BURKHOLDERIA PSEUDOMALLEI INDUCES MOUSE MACROPHAGE ACTIVATION VIA MYD88-DEPENDENT PATHWAY

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ABSTRACT

Burkholderia pseudomallei, the causative agent of melioidosis, is a facultative intracellular gram-negative bacterium that can survive and multiply inside the macrophages. The signaling pathway by which *B. pseudomallei* activates macrophage has not been elucidated. This study demonstrated that *B. pseudomallei* was able to induce gene expression through MyD88-dependent pathway (e.g. *ικbζ, il-6, tnf-α, socs3*) but failed to activate MyD88-independent pathway (e.g. *inos, ifn-β, irg1, socs1*). Moreover, in the presence of cytochalasin D, the gene expression in *B. pseudomallei*-infected macrophages was not affected, indicating that the activation of these genes was likely triggered via surface receptors of the macrophages. Examination of signaling by interaction of LPS to TLR showing that neutralizing antibody of TLR4 failed to block the macrophages activation by *B. pseudomallei* LPS, suggesting that *B. pseudomallei* LPS might use different types of TLR rather than TLR4 to activate the signaling. IFN-γ not only enhanced gene expression of MyD88-dependent pathway but also restored the gene expression of the MyD88-independent pathway and inhibited intracellular survival of *B. pseudomallei* in the infected macrophages. These results suggest that the MyD88-independent pathway is an essential pathway controlling *B. pseudomallei* survival in macrophages.

KEY WORDS: *BURKHOLDERIA PSEUDOMALLEI*/ IκBζ/ IL-6/ resveratrol/ MyD88-dependent/ MyD88-independent

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เบอร์โคลเดอเรีย สูโดมาลลิไอกระตุ้นมาโครฟาจของหนูโดยการส่งสัญญาณผ่าน MYD88-DEPENDENT PATHWAY (*BURKHOLDERIA PSEUDOMALLEI* INDUCES MOUSE MACROPHAGE ACTIVATION VIA MYD88-DEPENDENT PATHWAY)

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บทคัดย่อ

เชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ (*Burkholderia pseudomallei*) เป็นเชื้อแบคทีเรียชนิดแกรมลบซึ่งเป็นสาเหตุของโรคเมลิออยโดซิส เชื้อ *B. pseudomallei* เป็นแบคทีเรียชนิด facultative intracellular ที่สามารถเจริญเติบโตและเพิ่มจำนวนได้ทั้งในเซลล์ phagocytic และ non-phagocytic แต่ยังไม่มีการศึกษาถึงกลไกการส่งสัญญาณในเซลล์มาโครฟาจที่ถูกกระตุ้นด้วยเชื้อแบคทีเรียนี้ ในการศึกษานี้พบว่าเชื้อ *B. pseudomallei* สามารถกระตุ้นการแสดงออกของยีนผ่าน MyD88-dependent pathway (*ικbζ, il-6, tnf-α, socs3*) แต่ไม่สามารถกระตุ้นการแสดงออกนี้ผ่าน MyD88-independent pathway (*inos, ifn-β, irg1, socs1*) ซึ่งการแสดงออกของยีนเหล่านี้ถูกกระตุ้นผ่านตัวรับสัญญาณบนผิวของเซลล์มาโครฟาจ นอกจากนี้ในมาโครฟาจที่การทำงานของตัวรับสัญญาณ TLR4 ถูกยับยั้ง *B. pseudomallei* LPS ยังสามารถกระตุ้นให้เกิดการส่งสัญญาณได้ ซึ่งอาจกล่าวได้ว่า *B. pseudomallei* LPS อาจจะใช้ตัวรับสัญญาณอื่นแทนที่จะใช้ตัวรับสัญญาณ TLR4 นอกจากนั้นยังพบว่า IFN-γ สามารถที่จะเพิ่มระดับการแสดงออกของยีนทั้ง MyD88-dependent และMyD88-independent pathway อีกทั้งยังพบว่าเมื่อมีการแสดงออกของยีนโดยผ่าน MyD88-independent pathway แล้วมาโครฟาจจะสามารถทำลายเชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ ภายในเซลล์ได้ จึงสรุปว่า MyD88-independent pathway มีความสำคัญในการควบคุมและทำลายเชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอภายในเซลล์มาโครฟาจ

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LIST OF ABBREVIATIONS

-/-	knock out
/	per
%	percent
*	significance level
ADMEM	Advanced Dulbecco's modified Eagle's medium
AP-1	activating protein-1
APS	ammonium persulfate
ASA	Ashdown's selective agar
Arp	actin-related protein
bimA	*B. pseudomallei* intracellular motility
bp	base pair
BPSA	*Burkholderia pseudomallei* selective agar
BSA	bovine serum albumin
Bsa	*Burkholdoria* secretion apparatus
ºC	degree Celsius
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
cm^2	cubic centrimeter
CO_2	carbon dioxide
CpG	cytidine-phosphate-guanosine
CPS	capsule polysaccharide
DMEM	Dulbecco's modified Eagle's medium
dsRNA	double-stranded RNA
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
et al	et. all (Latin), and others

LIST OF ABBREVIATIONS

(Continued)

FBS	fetal bovine serum
g	gram
G-CSF	granulocyte-colony-stimuating factor
h	hour (s)
H_2O_2	hydrogen peroxide
H_2SO_4	sulfuric acid
IFN-γ	interferon-gamma
IFN-β	interferon-beta
IFNGR1	interferon-gamma receptor 1
IHA	indirect hemagglutination assay
IκBζ	ikappaB-zeta
IKK	IκB kinase
IL	interleukin
IL-6R	interleukin-6 receptor
iNOS	inducible nitric oxide syntase
IP-10	IFN-γ-inducible protein 10
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
IRGs	immune responsive genes
IRG1	immune responsive gene 1
JAK	janus kinase
kDa	kilodalton
KO	knockout
LBP	LPS-binding protein
LPS	lipopolysaccharide
LRR	leucine-rich repeat
M	molar
Mal	MyD88 adaptor-like
MAMP	microorganism-associated molecular patter

LIST OF ABBREVIATIONS

(Continued)

MAPK	mitogen-activated protein kinase
ME	mercaptoethanol
min	minute
MLK3	mixed-lineage kinase3
MNGC	multinucleated giant cell
ml	milliliter
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribosomal nucleic acid
M.Sc	Master of Science
MyD88	myeloid differentiation factor 88
NF-κB	nuclear factor-kappa B
NK	natural killer cell
nm	nanometer
ng	nanogram
NOD	nucleotide-binding oligomerization domain
O.D.	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDC	plasmacytoid dendritic cells
pg	pictogram
PRR	pattern recognition receptor
QS	quarum sensing
Refs	references
RIG-I	retinoic acid-inducible gene I
RIP1	receptor interacting protein 1
RNA	ribosomal nucleic acid
rpm	round per minute
RT	room temperature

LIST OF ABBREVIATIONS

(Continued)

RT-PCR	reverse transcriptase-polymerase chain reaction
SARM	sterile α and armadillo motifs
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second
SD	standard deviation
SH2	Src-homology 2
SOCS	suppressor of cytokine signaling
ssDNA	single-stranded RNA
STAT	signal transducer and activator of transcription
Streptavidin – HRP	streptavidin conjugated to horseradish-peroxidase
TAK	TGF-β-activated kinase
Taq	*Thermus aquaticus*
TBE	tris-borate EDTA buffer
TBK1	TANK-binding kinase 1
TEMED	N, N, N', N'- tetramethylethylene diamine
TFP	type IV pili
TIRAP	TIR domain-containing adaptor protein
TIR	Toll/IL-1 receptor homologous
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF-α	tumor necrosis factor-α
TNF-R	tumor necrosis factor receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein inducing IFN-β
TSA	tryptic soy agar
TSB	tryptic soy broth

LIST OF ABBREVIATIONS

(Continued)

TTSS	type III secretion system
U	unit
UV	ultra violet
µg	microgram
µl	microliter
µM	micromolar
V	volt
WASP	Wiskott-Aldrich syndrome protein
w/v	weight per volume

CHAPTER I

INTRODUCTION

Burkholderia pseudomallei is the causative agent of melioidosis which affects humans and animals in southeast Asia, particularly northeastern Thailand and northern Australia. In humans, the disease is usually acquired by skin inoculation or inhalation of the dust contaminated with this bacterium (1). The clinical features of melioidosis vary greatly from acute fatal sepsis to localized chronic infections (2). In Thailand, 95% of splenic abscesses patients are caused by this bacterium (3).

B. pseudomallei is a facultative intracellular Gram-negative bacillus which can survive and multiply in both phagocytic and non-phagocytic cells. After internalization, *B. pseudomallei* can escape membrane-bound phagosome into the cytoplasm (4). Inside the cell, this bacterium can induce cell- cell fusion resulting in multinucleated giant cell formation (5, 6). This unique ability may facilitate the bacterium to spread from one cell to another. Generally, macrophage has an ability to kill the invading pathogen. However, the mechanisms by which *B. pseudomallei* survives and multiplies inside the cells are not fully understood. Additionally, there is an evidence suggesting that the macrophages infected with this bacterium fail to produce an inducible nitric oxide synthase (iNOS) which is a key enzyme in antibacterial activity of the macrophages (7). The failure to activate iNOS expression can be explained by the fact that the macrophages infected with *B. pseudomallei* were unable to stimulate high level of interferon beta (IFN-β) production known to be produced by the cells infected by other gram negative bacteria such as *S. enterica* serovar Typhi (*S. typhi*) (8). The production of IFN-β from the infected macrophage is essential for activation of interferon regulatory factor-1 (IRF-1) expression, which is a transcription factor required for iNOS gene transcription.

Toll-like receptor (TLR) is a pattern recognition receptor which plays a major role in an innate immune system against the microorganisms (9). There are at least 11 members of TLR family in humans that have been identified (10). As many as 13 can be found in a search of the mouse structure genome (10). Each recognizes a different component of microbe structures. For example, TLR4 is involved in the recognition of lipopolysaccharide (LPS) (11). However, the intracellular signaling pathway of all TLRs shares common molecules. TLR signaling pathways can be divided into two groups ,(1) MyD88-dependent, (2) MyD88-independent pathway. These two signaling pathways use a different set of adaptor molecule to activate NF-κB leading to different cytokine production. In MyD88-deficient mice, the animals were unable to produce TNF-α, IL-12p40 and IL-6, which suggested that these cytokines were regulated through MyD88-dependent pathway. In contrast, IFN- β production was uneffected in case of MyD88-deficient mice, suggesting that the production of IFN-β did not require MyD88-dependent pathway (12). MyD88 pathway is also known to regulate several essential protein expression including IκBζ. Among them, a new nuclear IκBζ protein plays role as a transcription factor of IL-6 (13). In IκBζ-deficient mice, the animals failed to produce IL-6, indicating that a regulation of IL-6 requires IκBζ (13). In addition, MyD88-deficient mice failed to induce expression of IκBζ after stimulation indicating that regulation of IκBζ requires MyD88-dependent (14). Accordingly, IL-6 was also undetectable in MyD88-deficient mice. This finding shows that the MyD88-mediated signal pathway is required for an induction of IL-6 production through the activation of IκBζ expression (15). Typical lipopolysaccharide (LPS) of the gram-negative bacteria serves as a ligand of TLR4. On the other hand, the LPS isolated from *B. pseudomallei* is known to have an unusual chemical structure in the acid stable inner core region attached to the lipid A moiety (16). This unusual LPS structure has also been shown to be a weak macrophages activator compared with the LPS isolated from the other gram-negative bacteria, such as *S. typhi* and *Escherichia coli* (16, 17). However, whether *B. pseudomallei* LPS is an agonist for TLR2 or TLR4 will be also investigated in this study.

In the present study, the intracellular signaling pathway of the macrophages infected with *B. pseudomallei* was investigated. The gene expression that regulates through MyD88-dependent pathway such as IκBζ, IL-6, TNF- α, SOCS3 and MyD88-

independent pathway such as iNOS, IFN- β, IRG1, SOCS1 was elucidated in the *B. pseudomallei*-infected macrophages. Moreover, resveratrol which is a specific inhibitor of MyD88-independent was used to identify the intracellular signaling. The information obtained from this study will provide a better understanding of how *B. pseudomallei* can modulate the macrophages in order to survive inside the host.

CHAPTER II
LITERATURE REVIEW

1. Melioidosis and *Burkholderia pseudomallei*

1.1 History

In 1912, Whitmore and Krishnaswami discovered a new bacterium that caused a glanders-like disease. This bacterium could be cultured on peptone agar and potato slopes and distinguished from the organism causing glanders by ability to growth and motility (2). In 1932, Stanton and Fletcher (18) called this disease "melioidosis". The bacterium has been variously known as *Bacillus pseudomallei, Bacillus whitmorii, Malleomyces pseudomallei* and *Pseudomonas pseudomallei* and finally named *Burkholderia pseudomallei* by Walter Burkholder in 1992 (19).

1.2 Melioidosis

During half of the 20th century, melioidosis emerged as an infectious disease of major public health problem in southeast Asia and northern Australia (2). There are several risk factors for development of melioidosis. First, 60 % of patients with diabetes mellitus (mainly type 2) have a high incidence of melioidosis. It was suggested that insulin may have a direct effect on *B. pseudomallei* (20). Thalassemia, renal disease, and exposure to surface soil or water were also reported to be associated with an increased risk of this disease (21). The other factors such as excessive alcohol consumption, chronic lung disease and chronic renal disease are also considered as the risk factors for developing melioidosis (22, 23). In Thailand, *B. pseudomallei* accounts for up to approximately 20% of community-acquired bacteremia (24). In Australia, this bacterium is the most common cause of fatal community-acquired bacteremic pneumonia (25, 26). The highest incidence of disease occurs during rainy seasons (24), possibly due to the fact that the organism raise the underlying soil

to the surface, thus enhancing the potential of exposure to humans and animals. The manifestations of melioidosis are commonly represented by acute, sub-acute and chronic illnesses. However the incubation periods of disease are not well defined. They range from as little as a few days to upwards of 26 years (27-29).

1.2.1 Acute melioidosis : The incubation period of the acute form is 2-5 days and the disease can be subdivided into 2 groups.

I) Acute pulmonary symptoms, which appear rapidly, are characterized by high fever and pulmonary distress followed by the appearance of visceral abcesses and death within a few days, if left untreated.

II) Acute septicemic is also rapidly fatal. The mortality of septicemic form is high following shock, respiratory failure, and multiorgan failure (24, 30).

1.2.2 Sub-acute melioidosis : It is characterized as a prolonged febrile illness. The disease may be focal or disseminated with abscesses in many organs. The clinical manifestations vary from weeks to months or years. Symptoms of sub-acute melioidosis are milder than the acute form and often occur in individuals after they have left an endemic area (31).

1.2.3 Chronic pulmonary melioidosis : Patients have a fever, weight loss, pleuritic chest pain, a productive cough, sometimes with haemoptysis and hepatosplenomegaly (32).

Melioidosis carries a potentially high mortality rate. *B. pseudomallei*, a causative agent of melioidosis , is intrinsic antibiotic resistance and has a wide host range. After the incident " anthrax letters" in the United States, *B. pseudomallei* has been considered an important potential bioweapon (2). However, the potential risk of *B. pseudomallei* as a bioweapon is uncertain.

1.3 Epidimiology

B. pseudomallei is widely distributed in water and soil in the tropical areas. The region is generally restricted to the latitudes 20ºS and 20ºN in southeast Asia and northern Australia (19). Melioidosis was first recognized within Australia from an outbreak in sheep in 1949 in Winton, northern Queenland (33, 34). The first human case ever reported was a diabetic who died from septicemic melioidosis in Townsville in 1950 (35). Another case was described from the northern Territory of Australia

occurred in 1960 (36). This infectious disease is now endemic in the northeastern of Thailand (24) but it rarely occurred in other regions of the country.The infectious disease also occurred in a wide variety of animals species such as camels, horses, sheep, cattle, goats, pigs and kangaroos(33, 37-40). Moreover, several reports demonstrated that the differences in the density of *B. pseudomallei* in the environment or differences in phenotypes and genotypes of *B. pseudomallei* led to differences in virulence of the local strains (41). The cases of melioidosis were also reported outside the endemic areas. The possibility of disease ranged from the traveling to areas of endemic (2). However, during tsunami, there was a report indicating that survivors in Takuapa Hospital, Phangnga developed pneumonia caused by infection of *B. pseudomallei* (42). After that, there was a comparison of 6 patients with tsunami-related melioidosis with the individuals who acquired melioidosis in northeastern Thailand. The patients in the northeastern Thailand have a more severe form of disease and a higher rate of complications and death (42) and could possibly be related to the fact that bacteria concentration in soil specimens is greater in the northeastern (41). Thus, the risk of melioidosis for tsunami survivors will depend on the presence and concentration of *B. pseudomallei* in the soil combined with extensive of aspiration and wound inoculation (42).

1.4 Transmission

In northeastern Thailand (23) and northern Australia (25), 75 and 85 % of cases occurred respectively during rainy season. In humans, infection is usually caused by direct contact with muddy soil, surface water in endemic area or from percutaneous inoculation of *B. pseudomallei* (44-46). Inhalation or ingestion of contaminated soil or water can be occurred but less cases. However, there were the cases of transmission from animal to humans and from person to person via sexual transmission (47).

1.5 Treatment

The successful treatment of melioidosis patients is difficult because *B. pseudomallei* is inherently resistant to a variety of antibiotics including beta-lactams, aminoglycosides, macrolides and polymyxins (48, 19). The co-ordination of beta-

lactamase expression contributes to the resistance to ceftazidime and carbapenem. Piperacillin and tazobactam might be an alternative choice of treatment (49).

