenic gram-negative *B. pseudomallei* infection, *nlrc3* was observed in infected human lung epithelial cells. In the resting stage, NLRC3 mRNA was constitutively expressed in human lung epithelial cells. During wild-type *B. pseudomallei* and LPS mutant infection, the *nlrc3* expression was comparable to uninfected cells (Figure 16).

Wild-type *B. pseudomallei* induced *nlrc5* expression in infected human lung epithelial cells at 12 hours post infection.

Next, we initially examined the expression of NLRC5 in human lung epithelial cells following *B. pseudomallei* infection. Our data showed that human lung epithelial cells did not express *nlrc5* in uninfected condition. The wild-type *B. pseudomallei* was able to induce its expression in the late time point, 12 hours post infection (Figure 17).

NLRP12 expression was induced in the early phase of infection in wild-type *B. pseudomallei* infected human lung epithelial cells.

In order to investigate the contribution of NLRP12 in response to *B. pseudomallei* infection in human lung epithelial cells, *nlrp12* expression was preliminary observed during the infection. The

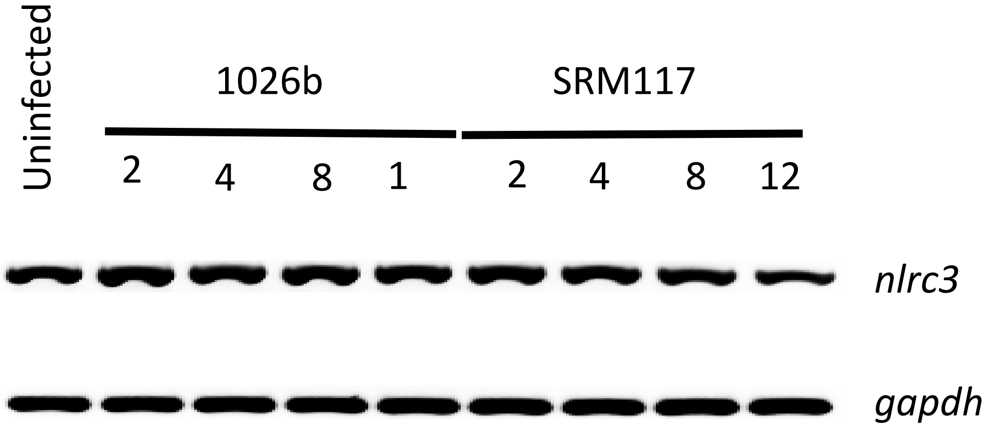


Figure 16. The wild type *B. pseudomallei* was able to maintain NLRC3 mRNA expression in infected alveolar epithelial cells. 549 cells were infected with wild type or LPS-mutant *B. pseudomallei* for 2 hours at MOI of 10. Cell pellets were collected and lysed at indicated time. Gene expressions were determined by RT-PCR.

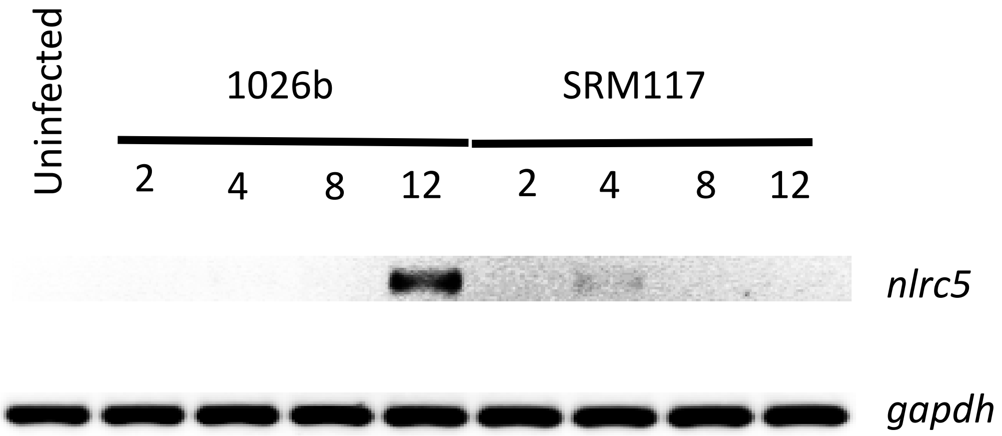


Figure 17. NLRC5 mRNA expression was inducible in human lung epithelial cells during the infection of wild-type *B. pseudomallei*. A549 were infected with wild type or LPS-mutant *B. pseudomallei* at MOI of 10. Cell pellets were collected and lysed at indicated time. Gene expressions were determined by RT-PCR.

