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การควบคุมการสร้าง Suppressor of Cytokines Signaling (SOCS) โดยเชื้อ *Burkholderia pseudomallei* ในเซลล์แมโครฟาจของหนู

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เมษายน 2550

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Abstract

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Project Title: Regulation of Suppressor Cytokines Signaling (SOCS) by *Burkholderia pseudomallei* in mouse macrophage cell line

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Burkholderia pseudomallei, the causative agent of melioidosis, is a facultative intracellular gram-negative bacterium that is able to survive and multiply in macrophages. It has been reported that *B. pseudomallei* was able to escape macrophage killing by interfering with the expression of inducible Nitric Oxide Synthase (iNOS). The results from this study demonstrated that both viable or heat-killed *B. pseudomallei* was able to activate the expression of suppressor of cytokine signal-3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) but not SOCS1 in a mouse macrophage cell line (RAW 264.7). This bacterium could directly induce the expression of these negative regulators. However, the presence of cytochalasin D was able to prevent SOCS3 and CIS expression indicating that the signaling for SOCS3 and CIS expression may be initiated inside the macrophages. The expression of SOCS3 and CIS in *B. pseudomallei*-infected macrophages directly correlated with a decreased gamma interferon (IFN-γ) signaling response, as indicated by a reduction in Y701-STAT-1 phosphorylation (pY701-STAT-1). Moreover, a reduction in the expression of IFN-γ-induced proteins, such as interferon regulatory factor 1 (IRF-1), was observed in *B. pseudomallei*-infected macrophages that were treated with IFN-γ. Since pY701- STAT-1 and IRF-1 are essential transcription factors for regulating iNOS expression, the failure to activate these factors could also result in depression of iNOS expression and loss of the macrophages killing capacity. Furthermore, the induction of these

negative regulators was most probably triggered from within rather than at the cell surface of mouse macrophage cell line (RAW 264.7) suggesting that macrophage activation most likely requires the interaction of bacteria with a putative host cell cytoplasmic component(s). Taken together, the data indicate that the activation SOCS3 and CIS expression in *B. pseudomallei*-infected macrophages interfered with IFN-γ signaling, thus allowing the bacteria to escape killing by these phagocytic cells. In addition, the present study, we also demonstrated that *B. pseudomallei* was able to induce gene expression through MyD88-dependent pathway (e.g. *iκbζ, il-6, tnf-α, socs3*) but failed to activate MyD88-independent pathway (e.g. *inos, ifn-β, irg1, socs1*). IFN-γ 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.

Keywords : *B. pseudomallei*, Melioidosis, SOCS3, CIS, IFN-γ

บทคัดย่อ

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ชื่อโครงการ : การควบคุมการสร้าง Suppressor of Cytokines Signaling (SOCS) โดยเชื้อ *Burkholderia pseudomallei* ในเซลล์แมคโครฟาจของหนู

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เชื้อเบอร์โคลเดอเรีย สูโดมาลลิไอ (*Burkholderia pseudomallei*) เป็นเชื้อแบคทีเรียชนิดแกรมลบซึ่งเป็นสาเหตุของโรคเมลิออยโดซิส เชื้อ *B. pseudomallei* เป็นแบคทีเรียชนิดfacultative intracellular ที่สามารถเจริญเติบโตและเพิ่มจำนวนได้ทั้งในเซลล์ phagocytic และ non-phagocytic เชื้อนี้มีความสามารถในการหลบหลีกจากการทำลายโดยเซลล์แมโครฟาจโดยเมื่อเชื้อนี้ถูกเซลล์ phagocytosed จะไม่กระตุ้น ให้เซลล์สร้างเอนไซม์ inducible nitric oxide synthase (iNOS) ซึ่งทำหน้าที่สำคัญในการสร้าง reactive nitrogen intermediate (RNI) ที่มี คุณสมบัติในการทำลายจุลชีพ ทั่วไป ในการศึกษานี้พบว่าเชื้อ *B. pseudomallei* ทั้งมีชีวิตและไม่มีชีวิตสามารถกระตุ้น เซลล์แมโครฟาจของหนู (RAW 264.7) ให้สร้างโปรตีน suppressor of cytokine signaling (SOCS) 3 และ cytokine-inducible Src-homology 2 (CIS) แต่ไม่สามารถกระตุ้นให้เกิดการสร้าง SOCS1 ได้ การแสดงออกของ SOCS3 และ CIS จะเกิดขึ้นได้ต่อเมื่อเชื้อ *B. pseudomallei* สามารถเข้าสู่เซลล์แมโครฟาจแล้ว ระดับการแสดงออกของ SOCS3 และ CIS ในเซลล์แมโครฟาจที่ได้รับเชื้อ *B. pseudomallei* สัมพันธ์กับการลดลงของระดับphosphorylation ของ signal transducer and activator of transcription (STAT)-1(pY701-STAT-1)

ซึ่งบ่งชี้ถึงการส่งสัญญาณที่ลดลงของ IFN-γ เมื่อระดับการส่งสัญญาณของ IFN-γ ลดลงจึงทำให้เซลล์แมโครฟาจไม่สามารถสร้างโปรตีนที่มีความสำคัญต่อกระบวนการการทำลายจุลชีพได้ อาทิเช่น interferon regulatory factor (IRF)-1 และ iNOS จึงทำให้เชื้อ *B. pseudomallei* สามารถเจริญเติบโตและเพิ่มจำนวนในเซลล์แมโครฟาจได้ ในการศึกษานี้ยังพบว่าเชื้อ *B. pseudomallei* สามารถกระตุ้นการแสดงออกของยีนผ่าน MyD88-dependent pathway (*iκbζ, il-6, tnf-α, socs3*) แต่ไม่สามารถกระตุ้นการแสดงออกนี้ผ่าน MyD88-independent pathway (*inos, ifn-β, irg1, socs1*)นอกจากนั้นยังพบว่า IFN-γ สามารถที่จะเพิ่มระดับการแสดงออกของยีนทั้ง MyD88-dependent และ MyD88-independent pathway อีกทั้งยังพบว่าเมื่อมีการแสดงออกของยีนโดยผ่าน MyD88-independent pathway แล้วแมโครฟาจจะสามารถทำลายเชื้อ *B. pseudomallei* ภายในเซลล์ได้ จึงสรุปว่า MyD88-independent pathway มีความสำคัญในการควบคุมและทำลายเชื้อ *B. pseudomallei* ภายในเซลล์แมโครฟาจ

Introduction

Burkholderia pseudomallei is the causative agent of melioidosis which affects humans and animals in southeast Asia, particularly northeastern Thailand and northern Australia. *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 (23). Inside the cell, this bacterium can induce cell- cell fusion resulting in multinucleated giant cell formation (20, 25). 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 (47). 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*) (48). 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. Addition of exogenous IFN-β or IFN-γ at the time of bacterial challenge restored the macrophages ability to activate both IRF-1 and iNOS expression, which resulted in enhanced killing of intracellular *B. pseudomallei* (34, 48, 49). These results demonstrated that under the appropriate conditions, IFNs play an essential role in stimulating the antimicrobial activity of macrophages in response to *B. pseudomallei* infection.

One common property of IFNs is to induce a transcriptional response through a JAK-STAT signal transduction pathway (13). Once STAT-1 is phosphorylated, it is translocated into the nucleus to regulate transcription of promoters containing an IFN-γ-activated site (GAS) element (12, 16). Several IFN-γ responsive genes, including IRF-1 and iNOS, are known to function in macrophage antimicrobial activity (13, 19). However, the macrophage response to IFNs can be influenced by the expression of

SOCS proteins. Eight members of the SOCS protein family have been identified that serve as negative effectors of cytokine signaling via the JAK/STAT pathway, thus attenuating macrophage response (2, 8). SOCS1, SOCS3, and CIS have been shown to inhibit Y701-STAT-1 phosphorylation (pY701-STAT-1) in response to IFN-stimulation (5, 39). In addition to cytokines, pathogens and their products can induce SOCS expression. For example, lipopolysaccharide (LPS) could induce SOCS1, SOCS3, and CIS expression macrophages (11, 27, 35, 41). Some pathogens could interfere with the host immune response by activating SOCS expression leading to suppression of macrophage activation. For example, persistent *Listeria monocytogenes* infection of macrophages diminished IFN-γ-stimulated transcription and phosphorylation of STAT-1 (42). The decreased IFN-γ signaling also correlated with the induction of SOCS3 expression in the *L. moncytogenes*-infected macrophages. In the present study, we demonstrated that *B. pseudomallei* is able to stimulate SOCS3 and CIS expression in macrophages. The expression of these negative regulators in *B. pseudomallei*-infected macrophages correlated with a decrease in Y701-STAT-1 phosphorylation, which is normally stimulated by exogenous IFN-γ and is essential for IRF-1 and iNOS production, thereby allowing *B. pseudomallei* to escape killing by the macrophages.

Toll-like receptor (TLR) is a pattern recognition receptor which plays a major role in an innate immune system against the microorganisms (43). There are at least 11 members of TLR family in humans that have been identified (3). As many as 13 can be found in a search of the mouse structure genome (3). Each recognizes a different component of microbe structures. For example, TLR4 is involved in the recognition of lipopolysaccharide (LPS) (32). 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 (22). 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 (52). In IκBζ-deficient mice, the animals failed to produce IL-6, indicating that a regulation of IL-6 requires IκBζ (52). In addition, MyD88-deficient mice failed to induce expression of IκBζ after stimulation indicating that regulation of IκBζ requires MyD88-dependent (51). 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 (38).

Material and Methods

Reagents and cell culture

Mouse macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC, Rockville, MD). If not indicated otherwise, the cells were cultured in Dulbeccco's modified Eagles' medium (DMEM) (HyClone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT) at 37^{o}C under a 5% CO_2 atmosphere.

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. *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 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.

Heat-killed bacteria preparation

Non-viable *B. pseudomallei* were prepared by heating the bacteria (10^8 CFU/ml) in boiling water bath at for 15 min. The heat-killed bacteria were washed

3 times with PBS and complete killing was ascertained by incubating the cultured in tryptic soy agar for 48 h and the bacterial colonies was counted.

Infection of mouse macrophage (RAW 264.7)

Mouse macrophages (1×10^6 cells) were cultured in a 6-well plate overnight before exposure to bacteria at multiplicity of infection (MOI) of 2:1 for 1 h. To remove extracellular bacteria, the cells were washed 3 times with 1 ml of PBS before replacing with DMEM containing 250 μg/ml kanamycin (Gibco Labs). At 4 hours after infection, the infected cells were lyzed before subjecting to immunoblotting or RT-PCR.