Ceftazidime is a choice of antibiotic which becomes the antibiotic for initial intensive therapy for melioidosis (50). The use of ceftazidime was associated with a 50% reduction in mortality (51). A combination of ceftazidime with TMP-SXT was also demonstrated to be effective and it appeared that high-dose intravenous ceftazidime with or without TMP-SXT was a choice for severe melioidosis (52).

In addition, Cefoperazone-sulbactam is as effective as ceftazidime for less severe melioidosis. This antibiotic could decrease mortality rates approximately 14-18% after treatment. For maintenance treatment, co-trimoxazole and doxycycline are the oral standard combination regimen and should be administered for a total of 12-20 weeks to limit the incidence of relapse (53, 52). Coamoxiclave can be substituted for used in children and pregnant woman (53). Clinafloxacin is the most active among the fluoroquinolines (49) but its adverse reaction prevents human use (53).

Besides antibiotics, other treatments have been reported to be successful in melioidosis treatment. For example, granulocyte-colony-stimulating factor (G-CSF) was reported to significantly reduce a mortality rate in melioidosis patients from 95% to 10% (54). A recent study demonstrating some protection in animal models following vaccination with *B. thailandensis* and other attenuated strains was demonstrated (55, 56). Another alternative including the use of DNA vaccines against the *fliC* flagellin structural gene and the use of attenuated *B. pseudomallei* mutants is under investigation (57, 2).

1.6 Diagnosis

Diagnosis of melioidosis remains the “gold standard” and requires a positive culture of *B. pseudomallei*. Isolation of *B. pseudomallei* is more difficult in clinical specimens from nonsterile body sites (58). Nowadays Ashdown’s selective agar (ASA) is still the medium of choice for the isolation and presumptive identification of *B. pseudomallei* in endemic area, the bacterium appears as wrinkled circular purple colonies on ASA within 48 h (59). A new selective agar (*Burkholderia pseudomallei* selective agar, BPSA) which provides large wrinkled colonies faster than ASA has also been used in diagnostic laboratories. The colonies of *B. pseudomallei* are more

visible the ability of grow is high in supplementary tests. This is important for public health control strategies that rely on rapid isolation of *B. pseudomallei*. In routine diagnostic laboratory, the commercial API 20NE (98%) or 20E (99%) biochemical kit is reliable in identifying *B. pseudomallei* isolates (60). Futhermore, many other techniques useful in diagnosing a melioidosis include antigen and antibody detection in specimens or culture supernatant, molecular techniques and rapid culture methods (2).

1.6.1 Antigen detection.

Antigen tests have been developed for use on direct specimens or in blood culture supernatant. Latex agglutination for culture identification and direct immunofluoresence have been used for diagnostic (in direct specimens such as sputum, urine, or pus) and research laboratories (2). In endemic area, immunofluoresence from direct specimen is the most promising way to reduce the time of diagnosis. The result from immunofluoresence can be obtained within an hour. A monoclonal antibody latex agglutination test which detects the 200 kDa protein of *B. pseudomallei* is currently the only test widely use in Thailand (2). It was shown to agglutinate blood culture fluid positive for *B. pseudomallei*, including strains with atypical LPS patterns, with a sensitivity of 95%. This test was also highly specific (99.7%) and did not agglutinate *ara*$^{+}$ *B . thailandensis* strains (61).

1.6.2 Antibody detection.

Indirect hemagglutination assay (IHA) is the most widely used method. However, this test has poor sensitivity and specificity (62). In Thailand, the use of the IHA become problematic in areas of endemicity where rates of seropositive background may be up to 30 to 47% in various populations (63). Studies of the clinical performance of IHA are difficult to interpret as a result of different thresholds used for bacteria strains used to formulate the whole-cell antigen which are not strandardized (2). However, there was evident that the sensitivity of IHA is limited in patients with acute septic illnesses (64). Furthermore, there is some heterogenecity among the strains in lipopolysaccharide (LPS) which is a major component in the crude *B. pseudomallei* antigens used in the IHA test. The antibodies against atypical LPS may not cross-react against the IHA antigens prepared from typical LPS. The specificity of this method depends on which organisms used to prepare the IHA assay reagent (65).

Other *B. pseudomallei* specific antigens have been identified and characterized such as 36-kDa exotoxin (18, 66) and 19.5-, 40- (45, 67), 200 kDa protein and LPS (68). However, these antigens have yet to be evaluated for their clinical usefulness in a large- scale field trial (69). ELISA test based on LPS and 36- and 200 kDa proteins have been validated in a clinical context which the antigen was found to be highly specific for the detection of *B. pseudomallei* antibody (2). The results showed that the detection of specific IgG, but not IgM, antibody appears to be more sensitive (74-82%) and specific (75- 80%) than the IHA (69, 70).

1.6.3 Molecular methods

The detection of genetic materials in different clinical specimens has been used successfully for the diagnosis of a large number of infectious diseases of man and animals (71). There are a variety of molecular methods described to detect *B. pseudomallei*. The hybridization is one method that did not have sufficient sensitivity for *B. pseudomallei* infection and is not practical for rapid diagnosis in laboratories in endemic area (72, 73). Additionally, few methods have been tested in the field. Some of them are DNA amplification methods by polymerse chain reaction (PCR) and they were developed for detection of *B. pseudomallei* in clinical specimens (71). The evalution of different sets of primers including the regions in the 23 S rRNA, 16S RNA, the junction between 16S and 23S RNA and specific sequences designed from a specific DNA probe have been studied. Using the different primers, a few of bacteria as a single bacterium presented in the clinical specimens can be successfully amplified and detected (69). The detection of a region of the 16S RNA demonstrated a sensitivity of 100% but low specificity in a small clinical study (74). It has been demonstrated that the nested PCR was highly sensitive. This method could detect two organisms present in the reaction (73). The role of a PCR for the type III secretion system as well as other 16S mRNA-specific primers in clinical and environmental specimens are examined (75). However, a seminested PCR assay was based on the sequences of the ribosomal protein subunit 21 (rpsU) gene 10 and a nested PCR assay targeting the gene encoding the filament forming flagellin (*fli*C) for the identification of *B. pseudomallei* have been developed (76). The result demonstrated that the combination of PCR assays had high sensitivity and specificity for the detection of *B.*

pseudomallei DNA in formalin fixed, paraffin embedded spleen tissues from infected mice (76).

1.7 General characteristics of *B. pseudomallei*

B. pseudomallei is a gram-negative bacillus with bipolar staining (2). The bacterium is a motile, aerobic and non-spore-forming bacterium (1). This microorganism is a soil saprophyte and can be recovered readily from water and wet soil in rice paddy fields in endemic areas. Sequenced genome of this bacterium by the Wellcome Sanger Centre is completely analyzed. The genome, 7.24 Mb, is divided unequally between two chromosomes (4.07 Mb and 3.17 Mb) with a G+C content of 68% (1). This bacterium is distinguished from *Burkholderia mallei* by its motility on a hanging drop but in semisolid media this finding is less reliable (77). On culture, this organism grows aerobically on most agar media and produces clearly visible colonies within 24 h at 37ºC. The colonial morphology is mostly smooth like the cornflower heads and dry or wrinkled colonies on further incubation (2, 1). A closely related but less virulent, *B. thailandensis* , has been identified (78). This bacterium is unable to cause the human disease (79). These findings also were supported in the animal models. Unlike *B. pseudomallei*, *B. thailandensis* is resistant to aminoglycosides but sensitive to tetracycline, ceftazidime and trimetroprim. Ultimately, *B. thailandensis* can grow at temperature ranging from 25 to 42 ºC on culture medium. The colonial morphology of *B. thailandensis* is smooth and glossy with a pink pigmentation (80).

1.8 Virulence factors

Similar to other gram-negative facultative intracellular pathogens, *B. pseudomallei* has evolved mechanisms to survive and proliferate within the host cells for a long period of times (4). The ability of *B. pseudomallei* to expliot a facultatively intracellular existence may be an important property in the pathogenesis of melioidosis (6). Several *B. pseudomallei* pathogenic determinants have been elucidated.

An extracellular polysaccharide capsule -3)-2-*O*-acetyl-6-deoxy-β-D-manno-heptopyranase-(1- has been shown to be an essential virulence determinant in animal model (81). Capsule production by *B. pseudomallei* contributed to reduce activation of the complement cascade, which is an essential components of innate

immunity, by reducing the levels of complement factor C3b deposition and phagocytosis (82). Capsular polysaccharides are often shed during the growth of the organism as well as during complement attack (82, 83). Shedding of the capsule may have a role in preventing a complement attack against invading *B. pseudomallei* (82).

Lipopolysaccharide (LPS) is a major component of the outer membrane of gram-negative bacteria. *B. pseudomallei* LPS expressed two distinct somatic *O*-antigens (PS) on their surface. The type I antigen consists of a high-molecular weight unbranced 1,3-linked homopolymer of 2-*O*-acetylated 6-deoxy-β-D-manno-heptopyranosyl residues, while the type II antigen is an unbranced heteropolymer consists of (-3)-β- D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranosyl-(1-disaccharide repeats (L-6dTal*p*: ~33% *O*-4 acetylated and *O*-2 methylated; ~66% *O*-4 acetylated) (84, 85). Using the infant diabetic rat model, Type II PS is probably a significant determinant in the pathogenesis of melioidosis since the LD_{50} value of a type II PS mutant is approximately 140 fold higher than the wild-type strain (86). The chromosomal region required for the synthesis of the O-polysaccharide of type II PS has been identified and is responsible for the serum resistance phenotype of organism that might play a role in the pathogenesis (86). *B. pseudomallei* LPS differ from the LPS of other gram-negative bacteria. It displays a weaker pyrogenic activity in rodents compared with enterobacterial LPS, but stronger mitogenic activity in murine splenocytes (16). *B. pseudomallei* LPS exhibit weaker and slower of activation of mouse macrophage cell line (87).

Several enzymes produced from *B. pseudomallei* have also been demonstrated as a virulence factor involving in pathogenesis of melioidosis (88, 89). For example, 36 kDa protein which contains proteolytic activity, has been identified (90). This enzyme is characterized as a metallogenic and serine- like protease (91). The serum from melioidosis patients also contain the antibody against these protein. *B. pseudomallei* strain that lacks this protease exhibited significantly less lung damage than the wild-type. These results indicated that 36 kDa protein may contribute to the pathogenesis of melioidosis (92).

Three type III secretion system (TTSS) gene clusters, which include two TTSS clusters (TTSS1 and TTSS2) homologous to the *Ralstonia solanacearum* plant pathogen TTSS (93, 94) and a third TTSS cluster (TTSS3) homologous to the SPI-1

animal pathogenecity island of *Salmonella typhimurium* (95), have been identified in the sequence genome of *B. pseudomallei*. The *B. pseudomallei bsa* (*Burkholdoria* secretion apparatus) (BipB, BipC, and BipD) (96) locus encodes homologues of *Salmonella* Sip translocator proteins (SipB, SipC, and SipD) which are the proteins required for injection of effector proteins and invasion of epithelial cells *in vitro* (97, 98). Similar to *Salmonella*, *B. pseudomallei* also produce BopE protein which is a protein encoded within the *bsa* locus. This protein is secreted via the Bsa apparatus and influences in cytoskeletal rearrangements, thus facilitating host cell invasion (99). Bacteria mutant in the Bsa and BopE are also confined to the endosome leading to the inability of the mutant bacteria to gain access to cell actin. This system also plays a part in mediating endosomal membrane lysis (96). The other putative *bsa* –encoded effector proteins (BopA and BopB) have also been identified (96). BopA is a homologous of the *Shigella* type III secreted protein IscB. This protein mediates cell-to-cell spreading by lysing the double membrane surrounding leading to actin-based protrusions which project the bacteria into adjacent cells (100). BopB is predicted to be encoded at one end of the *bsa* locus by the seventh predicted gene downstream of *bopA*. It contains an amino acid motif (CX_5R) that is conserved in the catalytic domains of numerous phosphatases (90). A recent report suggests a role for the TTSS3 cluster for the survival and persistence of bacteria within a murine macrophage-like cell line and for the disappearance of *B. pseudomallei* from endocytic vacuoles (96). On the other hand, *B. thailandensis* strain does not contain some TTSS (94) which suggests that TTSS may contribute to pathogenesis of melioidosis. However, the TTSS3 mutants in *B. pseudomallei* strains are not significantly attenuated (101, 102). The growth of *B. thailandensis* in the presence of arabinose was determined by microarray. It led to downregulation of TTSS3 via the putative positive regulator *bsa*N. These results suggested that the loss of the ability to assimilate arabinose is linked to the increased virulence of *B. pseudomallei* in humans and animals (103).

Flagella are commonly recognized as important virulence determinants expressed in several pathogenic bacteria. They are believed to play a role in the ability of the bacteria to disseminate from sites of localized infection to other organs of the body via the blood circulatory system (104, 105). Transposon mutagenesis of *B. pseudomallei* has the genes which involves in motility. *FliC* is one of gene which

encodes flagellin (104). The *fliC* gene and the flagellin protein were detected in 108 *B. pseudomallei* isolates obtained from human patients, animals, and soil (106). In animal models, (107) the antibodies raised against the *B. pseudomallei* flagellin markedly reduced the motility of the bacterium and provided passive protection against *B. pseudomallei* infection. The role of flagella as virulence determinants in *B. pseudomallei* was investigated. In BALB/c mice that were infected intranasally with *B. pseudomallei fliC* mutant, the LD_{50} was significantly higher comparing with wild-type *B. pseudomallei* indicating that flagella contribute to pathogenecity of *B. pseudomallei* (106, 108, 109).

Quarum sensing (QS), a cell-density-dependent communication system utilized by many gram-negative bacteria, has been identified in *B. pseudomallei*. These factors are involved in both positive and negative regulation of putative virulence factors (110). Recent report demonstrated that *B. pseudomallei*-QS is also involved in the *in vivo* pathogenicity of *B. pseudomallei* (110). In addition, RpoS (σ^s), a global regulatory factor known to mediate the stationary-phase expression of a large number of genes, is shown to resist a stress condition and prolonged nutrient deprivation of *B. pseudomallei* (111, 112). In *Salmonella typhimurium* , the RpoS has been reported to control the expression of *Salmonella* plasmid virulence (*spv*) genes, which are required for systemic infection (113-115). *S. typhimurium rpoS* mutant showed significantly reduced virulence in animal model (116). The possible involvements of RpoS in the pathogenecity of *B. pseudomallei* have been reported. *B. pseudomallei* lacking *rpoS* failed to induce MNGC formation suggesting that the RpoS of *B. pseudomallei* was involved in this process. In the presence of IFN-γ, the level of iNOS in the *rpoS* mutant-infected mouse macrophages was markedly increase compared with the wild-type. These results demonstrated that RpoS of *B. pseudomallei* may be involved in the regulation of iNOS expression in the IFN-γ activated macrophages (117).

Adherence of bacteria mediated by carbohydrate molecules, pilus, and nonpilus, is also considered to be an important virulence factor (118). Type IV pili (TFP) has been demonstrated to be an essential virulence factor in many gram-negative bacteria and are divided into two subclasses, IVA and IVB, based on the presence of conserved motifs (119). Current report (118) describes the identification of multiple TFP-associated loci in *B. pseudomallei* and shows that a TFP gene

homologue is required for efficient adherence of *B. pseudomallei* to cultured cells and for virulence *in vivo*. Deletion of *pilA* which encodes type IVA of *B. pseudomallei* results in the reduction of *B. pseudomallei* adherence to human epithelial cells comparing with the wild-type (118).

1.9 Intracellular survival of *B. pseudomallei*

B. pseudomallei can survive and multiply in both phagocytic and non-phagocytic cells (4). The exact mechanism of this bacteria to evade the macrophage killing are poorly understood. Recent report suggests that this organism can invade macrophages without substantially activating inducible nitric oxide synthase (iNOS), which plays an essential role in the killing of intracellular bacteria (7). The inability of *B. pseudomallei*-infected macrophages to activate iNOS expression may be related to the fact that the infected macrophage failed to stimulate high level of IFN-β production. This cytokine is a key cytokine that regulates iNOS expression by activating the expression of interferon regulatory factor-1 (IRF-1), a transcriptional activator required for iNOS gene transcription (8). However, exogenous IFN-γ or IFN-β could enhance and restore its ability to kill intracellular *B. pseudomallei* by activating iNOS expression (7, 8, 120).

1.10 Modulation of cell cytoskeletal by *B. pseudomallei*

After invasion of host cells, *B. pseudomallei* is able to escape from endocytic vacuoles including lysing the endosomal membrane (121) and utilizes the power of actin polymerization to propel itself within host cells (6, 122). Intracellular actin-based motility is believed to underlie the ability of *B. pseudomallei* to spread from cell-to-cell and promote multinucleated giant cell formation (MNGC) (6). The actin-based intracellular motility of intracellular pathogens is limited to a few members of the bacteria including *Shigella, Shigella, Listeria* and *Rickettsia* (123). The intracellular motility of these organisms is strictly coupled to a polarized actin polymerization process at one pole of the bacterium leading to the formation of actin comet or rocket tail (124). Although recent report demonstrated that *B. pseudomallei* can induce actin rearrangement initiated at one pole of the bacterium, leading to actin tail formation and actin-associated peripheral membrane protrusions (6), the molecular

mechanism of the actin polymerization by *B. pseudomallei* is largely unknown. Recent report that the Arp2/3 complex of seven polypeptides, which are normally located in areas of dynamic actin assembly in the cell, is localized throughout actin tails formed by this bacterium (125) suggests that Arp2/3 complex may contribute to actin-based motility (123). The recent report showing that the ability of *B. pseudomallei* to induce the formation of actin tails require BimA, a protein that contains proline-rich motifs and WH-2-like domains. This protein shares homology at the C terminus with the *Yersenia* type V secreted adhesion YadA. Mutation of *bimA* in *B. pseudomallei* abolished actin-based motility of the pathogen in a macrophage-like cell line J774.2 (122). Actin tail formation could be restored by inducible expression of the *bimA* gene on plasmid, indicating the essential role for this gene in actin tail formation (122).