result in Figure 18 demonstrated that human lung epithelial cells was slightly expressed *nlrp12* mRNA. The transient *nlrp12* expression was observed in wild-type *B. pseudomallei* infected cells. The rapid mRNA expression in infected cells was induced in 2 hours post infection of wild-type *B. pseudomallei*. In LPS mutant infection, the *nlrp12* expression was comparable indicating the no contribution of LPS in inducing *nlrp12* transcription.

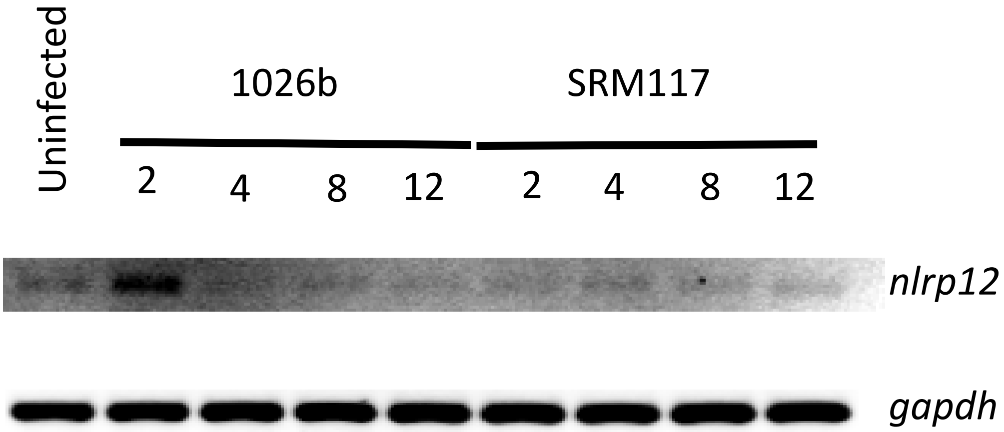


Figure 18. NLRP12 mRNA expression was strongly induced in early time-point of wild-type *B. pseudomallei* infection in human lung epithelial cells. A549 were infected with wild type or LPS-mutant *B.pseudomallei* at MOI of 10. Cell pellets were collected and lysed at indicated time. Gene expressions were determined by RT-PCR.

Discussion

In this study, we demonstrated the different pattern of intracellular bacterial growth profile of virulent wild type (1026b) and nonvirulent LPS mutant (SRM117) *B. pseudomallei* in infected A549 cells. The higher level of bacteria internalization was detected in 2 hours post infection in LPS mutant *B. pseudomallei* infected A549 comparing to internalization level of wild type strain (Figure 1). The LPS mutant strain was lacked the O-antigenic polysaccharide moiety in lipopolysaccharide part resulting in exposing the lipid portion of LPS to the environment (15). The hydrophobic interaction between cell membrane and mutated LPS might contribute to slightly higher level of bacteria internalization that was observed in the early time point. However, the dramatically decrease in either wild type or LPS mutant strain was showed in 4 hours post infection indicating the activation of defense mechanism within the human lung epithelial cells. In addition, the greater number of bacterial survival in wild type infected A549 suggested the important of O-antigenic polysaccharide moiety in camouflaging the antigenic part of *B. pseudomallei* LPS. This result was consistent with LPS mutant infected mouse macrophages cell line RAW264.7 that showed lower intracellular survival due to suppression of replication cycle in the early time point (20). In animal models experiment, higher LD50 of SRM117 was applied to reach the same severity as 1026b parental strain (15).