Cell invasion and intracellular bacterial survival assay

To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed. Briefly, after killing extracellular bacteria with 250 μg/ml kanamycin, the cells were washed 2 times with 1 ml PBS and incubated in the supplemented medium containing 20 μg/ml kanamycin to inhibit the growth of residential extracellular bacteria. At the indicated time, the intracellular bacteria were liberated by lyzing the macrophages with 1 ml 0.1% Triton X-100. One hundred microliters of 0.1% Triton X-100 from each well was added to 900 μl TSB to make a 10-fold serial dilution before plating the released bacteria in 20 ml TSA. The number of intracellular bacteria, expressed as CFU, was determined by bacterial colony counting after incubated at 37 °C for 48 h. All invasion and bacterial survival were performed in duplicate.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

In order to determine mRNA expression of the SOCS, the gene expression of MyD88-dependent pathway (IκB-ζ, IL-6, TNF-α) and MyD88-independent pathway (iNOS, IFN-β, IRG1), mRNA of the infected macrophages

was purified as described by manufacture (Eppendrof, Hamburg, Germany). The extracted mRNA was subsequently used for cDNA synthesis (Eppendrof, Hamburg, Germany). The PCR reaction was conducted by using cDNA as template for SOCS1, SOCS2, SOCS3, CIS, IκB-ζ, IL-6, TNF-α, iNOS, IFN-β, IRG1. Sequences of primers were as followed:

Primers	**Sense 5' → 3'**	**Antisense 5' → 3'**
SOCS1	CACCTTCTTGGTGCGCG	AAGCCATCTTCACGCTGAGC
SOCS2	AAGCCCAGAAGCCCCAC	GTTGGTAAAGGCAGTCCCCA
SOCS3	AGTGTCACCCACAGCAAGTT	AATCCGCTCTCCTGCAGCTT
CIS	GAACCGAAGGTGCTAGACCCT	TGTACCCTCCGGCATCTTCT
IRG1	GGTATCATTCGGAGGAGCAA	ACAGAGGGAGGGTGGAATCT
iNOS	GCAGAATGTGACCATCATGG	ACAACCTTGGTGTTGAAGGC
IFN-β	TCCAAGAAAGGACGAACATTCG	TGAGGACATCTCCCACGTCAA
TNF-α	GTAGCCCACGTCGTAGCAAA	CCCTTCTCCAGCTGGGAGAC
IL-6	GGTCTCTGGGAAATCGTG GA	GCTGACCCTAGAGCATCCTG
IκB-ζ	TGTTGCCTTCTCACTTCGTG	TGGTCCATCATCTGTGGAGA

The amplified products were electrophoresed on 2% agarose gel before visualized by ethidium bromide staining.

Immunoblotting

Different mouse macrophage preparations were lyzed in buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates containing 30 μg of protein were electrophoresed on SDS-PAGE at 12% and 8% polyacrylamide, respectively, and then electrotransferred to nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany). For SOCS, the membrane was blocked with 10% milk for 1 h before incubating overnight with polyclonal antibody to mouse SOCS1 or SOCS3 (Santa Cruz, Santa Cruz, CA). For iNOS, 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. Blots were then reacted with horseraddish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostic, Mannheim, Germany).

Gamma interferon stimulation

1. Co-stimulation

The mouse macrophages were infected with bacteria at MOI 2:1 as described above. At the time of infection, IFN-γ 10 U/ml was added to cultures. After infection for an hour, extracellular bacteria were removed and washed 2 times with 1 ml PBS before culturing in the medium containing 250 μg/ml kanamycin (Gibco) for 2 h. The infected cells were washed twice with PBS before cultured in the medium containing 20 μg/ml kanamycin to inhibit the growth of any remaining of bacteria. At the time indicated, the cells were lyzed for analysis of intracellular survival of bacteria or immunoblotting.

2. Post-stimulation

The mouse macrophages were infected with bacteria as described above. After infection 10 U/ml of IFN-γ was added and kept for 15 min before replacing with fresh medium containing kanamycin. At time indicated, the cells were lyzed for analysis of intracellular survival of bacteria or immunoblotting.

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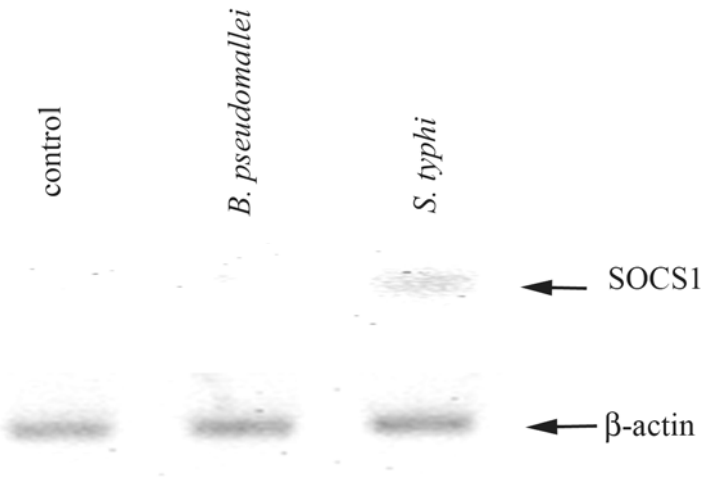
Results

Expression of SOCS mRNA in mouse macrophages infected with *B. pseudoamallei*

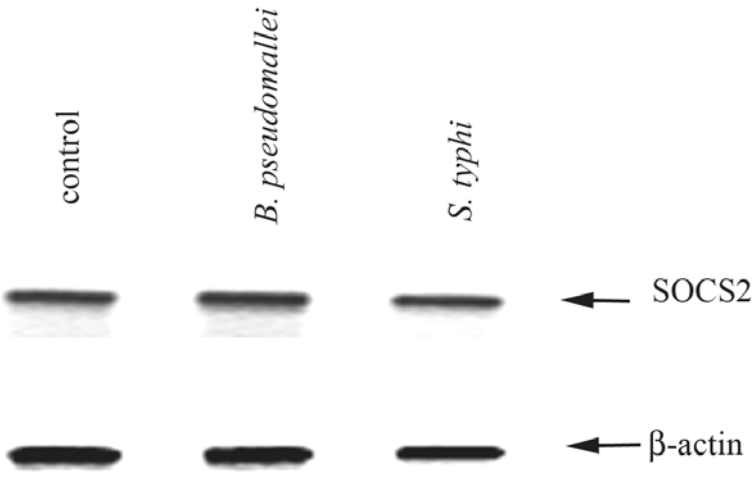
To investigate mRNA expression of SOCS (SOCS1, SOCS2, SOCS3 and CIS) in mouse macrophages infected with *B. pseudomallei* (or S. *typhi* as comparison bacteria), RT-PCR was performed as described in Material and Methods. As shown in Fig. 1A, the cells infected with *B. pseudomallei* were unable to upregulate SOCS1 mRNA expression after four hours of infection while the cells infected with *S. typhi*, showed high level of SOCS1 mRNA. In contrast to SOCS1, SOCS2 mRNA was observed even in the non-infected cells and the level of SOCS2 expression was not changed in the cells infected with either bacteria. These results suggest that SOCS2 may be constitutive expressed in mouse macrophages cell line and was not regulated by *B. pseudomallei* or *S. typhi* (Fig. 1B). Expression of SOCS3 mRNA was not observed in the non-infected cells but highly expressed in the cells that infected with *B. pseudomallei* or *S. typhi* (Fig. 1C). As same as SOCS-3, expression of CIS could also be observed in the cells infected with *B. pseudomallei* and *S. typhi* (Fig. 1D).

Expression of SOCS proteins in mouse macrophages infected with *B. pseudomallei*

In order to confirm the expression of SOCS1 and SOCS3 in the macrophages infected with *B. pseudomallei* and *S. typhi*, immunoblotting with specific antibody against SOCS1 and SOCS3 was performed as described in Material and Methods. As shown in Fig 2A, the macrophages infected with *S. typhi* were able to express high level of SOCS1 after 4 h of infection. In contrast, *B. pseudomallei* was unable to upregulate SOCS1 protein expression. However, high level of SOCS3 protein expression was observed in the cells infected with *B. pseudomallei* and *S. typhi* (Fig. 2 B).

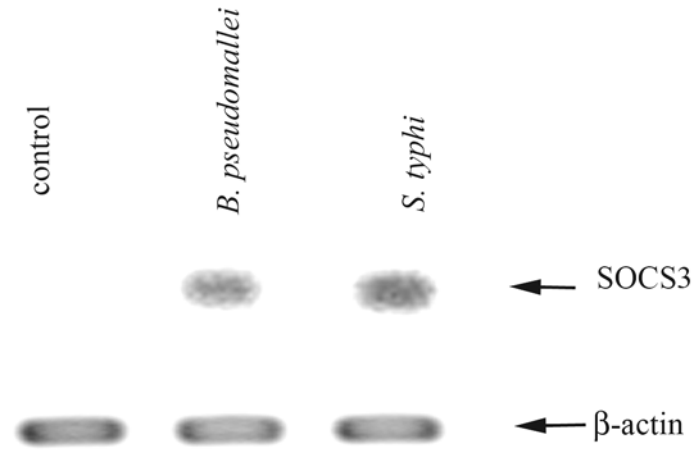

control
B. pseudomallei
S. typhi
SOCS1
β-actin

A

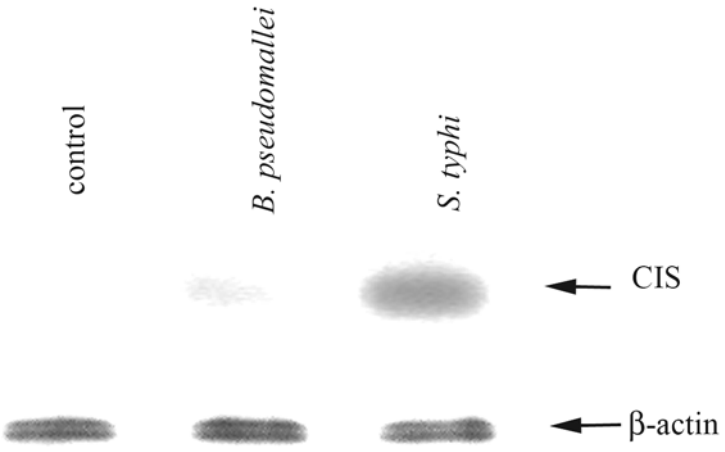

control
B. pseudomallei
S. typhi
SOCS2
β-actin

B

.