2. Toll-like receptor (TLR)

Toll-like receptor (TLR) is a pattern recognition receptor which is important in innate immune system against the pathogenic microorganisms (9). At least 11 members of TLR family in humans have been identified and 13 can be found in searches of the mouse genome (10). TLRs 1-9 are conserved between both species (126). The different TLRs recognize the specific microbial components derived from pathogens, including bacteria, fungi, protozoa and viruses and then activatecytokine production. With the exception of TLR3, TLR7 and TLR9 that are expressed in the endosome, all TLRs are expressed on the cell surface (Figure 1).

2.1 Structure of TLR

The discovery of the TLR family began with the identification of Toll receptor expressed in insects and found to be essential for establishing dorsoventral polarity during embryogenesis (127). The TLRs are type I integral membrane glycoproteins and are divided into two regions, the extracellular and the cytoplasmic region. The cytoplasmic portion of TLRs shows high similarity to the IL-1 receptor family and is termed a Toll/IL-1 receptor (TIR) domain. In contrast, the extracellular portions of these receptors are structurally unrelated (9). The extracellular region of

TLRs cantains leucine-rich repeat (LRR) motifs whereas the IL-1 receptors possess three immunoglobulin-like domains (Figure 2) (10).

2.2 TIR-domain containing adaptors

TLR signaling pathway is mediated through adaptor proteins. Four adaptor proteins including myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor protein (TIRAP) or MyD88 adaptor-like (Mal), TIR domain-containing adaptor inducing interferon (IFN)-β (TRIF) and TRIF-related adaptor molecule (TRAM) have been well characterized (128). The characterization of TIRAP has established the essential roles of these adaptors in TLR signaling. MyD88 is an essential for most TLR. The signaling through MyD88 leads to the activation of inflammatory cytokines production (e.g. IL-6 and TNF-α). However, stimulation of TLR3 or TLR4 could also result in the induction of type I IFNs in a MyD88-independent manner. In addition, TIRAP involves in both TLR2 and TLR4 mediated activation of the MyD88-dependent pathway except for TRAM and TRIF which are the specific adaptor in MyD88-independent pathway of TLR4 (Figure 3) (10). The fifth TIR domain-containing protein called SARM (Sterile alpha and HEAT/Armadillo motif protein) has been recently identified (128). This adaptor is encoded an ortholog of *Drosophilla* and *C. elegans* proteins but its role in TLR signaling is currently unclear (128).

2.2.1 MyD88

After activation, MyD88 is recruited to the C-terminal TIR domain of the TLR which lead to mediation downstream signaling through recruitment of IL-1R-associated kinase (IRAK) to its N-terminal “death domain” (DD) (129, 130). Activation of TLR through MyD88 resulting in the production of inflammatory cytokines e.g. TNF-α and IL-6. In MyD88 KO mice, the animals were insensitive to LPS-induced death and failed to secrete proinflammatory cytokines such as IL-6, TNF-α, IL-1 (130).

2.2.2 TIRAP/Mal

TIRAP or Mal was identified in the second group of TLR adaptor. It possesses a C-terminal TIR domain but lacking the N-terminal DD. The role of TIRAP was revealed by generating KO mice. The production of inflammatory cytokines in

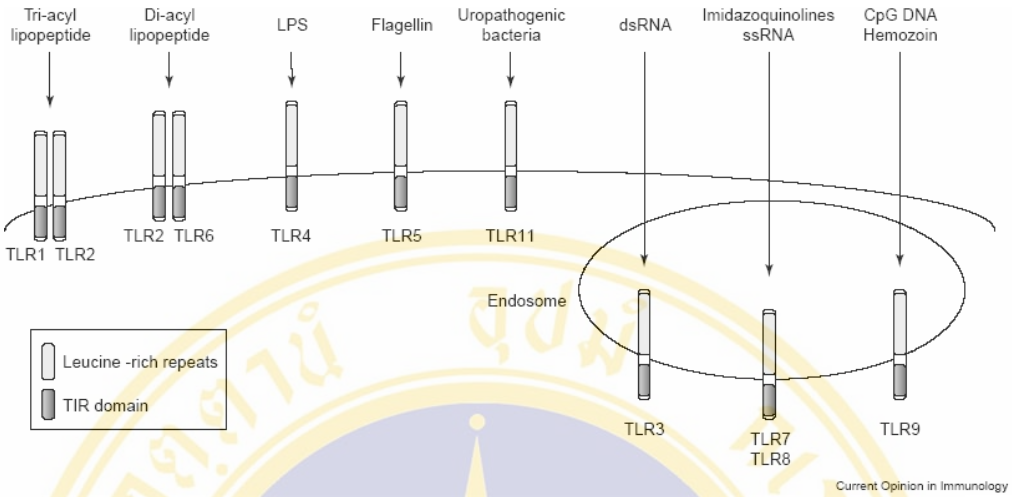

Figure 1. A schematic representation of ligands for Toll-like receptors. TLR2 is essential in the recognition of lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate differences between triacyl and diacyl lipopeptides, respectively. TLR3 is essential in the recognition of viral dsRNA. TLR4 is the receptor for LPS. TLR5 recognizes flagellin. TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR9 is essential in CpG DNA recognition (131).

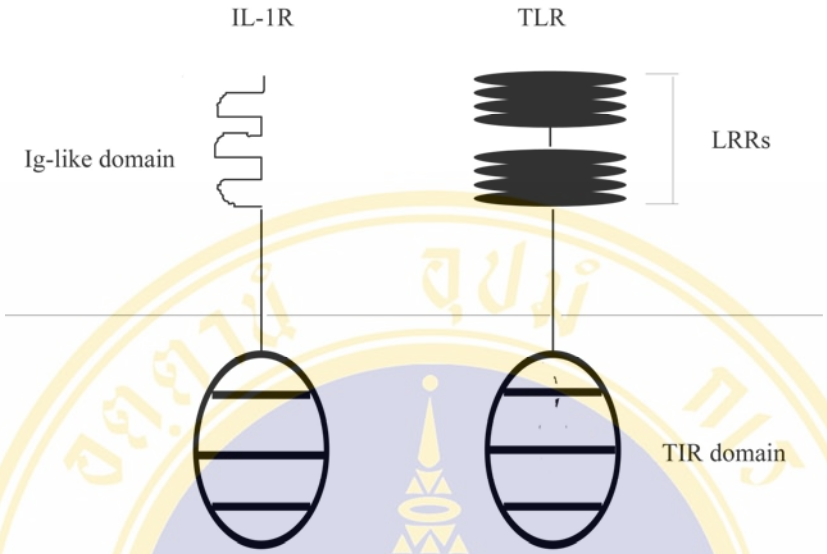

Figure 2. TLR structure. Toll-like receptors (TLRs) and interleukin-1 receptors (IL-1Rs) have a conserved cytoplasmic domain or Toll/IL-1R (TIR) domain. They can be divided into two domains, the extracellular domain and the cytoplasmic domain. Both receptors have similar TIR domain, that is characterized by the presence of three highly homologous region. Despite the similarity of the cytoplasmic domains of these molecules, their extracellular domains differ. The extracellular region of TLRs cantains leucine-rich repeat (LRR) motifs, whereas the IL-1 receptors possess three immunoglobulin-like domains.

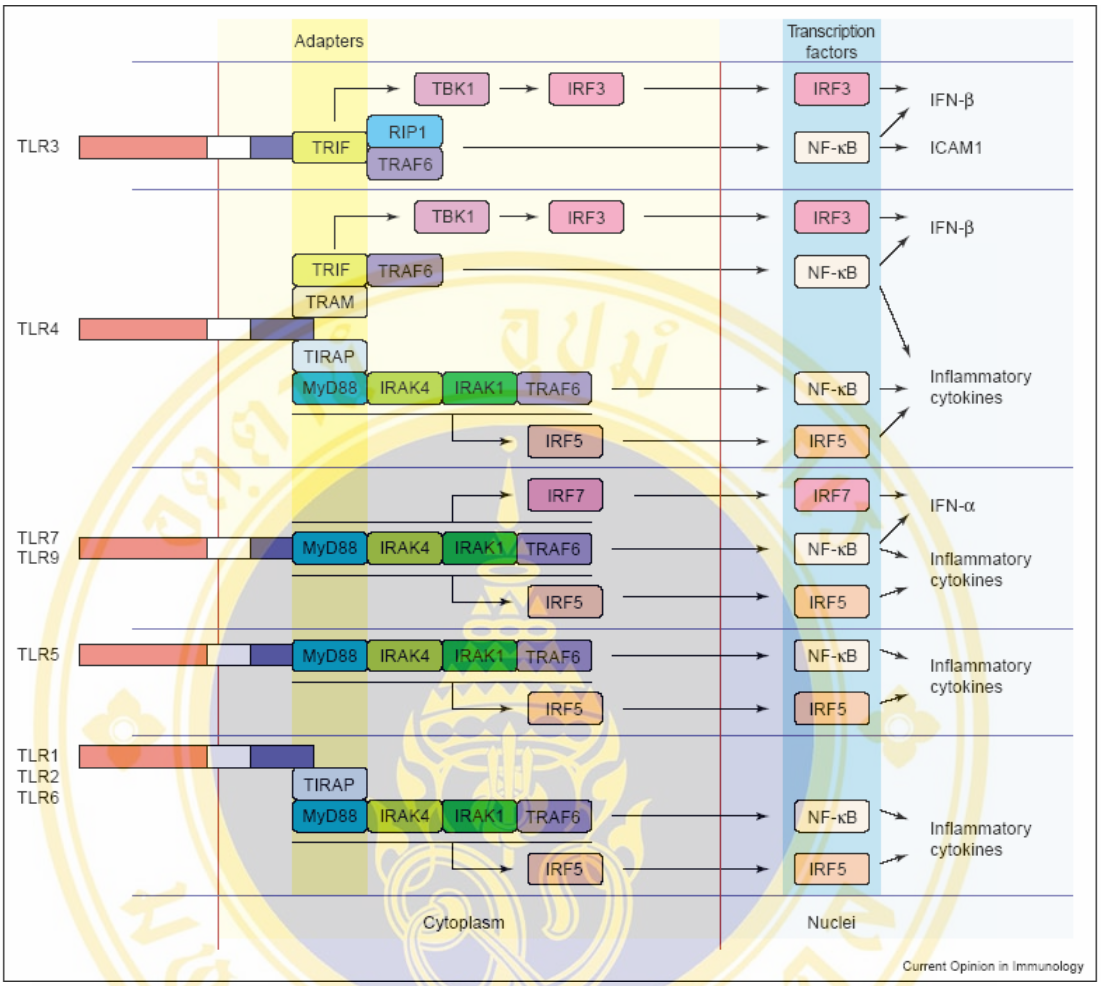

Figure 3. A schematic diagram of TLR signaling pathways (131).

response to LPS was found to be defective in TIRAP-deficient mice but the expression of IFN-inducible genes and the delayed activation of NF-κB was still observed (132, 133). These indicated that TIRAP is essential for the MyD88-dependent but not the MyD88-independent pathway of TLR4 (10).

In responses to other TLR ligands such as double stranded RNA (for TLR3), flagellin (for TLR5), R-848 (for TLR7) and CpG DNA (for TLR9), were indistinguishable between wild-type and TIRAP-deficient macrophages which suggested that TIRAP is not essential for these TLRs (134). Nevertheless, the response to TLR2 ligands (peptidoglycan and MALP-2), proinflammatory cytokines were produced from wild-type cell in a dose-dependent manner and TIRAP-deficient cells showed severely impaired production in response to the stimulation. The results demonstrated that TIRAP plays an important role in the MyD88-dependent immune response which shared by TLR2 and TLR4 (134).

2.2.3 TRIF

The analysis of TIRAP-deficient mice suggested that other molecules were responsible for the MyD88-independent pathway downstream of TLR3 or TLR4 that leaded to the identification of a third TIR domain-containing adaptor,TRIF (133). TRIF is larger than either MyD88 or TIRAP/Mal, which have 296 and 232 amino acids respectively. Overexpression of TRIF also results in NF-κB activation. Unlike MyD88 or TIRAP/Mal, overexpresion of TRIF leads to the strong expression of an IFN-β gene (133, 135). These results showed that TRIF is a unique and potent activator of the IFN-β promoter. The physiological role of TRIF was subsequently revealed by the targeted deletion of *Trif* in mice. TRIF-deficient mice showed both impaired activation of IFN-regulatory factor 3 (IRF3) and decreased expression of IFN-inducible genes in response to TLR3 and TLR4 ligands (136). The results suggested that TRIF was an essential component for TLR3 and TLR4 to activate MyD88-independent pathway which subsequently led to the IFN-β production. Besides, TRIF-deficient mice showed defective production of IFN-β and IFN-inducible gene products in the response to TLR4 ligand but not to other TLR ligands.On the other hands, TLR4-mediated activation of the MyD88-dependent manner was not impaired (133, 137). These findings indicate that TLR4 requires both MyD88-dependent and MyD88-independent/TRIF-dependent signals to induce the

expression of inflammatory cytokines. On the other hand, the activation of MyD88-dependent pathway alone is sufficient to induce inflammatory cytokine production in response to the other TLR ligands including TLR2, TLR5, TLR7 and TLR9 which cannot activate the MyD88-independent/TRIF-dependent pathway (10).

2.2.4 TRAM

A fourth TIR domain-containing adaptor, TRAM has 235 amino acid in length and shares sequence similarity to TRIF. In both humans and mouse, TRAM contains a cysteine at the position 117 of amino acid when other adaptors and TLRs have a conserved proline. The current studies indicate that TRAM associates with TRIF in TLR4 not TLR3. The inhibition of TRAM expression by siRNA demonstrated its important role in induction of IFN-β and IFN-inducible genes through TLR4 (138, 139). The essential role of TRAM in the MyD88-independent cascade of TLR4-induced signal was also demonstrated in TRAM-deficient mice. In response to TLR4 ligands, TRAM-deficient mice showed impaired activation of IRF3 and reduced expression of IFN-inducible genes. TRAM-deficient mice showed a normal response to TLR3 stimulation (140), indicating that TRAM is involved specifically in the only activation of MyD88-independent/TRIF-dependent signaling pathway through TLR4. In addition, TRAM-deficient mice are also defective in their production of inflammatory cytokines in response to LPS, in spite of the fact that the activation of NF-κB in early phase is normal (140). This indicates that TRAM and TRIF are involved in the TLR4 mediated induction of inflammatory cytokines.

2.3 Signaling pathways of TLR in response to cytokine production

The characterization of TIR domain-containing adaptors has established the essential roles of these adaptors in TLR signaling (9). The intracellular signaling of the TLRs can be divided into two major pathways as (1) MyD88-dependent and MyD88-independent pathway. These two signaling pathways regulate the various cytokine productions.

2.3.1 MyD88-dependent signaling

MyD88-dependent pathway is analogous to signaling pathways through the IL-1 receptor. MyD88 possesses a C-terminal TIR domain and N-terminal death domain. It associates with the cytoplasmic TIR domain of TLRs (11). After recruited

to the TIR domain, MyD88 recruites members of IL-1R-associated kinase (IRAK) family (IRAK-1 and IRAK-4). Then MyD88 recruits IRAK-4 to TLRs and facilitates IRAK-4-mediated phosphorylation of IRAK-1. Activated IRAK-1 then associates with adaptor tumour necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) leading to the activation of two distinct signaling pathways (11). One pathway leads to an activation of AP-1 transcription factors via an activation of MAP kinases. Another pathway activates the TAK1/TAB complex, which in turn promotes downstream activation of the IκB kinases (IKK), IKKα and IKKβ. Once activated, the IKK complex directly phosphorylate and subsequently degrade the inhibitory IκB family which lead to NF-κB translocated into the nucleus and turn on the gene responsible for inflammatory cytokine production (141).

2.3.2 MyD88-independent signaling

With the exception of TLR3, all other TLR pathways use MyD88-dependent signaling pathway to activate NF-κB. However, the TLR4 ligand, LPS, can trigger activation of NF-κB in MyD88-deficient mice with delayed kinetics compared with wild-type cells. On the other hand, this signaling in most other TLRs is completely ineffective to activate NF-κB in MyD88-deficient mice (142). In contrast to the MyD88 dependent pathway, the MyD88-independent pathway induces expression of IFN-inducible genes, such as IP-10, glucocorticoid-attentuated response gene 16 (GARG16), immunoresponsive gene 1 (IRG1) through an activation of the transcription factor, IFN regulatory factor 3 (IRF3) and co-stimulatory molecules and their dendritic cells (DCs) mature in response to LPS (10, 143, 144, 12).