Nod-like receptors (NLRs) which are the intracellular sensors that responsible in detecting the cytosolic bacterial infection including *B. pseudomallei* (21). A great number of publications suggests that activation of NLRs have been observed following *B. pseudomallei* infection (13, 22). Some subset of NLRs has distinct defense mechanism by forming multiprotein complexes called “Inflammasomes” to activate cysteine protease caspase-1. The results of inflammasome activation is including IL-1β and IL-18 production, and pyroptosis, a special form of cell death (4, 6). In order to understand the pattern of inflammasome activation, the *il-1β* expression under virulent and nonvirulent *B. pseudomallei* infection in A549 was detected (Figure 2). The upregulation of *il-1β* expression after either wild type or LPS mutant infection suggested the possible inflammasome activation. The result was also related to the LPS mutant intracellular survival pattern in Figure 1 since the strongly upregulation of *il-1β* expression was observed in 4 hours post infection. However, the translational IL-1β production data showed the unexpected pattern. The result in Figure 3 suggested the intracellular production but not extracellular secretion of IL-1β in either wild type or LPS mutant infection. In the process of IL-1β secretion, IL-1β was firstly produced in the form of pro-IL-1β and required caspase-1 to cleave into active form. It was possible that the detectable intracellular IL-1β was inactive form since ELISA was unable to distinguish between both form of IL-1β. There were several studies suggesting the remaining intracellular IL-1β during bacterial infection (23, 24). Even though several cell types were capable to produce the precursor of IL-1β, the secretion was predominantly limited to certain type of cell lineage such as monocytes and macrophages. Notably, intracellular IL-1β production in wild type *B. pseudomallei* infected A549 is higher than LPS mutant infected cells. IL-18 was also another reported cytokine that was secreted during inflammasome activation. However, IL-18 secretion also showed the same pattern as IL-1β that was unable to detect in cell supernatants at any time point (Figure 4).

Pyroptosis is a distinct type of cell death that induces high level of inflammation. The characteristic of pyroptosis are rapid cell membrane rupture and release all of cytosolic content. Therefore, pyroptosis is considered as the effective method to cut off the replication cycle of intracellular bacteria by eliminating the niches of those bacteria (16). The previous study suggested that caspase-1 of inflammasome complex plays a crucial role in defense against

B.pseudomallei in the mouse model. The former results suggested that caspase-1$^{-/-}$ mice were highly susceptible to *B.pseudomallei* infection. Higher bacterial burden and less IL-1β secretion were observed in infected caspase-1$^{-/-}$ mice (25). In wild type *B.pseudomallei* infection, pyroptosis level was lower than LPS mutant infection as shown in Figure 5 suggesting the possible correlation of pyroptosis and limiting of intracellular bacteria growth (Figure 1). Since there is no available specific inhibitor of caspase-1, pan-caspase inhibitor (Z-VAD-FMK) was applied to investigate the role of inflammasome activation in *B.pseudomallei* infected A549. However, the results in Figure 6 indicated the non-significant role of caspases in intracellular bacterial survival. The alteration of survival growth was not observed either in wild type or LPS mutant infected cells. Caspase-1 activation was also undetected by using colorimetric caspase-1 activity assay (Figure 7).

Next, we further examined the role of the distinct inflammatory responses in human lung epithelial cells (A549) against the intracellular airborne bacteria, *B. pseudomallei*. Unlike murine macrophages, our data established the different inflammasome responses in human lung epithelial cells during *B. pseudomallei* infection. We also emphasized the role of the caspase-4 non-canonical inflammasome, not canonical NLRP3/ASC and NLRC4 inflammasome, in restriction of intracellular *B. pseudomallei* in lung epithelial cells. Although several reports indicate the importance of canonical NLRP3/ASC and NLRC4 inflammasome in the modulation of lung immunity against gram-negative bacteria, more than a half of studies only emphasize their role in hematopoietic cells (26). The canonical NLRP3/ASC and NLRC4 have the non-redundant function in murine melioidosis model. The NLRP3$^{-/-}$ and ASC$^{-/-}$ mice macrophages secrete significantly low level of IL-1β whereas the NLRC4$^{-/-}$ macrophages are unable to undergo pyroptosis resulting in high bacterial burden (13). According to our results as shown in