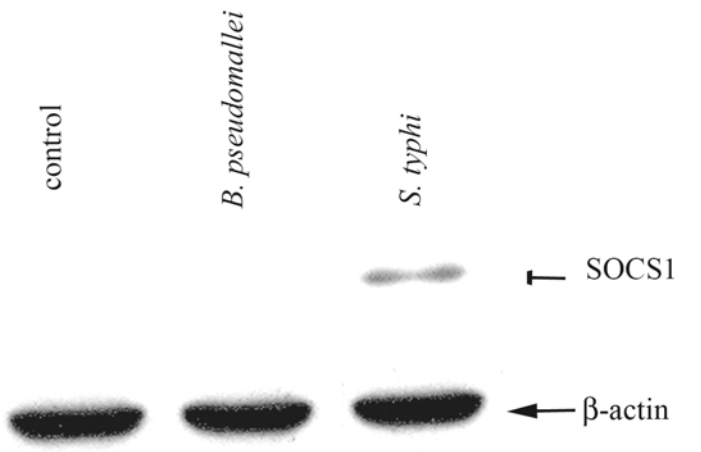


C

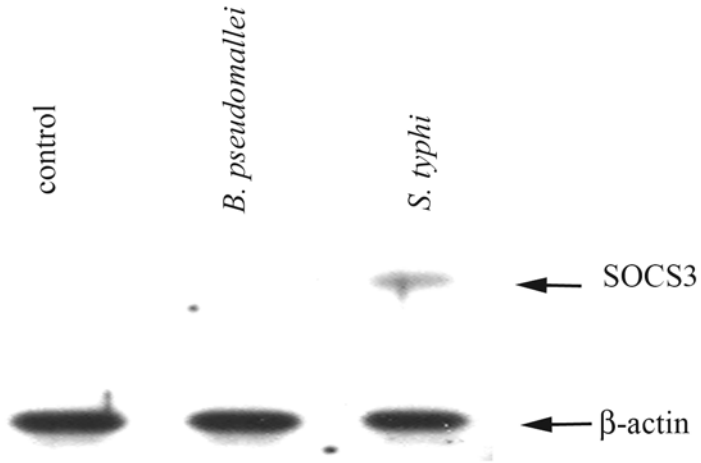


D

Figure 1 Expression of SOCS-1 (A), SOCS-2 (B), SOCS-3 (C) and CIS (D) mRNA in *B. pseudomallei*- and *S. typhi*-infected cells. The mouse macrophages cell line ($1x10^6$ cells/well) was infected with *B. pseudomallei* or *S. typhi* at MOI of 2:1. After four hours of infection, the cells were lysed and the SOCS mRNA expression was determined by RT-PCR.



A



B

Figure 2 Expression of SOCS-1 (A) and SOCS-3 (B) proteins in *B. pseudomallei*- and *S. typhi*-infected cells. The mouse macrophages cell line ($1x10^6$ cells/well) was infected with *B. pseudomallei* or *S. typhi* at MOI of 2:1. After four hours of infection, the cells were lysed and the SOCS proteins expression was determined by immunoblotting.

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Kinetics of SOCS1 and CIS mRNA expression in mouse macrophages infected with *B. pseudomalllei*

To investigate the kinetics of mRNA expression of SOCS1, SOCS2, SOCS3 and CIS, the mouse macrophages cell line were infected with *B. pseudomallei* or *S. typhi* at MOI of 2:1. Extracellular bacteria were removed as described in Material and Methods. At 1, 2 and 4 hours after infection, the infected cells were harvested and the mRNA expression of SOCS1, SOCS2, SOCS3 and CIS were determined by RT-PCR. The results showed that the expression of both SOCS3 and CIS was observed within 1 h after infection and the levels gradually increased with time of infection. In contrast, the macrophages similarly infected with *S. typhi* could activate not only SOCS3 and CIS but also SOCS1 mRNA expression within 1 h of infection (Fig. 3).

Kinetics of SOCS1 and CIS protein expression in mouse macrophages infected with *B. pseudomalllei*

Mouse macrophage cell line was infected with *B. pseudomallei* or *S. typhi* at MOI of 2:1. Four, 6 and 8 hours after infection, protein expression of SOCS1, SOCS3 and CIS in the infected cells were determined by immunoblotting. The result showed that the expression of both SOCS3 and CIS but not SOCS1 in *B. pseudomallei*-infected macrophages was gradually increased from 4 through 8 h of infection. In contrast to *B. pseudomallei*, mouse macrophages infected with *S. typhi* was not only able to activate expression of SOCS3 and CIS protein but *S. typhi* also activated the expression of SOCS1 protein (Fig. 4). These results correlated with the results from RT-PCR which is shown in figure 3.

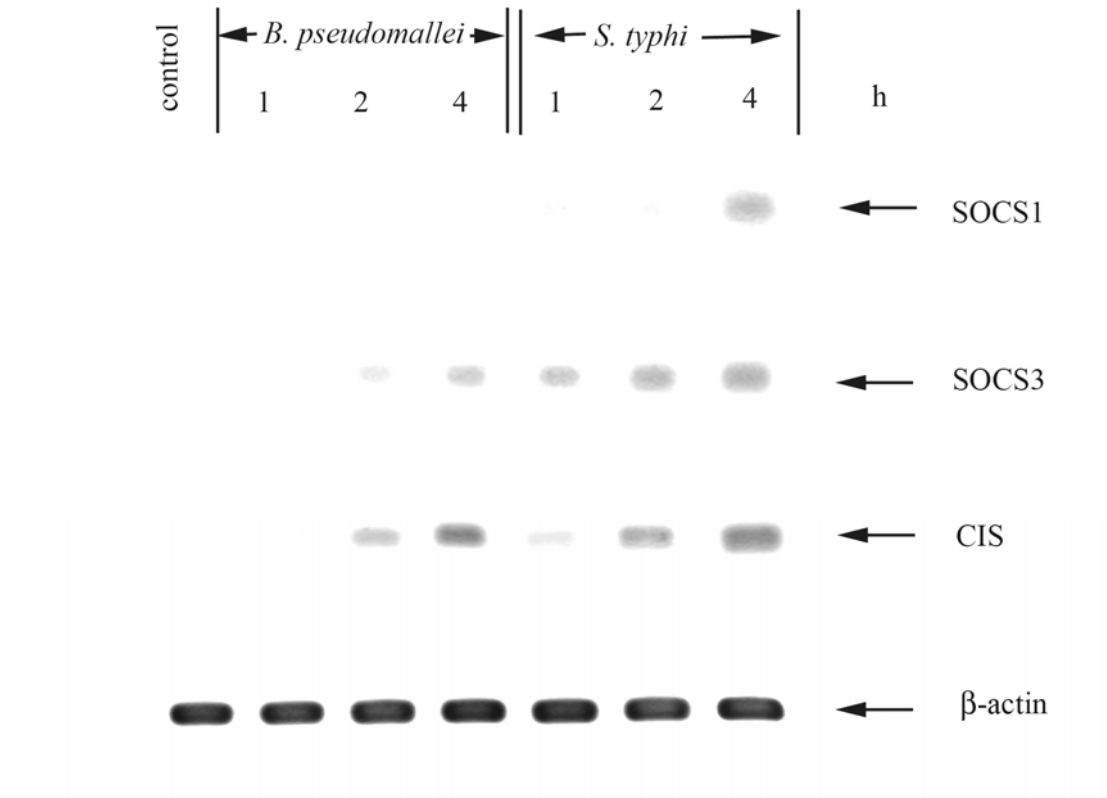

Figure 3 *B. pseudomallei*-induced SOSC3 and CIS expression in macrophages. Mouse macrophages were infected with either *B. pseudomallei* or *Salmonella* (used as reference) at MOI of 2:1. At different time intervals, the infected cells were lyzed and the levels of SOCS mRNA was determined by RT-PCR.

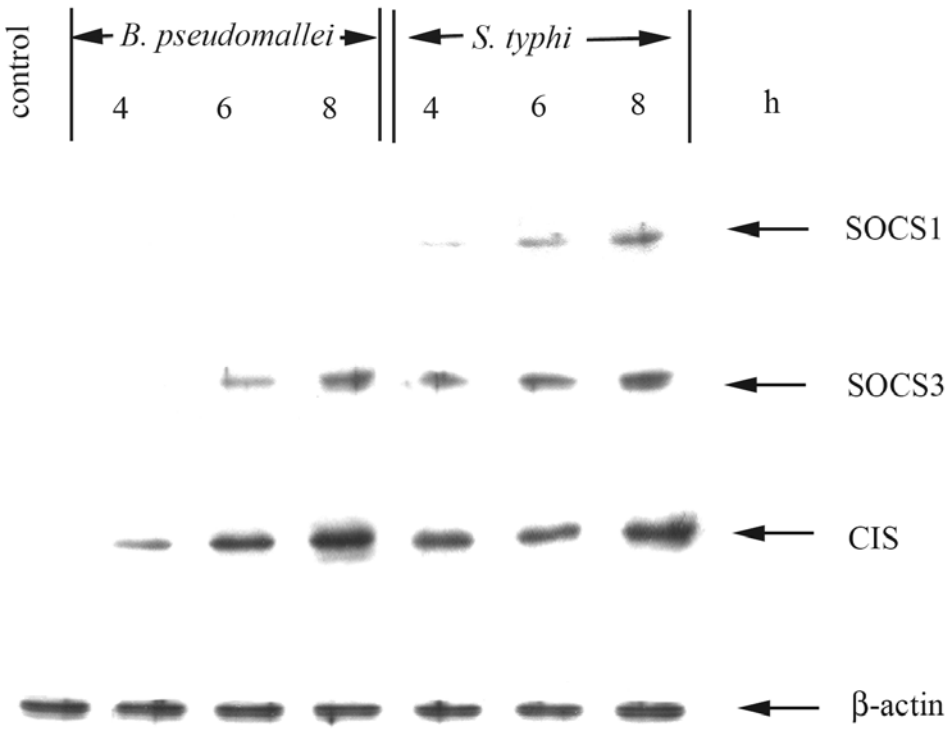

Figure 4 *B. pseudomallei*-induced SOSC3 and CIS expression in macrophages. Mouse macrophages were infected with either *B. pseudomallei* or *Salmonella* (used as reference) at MOI of 2:1. At different time intervals, the infected cells were lyzed and the levels of SOCS protein was determined by immunoblotting.

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Kinetics of SOCS1 and CIS mRNA expression in mouse macrophages activated with heat-killed *B. pseudomalllei*

In order to investigate the activation of SOCS proteins by heat-killed bacteria, the macrophages were treated with heat-killed *B. pseudomallei* or heat-killed *S. typhi* at MOI of 10:1. After 1, 2 and 4 hours of activation, the activated cells were harvested and the mRNA expressions of SOCSs were determined by RT-PCR. The results showed that heat-killed *B. psuedomallei* could activate the expression of SOCS3 and CIS within 1 hour after cell treated and gradually increased with time of infection. In contrast, the macrophages similarly infected with *S. typhi* could activate not only SOCS3 and CIS but also SOCS1 mRNA expression within 1 h of infection (Fig 5).