2.4 TLR family members

2.4.1 TLR1, TLR2 and TLR6

TLR2 recognizes a broad range of microbial products including peptidoglycan from Gram-positive bacteria, bacterial lipoproteins, mycobacterial cell-wall lipoarabinomannan, glycosylphosphatidylinositol lipid from *Trypanosoma cruzi* , a phenol-soluble modulin produced by *Staphylococcus epidermidis*, and yeast cell walls (11). In addition, TLR2 recognizes LPS from non-enterobacteria such as *Leptospira interrogans*, *Porphyromonas gingivalis* and *Helicobacter pyroli* (145-147). These LPS structures are different from LPS of other bacteria Gram-negative

recognized by TLR4 in the number of acyl chains in the lipid A component (148). There are two possible aspects to explain the mechanisms by which TLR2 recognizes a broad range of microbial components explain. Firstly, TLR2 forms heterodimer with either TLR1 or TLR6 which have structural related to that of the TLR2 (149, 150). The report indicated that macrophages from TLR6-deficient mice could not activate production of inflammatory cytokines in response to mycoplasma-derived diacyl lipopeptides. These cells could activate normal production of inflammation cytokines in response to triacyl lipopeptides derived from Gram-negative bacteria (150). In contrast, macrophages from TLR1-deficient mice could activate normal response to mycoplasma-derived diacyl lipopeptides, but not to triacyl lipopeptides (151). Secondly,TLR2 could collaborate with distinct types of receptors such as dectin-1, a lectin family receptor for the fungal cell wall component β-glucan (152). Thus, a wide range of microbial products could activate TLR2 cooperated with several other proteins (11).

2.4.2 TLR3

TLR3-deficient mice decreased the production of type I IFN in response to viral RNA and polyinosinic-polycytidylic acid (poly (I:C)) responses to type I IFN production. This result suggested that TLR3 is involved in the recognition of dsRNA (153).

2.4.3 TLR4

TLR4 is an essential receptor for LPS recognition (154, 155). Recognition of LPS by TLR4 is complicated and requires several accessory molecules such as LBP, CD14 and MD-2 (156-158). In addition, TLR4 is involved in the recognition of taxol, a diterpene purified from the bark of the western yew (*Taxus brevifolia*) (159, 160) and endogenous ligands such as heat shock proteins (HSP60 and HSP70), extra domain A of fibronectins, oligosaccharides of hyaluronic acid, heparin sulfate and fibrinogen.Anyway, all of these endogenous ligands require very high concentrations to activate this TLR (11).

2.4.4 TLR5

TLR5 recognized a conserved domain of bacterial flagellin from both Gram-positive and Gram-negative bacteria. In contrast to other TLR, TLR5 is

expressed on the basolateral, not the apical side of intestinal epithetlial cells (161). Thus, it recognized the flagellin from pathogenic bacteria e.g. *Salmonella* (161).

2.4.5 TLR7 and TLR8

TLR7 and TLR8 are highly conserved proteins which recognizes the same ligand in some cases. In details, TLR7 recognizes synthetic compounds, imidazoquinoline, which are clinically used for treatment of genital warts associated with viral infection (162). Murine TLR7 can recognize another synthetic compound such as loxoribine, an anti-viral and anti-tumor activities (163, 164). Imidazoquinoline and loxoribine have similar structure related to guanosine nucleoside. The recent report shows that TLR7 and human TLR8 recognize guanosine or uridine-rich single-stranded RNA (ssRNA) from viruses such as human immunodeficiency virus, vesicular stomatitis virus and influenza virus (165-168).

2.4.6 TLR9

TLR9 is a receptor for CpG DNA (168). Bacterial DNA contains unmethylated CpG motif which confer its immunostimulatory activity. At least two types of CpG DNA (A/D-type CpG DNA and B/K-type CpG DNA) were identified to induce inflammatory cytokines. B/K-type CpG DNA is a potent inducer of inflammatory cytokines such as IL-12 and TNF-α, A/D-type CpG DNA has a greater ability to induce IFN-α from PDC but less ability to induce IL-12 (169, 170). Recently, TLR9 recognition of A/D-type CpG DNA leads to the induction of an anti-viral cytokine IFN-α in PDC. TLR9 is also involved in viral recognition (171, 172). In addition, TLR9 is presumably involved in pathogenesis of autoimmune diseases (11).

2.4.7 TLR11

TLR11 has been shown to be expressed in bladder epithelial cells and mediate resistance to infection by uropathogenic bacteria in mouse (173). The study in TLR11-deficient mice indicates that they are highly susceptible to uropathogenic bacterial infection. However, the ligand of TLR11 has not yet been identified. And TCR in human TLR11 has not been identified (173), suggesting that the human TLR11 protein was futile in the human environment and became lost through evolution (11).

3. Cytokines

Cytokines is essential in the communication between cells of multicellular organisms (174). They regulate the survival, proliferation, differentiation, and function of immune cells. Cytokines, including interleukins, interferons and hemopoietins, can induce cell signaling by activation of the Janus kinases leading to phosphorylation of signal transducer molecule such as signal transducer and activator of transcription (STAT) family (174).

3.1 Interleukin-6 (IL-6)

IL-6 is a cytokine that functions in both innate and adaptive immunity (176). It is synthesized by a number of cell types including fibroblast, macrophages, dendritic cells, T and B lymphocytes, endothelial cells, glia cells and keratinocytes (166). IL-6 has several diverse functions. In innate immunity, it stimulates the synthesis of acute phase response proteins by hepatocytes and thus contributes to the systemic effects of inflammation (176). In adaptive immunity, IL-6 stimulates terminal differentiation of B cells to antibody producing plasma cells, differentiation of monocytes to macrophages, and growth of hematopoetic stem cells (166). IL-6 uses the common receptor subunit gp130 for signal transduction (175). After IL-6 bind to the surface IL-6 receptor (IL-6R)α, gp 130 is dimerized and formed the receptor complex before activating two intracellular signaling pathways included JAK-STAT pathway and the CCAAT/enhancer binding protein (C/EBP) pathway (177, 178).

3.2 Tumor necrosis factor-alpha (TNF-α)

TNF-α is the principal mediator of the acute inflammatory response to gram-negative bacteria and other infectious microbes and it is responsible for many of the systemic complications of severe infections. TNF-α was originally identified as a substance present in the serum of animals treated with bacterial endotoxin that caused the necrosis of tumors *in vivo* (176). TNF-α is mainly expressed by activating macrophages, NK-cells, T-cells, fibroblasts, astrocytes, Kupffer cells, smooth-muscle cells, keratinocytes and tumor cells (179). The signal of TNF transduces through two distinct receptors including TNF-R1 and TNF-R2 which expressed only on immune and endothelial cells (108, 181). Cytokine binding to TNF receptor leads to

recruitment and initiate signaling cascades resulting in the activation of effector proteins (e.g. caspases) and protein kinases (MAPK, IKK) followed by the activation of transcription factors (AP-1, NF-κB) (179).

3.3 Type I interferons (IFNs)

Type I interferons consist of seven classes including IFN-α, IFN-β, IFN-ε, IFN-κ, IFN-ω, IFN-δ and IFN-τ (182). These cytokines particularly IFN-α and IFN-β are the first line of defence against viral infection (183). IFN-α and IFN-β are produced by several cell types including macrophages, fibroblasts, NK cells, T cells, dendritic cels and plasmacytoid monocytes (184-188). However, macrophages are the major cellular source of IFN-α/β in the immune system (189). Several reports demonstrated a low-level constitutive expression of IFN-α/β in resting macrophages (184, 190), their production are upregulated upon infection with viruses, stimulation with dsRNA or exposure to microbial pathogens or microbial products (191-194). IFN-α/β are a key autocrine and paracrine immunoregulatory cytokines. Futhermore, autocrine IFN-α/β was essential in the induction of iNOS by LPS stimulation (195). Expression of IFN-β gene is rapidly induced by TLR4 activation in an MyD88-independent manner (196). Binding of these cytokines to cell surface receptors generate an intracellular signal through the JAKs. Activated JAKs phosphorylate STAT-1 which then translocate to nucleus and regulate cytokine-dependent transcription (197). Besides viral infection, type I IFN is also involved in antibacterial activity by regulating iNOS expression in infected macrophages.

CHAPTER III
MATERIALS AND METHODS

1. Cell culture

Mouse macrophage cell line (RAW264.7), obtained from American Type Culture Collection (ATCC, Rockville MD), was used through our the study. Unless indicated otherwise, cells were cultured and maintained in 25 or 75-cm^2 flask (Costar, Cambridge, MA) with Advanced Dulbecco's modified Eagle's medium (ADMEM) (Gibco Labs, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) and 1% L-glutamine (Gibco Labs) (see Appendix). The cultures were incubated at 37 °C under 5% CO_2 atmosphere and subcultured at 80-90% confluency before being detached from plastic surface by using cell scraper. The cell suspension was then centrifuged at 1000 rpm for 5 min and the packed cells were resuspended in 1 ml of the medium. Thereafter, the cell suspension was transferred to fresh medium and cultured as described.

2. Bacterial strains

B. pseudomallei strain 844 was originally isolated from a patient admitted at Srinagarind Hospital in the melioidosis endemic Khon Kaen province of Thailand. This bacterium was originally identified as *B. pseudomallei* based on its biological characteristics, colonial morphology on selective media, antibiotic sensitivity profiles and reactivity with polyclonal and monoclonal antibodies (198, 16, 79). *Salmonella enterica* serovar Typhi (*S. typhi*), a prototype of intracellular bacteria used for comparison was originally isolated from a patient admitted at Ramathibodi Hospital (Mahidol University, Bangkok Thailand). All bacterial strains were routinely subcultured from stock and kept in 20% glycerol at -70 °C. For used in the experiments, they were cultured in Trypticase soy broth (TSB) (see Appendix) at 37 °C with shaking 150 rpm. The overnight cultures were washed twice in phosphate-

buffered saline (PBS) pH 7.0 (see Appendix) and adjusted to a desired concentration (10^8 CFU/ml) by measurement of the optical density at 650 nm and estimated from the calibrated standard curve.

3. Preparation heat-killed bacteria

Heat-killed bacteria were prepared by heating the bacteria (10^8 colony forming unit (CFU)/ml) in the boiling water bath at 100 °C for 15 min. The heat-killed bacteria were washed 3 times with PBS and then resuspended in 1ml PBS before kept at -20 °C as stock. The complete killing was ascertained by colony counting after culturing in tryptic soy agar (TSA) (see Appendix) for 48 h. For used in the experiment, they were centrifuged at 15,000 rpm for 5 min before resuspended in 1 ml of the medium.

4. Preparation of LPS

LPS was extracted from *B. pseudomallei* by the modified phenol-chloroform-petroleum ether method (16) and characterized by SDS-PAGE and immunoblotting (198). The LPS from *Escherichia coli* strain 0111:B4 (Sigma, St Louis, MO) was used as reference. The carbohydrate content of these LPSs was determined by the orcinol-sulphuric acid method using glucose as standard (199).

5. Infection of mouse macrophage cell line (RAW 264.7)

An overnight culture of mouse macrophages (10^6 cells) in a 6-well plate was co-cultured with bacteria at a multiplicity of infection (MOI) of 2:1 for 1 h. To remove extracellular bacteria, the cells were washed twice with 1 ml of PBS and residual bacteria were killed by incubating in ADMEM containing 250 μg/ml kanamycin (Gibco Labs) for 2 h. Thereafter, the infection was allowed to continue in the medium containing 20 μg/ml of kanamycin until the experiment was terminated (6, 7).

6. Blocking mouse macrophage cell line (RAW 264.7) by using neutralizing antibody against TLR4

Mouse macrophages (10^6 cells) were pre-incubated with different concentrations of anti-mouse Toll-like receptor 4 (TLR4)/MD-2 (e-bioscience, San Diego, CA) for 2

h at 37 °C. The cells were then exposed to *B. pseudomallei* LPS (100 ng/ml) or *E. coli* LPS (10 ng/ml) for 8 h. The level of secreted IL-6 and TNF-α in the supernatant were determined by ELISA.

7. Intracellular survival

To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed as previously described (6). The intracellular bacteria were liberated by lysing the macrophages with 0.1% Triton X-100 and the released bacteria were plated on tryptic soy agar. The number of intracellular bacteria, expressed as CFU, was determined by bacterial colony counting.

8. Reverse transcription- polymerase chain reaction (RT-PCR) assay

8.1 Total cellular RNA extraction

Total RNA was extracted from the infected cells according to the manufacturer's instruction (Eppendorf, Hamburg). In brief, the infected cells were washed twice with 1 ml PBS and lyzed directly in the culture well with 350 µl of lysis buffer and then transferred to a sterile 1.5 ml microcentrifuge tube followed by centrifugation at 14,000 rpm for 5 min. The supernatant was mixed with 350 µl of 70% ethanol and the mixture was gentle mixed before adding 200 µl of Perfect RNA Binding Matrix solution. The mixture was transferred to a Perfect RNA spin column and centrifuged at 14,000 rpm for 30 sec. The supernatant was discarded and the pellet was washed with wash solution I before being centrifuged at 14,000 rpm for 30 sec. After removing wash solution I, the spin column was transferred to a fresh collection tube before wash solution II was added twice and centrifuged at 14,000 rpm for 15 sec. The spin column was transferred to a new collection tube before 50 µl of molecular biological grade water was added. The samples were incubated at 50 °C for 5 min, vortex for 5 sec and centrifuged at 14,000 rpm for 2 min. Total RNA concentration was determined by using photometrically at 260/280 nm (Multiskan Spectrum, ThermoLabsystems, Helsinki).

8.2 First-strand cDNA synthesis

First-strand cDNA was synthesized from the mRNA by using iScript^{TM}cDNA synthesis kit (Biorad, Hercules, CA). According to manufacturer's protocol, 0.5 µg of total RNA was submitted to 20 µl cDNA synthesis. One reaction contained 4 µl of 5X iScript reaction mix, 1 µl of iScript reverse transcriptase, RNA template 0.5 µg and nuclease-free water to make final volume 20 µl. Thereafter, the reaction mixture was incubated at 25 °C for 5 min , 42 ˚C for 30 min and 85 ºC for 5 min. The reaction mixture was then kept at -20 °C until used.

8. 3 Polymerase-chain reaction (PCR)

The PCR reaction was performed in a 25 µl total volume using 10X PCR buffer (Invitrogen, Carlsbad, CA), 20 µM of specific primer, 5U/µl *Thermus aquaticus* (*Taq*) DNA polymerase (Invitrogen, Carlsbad, CA) and DNase-RNase free water (Gibco Labs). The nucleotide primers specific for all genes of interested and their expected size used in the PCR amplification are shown in Table 1. The TGradiant thermalcycler 96 (Biometra, Goettingen) was used for PCR amplification. The condition for all PCR amplification are shown in Table 2.

8.4 Detection of PCR products

All amplified products were electrophoresed in 1.5% or 4% agarose gel (see appendix) and stained with ethidium bromide (Plusone, Amersham Pharmacia Biotech). The gels were electrophoresed in 1X TBE buffer (see appendix) at a constant voltage (90 volts) for 60 min. Quantitative analysis of PCR band was done by using Gel Documentation (GeneSystem).

9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

9.1 Preparation of separating gel

Electrophoresis was performed on 8x10x0.1 cm gel. A 5 ml of a 8 % acrylamide solution in 3.0 M gel buffer pH 8.8 containing 1% sodium dodecyl sulfate (SDS) (Plusone, Uppsala, Sweden), 0.05% (v/v) N, N, N', N'-tetrametyhlethylene diamine (TEMED) (Plusone) and 0.05% ammonium persulfate (APS) (Plusone) were carefully added and allowed to polymerize at RT for at least 1 h. The gel prepared as described could be kept at 4 °C with distilled water covering the top surface if the

Table 1 : Primer specifications for RT-PCR.

Gene	Primer	Sequence (5'→3')	Size (bps)	(Refs)
IL-6	Forward	ggt ctc tgg gaa atc gtg ga	452	-
	Reverse	gct gac cct aga gca tcc tg		
IκB-ζ	Forward	tgt tgc ctt ctc act tcg tg	301	-
	Reverse	tgg tcc atc atc tgt gga ga		
IRG1	Forward	ggt atc att cgg agg agc aa	416	-
	Reverse	aca gag gga ggg tgg aat ct		
IFN-β	Forward	tcc aag aaa gga cga aca ttc g	312	(196)
	Reverse	tga gga cat ctc cca cgt caa		
iNOS	Forward	gca gaa tgt gac cat cat gg	414	-
	Reverse	aca acc ttg gtg ttg aag gc		
TNF-α	Forward	gta gcc cac gtc gta gca aa	350	-
	Reverse	ccc ttc tcc agc tgg gag ac		
SOCS1	Forward	cac ctt ctt ggt gcg cg	65	(200)
	Reverse	aag cca tct tca cgc tga gc		
SOCS3	Forward	atg gtc acc cac agc aag tt	140	(201)
	Reverse	aat ccg ctc tcc tgc agc tt		
β –actin	Forward	cca gag caa gag agg tat cc	436	(202)
	Reverse	ctg tgg tgg tga agc tgt ag		

Table 2: PCR condition

Gene	Condition
IL-6, IκBζ, TNF-α, SOCS3 and *β –actin,*	94 °C for 5 min for one cycle, 94 °C for 45 sec, 58 °C for 1 min and 72 °C for 1 min for 30 cycles (202)
IRG1	94 °C for 5 min for one cycle, 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 2 min for 30 cycles
IFN-β	94 °C for 5 min for one cycle, 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec for 30 cycles (196)
iNOS	94 °C for 5 min for one cycle, 94 °C for 45 sec, 55 °C for 30 sec and 72 °C for 45 sec for 30 cycles
SOCS1	94 °C for 5 min for one cycle, 94 °C for 45 sec, 55 °C for 1 min and 72 °C for 1 min for 30 cycles (200)

electrophoresis was not performed immediately. The comb was gently inserted on the top of the gel to make well for sample application. In either situation, 5 ml of 30% acrylamide in 0.5 M gel buffer pH 6.8 (see appendix) to be used as stacking gel was layered over the separating gel 30 min before used. After polymerization, the comb was carefully removed, samples then electrophoresed immediately.