Figure 8, Figure 9 and Figure 12, the human lung epithelial cells (A549) also express the wide range of inflammasome machinery including NLRP3, ASC, NLRC4, and caspase-4 (27, 28). The upregulation of the canonical inflammasome components was observed implying the possible involvement of this pathway against *B. pseudomallei* in human epithelial cells (Figure 8 and Figure 9). Although delayed response of caspase-1 activation was detected in wild-type infected cells (Figure 8), one interesting finding was that we were unable to find the concrete contribution of these canonical inflammasomes in human lung epithelial cells during *B. pseudomallei* infection. The intracellular bacterial growth and pyroptosis level in NLRP3 and ASC depleted lung epithelial cells were comparable with the negative siRNA treated cells (Figure 10). Even though NLRC4 depleted cells showed decreasing pyroptosis level suggesting its role in regulation of pyroptosis during *B. pseudomallei* infection, the contribution was not enough to affect the intracellular bacterial growth (Figure 11).

Notably, non-canonical inflammasome caspase-4, not canonical pathway, significantly contributed to *B. pseudomallei* restriction in human lung epithelial cells (Figure 13 and Figure 14). Caspase-4 is the orthologs of murine caspase-11 and reports to express in various type of cells, including epithelial lineages (8, 29). Our results correlate with the study in melioidosis murine system, which Casp11$^{-/-}$ mice suffer from lethal challenge infection either from highly pathogenic *B. pseudomallei* or from less virulent *B. thailandensis* (7). Furthermore, the higher bacterial burden is observed in human caspase-4 depleted intestinal epithelial cells suggesting their function in intestinal epithelial defense against enteric bacteria (8, 30). Similar to murine caspase-11, human caspase-4 can detect intracellular LPS of gram-negative bacteria by direct interaction with CARD-domain of caspase-4 resulting in non-canonical inflammasome activation (29). We confirmed the contribution of human caspase-4 in human lung epithelial cells and *B.*

pseudomallei LPS as a virulent factor using LPS-mutated *B. pseudomallei* strain SRM117. The LPS-mutated strain deficient in type II O-polysaccharide antigen (O-antigen) leaving the core-oligosaccharide and the lipid A moiety embedded in outer membrane of bacteria (15). Unlike parental strain *B. pseudomallei* 1026b, LPS-mutated *B. pseudomallei* is more susceptible to macrophage killing and establishes lower LD50 *in vivo* (20, 31). As expected, human caspase-4 of LPS-mutated *B. pseudomallei* infected lung epithelial cells tremendously activated in the early time point and remained noticeable all along observing period (Figure 12). This result may be explained by the fact that the length of O-antigen and polysaccharide core affects the binding efficiency of inflammatory caspase-4. The shorter in length, the stronger binding is observed (29). Since LPS-mutated *B. pseudomallei* possess the shorter LPS structure, caspase-4 activation was remarkable observed and contributed to less aggressive intracellular growth pattern. It is worth to mention that the extensively caspase-4 activation in LPS-mutated *B. pseudomallei* infected lung epithelial cells is due to their higher internalization rate. Our data also suggest the possible role of O-polysaccharide antigen as the virulent factor in interference of inflammatory caspase-4 sensing. In addition, the increasing intracellular growth of LPS-mutated *B. pseudomallei* in caspase-4 depleted lung epithelial cells was solidly indicated the bacterial killing role of caspase-4 in *B. pseudomallei* restriction (Figure 13).