Kinetics of SOCS1 and CIS protein expression in mouse macrophages activated with heat-killed *B. pseudomalllei*

Mouse macrophages were treated with bacteria with MOI 10:1. Four, 6 and 8 hours after activation, protein expression of SOCS1, SOCS3 and CIS in the activated macrophages were determined by immunoblotting. The result showed that the expression of both SOCS3 and CIS in heat-killed *B. pseudomallei* treated macrophages was gradually increased from 4 through 8 h of activation. In contrast, heat-killed *S. typhi* was not only able to expressed SOCS3 and CIS protein but also activate the expression of SOCS1 protein in (Fig 6). These results are similar to the results from the cells infected with the living bacteria.

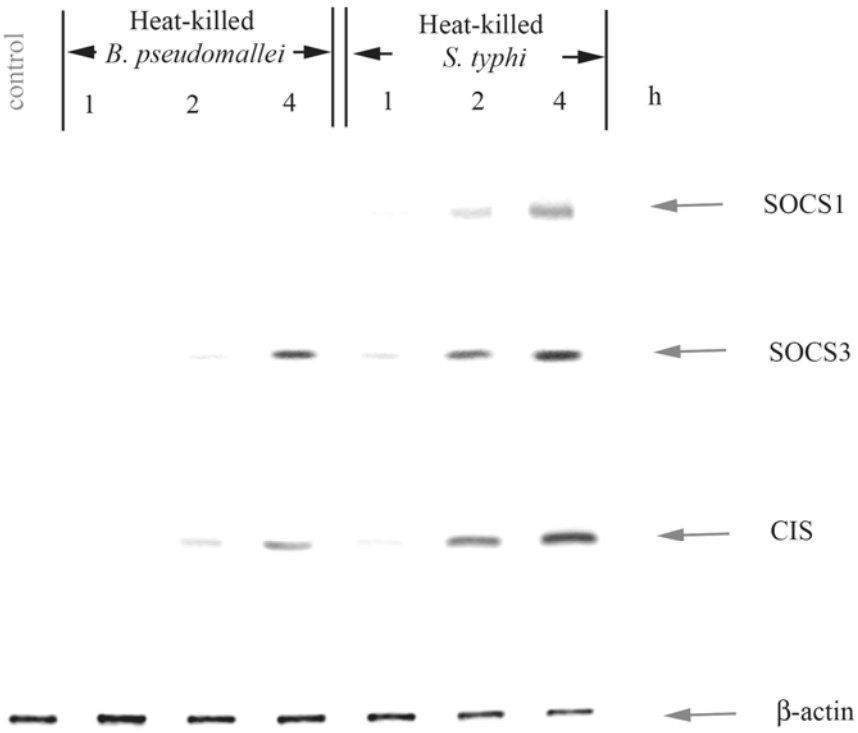

Figure 5 Heat-killed *B. pseudomallei*-induced SOCS3 and CIS expression in mouse macrophages. Mouse macrophages were treated with either heat-killed *B. pseudomallei* or heat-killed *Salmonella* (used as reference) at MOI of 10:1. At different time intervals, the infected cells were lyzed and the levels of SOCS mRNA was determined by RT-PCR.

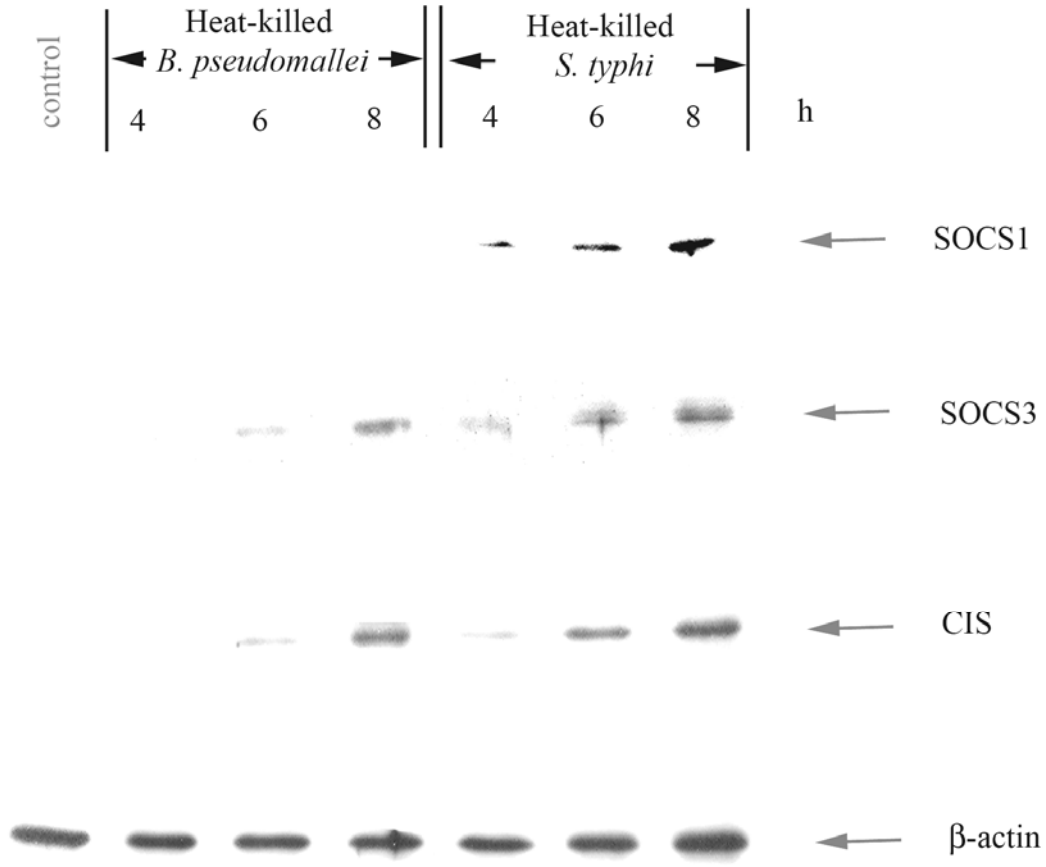

Figure 6 Heat-killed *B. pseudomallei*-induced SOCS3 and CIS expression in mouse macrophages. Mouse macrophages were treated with either heat-killed *B. pseudomallei* or heat-killed *Salmonella* (used as reference) at MOI of 10:1. At different time intervals, the infected cells were lyzed and the levels of SOCS proteins was determined by immunoblotting.

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Expression of SOCS3 and CIS in *B. pseudomallei*-infected macrophages requires internalization of the bacteria

To further investigating whether internalization was required to trigger SOCS expression, the mouse macrophages were pretreated with 2 μg/ml cytochalasin D (Sigma) for 2 h before exposed to *B. pseudomallei*. At 4 and 8 h after infection, the mRNA and protein extracts from the infected cells after infection were analyzed by RT-PCR and immunoblotting, respectively. As shown in Figure 7, the level of SOCS3 and CIS mRNA expression was relatively lower in the cells that were pretreated with cytochalasin D when comparing with untreated infected macrophages. In contrast to SOCS3 and CIS expression, the level of TNF-α gene expression was not altered in the macrophages which were pretreated with cytochalasin D. The SOCS3 and CIS protein expression in the *B. pseudomallei*-infected macrophages was also determined by immunoblotting. Similar to mRNA expression, cytochalasin D was also able to inhibit SOCS3 and CIS protein expression which determined by immunoblotting (Figure 8). It should be noted that cytochalasin D, at concentration used, was able to significantly inhibit invasion of *B. pseudomallei* (data not shown). The inhibition of internalization of non-viable bacteria was also observed. The cytochalasin D-pretreated mouse macrophages were treated with heat-killed *B. pseudomallei* at MOI of 10:1. At 4 and 8 h after infection, the mRNA and protein extracts from the infected cells after infection were analyzed by RT-PCR and immunoblotting, respectively. As shown in Figure 9, the level of SOCS3 and CIS mRNA in *B. pseudomallei*-infected cytochalasin D-treated macrophages was lower than the untreated infected macrophages. Similar to mRNA expression, cytochalasin D was also able to inhibit SOCS3 and CIS protein expression which determined by immunoblotting (figure 10).

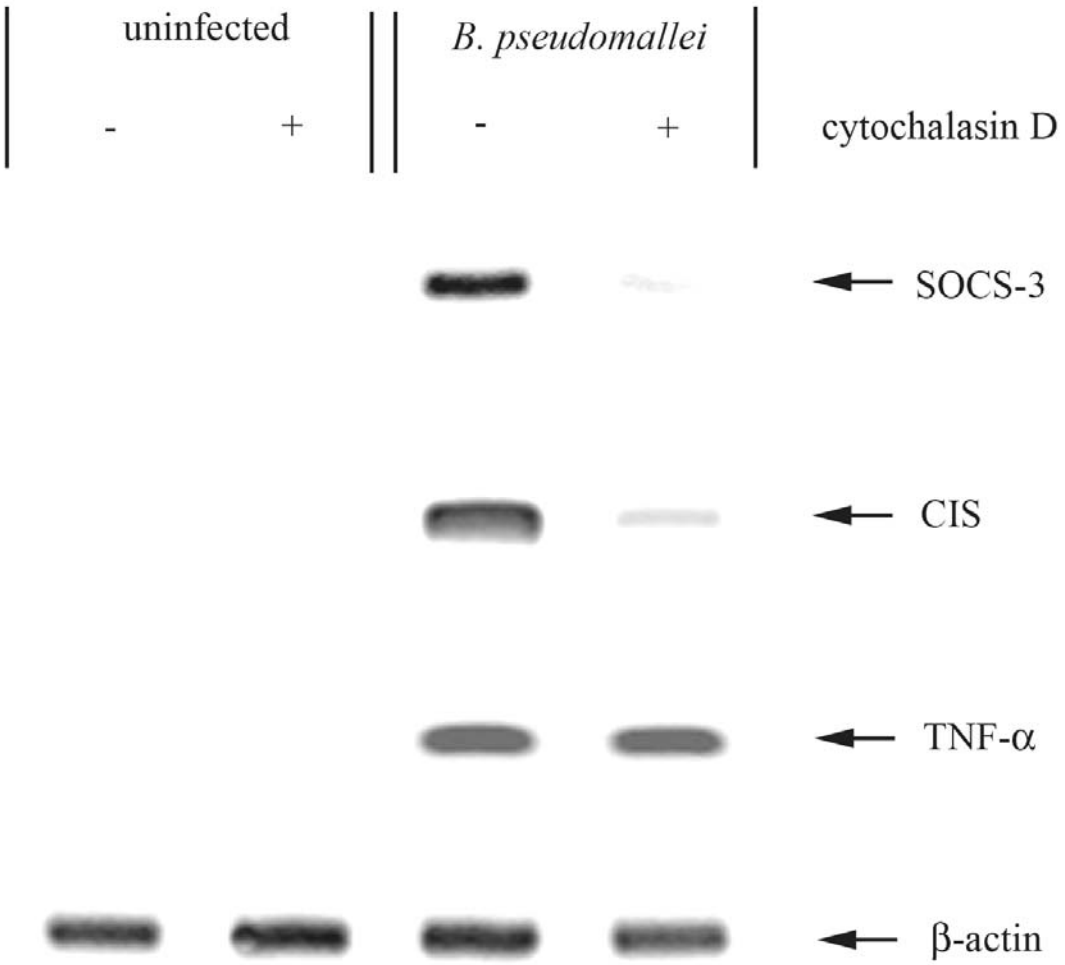

Figure 7 Expression of SOCS3 and CIS mRNA in mouse macrophages required invasion of *B. pseudomallei*. Cytochalasin D (2 μg/ml) was added to the macrophage culture 2 h prior to the time of infection. The pretreated macrophage culture was then infected with *B. pseudomallei* at MOI of 2:1. Four hours after infection, the cells were harvested and total mRNA was extracted and subjected to RT-PCR assay. TNF-α expression was used as a positive control.