9.2 Preparation of samples

Cell samples were centrifugation at 10,000 rpm. Supernatant was discharged and the pellet was solubilized in lysis buffer (see appendix) containing 0.0625 M Tris-HCl (USB), pH 6.8, 1% SDS, 10% glycerol (Sigma), 5% 2-mercaptoethanol (2-ME) (BDH) and bromophenol blue. The solution was sonicated on ice for 1 min before heated 5 min in the heat box. The samples were centrifuged at 10,000 rpm, 5 min, 4 °C and the supernatant was subjected to electrophoresis.

9.3 Electrophoresis

The electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, CA) was prepared with cathode at the top and the anode at the bottom chambers. Electrophoresis was carried out at the RT with a constant current of 20 mA per gel in descending direction until the blue dye marker reached the bottom of the gel, approximately 1 h to complete this process.

10. Immunobloting

After electrophoresis, the gels were electrotransferred to nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, England) at a constant voltage (80 volts) for 3 h. The membrane was blocked with 5% blocking solution (Roche Diagnostics, Mannheim, Germany) for 1 h before incubating at 4 °C overnight rabbit anti-mouse iNOS antibody (Santa Cruz, Santa Cruz, CA) in 10% blocking solution. The concentrations of each antibody was used as manufacturer's recommendation. The membranes were washed 3 times with 0.1% Tween20 in PBS for 10 min. After that, the blots were then allowed to react with horseradish peroxidase (HRP)-conjugated swine anti-rabbit IgG (Pierce, Rockford, IL) or HRP-conjugated goat anti-rabbit IgG (Pierce). Protein bands were detected by enhanced chemiluminescence as

recommended by the manufacturer (Roche Diagnostics) which exposed to hyperfilm (Amersham Biosciences).

11. Enzyme- linked immunosorbent assay (ELISA)

The level of cytokines were determined by using ELISA assays. The culture supernatant obtained from the stimulated or unstimulated cell cultures were kept at -80 °C until used.

11.1 ELISA for mouse IL-6

IL-6 in the supernatant was measured by using BD OptEIA™ Mouse IL-6 ELISA Set (BD Biosciences, San Diego, CA). The 96-well plates (NUNC™ Brand Products, Roskilde, DK) were coated with 100 µl of anti-mouse IL-6 antibody (diluted 1:250 in coating buffer) (see appendix) and incubated overnight at 4°C. The plates were washed 3 times with washing buffer (PBS pH 7.0 supplemented with 0.05% Tween-20) (see appendix), then 200 µl of assay diluent (PBS supplemented with 10% FBS) (see appendix) was added to each well and incubated at RT for 1 h. Plates were then washed 3 times with washing buffer and 100 µl of samples or recombinant mouse IL-6 standard (diluted in assay diluent) including negative control were added in duplicate. The plates were incubated for 2 h at RT before washed 5 times with washing buffer and 100 µl of working detector (biotinylated anti-mouse IL-6 and avidin-horseradish peroxidase conjugate diluted 1:250 in assay diluent) was added to each well. After incubating at RT for 1 h, the plates were washed 7 times with washing buffer and 100 µl of substrate solution was subsequently added to each well. The plates were then incubated for 30 min at RT in dark. The reaction was stopped by addition 50 µl of stop solution (see appendix). The intensity of color was determined immediately by measurement with microplate reader (Softmax) at absorbance 450 nm. A standard curve was prepared for each microtiter plate and used to quantify the amount of IL-6 present in test samples.

11.2 ELISA for mouse TNF-α

The production of TNF- **α** in culture supernatant was analyzed using commercially available ELISA kit (R&D systems, Inc., Minneapolis, MN). The captured antibody (mouse TNF- **α**) was diluted to a working concentration of 0.8 µg/ml in PBS (see Appendix). The 96-well plates were coated with 100 µl of captured

antibody at RT overnight. The plates were then washed 6 times with washing buffer (see Appendix), blocked with 300 µl of Reagent Diluent to each well (see Appendix) and incubated further at RT for 1 h. The samples were then aspirated and washed 6 times with washing buffer. The samples or standards (100 µl) were added and incubated for 2 h at RT before washed 6 times with washing buffer. The one-hundred µl of detection antibody (at concentration of 75 ng/ml) was added to each well and incubated 2 h at RT before washing 6 times with washing buffer and 100 µl of Streptavidin –HRP (diluted 1:200 in reagent diluent) was added to each well. The plates were incubated for 20 min at RT in dark followed by washing 6 times. The substrate solution (100 µl) (see Appendix) was then added to the samples and incubated 20 min at RT in dark. The reaction was terminated by adding stop solution (50 µl) (see Appendix) into the samples. The intensity of color was determined immediately using microplate reader (Softmax) at absorbance 450 nm. A standard curve was prepared for each microtiter plate and used to quantify the amount of TNF-α present in testing samples.

12. Statistical analysis

All statistics reported were performed using SigmaPlot 8.0 (SPSS). The results were expressed as the mean ± standard deviation (s.d.) from three experiment, each run in duplicate.

CHAPTER IV
RESULTS

1. *B. pseudomallei* activates IκBζ, IL-6 and TNF-α gene expression in mouse macrophage

To investigate the expression of IκBζ, IL-6 and TNF-α mRNA, mouse macrophages were infected with bacteria at MOI of 2:1 as described in Materials and Methods. At different time intervals, the expression of IκBζ, IL-6 and TNF-α mRNA from *B. pseudomallei*-infected mouse macrophages were determined by RT-PCR. The result in Figure 4A showed that *B. pseudomallei* was able to rapidly activate mRNA expression of IκBζ within 15 min before peaking at 30 min after infection. As shown in Figure 4B, *B. pseudomallei* induced the expression of IL-6 and TNF-α mRNA at 30 min. The mRNA expression of IκBζ, IL-6 and TNF-α in mouse macrophages infected with *S. typhi* was also used for comparison. Similar results were observed as shown in Figure 5A and 5B.

2. Heat-killed *B. pseudomallei* induces the expression of IκBζ, IL-6 and TNF-α mRNA in mouse macrophage

To further determine whether IκBζ, IL-6 and TNF-α gene expression depend on the viability of bacteria, the IκBζ, IL-6 and TNF-α mRNA expression from the macrophages exposed to heat-killed *B. pseudomallei* were analyzed. The macrophages were treated with heat-killed bacteria at concentration similar to MOI of 10:1. At 0.5, 1, 2 and 4 h after activation, the treated cells were collected for RNA extraction before subjected to RT-PCR. Results presented in Figure 6 showed that heat-killed *B. pseudomallei* could also activate the IκBζ, IL-6 and TNF-α mRNA expression with similar profiles as living *B. pseudomallei*.

A

B

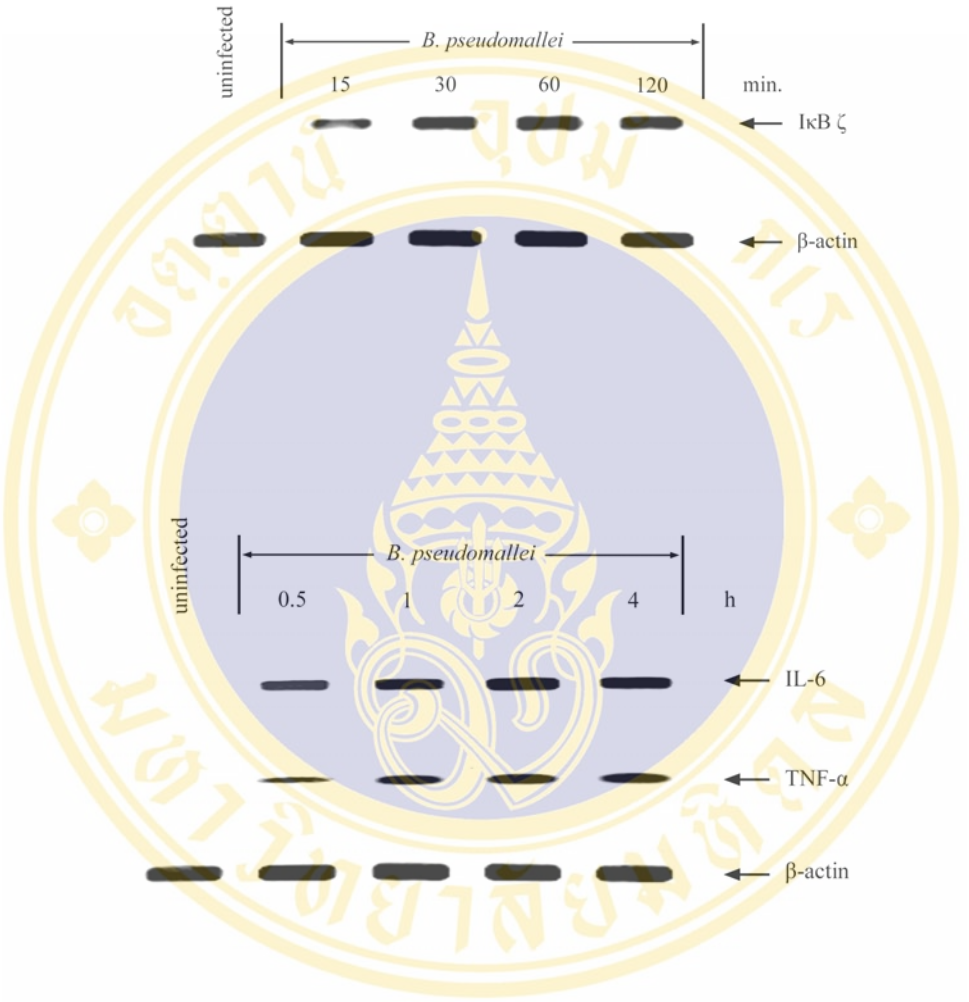

Figure 4. Kinetics of IκBζ, IL-6 and TNF-α mRNA expression in *B. pseudomallei*-infected mouse macrophages (RAW264.7). The macrophages were infected with the bacteria at MOI of 2:1. At various time intervals, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

A

B

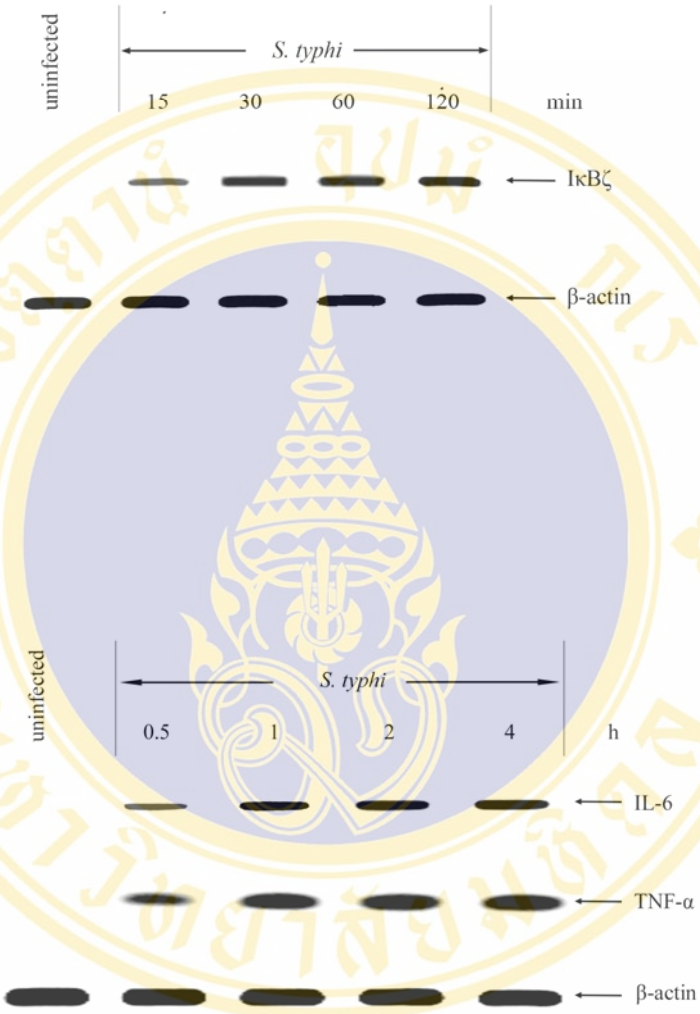

Figure 5. Kinetics of IκBζ, IL-6 and TNF-α mRNA expression in *S. typhi*-infected mouse macrophages (RAW264.7). The macrophages were infected with the bacteria at MOI of 2:1. At different time intervals, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

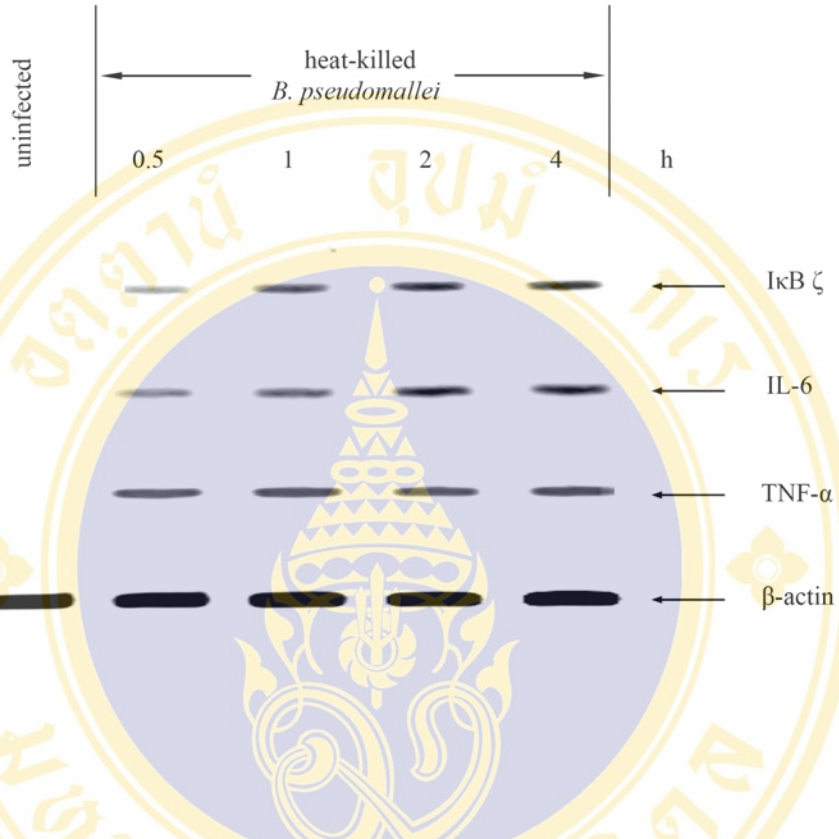

Figure 6. Heat-killed *B. pseudomallei* induce IκBζ, IL-6 and TNF-α mRNA expression in mouse macrophages. The macrophages were exposed to heat- killed *B. pseudomallei* at concentration similar to MOI of 10:1. At 0.5, 1, 2 and 4 h after infection, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

3. Kinetics of IL-6 and TNF-α production from mouse macrophages activated by *B. pseudomallei* and *S. typhi*

To determine the ability of *B. pseudomallei* to activate the cytokine secretion, mouse macrophages were infected with *B. pseudomallei* or *S. typhi* at MOI of 2:1. At 2, 4, 6, and 8 h after infection, the levels of IL-6 and TNF-α in supernatant were determined by ELISA as described in Materials and Methods. For comparison, *S. typhi* was used as a positive control. As shown in Figure 7 and 8, the levels of IL-6 and TNF-α produced by the *B. pseudomallei*-infected macrophages were only slightly higher than those produced by the uninfected macrophages. In contrast, the levels of IL-6 and TNF-α in cells infected with *S. typhi* were markedly higher than those of *B. pseudomallei*-infected macrophages throughout the time of observation. It should be noted that the level of IL-6 and TNF-α production was not directly correlated with the expression of the IL-6 and TNF-α gene in *B. pseudomallei*-infected macrophages.

4. Activation of IL-6 production by heat-killed *B. pseudomallei* is dose dependent

To further investigate the production of IL-6 activated by heat-killed bacteria, mouse macrophages were treated with heat-killed *B. pseudomallei* or *S. typhi* at concentration equivalent to MOIs of 1:1, 10:1 and 100:1. Eight hours after exposure, the supernatant was collected and then the level of IL-6 was determined. As shown in Figure 9, heat-killed *B. pseudomallei* was able to activate only low level of IL-6 even when MOI as high as 100:1 was used. In contrast, *S. typhi* was able to stimulate high level of IL-6 even at MOI of 10:1.