Recently, the accumulating numbers of studies suggest that not only proinflammatory responsible function, some NLRs also act as an immune regulators. NLRC3, NLRC5, and NLRP12 can negatively regulate intracellular signaling pathways in different ways (14). NLRC3 is reported to reduce type I interferon production by interfering binding between STING/TANK-binding kinase 1 (TBK1) (18). NLRC3 can associated with both STING and TBK1 and further inhibit the initiation of antibacterial gene response. The presence of *nlrc3* expression during *B. pseudomallei* infection

(Figure 16) might contribute to the higher survival rate of wild-type strain in human lung epithelial cells. Moreover, NLRC3 also can disrupt NLRP3/ASC canonical inflammasome formation by competitive binding to ASC and pro-caspase-1 (32). The comparable *nlrc3* expression level possibly benefit to intracellularly replicate of *B.pseudomallei* in human lung epithelial cells. Besides NLRC3, NLRC5 and NLRP12 were also induced in *B. pseudomallei* infected lung epithelial cells (Figure 17 and Figure 18), suggesting that these negative regulators of NLRs may involve in the intracellular replication of *B. pseudomallei* infection of lung epithelial cells.

References :

1. Currie BJ, Jacups SP. Intensity of rainfall and severity of melioidosis, Australia. Emerg Infect Dis. 2003; 9:1538-42.
2. Liu X, Pang L, Sim SH, Goh KT, Ravikumar S, Win MS, Tan G, Cook AR, Fisher D, Chai LY. Association of melioidosis incidence with rainfall and humidity, Singapore, 2003-2012. Emerg Infect Dis. 2015;21:159-62.
3. Cheng AC, Currie BJ. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev. 2005; 18:383-416.
4. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140(6):805-20.
5. Martinon F, Burns K, Tschopp J. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell. 2002;10:417-26.
6. Lamkanfi M, Dixit VM. Mechanisms and functions of inflammasomes. Cell. 2014;157(5):1013-22.
7. Aachoui Y, Leaf IA, Hagar JA, Fontana MF, Campos CG, Zak DE, *et al*. Caspase-11 protects against bacteria that escape the vacuole. Science. 2013;339(6122):975-8.
8. Knodler LA, Crowley SM, Sham HP, Yang H, Wrande M, Ma C, *et al*. Noncanonical inflammasome activation of caspase-4/caspase-11 mediates epithelial defenses against enteric bacterial pathogens. Cell Host Microbe. 2014;16(2):249-56.
9. Jones AL, Beveridge TJ, Woods DE. Intracellular survival of *Burkholderia pseudomallei*. Infect Immun. 1996;64:782-90.

10. Kespichayawattana W, Rattanachetkul S, Wanun T, Utaisincharoen P, Sirisinha S. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect Immun. 2000;68:5377-84.
11. Phewkliang A, Wongratanacheewin S, Chareonsudjai S. Role of *Burkholderia pseudomallei* in the invasion, replication and induction of apoptosis in human epithelial cell lines. Southeast Asian J Trop Med Public Health. 2010;41:1164-76.
12. Sun GW, Lu J, Pervaiz S, Cao WP, Gan YH. Caspase-1 dependent macrophage death induced by *Burkholderia pseudomallei*. Cell Microbiol. 2005;7:1447-58.
13. Ceballos-Olvera I, Sahoo M, Miller MA, Del Barrio L, Re F. Inflammasome-dependent pyroptosis and IL-18 protect against *Burkholderia pseudomallei* lung infection while IL-1beta is deleterious. PLoS Pathog. 2011;7(12):e1002452.
14. Kufer TA, Sansonetti PJ. NLR functions beyond pathogen recognition. Nat Immunol. 2011;12(2):121-8.
15. DeShazer D, Brett PJ, Woods DE. The type II O-antigenic polysaccharide moiety of *Burkholderia pseudomallei* lipopolysaccharide is required for serum resistance and virulence. Mol Microbiol. 1998;30(5):1081-100.
16. Bergsbaken T, Fink SL, Cookson BT. Pyroptosis: host cell death and inflammation. Nat Rev Microbiol. 2009;7(2):99-109.
17. Wang J, Sahoo M, Lantier L, Warawa J, Cordero H, Deobald K, *et al.* Caspase-11-dependent pyroptosis of lung epithelial cells protects from melioidosis while caspase-1 mediates macrophage pyroptosis and production of IL-18. PLoS Pathog. 2018;14(5):e1007105.
18. Zhang L, Mo J, Swanson KV, Wen H, Petrucelli A, Gregory SM, *et al.* NLRC3, a member of the NLR family of proteins, is a negative regulator of innate immune signaling induced by the DNA sensor STING. Immunity. 2014;40(3):329-41.
19. Uchimura T, Oyama Y, Deng M, Guo H, Wilson JE, Rampanelli E, *et al*. The Innate Immune Sensor NLRC3 Acts as a Rheostat that Fine-Tunes T Cell Responses in Infection and Autoimmunity. Immunity. 2018;49(6):1049-61 e6.
20. Arjcharoen S, Wikraiphat C, Pudla M, Limposuwan K, Woods DE, Sirisinha S, *et al*. Fate of a *Burkholderia pseudomallei* lipopolysaccharide mutant in the mouse macrophage cell line RAW 264.7: possible role for the O-antigenic polysaccharide moiety of lipopolysaccharide in internalization and intracellular survival. Infect Immun. 2007;75(9):4298-304.