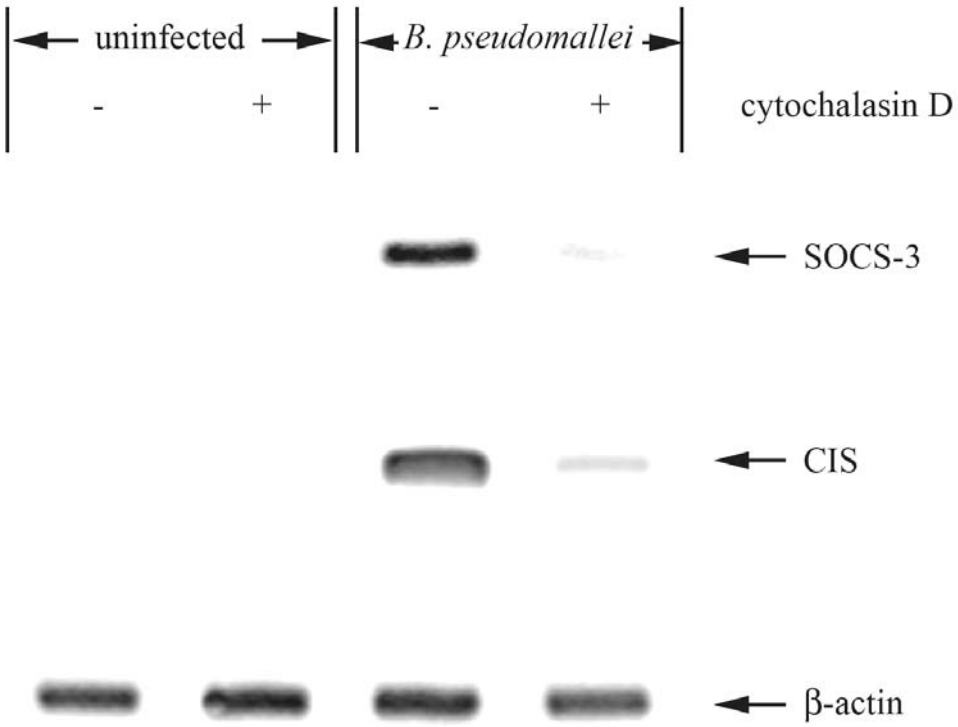

Figure 8 Expression of SOCS3 and CIS proteins in macrophages required invasion of *B. pseudomallei*. Cytochalasin D (2 μg/ml) was added to the macrophage culture 2 h prior to the time of infection. The pretreated macrophage culture was then infected with *B. pseudomallei* at MOI of 2:1. Eight hours after infection, the cells were harvested at 8 h after infection for immunoblotting.

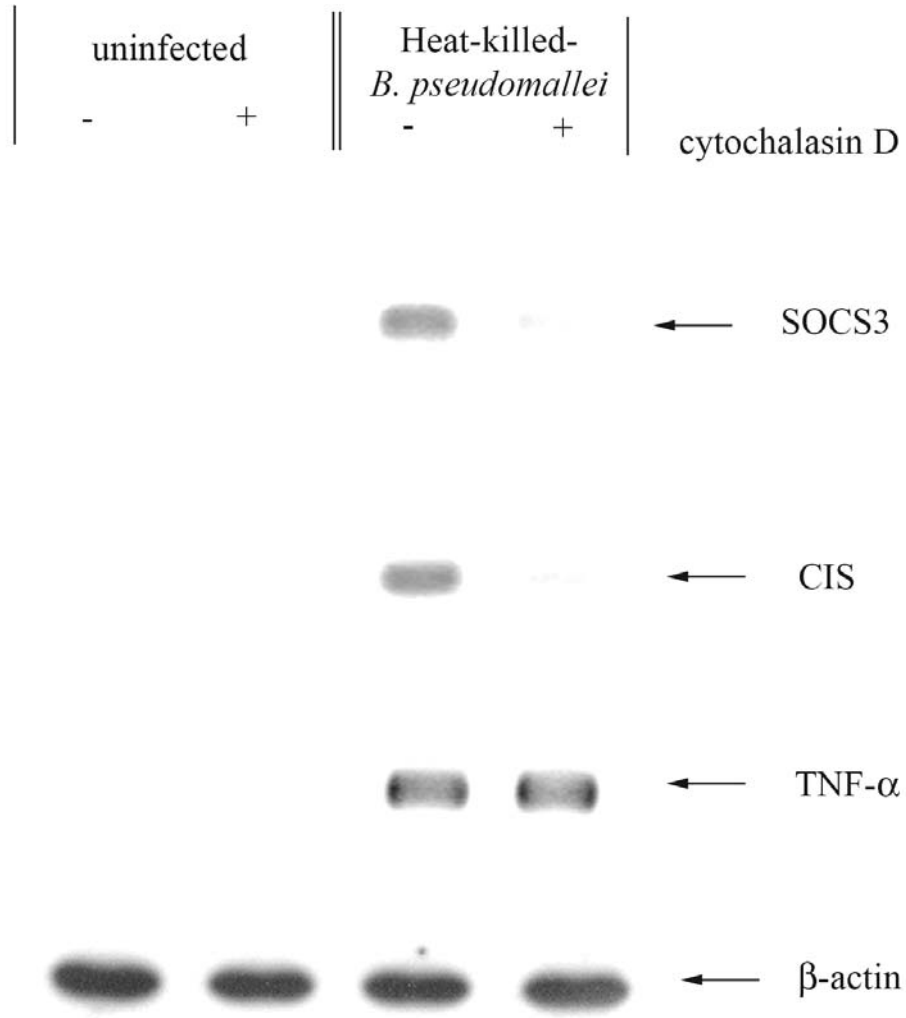

Figure 9 Expression of SOCS3 and CIS mRNA in heat-killed *B. pseudomallei* treated mouse macrophages required internalization. Cytochalasin D (2 μg/ml) was added to the macrophage culture 2 h prior to the time of infection. The pretreated macrophage culture was then treated with heat-killed *B. pseudomallei* at MOI of 10:1. Four hours after treated, the cells were harvested and total mRNA was extracted and subjected to RT-PCR assay. TNF-α expression was used as a positive control.

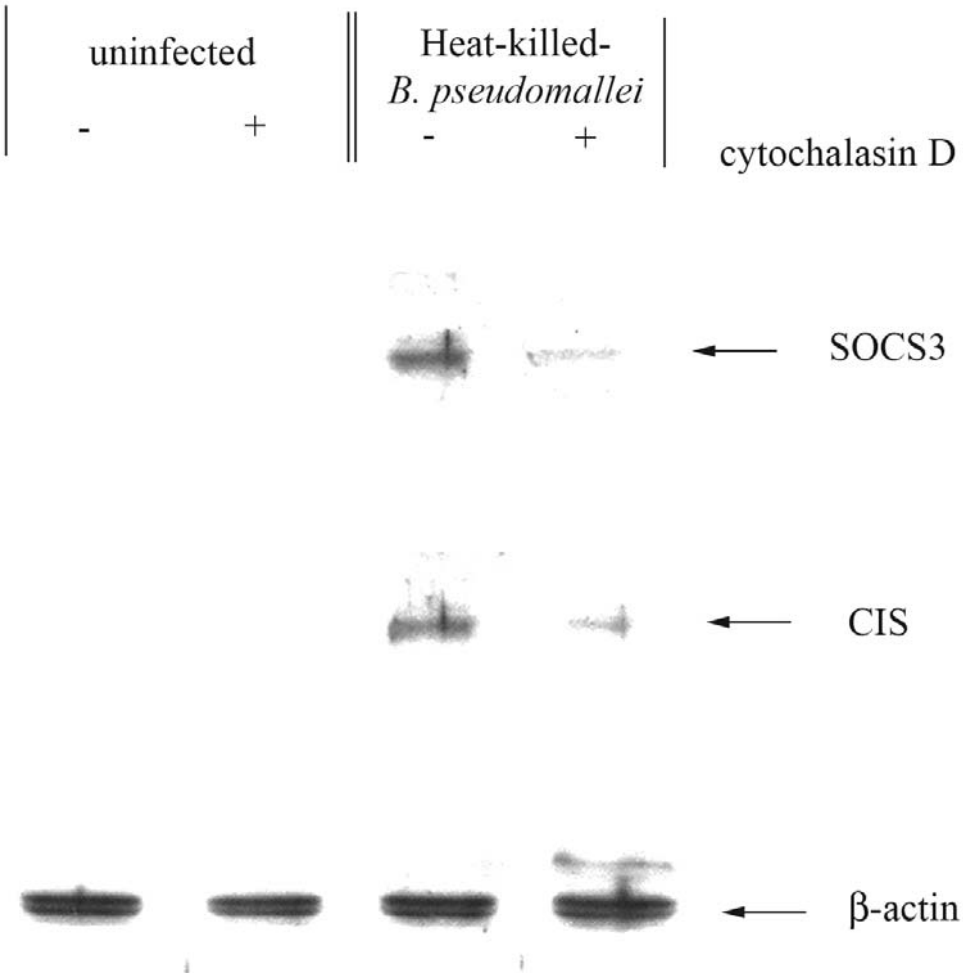

Figure 10 Expression of SOCS3 and CIS proteins in heat-killed *B. pseudomallei* treated mouse macrophages required internalization. Cytochalasin D (2 μg/ml) was added to the macrophage culture 2 h prior to the time of infection. The pretreated macrophage culture was then treated with heat-killed *B. pseudomallei* at MOI of 10:1. Eight hours after infection, the cells were harvested at 8 h after infection for immunoblotting.

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Chloramphenicol inhibits intracellular bacterial replication in *B. pseudomallei*-infected macrophages

In this experiment, *B. pseudomallei* was allowed to interact with the macrophage at MOI of 2:1 for 1 h before culturing in the medium containing 100 μg/ml of chloramphenicol. At this concentration, the chloramphenicol inhibited intracellular bacterial replication and under this condition, the number of intracellular *B. pseudomallei* determined at 4 and 8 h after the infection were $3x10^4$ and $3.1x10^4$, respectively, compared with $6x10^6$ and $6x10^7$ in the untreated macrophages (Figure 11).