5. *B. pseudomallei* directly activates the gene expression through MyD88-dependent pathway in mouse macrophages

Mouse macrophages were either treated with or without resveratrol, an inhibitor of MyD88-independent pathway before stimulating with either *B. pseudomallei* or *S. typhi* (for comparison) at MOI of 2:1 as described in Materials and Methods. Four hours after infection, the expression of genes known to be activated through MyD88-dependent pathway (IκBζ, IL-6, TNF-α and SOCS3 (201) and MyD88-

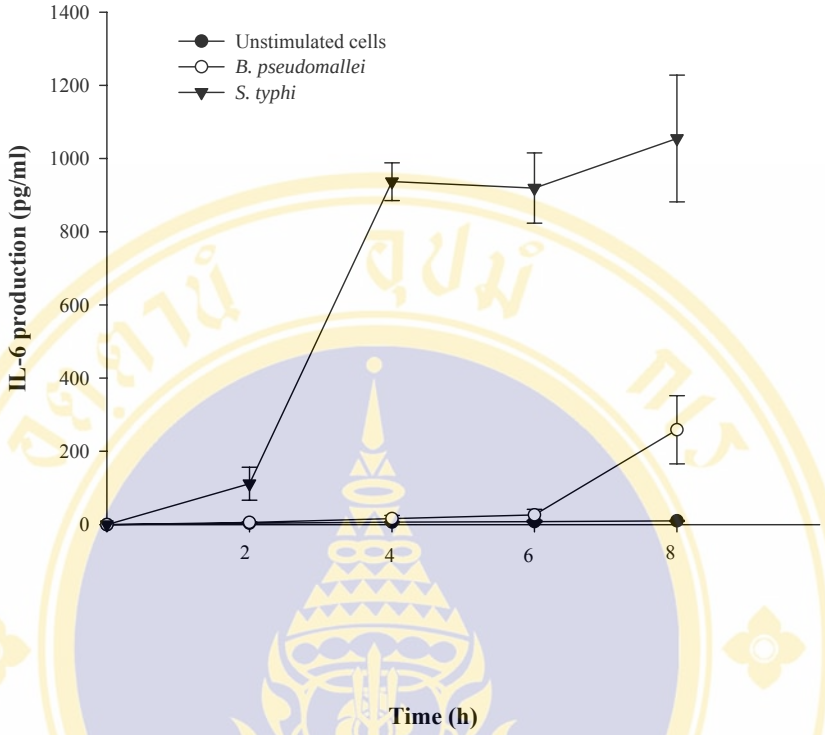

Figure 7. Kinetics of IL-6 production from mouse macrophage cell line (RAW 264.7). The mouse macrophages were infected with *B. pseudomallei* or *S. typhi* at MOI of 2:1. At 2, 4, 6, and 8 h after infection, the level of IL-6 in supernatant was analyzed by ELISA. Data represent the means and standard deviations from three separate experiments with duplicate samples.

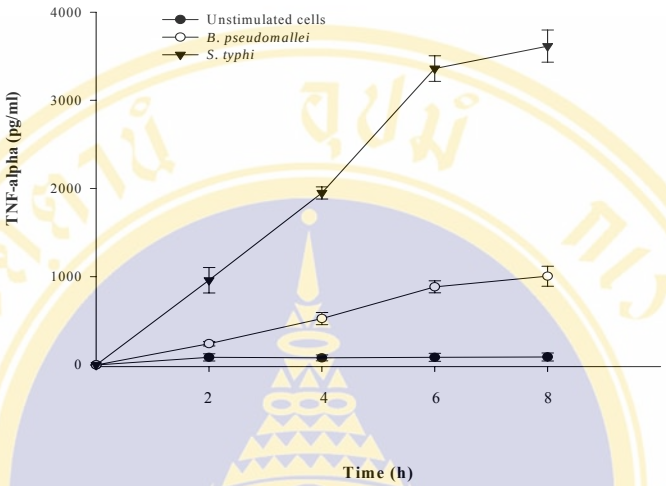

Figure 8. Kinetics of TNF-α production from mouse macrophage cell line (RAW 264.7). The mouse macrophages were infected with *B. pseudomallei* or *S. typhi* at MOI of 2:1. At 2, 4, 6, and 8 h after infection, the level of TNF-α in supernatant was analyzed by ELISA. Data represent the means and standard deviations from three separate experiments with duplicate samples.

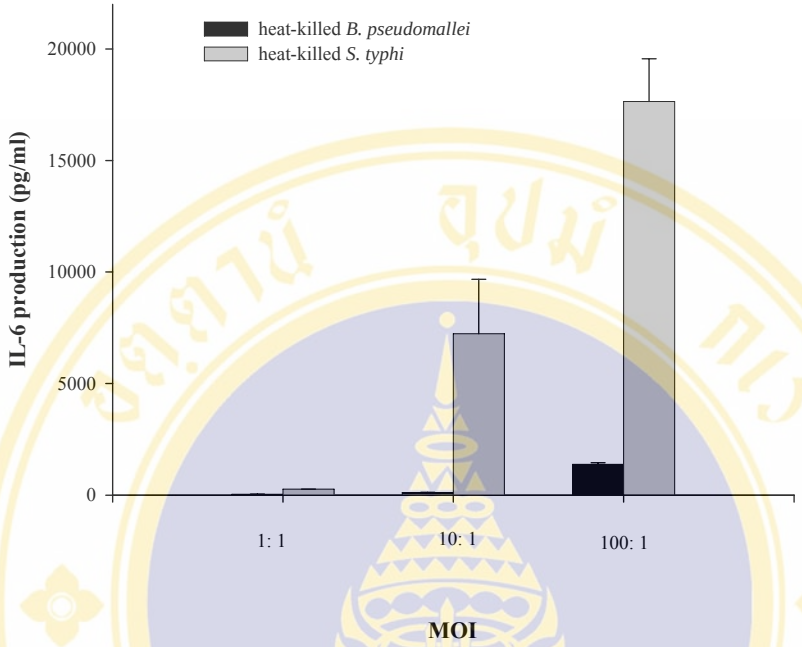

Figure 9. Production of IL-6 by heat-killed bacteria-treated macrophages. Mouse macrophages were treated with heat-killed *B. pseudomallei* or *S. typhi* at concentration similar to MOIs of 1:1, 10:1 and 100:1 for 8 h. The secreted IL-6 in supernatant was analyzed by ELISA. Data shown represent means and standard errors of 2 separate experiments, each carried out in duplicate.

independent pathway (iNOS, IFN-β, IRG1 and SOCS1 (200)) were determined by RT-PCR. *B. pseudomallei* was able to stimulate gene expression of MyD88-dependent pathway but failed to activate gene expression of MyD88-independent pathway (Figures 10, 11). As to be expected resveratrol was unable to interfere with gene expression of MyD88-dependent pathway. For *S. typhi* which could activate genes expression through both MyD88-dependent and MyD88-independent pathways, resveratrol inhibited iNOS, IFN-β, IRG1 and SOCS1, thus confirming that expression of these genes by *S. typhi* depends on MyD88-independent pathway. In order to confirm that resveratrol at concentration used was able to inhibit MyD88-independent, the protein expression of iNOS (used as a representative of MyD88-independent) was determined in *S. typhi*- infected macrophages. As shown in Figure 12, in the presence of resveratrol, the level of iNOS protein expression was markedly decreased indicating that resveratrol at concentration used was able to inhibit MyD88-independent pathway.

6. The inhibition of MyD88-independent pathway by resveratrol does not interfere intracellular survival of *B. pseudomallei*

In order to determine if the inhibition of MyD88-independent pathway by resveratrol would interfere with the ability of *B. pseudomallei* to survive inside the macrophages, the macrophages were pretreated with the inhibitor for 1h before infected with *B. pseudomallei*. Eight hours after the bacterial challenge, the number of intracellular *B. pseudomallei* was determined. As shown in Figure 13, the number of intracellular bacteria in the presence or absence of resveratrol was not significantly different. This result suggested that MyD88-independent pathway plays only a minor role, if any, in the controlling of intracellular survival and replication of *B. pseudomallei*.

7. Expression of IκBζ, IL-6 and TNF-α in *B. pseudomallei*-infected mouse macrophages does not require bacterial internalization

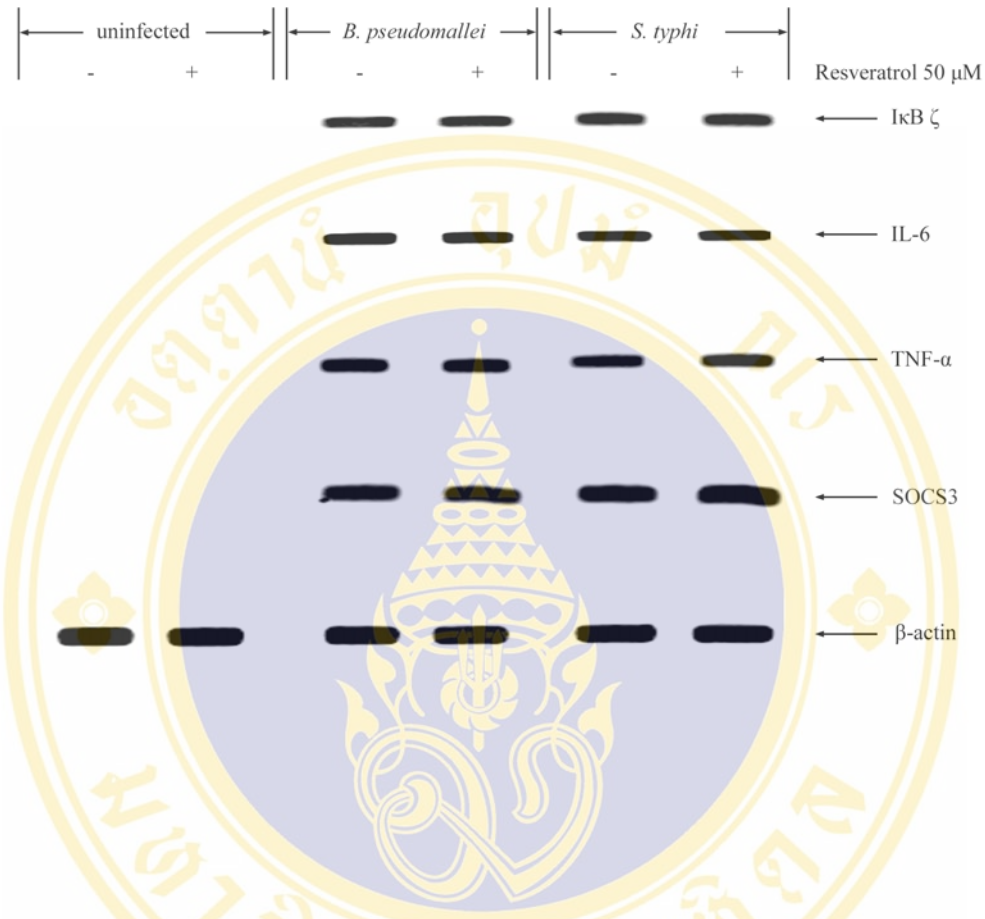

Figure 10. Expression of mRNAs via MyD88-dependent pathway in the bacterial infected macrophages. Mouse macrophages were treated with or without resveratrol (50μM) for 1 h before exposed with *B. pseudomallei* or *S. typhi* at MOI of 2:1. After 4 h of infection, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

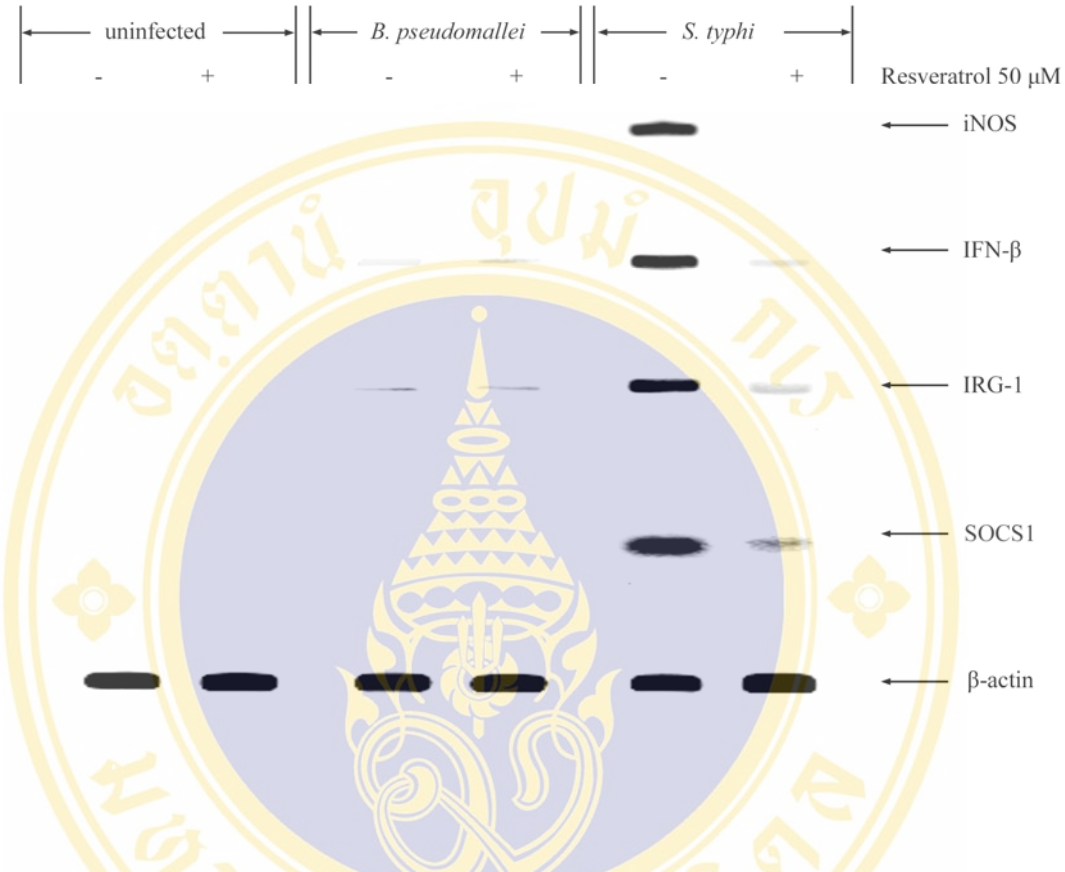

Figure 11. Expression of mRNAs via MyD88-independent pathway in bacterial infected macrophages. Mouse macrophages were treated with or without resveratrol (50μM) for 1 h before exposed with *B. pseudomallei* or *S. typhi* at MOI of 2:1. After 4 h of infection, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

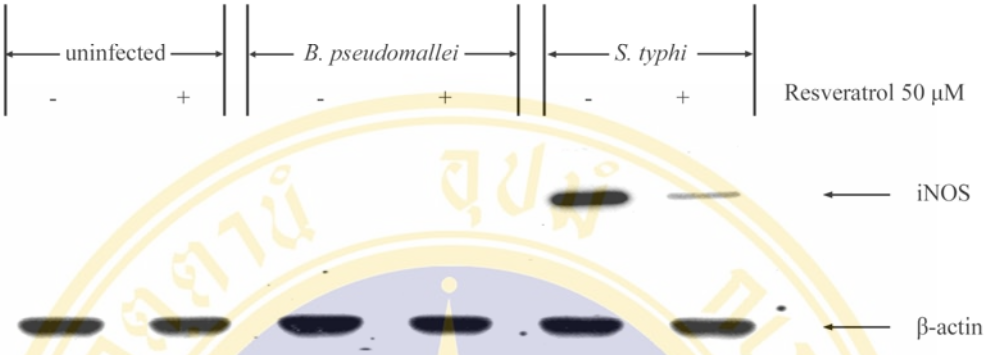

Figure 12. Ability of resveratrol to suppress iNOS protein in *S. typhi*-infected mouse macrophages. Mouse macrophages were pretreated with resveratrol (50µM) for 1 h before exposed with either *B. pseudomallei* or *S. typhi* at MOI 2:1. After 8 h of infection, cell lysates were analyzed for iNOS and β-actin immunoblots.

To further investigate whether internalization of bacteria is required to trigger IκBζ, IL-6, TNF-α and SOCS3 (201) expression, the mouse macrophages were pretreated with cytochalasin D (2 μg/ml) for 2 h before exposure to *B. pseudomallei*. Four hours after infection, the genes were determined by RT-PCR. IκBζ, IL-6 and TNF-α genes were activated in *B. pseudomallei*-infected mouse macrophages in both presence or absence of cytochalasin D (Figure 14) suggested that activation of these genes did not require internalization. In contrast, SOCS3 was decreased in the presence of cytochalacin D suggesting that the expression of SOCS3 by macrophages required internalization of *B. pseudomallei*. In order to prove that cytochalasin D at concentration used was able to inhibit internalization, the number of intracellular *B. pseudomallei* was determined by standard antibiotic protection assay. Results in presented Figure 15 showed that the number of intracellular bacteria was significantly decreased in the presence of cytochalasin D.

8. Neutralizing antibody against TLR4 fails to inhibit IL-6 and TNF-α production by *B. pseudomallei* LPS-activated mouse macrophages

Mouse macrophages were pretreated with antibody against TLR4 for 2 h before exposing to *B. pseudomallei* LPS (100 ng/ml). At 8 h of activation, the production of IL-6 and TNF-α in the supernatant were determined by ELISA kit. For comparison, *E. coli* LPS (10 ng/ml) was used as a positive TLR4 agonist control. As shown in Figures 16 and 17, neutralizing antibody against TLR4 was able to significantly reduce IL-6 and TNF-α production of *E. coli* LPS-activated macrophages. In contrast, TLR4 antibody failed to prevent IL-6 and TNF-α production from *B. pseudomallei* LPS. The results suggested that *B. pseudomallei* LPS did not activate macrophages through TLR4.