21. Silva EB, Dow SW. Development of *Burkholderia mallei* and *pseudomallei* vaccines. Front Cell Infect Microbiol. 2013;3:10.
22. Sahoo M, Ceballos-Olvera I, del Barrio L, Re F. Role of the inflammasome, IL-1beta, and IL-18 in bacterial infections. ScientificWorldJournal. 2011;11:2037-50.
23. Yang J, Hooper WC, Phillips DJ, Talkington DF. Regulation of proinflammatory cytokines in human lung epithelial cells infected with *Mycoplasma pneumoniae*. Infect Immun. 2002;70(7):3649-55.
24. Uchida Y, Shiba H, Komatsuzawa H, Takemoto T, Sakata M, Fujita T, *et al.* Expression of IL-1 beta and IL-8 by human gingival epithelial cells in response to *Actinobacillus actinomycetemcomitans*. Cytokine. 2001;14(3):152-61.
25. Breitbach K, Sun GW, Kohler J, Eske K, Wongprompitak P, Tan G, *et al.* Caspase-1 mediates resistance in murine melioidosis. Infect Immun. 2009;77(4):1589-95.
26. Chaput C, Sander LE, Suttorp N, Opitz B. NOD-Like Receptors in Lung Diseases. Front Immunol. 2013;4:393.
27. Gonzalez-Juarbe N, Bradley KM, Riegler AN, Reyes LF, Brissac T, Park SS, *et al*. Bacterial Pore-Forming Toxins Promote the Activation of Caspases in Parallel to Necroptosis to Enhance Alarmin Release and Inflammation During Pneumonia. Sci Rep. 2018;8(1):5846.
28. Kong H, Wang Y, Zeng X, Wang Z, Wang H, Xie W. Differential expression of inflammasomes in lung cancer cell lines and tissues. Tumour Biol. 2015;36(10):7501-13.
29. Shi J, Zhao Y, Wang Y, Gao W, Ding J, Li P, *et al*. Inflammatory caspases are innate immune receptors for intracellular LPS. Nature. 2014;514(7521):187-92.
30. Kobayashi T, Ogawa M, Sanada T, Mimuro H, Kim M, Ashida H, *et al.* The Shigella OspC3 effector inhibits caspase-4, antagonizes inflammatory cell death, and promotes epithelial infection. Cell Host Microbe. 2013;13(5):570-83.
31. Wikraiphat C, Charoensap J, Utaisincharoen P, Wongratanacheewin S, Taweechaisupapong S, Woods DE, *et al.* Comparative in vivo and in vitro analyses of putative virulence factors of *Burkholderia pseudomallei* using lipopolysaccharide, capsule and flagellin mutants. FEMS Immunol Med Microbiol. 2009;56(3):253-9.
32. Eren E, Berber M, Ozoren N. NLRC3 protein inhibits inflammation by disrupting NALP3 inflammasome assembly via competition with the adaptor protein ASC for pro-caspase-1 binding. J Biol Chem. 2017;292(30):12691-701.