Chloramphenicol inhibits SOCS3 and CIS expression in *B. pseudomallei*-infected macrophages

The results presented in Figure 12 and 13 showed that in the presence of chloramphenicol, there was no activation of SOCS3 and CIS mRNA and protein expression, respectively. In contrast, the level of TNF-α mRNA expression was unaltered. Previously, we demonstrated that both living and heat-killed *B. pseudomallei* was able to induce SOCS3 and CIS expression. In order to rule out the possibility that the chloramphenicol itself interfered with SOCS3 and CIS signaling pathway, the chloramphenicol-treated macrophages were first exposed to heat-killed *B. pseudomallei* using a ration equivalent to MOI of 10:1 and 4 h later, the level of SOCS3 and CIS mRNA was determined. The results showed that the level of SOCS3 and CIS mRNA expression was similar in the absence or presence of chloramphenicol (Figure 14), suggesting that Chloramphenicol, at the concentration used, did not directly interfere with the macrophages to activate SOCS3 and CIS expression.

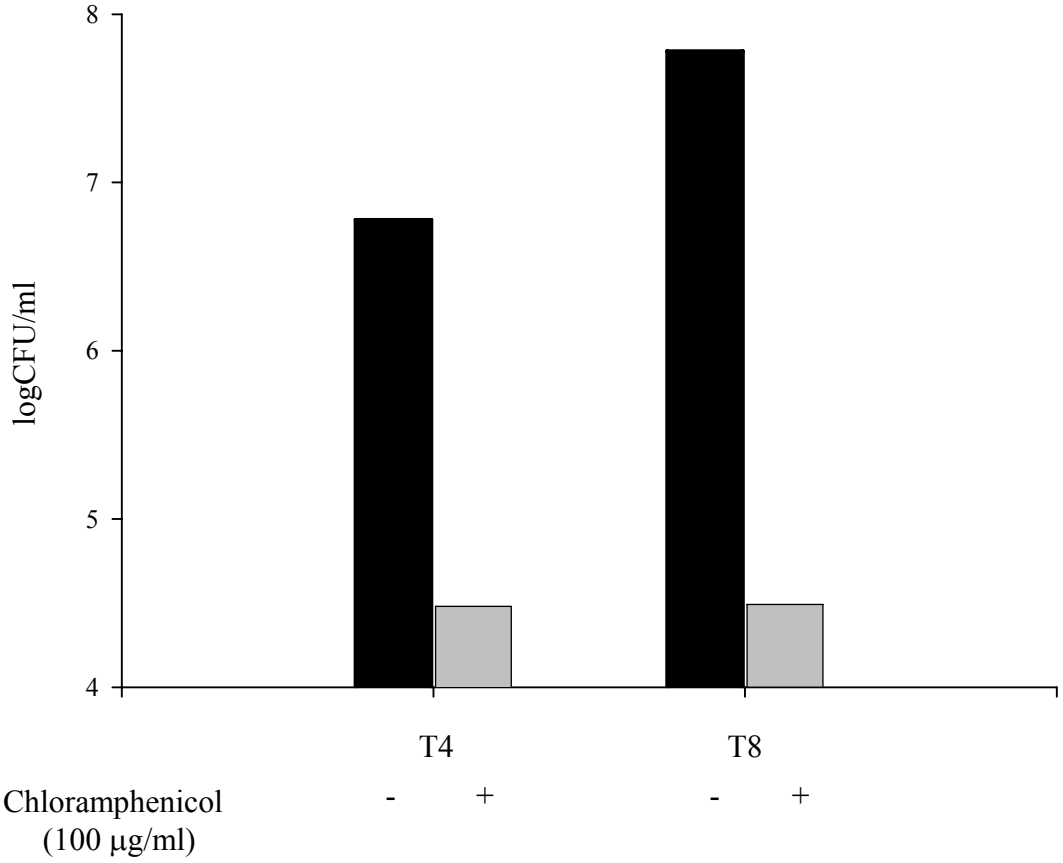

Figure 11 Chloramphenicol inhibits intracellular *B. pseudomallei* replication. Macrophage culture was infected with *B. pseudomallei* at MOI of 2:1 for 1 h before culturing in the medium containing 100 μg/ml of chloramphenicol. At time indicated, the infected cells were lyzed to determine the number of intracellular bacteria.

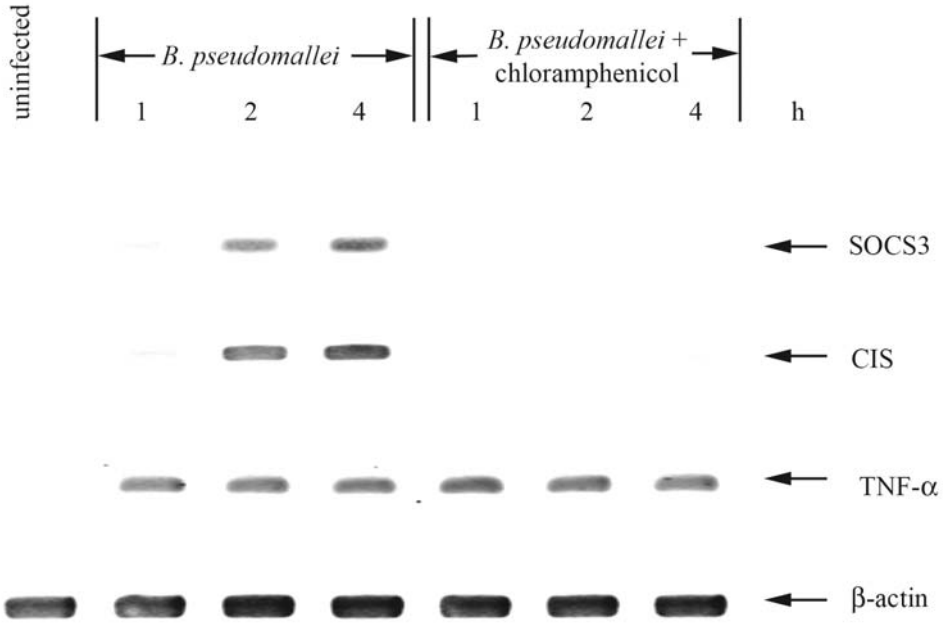

Figure 12 Activation of SOCS3 and CIS gene expression in *B. pseudomallei*-infected macrophages was inhibited by chloramphenicol. Mouse macrophages were exposed to *B. pseudomallei* at MOI of 2:1. To inhibit the replication of internalized bacteria, the macrophages were infected with *B. pseudomallei* as above for 1 h before culturing in the medium containing 100 μg/ml of chloramphenicol. At different time intervals, the infected cells were lyzed and the levels of SOCS mRNAs in the cell lysates were determined by RT-PCR. TNF-α expression was used as a positive control.

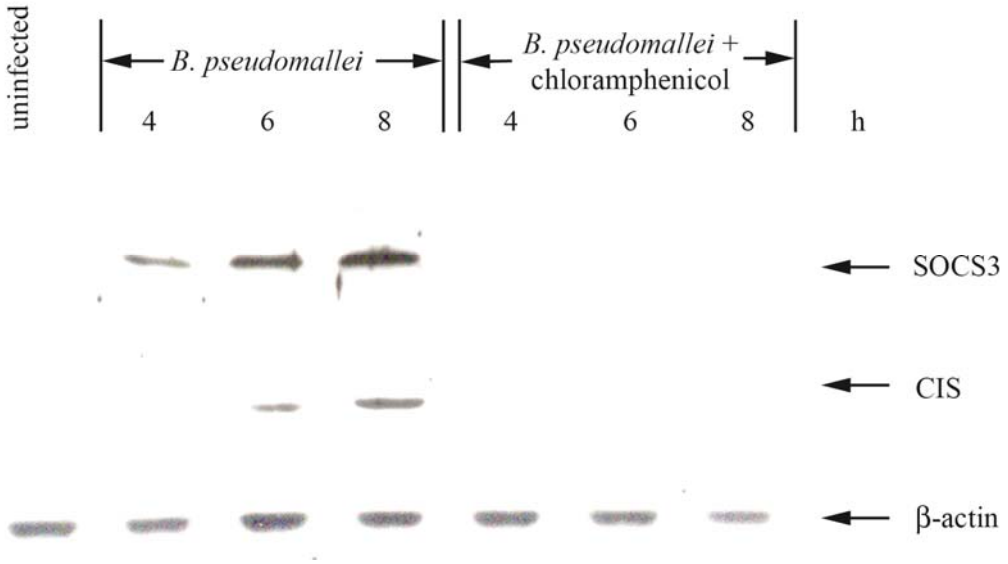

Figure 13 Activation of SOCS3 and CIS protein expression in *B. pseudomallei*-infected macrophages was chloramphenicol. Mouse macrophages were exposed to *B. pseudomallei* at MOI of 2:1. To inhibit the replication of internalized bacteria, the macrophages were infected with *B. pseudomallei* as above for 1 h before culturing in the medium containing 100 μg/ml of chloramphenicol. At different time intervals, the infected cells were lyzed and the levels of SOCS proteins in the cell lysates were determined by immunoblotting.