9. Ability of IFN-γ to enhance genes expression through MyD88-independent pathway in *B. pseudomallei*-infected mouse macrophages

To determine whether IFN-γ could facilitate *B. pseudomallei*-infected mouse macrophages to activate the gene expression through MyD88-independent

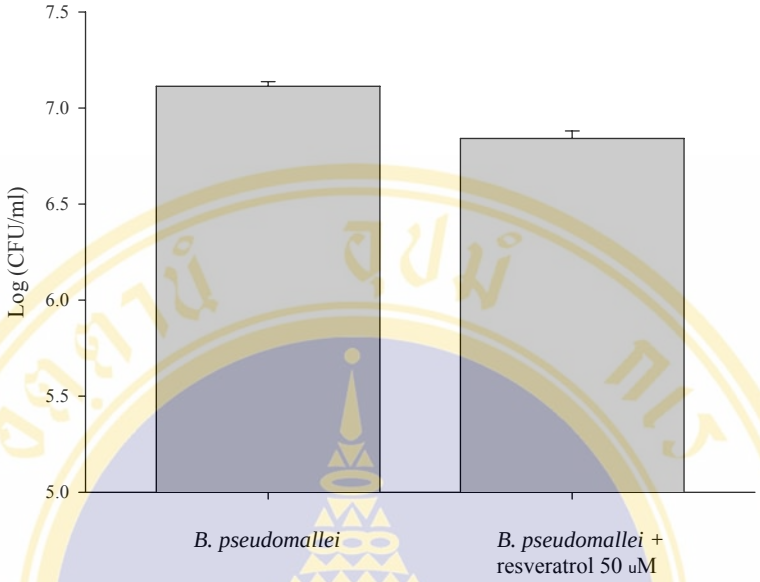

Figure 13. Resveratrol does not interfere with intracellular survival of *B. pseudomallei* inside the mouse macrophages. Mouse macrophages were treated with resveratrol (50μM) for 1 h before infecting with *B. pseudomallei* at MOI of 2:1. At 8 h after infection, the cells were lyzed with 1 ml 0.1% TritonX-100 and released bacteria were plated on TSA. The number of intracellular survival of bacteria was determined by bacterial colony counting. The data represent the mean and s.d. of 3 separate experiments, each carried out in duplicate.

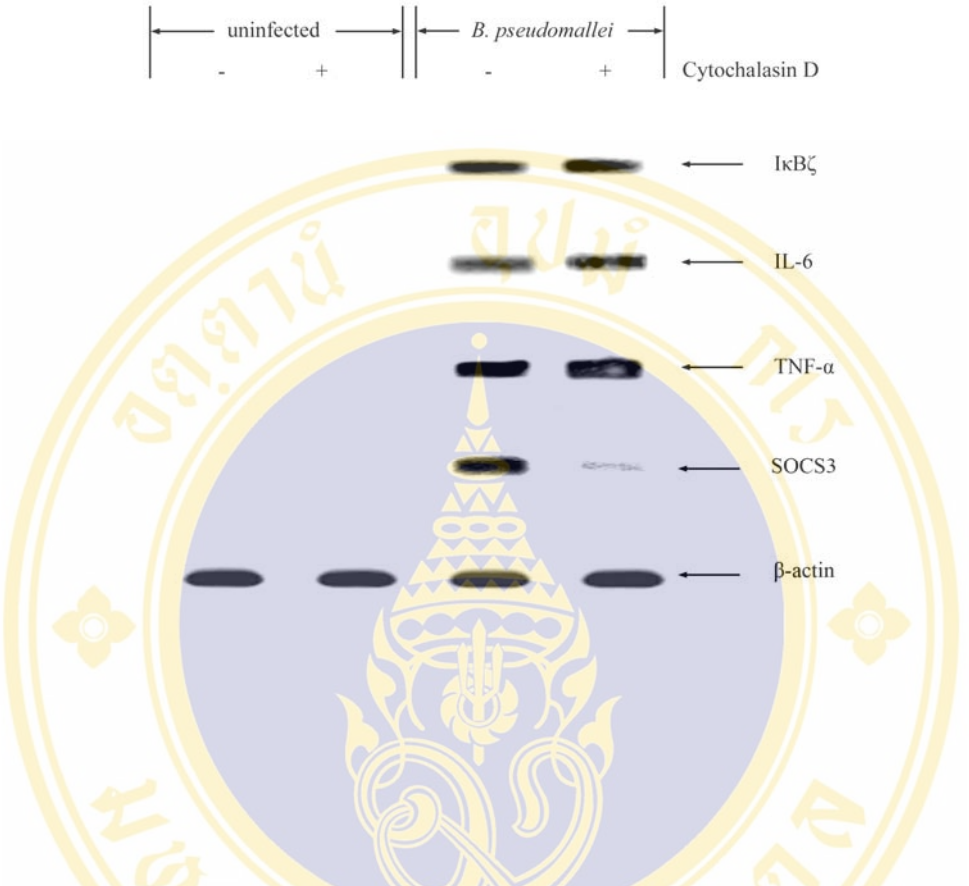

Figure 14. Expression of various mRNA in mouse macrophages does not require invasion of *B. pseudomallei*. Cytochalasin D (2 μg/ml) was added for the macrophage culture 2 h prior to the time of infection. The macrophage-treated culture was then infected with *B. pseudomallei* at MOI of 2:1. After 4 h, the cells were harvested and total mRNA was extracted and subjected to RT-PCR assay. β-actin mRNA expression served as an internal control.

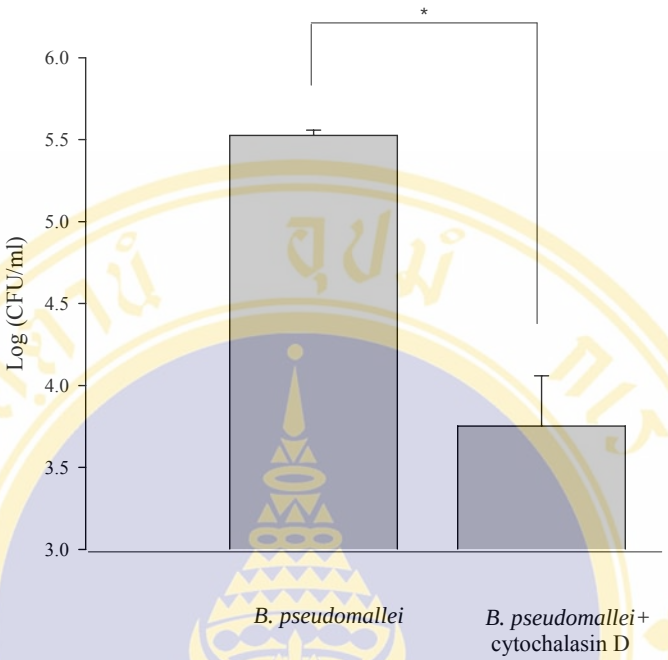

Figure 15. Invasion of *B. pseudomallei*-infected mouse macrophages inhibited by cytochalasin D. Cytochalasin D (2μg/ml) was added for 2 h in culture cells before exposed with *B. pseudomallei* at MOI of 2:1. Two hours later, the infected cells were lyzed with 1ml of 0.1% Triton X-100 and the release cells were plated on TSA. The number of intracellular bacteria was determined by bacterial colony counting. The data represent the mean and s.d. of 3 separate experiments, each carried out in duplicate. * $P<0.01$ by Student's *t*-test.

pathway,the mouse macrophages were pretreated with IFN-γ (10U/ml) overnight and then treated with resveratrol (50μM) for 1 h before exposure to *B. pseudomallei* at MOI of 2:1. After 4 h of infection, iNOS, IFN-β, IRG1 and SOCS1 genes expression were determined by RT-PCR. As shown in Figure 18, IFN-γ alone was able to activate low level of iNOS, IFN-β, IRG1 and SOCS1 expression. However, IFN-γ synergistically enhanced expression of these genes in *B. pseudomallei*-infected macrophages. The results suggested that IFN-γ was able to facilitate ability of *B. pseudomallei*-infected mouse macrophages to induce the genes dependent on MyD88-independent pathway. However, in this condition resveratrol was unable to inhibit the gene expression of MyD88-independent pathway in *B. pseudomallei*-infected macrophages that were pretreated with IFN-γ.

10. Inability of resveratrol to suppress the intracellular *B. pseudomallei* killing in mouse macrophages pretreated with IFN-γ

To determine whether or not resveratrol was able to interfere the intracellular survival in mouse macrophages pretreated with IFN-γ, the mouse macrophages were pretreated with IFN-γ (10U/ml) overnight. Then the mouse macrophages were treated with resveratrol (50μM) for 1 h before exposing to *B. pseudomallei* at MOI of 2:1. Eight hours after infection, the intracellular *B. pseudomallei* was determined by standard antibiotic protection assay. As shown in Figure 19, the number of intracellular bacteria was not significantly decreased in the combination of resveratrol and IFN-γ when compared with IFN-γ alone. This result suggested that resveratrol, at the concentration used, was not able to interfere intracellular survival of *B. pseudomallei*. These results also agree with the inability of resveratrol to inhibit gene expression of MyD88-independent pathway (as shown in Figure 18).

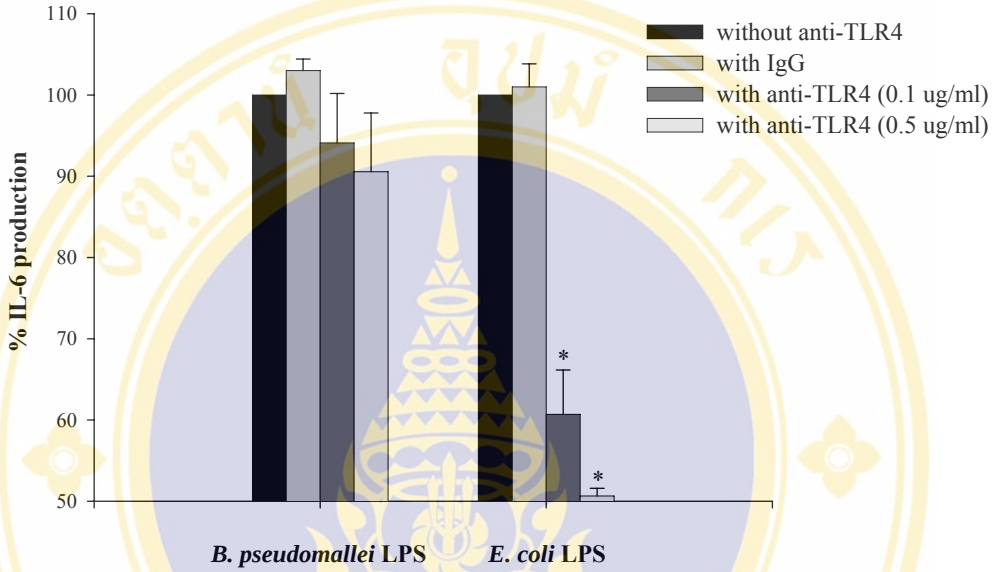

Figure 16. Neutralizing antibody against TLR4 fails to inhibit IL-6 production. Mouse macrophages were pretreated with antibody against TLR4 at concentration of 0.1 and 0.5 μg/ml for 2 h before activating with *B. pseudomallei* LPS (100 ng/ml). *E. coli* LPS (10 ng/ml) was used as a positive control. After 8 h of infection, the production of IL-6 was determined in the supernatant by ELISA assay. Data shown represent means and standard errors of 3 separate experiments, each carried out in duplicate. *$P<0.01$ by Students's *t*-test

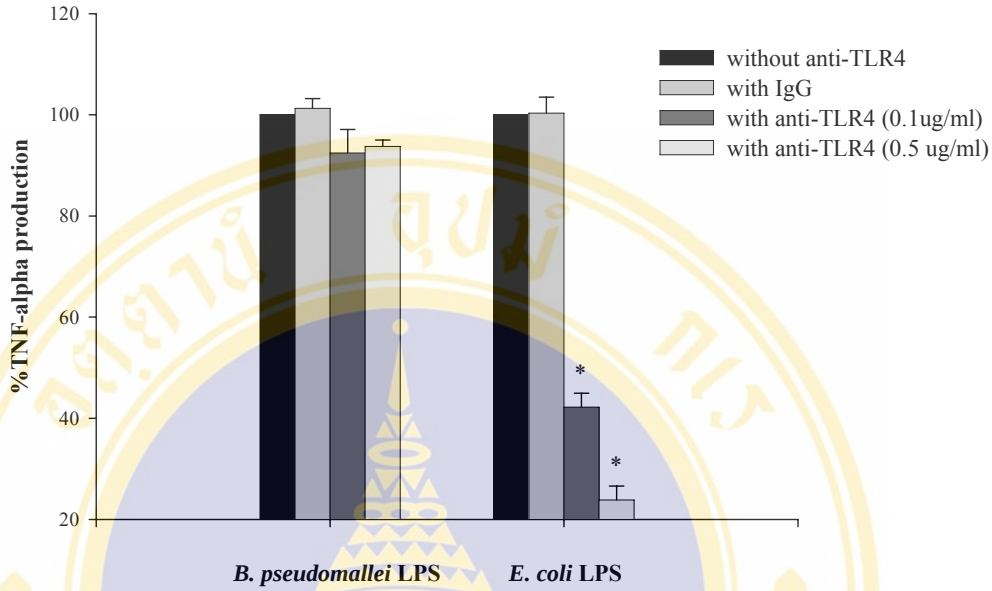

Figure 17. Neutralizing antibody against TLR4 fails to inhibit TNF-α production. Mouse macrophages were pretreated with antibody against TLR4 at concentration of 0.1 and 0.5 μg/ml for 2 h before activating with *B. pseudomallei* LPS (100 ng/ml). *E. coli* LPS (10 ng/ml) was used as a positive control. After 8 h of infection, the production of TNF-α was determined in the supernatant by ELISA assay. Data shown represent means and standard errors of 3 separate experiments, each carried out in duplicate. **P*<0.01 by Students's *t*-test

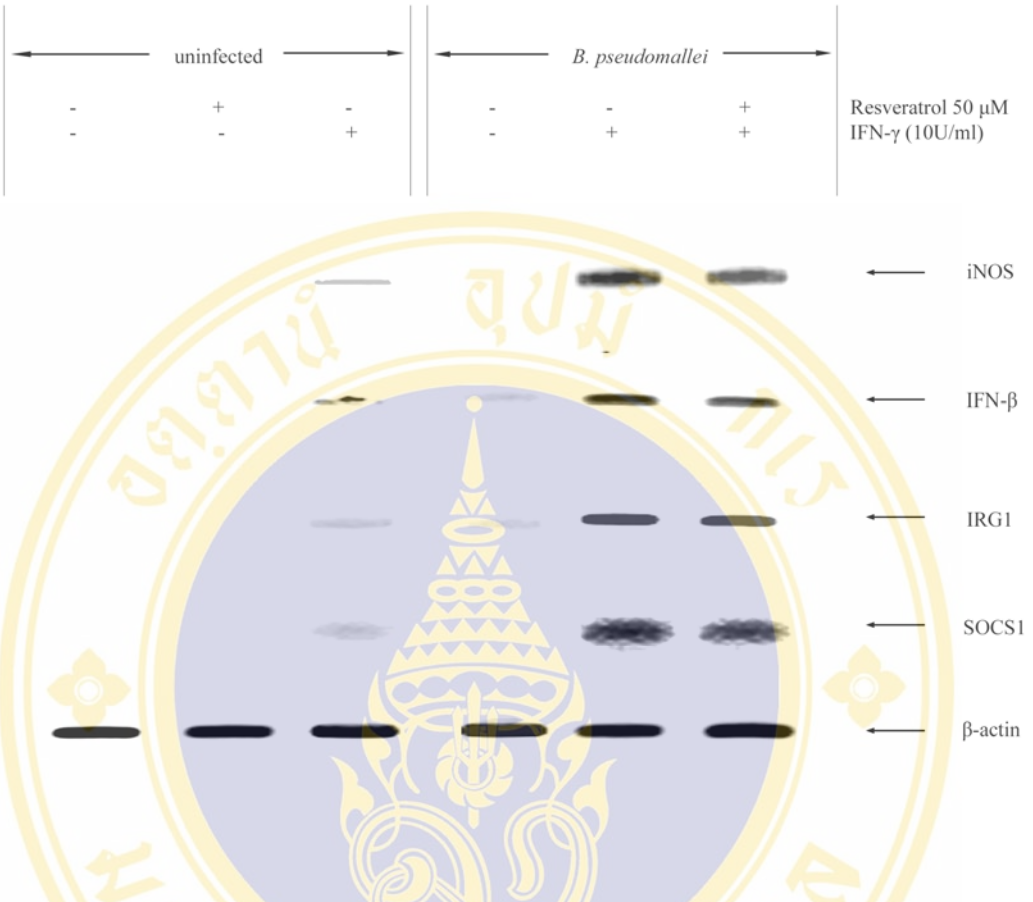

Figure 18. Ability of IFN-γ to enhance the genes expression through MyD88-independent pathway in *B. pseudomallei*-infected macrophages. Mouse macrophages were pretreated with IFN-γ (10U/ml) overnight. Then the mouse macrophages were treated with resveratrol (50μM) for 1 h before exposing to *B. pseudomallei* at MOI of 2:1. After 4 h of infection, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

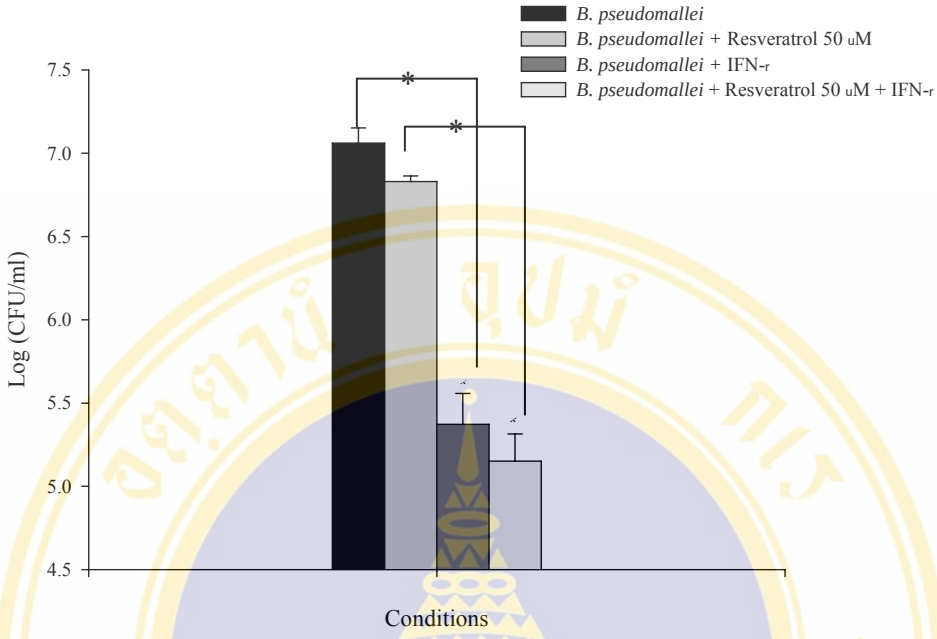

Figure 19. Resveratrol does not suppress intracellular *B. pseudomallei* killing in mouse macrophages pretreated with IFN-γ. Mouse macrophages were pretreated with IFN-γ (10 U/ml) overnight. Then the mouse macrophages were treated with resveratrol (50μM) for 1 h before exposing to *B. pseudomallei* at MOI of 2:1. At 8 h after infection, the cells were lysed with 1 ml 0.1% TritonX-100 and released bacteria were plated on TSA. The number of intracellular survival of bacteria was determined by bacterial colony counting. The data represent the mean and s.d. of 3 separate experiments, each carried out in duplicate. *P<0.01 by Students's t-test

CHAPTER V
DISCUSSION

In the innate immunity, Toll-like receptors (TLRs) is a skillful system that detects invasion of microbial pathogens (9). Once activated, the intracellular domains of TLRs interact with the adaptor molecule such as MyD88 and initiate a common signaling cascade resulting in activation of MAPKs and the transcription factor NF-κB leading to the induction of several genes that function in host defense including inflammatory cytokines, chemokines and effector molecules such as iNOS (11, 196). The intracellular signaling pathway of TLR can be divided into two distinct pathways based on the requirement of MyD88 adaptor molecules. All TLRs, except TLR3 use MyD88 (MyD88-dependent) pathway for signaling. In contrast to others, TLR4 contains both MyD88-dependent and MyD88-independent pathway for signaling. In MyD88-deficient mice, the animals failed to produce the proinflammatory cytokines such as TNF-α, IL-6, IL-1β (203, 204). IκBζ, an essential transcription factor of IL-6, is an inducible protein, which is mediated through TLR. IL-6, was completely abolished in MyD88-deficient mice indicating that signaling for IκBζ production depending on MyD88 pathway (14). On the other hand, type I IFN expression could also be induced in the absence of MyD88, suggesting that MyD88-independent pathway is involved in the regulation of type I IFN (203, 204).

Although the mechanisms underlying the macrophage activation by *B. pseudomallei* has not been fully elucidated, the recent report demonstrated that *B. pseudomallei*, unlike other gram-negative bacteria such as *S. typhi*, failed to stimulate IFN-β production (8). These results indirectly implied that *B. pseudomallei* may not activate MyD88-independent pathway of macrophages. For this study, we extended the finding to demonstrate that *B. pseudomallei* is capable to stimulate the gene expression only through MyD88-dependent pathway (e.g. IκBζ, IL-6, TNF-α)

(Figure 4) and failed to stimulate a number of gene known to require MyD88-independent pathway (IFN-β, IRG1, iNOS, SOCS1) (Figure 11). This is in contrast to *S. typhi* which is capable of activating the gene expressions utilizing both MyD88-dependent and -independent pathways (Figure 5, 10, 11). Additionally, heat-killed bacteria also activate the gene expression in a similar profiles to living bacteria, it suggesting that activation of these gene expressions did not require the viability of bacteria (Figure 6). The resveratrol is a polyphenol compound which specifically inhibits TBK1 and RIP1 molecules in MyD88-independent signaling pathway. In the presence of resveratrol, this inhibitor was unable to alter the gene expression of *B. pseudomallei*-infected macrophages (Figure 10, 11). It also did not interfere with the intracellular survivor of *B. pseudomallei* (Figure 13), therefore it can be understood that *B. pseudomallei* did not activate MyD88-independent pathway of macrophage.The gene and protein expression that regulated through MyD88-independent pathway of *S. typhi*-infected macrophages was suppressed by resveratrol, indicating that *S. typhi* was able to activate both MyD88-dependent and –independent pathways (Figures 10, 11, 12).

It can be perceived that the gene expression, which was induced by *B. pseudomallei,* was initiated by interaction of the bacteria to the surface receptors of the macrophages instead of using intracellular receptors. The inhibitor of internalization, cytochalasin D, was used. In the presence or absence of cytochalasin D, *B. pseudomallei* could activate the expression of IκBζ, IL-6 and TNF-α (Figure 14) but not SOCS3. This suggested that the expression of the former groups (IκBζ, IL-6, TNF-α) by *B. pseudomallei* was triggered via macrophage surface receptor. SOCS3 gene expression, on the other hand, was inhibited in the presence of cytochalasin D, suggesting that the expression of this gene may not involve surface receptors for initiation (201). Several intracellular receptors e.g. nod may be involved in the activation of the gene expression by *B. pseudomallei* as well. However, the type of intracellular receptor that *B. pseudomallei* required for activation of SOCS3 gene expression would be considered for future investigation.

The ability of *B. pseudomallei* to activate the gene expression through MyD88 suggested that TLR may be involved in signaling. Among the TLRs, TLR4 is a crucial receptor for signaling by most bacterial lipopolysaccharide (LPS). However, it has

been demonstrated that LPS isolated from *B. pseudomallei* exhibited an unusual chemical structure in the acid-stable inner core region attached to the lipid A moiety (16). This unusual structure of *B. pseudomallei* LPS may be associated with its relatively weaker and slower activator of macrophage (6). These results indirectly suggested that *B. pseudomallei* LPS may use different types of TLR. In this study, we demonstrated that TLR4 antibody failed to inhibit signaling in *B. pseudomallei* LPS-activated macrophages (Figures 16, 17) in order to show that *B. pseudomallei* LPS did not use TLR4 for signaling. It has been demonstrated that lipid A of nonenterobacterial species such as *P. gingivalis*, which differs both structurally and functionally from enterobacterial, requires TLR2 in stead of TLR4 for signaling (145). Consequently, it is possible that *B. pseudomallei* LPS could also use TLR other than TLR4, to mediate intracellular signaling. However, whether the *B. pseudomallei* LPS uses TLR2 for signaling or not needs to be investigated.

IFN-γ signaling is essential for the macrophage activation and elimination of invading microorganisms. The biological activities of IFNs are initiated by the binding of IFN-γ to their cognate receptors on the surface of cells. The binding of IFN-γ to its receptor induces a receptor dimerization Jak1 and Jak2 respectively (205-208). Later on, the activated Jaks phosphorylate a tyrosine-containing sequence, thereby forming paired binding sites for STAT1 that interact through their SH2 domains (209, 210). After dissociation from the receptor, STAT1 translocates to the nucleus and binds to the promoter, resulting in stimulation of transcription of several genes including IRF-1 and iNOS that are known to play an important role in controlling the fate of intracellular pathogens (211-213). As mentioned earlier, *B. pseudomallei*-infected macrophages failed to activate IRF-1, iNOS causing a failure to eliminate intracellular *B. pseudomallei* (7, 8, 201). However, exogenous IFN-γ could enhance IRF-1, iNOS expression and TNF-α release from the cells infected with *B. pseudomallei* (7, 201). These results also correlated with a decreased intracellular survival of *B. pseudomallei* inside macrophage pretreated with IFN-γ (Figure 19). In the presence of IFN-γ, this cytokine alone induce only low level of gene expression of both MyD88-dependent and -independent pathway (Figure 18). Nevertheless, IFN-γ synergistically enhances gene expression of both pathway in *B. pseudomallei*-infected macrophages. In the presence of resveratrol, this inhibitor was unable to inhibit gene expression of MyD88-

independent pathway (Figure 18), implying that IFN-γ may activate MyD88-independent pathway at point downstream of TBK1 and RIP1 or bypassing these molecules. According to the recent report of IFN-γ signaling, MyD88 bridged IFNGR1 to MLK3 which is a molecule involves in mitogen-activated protein kinase signaling cascade (214). However, whether or not MLK3 could activate MyD88-independent pathway downstream from TBK1 and RIP1 remaine to be investigated. Moreover, these results also suggested that MyD88-independent pathway is an essential pathway in controlling the macrophage killing of intracellular *B. pseudomallei*. Failure to activate this pathway lead to the ability of *B. pseudomallei* to survive inside the macrophage. However, if this pathway is restored such as by adding IFN-γ, the macrophage would exhibit the ability to suppress intracellular survival of the bacteria.

Although the level of gene expression stimulated by *B. pseudomallei* did not differ from *S. typhi* (Figure 4, 5), the level of secreted cytokine of *B. pseudomallei*-infected macrophages was lower than *S. typhi*-infected macrophages (Figure 7, 8, 9). These results suggest that *B. pseudomallei* may also modulate the protein secretion pathway of the infected macrophages (215).

CHAPTER VI
CONCLUSION

The results from this study demonstrated that *B. pseudomallei* was able to activate the gene expression of MyD88-dependent pathway (e.g. IκBζ, IL-6, TNF-α, SOCS3) in mouse macrophage cell line (RAW 264.7). It failed to activate the gene expression of MyD88-independent pathway (e.g. iNOS, IFN-β, IRG1, SOCS1). In the presence of resveratrol, a specific inhibitor of MyD88-independent pathway, the viability of intracellular *B. pseudomallei* remained unaltered, confirming that the MyD88-independent pathway was not involved in macrophage activation by *B. pseudomallei*. Heat-killed *B. pseudomallei* was also able to activate gene expression in similar profiles as living *B. pseudomallei*, indicating that the expression of these genes did not require viability of bacteria. Moreover, the expression of these genes in mouse macrophages did not require invasion of this bacteria which suggested that the gene expression induced by *B. pseudomallei* was triggered via the surface receptor of the macrophage. From these results, it is likely that TLR might be involved for signaling in *B. pseudomallei*-infected macrophages. In contrast to other gram-negative bacteria which triggers the signaling by the interaction of LPS to TLR4, neutralizing antibody of TLR4 failed to block the macrophages activation by *B. pseudomallei* LPS, suggesting that *B. pseudomallei* LPS might use the TLR other than TLR4. Exogenous IFN-γ was able to increase expression of both MyD88-dependent and –independent pathway which resulted in the inability of *B. pseudomallei* to multiply inside the macrophages. It is implying that MyD88-independent pathway play an essential role to suppress the intracellular survival in this bacterium. However, in the presence of resveratrol, this inhibitor was unable to inhibit the gene expression of MyD88-independent pathway in IFN-γ activated macrophages, suggesting that IFN-γ may activate MyD88-independent pathway at downstream of TBK1 and RIP1 or bypassed these molecules. Furthermore, the gene expression of MyD88-independent pathway was directly correlated with the ability of the infected macrophages to kill intracellular

B. pseudomallei, suggesting that MyD88-independent pathway is an important pathway for controlling *B. pseudomallei* survival. By understanding how *B. pseudomallei* exploits macrophages in order to survive and multiply inside the host cells, it is hoped that pathogenesis mechanism of melioidosis and effective vaccines for this disease will not be too far away.

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APPENDIX

APPENDIX

Reagent for cell and bacteria culture

Unsupplemented DMEM medium

Advanced D-MEM 1X
With D-glucose at 4500 mg/l
With NEAA
With sodium pyruvate at 110 mg/l
Without L-glutamine

Supplemented DMEM medium

Unsupplemented DMEM medium	94	ml
Heat-inactivated Fetal Bovine Serum	5	ml
(FBS was heated at 56ºC for 30 min)		
L-glutamine	1	ml

Freezing solution

Dimethyl sulfoxide (DMSO)	100	µl
Fetal bovine serum	900	µl

Phosphate-buffered saline (PBS) pH 7.0

NaCl	8	g
Na_2HPO_4	0.92	g
KH_2PO_4	0.2	g
KCl	0.2	g
Distilled water to a final volume of	1	liter

The solution was adjusted to pH 7.0 and sterilized by autoclaving

Trypticase soy broth (TSB)

Tryrpticase soy broth	30	g
Distilled water to a final volume of	1	liter

Dissolved by heating twice in microwave oven about 10 minutes

Sterilized by autoclaving and kept at 4 ºC until used

Trypticase soy agar (TSA)

Tryrpticase soy broth	30	g
Agar-agar	12	g
Distilled water to a final volume of	1	liter

Dissolved by heating twice in microwave oven about 10 minutes

Sterilized by autoclaving and kept at 4 ºC until used

Reagent for ELISA mouse IL-6

Coating buffer (0.1 M carbonate buffer, pH 9.5)

$NaHCO_3$	2.1	g
Na_2CO_3	0.89	g
Distilled water to a final volume of	250	ml

Freshly prepared and used within 7 days (4ºC storage)

Phosphate-buffered saline (PBS) pH 7.0

NaCl	8	g
Na_2HPO_4	1.16	g
KH_2PO_4	0.2	g
KCl	0.2	g
Distilled water to a final volume of	1	liter

Freshly prepared and used within 1 week (4ºC storage)

Wash buffer (PBS, pH 7.0 with 0.05% tween-20)

NaCl	8	g
Na_2HPO_4	1.16	g

KH_2PO_4	0.2	g
KCl	0.2	g
Tween-20	500	µl
Distilled water to a final volume of	1	liter

Freshly prepared and used within 3 days (4ºC storage)

Assay diluent (PBS, pH 7.0 with 10% FBS)

PBS (pH 7.0)	9	parts
FBS	1	part

Freshly prepared and used within 3 days (4ºC storage)

Substrate buffer (0.1 M acetate buffer) for TMB

Sodium acetate	0.8203	g
Distilled water to a final volume of	100	ml

Adjusting pH to 6.0 with 1N citric acid

Freshly prepared and used within 2 weeks (4ºC storage)

TMB (3,3′,5,5′- Tetramethybenzidine) solution

Tetra	0.01	g
DMSO	1	ml

3% hydrogen peroxide(H_2O_2)

Stock 35% H_2O_2	30	µl
Distilled water	320	µl

TMB substrate working solution

0.1 M acetate buffer	10	ml
TMB in DMSO	100	µl
3% H_2O_2	10	µl

Freshly prepared and used immediately

2N H_2SO_4 Stop solution

Reagent for ELISA mouse TNF-α

Reagent diluent (1% BSA in PBS, pH 7.2-7.4), 0.2 μm filtered

Phosphate-buffered saline (PBS) pH 7.2-7.4

NaCl	8	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.2	g
KCl	0.2	g
Distilled water to a final volume of	1	liter

Freshly prepared and used within 1 week (4ºC storage)

Wash buffer (PBS, pH 7.2-7.4 with 0.05% tween-20)

NaCl	8	g
Na_2HPO_4	1.16	g
KH_2PO_4	0.2	g
KCl	0.2	g
Tween-20	500	μl
Distilled water to a final volume of	1	liter

Freshly prepared and used within 3 days (4ºC storage)

Substrate solution – 1:1 mixture of

Color reagent A (H_2O_2)

Color reagent B (Tetramethylbenzidine)

2N H_2SO_4 Stop solution

Reagents for PCR

Tris-base-EDTA buffer (TBE, 10X)

Tris base	108	g
Boric acid	55	g
Na_2EDTA	8.3	g

Distilled water to a final volume of	1	liter
Adjusting pH to 8.0		

TBE running buffer (1X)

10X TBE buffer	100	ml
Distilled water	900	ml

Agarose gel preparation

1.5% agarose gel

Agarose (dry powder)	1.65	g
1X TBE buffer	110	ml

Dissolved by boiling in microwave and cool at RT

Reagents for SDS-PAGE

Stock acrylamide (30%w/v)

Acrylamide	150	g
N,N-bis-methylene acrylamide	4.5	g
Distilled water to a final volume of	500	ml

The solution was filtrated with Whatman filter paper No.1 and stored at 4ºC

Gel buffer 1.875 M pH 8.8

Trizma base (MW 121.14)	113.57	g
Distilled water to a final volume of	500	ml
Adjusting pH to 8.8		

Gel buffer 1.875 M pH 6.8

Trizma base (MW 121.14)	113.57	g
Distilled water to a final volume of	500	ml
Adjusting pH to 6.8		

Electrophoresis buffer

Trizma base	3	g
Glycine	14.4	g
SDS	1	g
Distilled water to a final volume of	1	liter

Towbin's buffer

Trizma base	9.1	g
Glycine	43.2	g
Methanol	600	ml
Distilled water to a final volume of	3	liters

Phosphate-buffered saline (PBS)

NaCl	8.9	g
Na_2HPO_4	1.28	g
$Na_2HPO_4.2H_2O$	0.156	g
Distilled water to a final volume of	1	liter

Sample buffer (5X) pH 6.8

Trizma base	0.3784	g
SDS	0.5	g
Glycerol	5	ml
2-mercaptoethanol (2-ME)	2.5	ml
1N HCL	1.5	ml
Bromophenol blue	0.005	g
Distilled water to a final volume of	10	ml

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