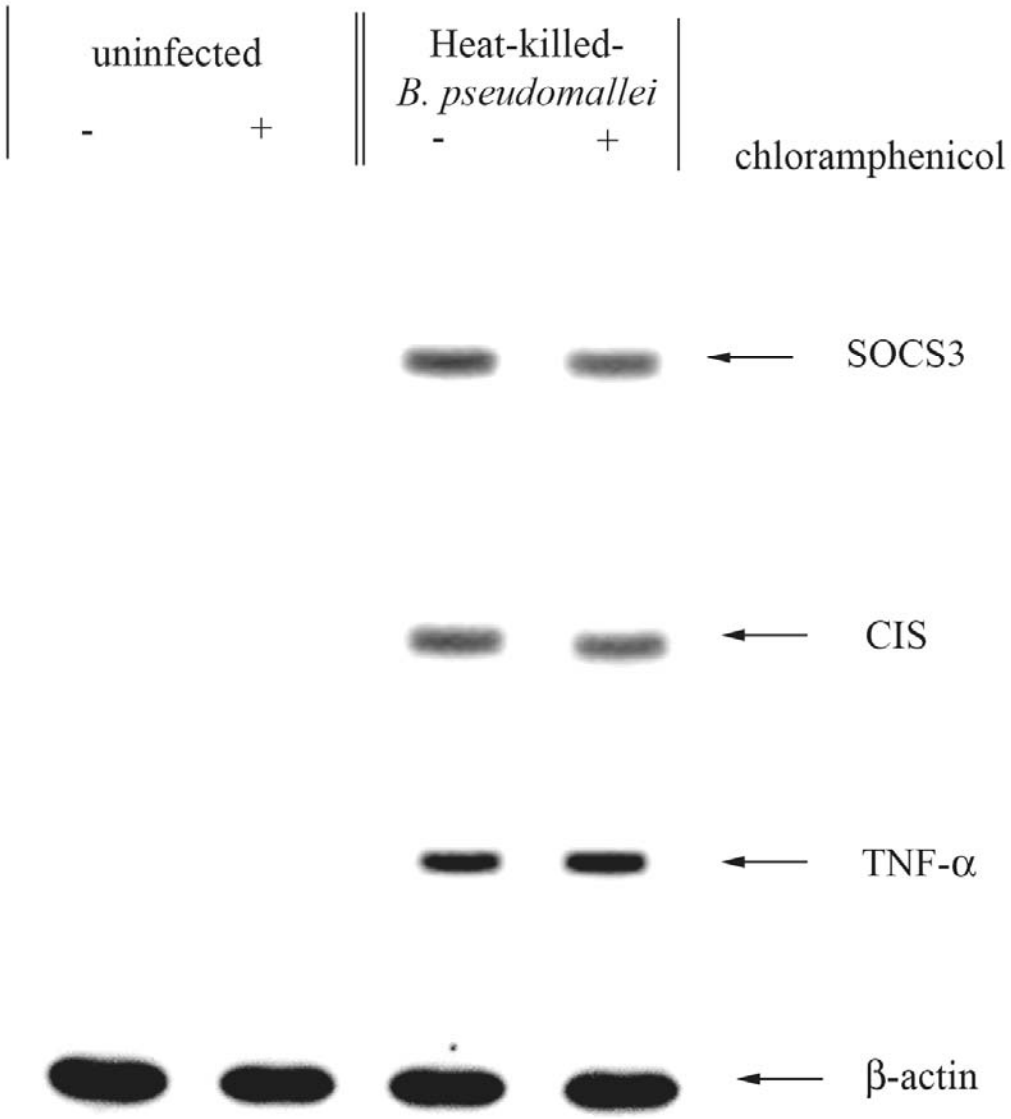

Figure 14 Expression of SOCS3 and CIS genes in heat-killed *B. pseudomallei* treated mouse macrophages. The pretreated macrophage culture was then treated with heat-killed *B. pseudomallei* at MOI of 10:1 for 1 h before culturing in the medium containing 100 μg/ml of chloramphenicol. Four hours after infection, the cells were harvested for RT-PCR.

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B. pseudomallei fails to activate phosphorylation of tyrosine 701 STAT-1 (Y701-STAT-1) in mouse macrophages

To determine the ability of *B. pseudomallei* to stimulate phosphorylation of Y701-STAT-1, the macrophages were infected with *B. pseudomallei* at MOI of 2:1 for one hour. At time indicated, the phosphorylation of Y701-STAT-1 in the infected cells was determined by immunoblotting. The result in Figure 15 showed that the phosphorylation of Y701-STAT-1 could not be observed in the mouse macrophages infected with *B. pseudomallei*. In comparison, the phosphorylation of Y701-STAT-1 could be detected within 2 h after infecting the mouse macrophages with *Salmonella*. IFN-β is known to be able to activate the phosphorylation of STAT-1. In order to determine if the phosphorylation of STAT-1 observed in the macrophages infected with *Salmonella* was due to the ability of *Salmonella* to stimulate IFN-β production, antibody against IFN-β (R&D) was used to neutralize the IFN-β produced by the cells infected with *Salmonella*. In the presence of this antibody, the levels of pY701-STAT1 were markly decreased (Figure 16). Therefore, the phosphorylation of Y701-STAT-1 in the *Salmonella* system was likely mediated through the production of IFN-β. Since *B. pseudomallei* failed to activate the production of IFN-β in the mouse macrophages, the absence of IFN-β may be responsible for the inability to stimulate the phosphorylation of Y701-STAT-1 in the mouse macrophages.

Exogenous IFN-γ added after _B. pseudomallei_ infection fails to activate phosphorylation of Y701-STAT-1

SOCS3 and CIS have been reported to interfere with the IFN-γ signaling by inhibiting Y701-STAT-1 phosphorylation. In order to determine whether the induction of SOCS3 and CIS in *B. pseudomallei*-infected macrophages would

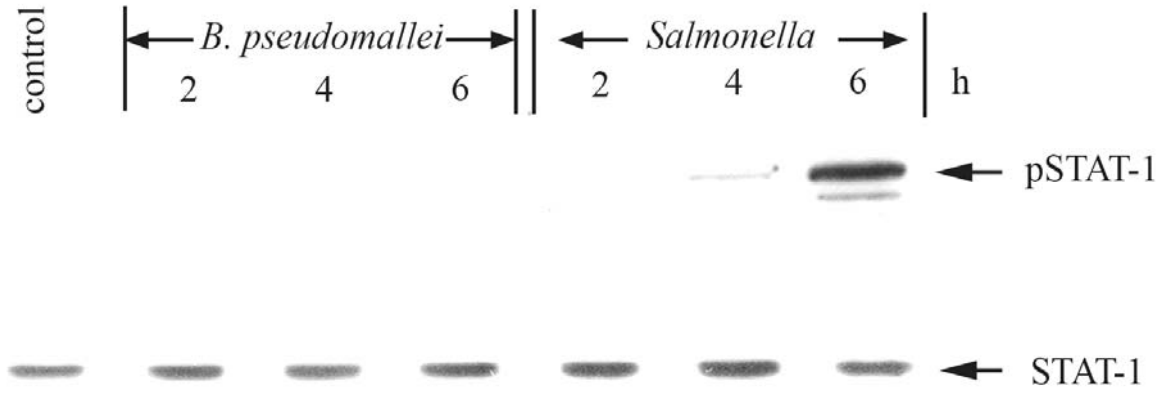

Figure 15 *B. pseudomallei* fails to induce Y701-STAT-1 phosphorylation. Mouse macrophages were infected with *B. pseudomallei* or *Salmonella* at MOI of 2:1 for 1 h. Phosphorylation of Y701-STAT-1 in the infected cell lysate was determined by immunoblotting at different time intervals. STAT-1 protein expression was used as a loading control.

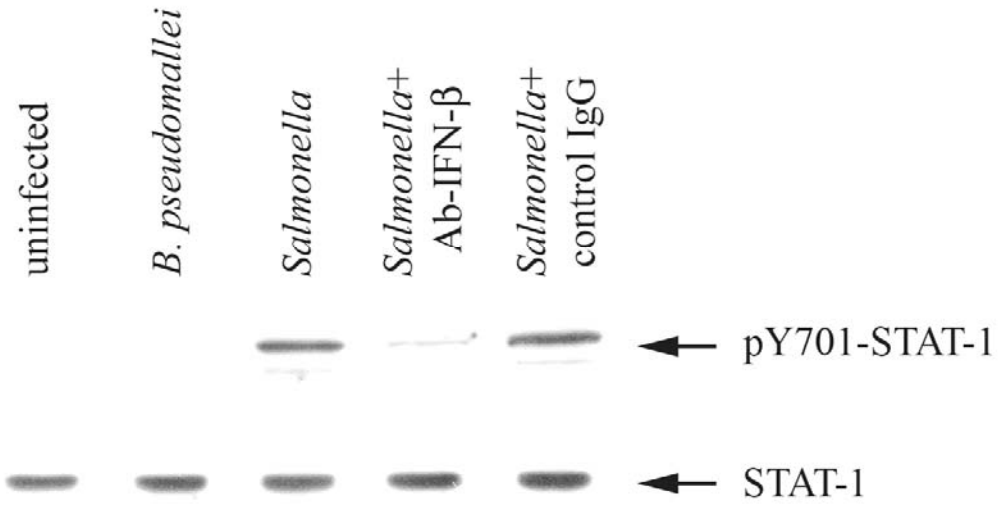

Figure 16 Phosphorylation of Y701-STAT-1 in the *Salmonella*-infected macrophages depends on IFN-β production. Mouse macrophages were infected with *Salmonella* at MOI of 2:1 in the presence of neutralizing antibody against IFN-β (10 μg/ml). After 4 h of infection, the infected cells were lyzed and the level of pY701-STAT-1 in the cell lysate was analyzed by immunoblotting.

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also adversely affect their ability to respond to subsequent IFN-γ activation, the level of pY701-STAT-1 was monitored in *B. pseudomallei*-infected macrophages that were exposed to IFN-γ at 1, 2 and 4 h post-infection. IFN-γ exposure at 1 h post-infection reduced the level of Y701-STAT-1 phosphorylation only slightly, relative to uninfected cells, while exposure at 2 and 4 h post-infection resulted in drastic reductions in the levels of pY701-STAT-1 (Figure 17). It should be noted that the observed decrease in IFN-γ induced Y701-STAT-1 phosphorylation in infected cells directly correlated with increased levels of SOCS3 and CIS as previously demonstrated.

IFN-γ fails to enhance IRF-1 and iNOS expression in the *B. pseudomallei*-infected macrophages

Previously, we demonstrated that IRF-1 and iNOS played an important role in regulating the bactericidal activity of macrophages against *B. pseudomallei*. The induction of both IRF-1 and iNOS expression in uninfected cells is known to require prior phosphorylation of Y701-STAT-1. Therefore, it was of interest to determine of the depressed pY701-STAT-1 level in *B. pseudomallei*-infected macrophages would also influence the expression of IRF-1 and iNOS. In order to address this question, IFN-γ was added to macrophage cultures either at the time of infection (co-stimulation) or at 4 h after infection (post-stimulation). Further 4 h after the addition of IFN-γ, the infected cells were lyzed and the IRF-1 and iNOS expression were then determined by immunoblotting. Consistent with previous report, IFN-γ exposure alone was able to activate IRF-1 but not iNOS expression in uninfected cells (Figure 18). While *B. pseudomallei* infection alone, in the absence of IFN-γ, failed to activate the expression of IRF-1 and iNOS. The expression of IRF-1 and iNOS was observed when IFN-γ was added to the cells at the time of infection (Figure 18). However, the levels of both proteins were noticeably reduced when IFN-γ was added 4 h after the cells were infected. These results suggested that the host cell response to IFN-γ was suppressed in *B. pseudomallei*-infected macrophages and that the suppression

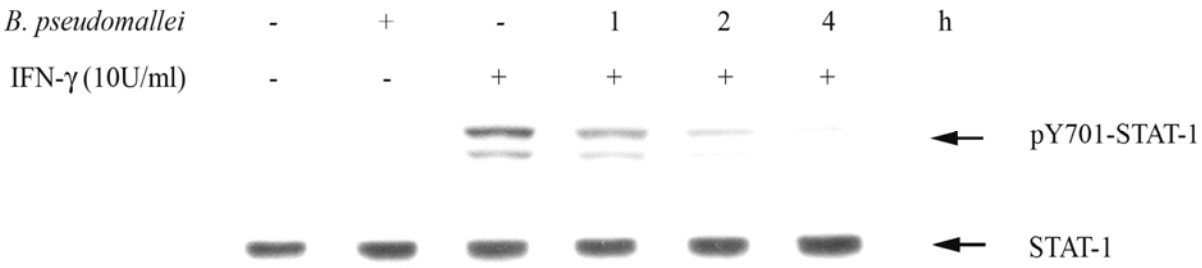

Figure 17 Failure of *B. pseudomallei*-infected macrophages to phosphorylate Y701-STAT-1 in response to IFN-γ stimulation. Mouse macrophages were infected with *B. pseudomallei* at MOI of 2:1 and incubated for 1, 2 and 4 h before IFN-γ (10 U/ml) was added. The cells were incubated further for one hour before the level of pY701-STAT-1 was determined by immunoblotting. Compared with the uninfected control culture, *B. pseudomallei* infection interfered with the macrophage response, particularly when the time interval between bacterial infections was prolonged (e.g., 2 and 4 h).

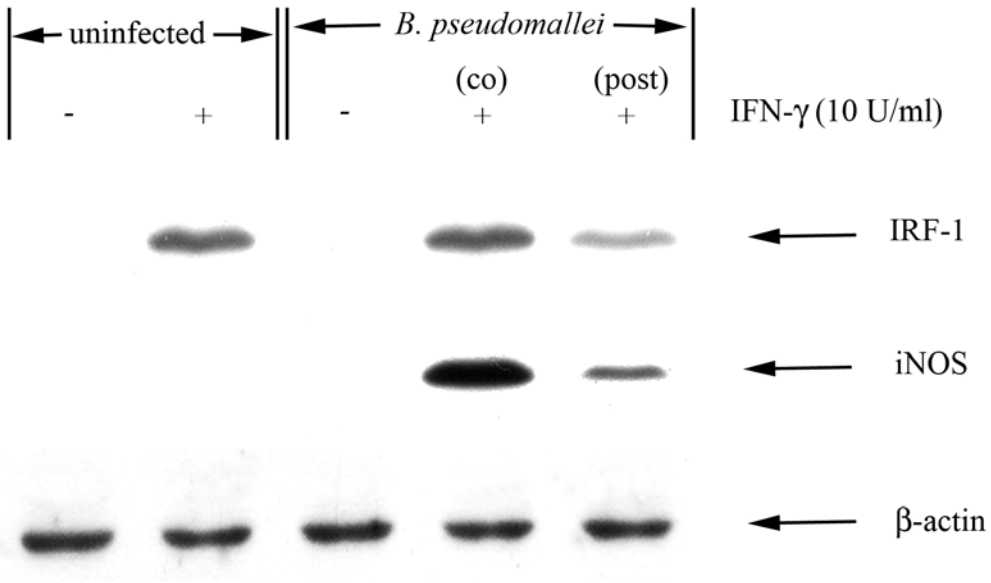

Figure 18 Failure of *B. pseudomallei*-infected macrophages to activate IRF-1 and iNOS expression in response to postinfection IFN-γ stimulation. Mouse macrophages were infected with *B. pseudomallei* at MOI of 2:1 as described in the Materials and Methods section. In the post-infection stimulation (post) experiment, IFN-γ (10 U/ml) was added 4 h after the cells were infected with *B. pseudomallei*. The infected cell cultures were incubated for one more hour before excess IFN-γ was removed. The cell cultures were then incubated for a further 3 h and the macrophages were lyzed and the levels IRF-1 and iNOS in the lysates were determined by immunoblotting. For the co-stimulation (co) experiment, the macrophages were infected with *B. pseudomallei* in the presence of IFN-γ (10 U/ml). After 1 h of incubation, the macrophages were washed 3 times with PBS and cultured for 3 more hours before the levels of IRF-1 and iNOS in the cell lysate were determined by immunoblotting.

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required time to establish. The suppression of both IRF-1 and iNOS correlated with both the decrease of the pY701-STAT-1 level shown in Figure 17.

IFN-γ fails to enhance killing of intracellular *B. pseudomallei* in infected macrophages

Previously, the addition of exogenous IFN-γ to the macrophages either prior to or at the time of *B. pseudomallei* infection could significantly enhance killing of intracellular *B. pseudomallei*, suggesting that this was likely attributable to the enhanced production of iNOS. It was of interest to determine whether a post-infection incubation had any effect on the observed IFN-γ-induced enhance killing. Co- and post-stimulation of *B. pseudomallei*-infected macrophages with IFN-γ was performed as described in Materials and Methods with the exception that the number of intracellular bacteria was determined 4 h after IFN-γ addition. As shown in Figure 19, addition of IFN-γ 4 h after infection failed to enhance the intracellular killing of *B. pseudomallei*. Viable intracellular *B. pseudomallei* levels were significantly higher in cells that received post-stimulation with IFN-γ relative to the cells that were co-stimulated at the time of infection (Figure 19). The failure of IFN-γ to stimulate killing of intracellular *B. pseudomallei*, when added to macrophages at 4 post-infection, correlated well with the reduced IRF-1 and iNOS expression observed in Figure 18.

***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, SOCS3) and MyD88-independent pathway (iNOS, IFN-β, IRG1, SOCS1) were

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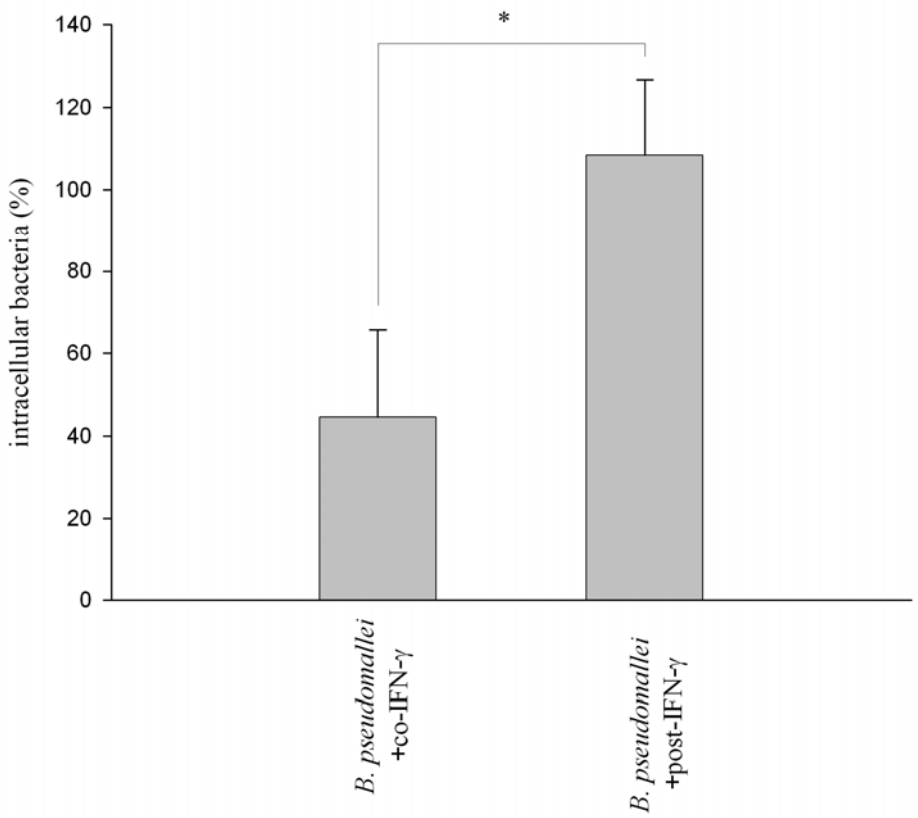

Figure 19 Inability of IFN-γ to enhance antimicrobial activity of *B. pseudomallei*-infected macrophages. The experimental protocol for this experiment was similar to the one described in Figure 18 with the except that the viability of intracellular bacteria was determined and the results of post stimulation and co-stimulation conditions were compared. The data indicate the means and s.d. of 3 separate experiments, each carried out in duplicate. * $P<0.01$ by Student's *t*- test.

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 20, 21). 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 22, 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.

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 23, 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*.

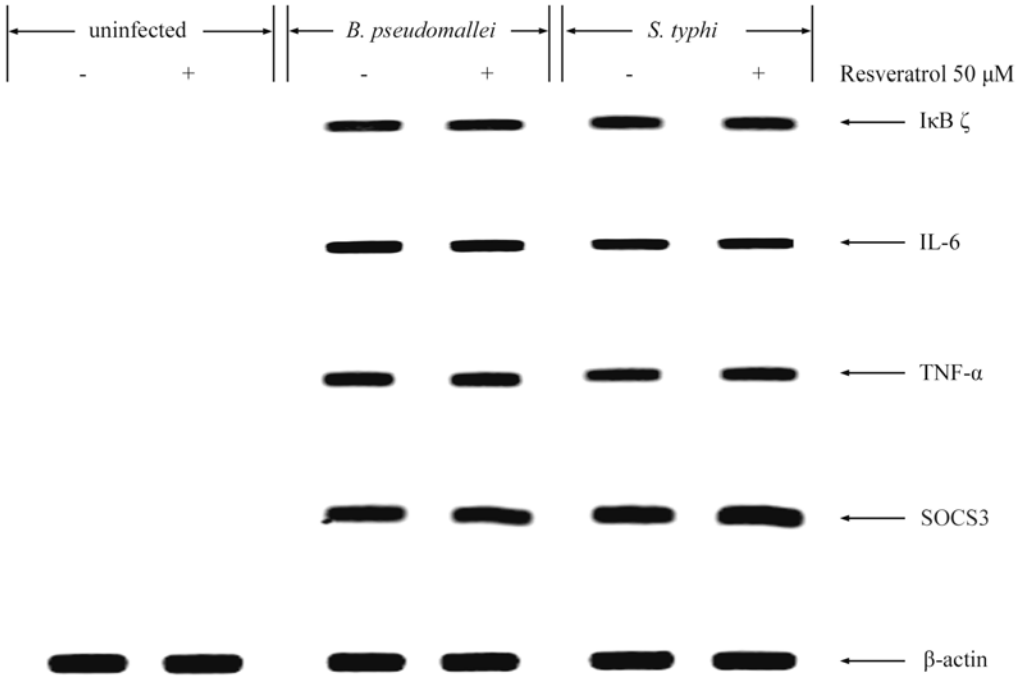

Figure 20 Expression of mRNAs via MyD88-dependent pathway in the bacterial infected macrophages. Mouse macrophages were treated with or without resveratrol (50μM) for 1 h before exposed with *B. pseudomallei* or *S. typhi* at MOI of 2:1. After 4 h of infection, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

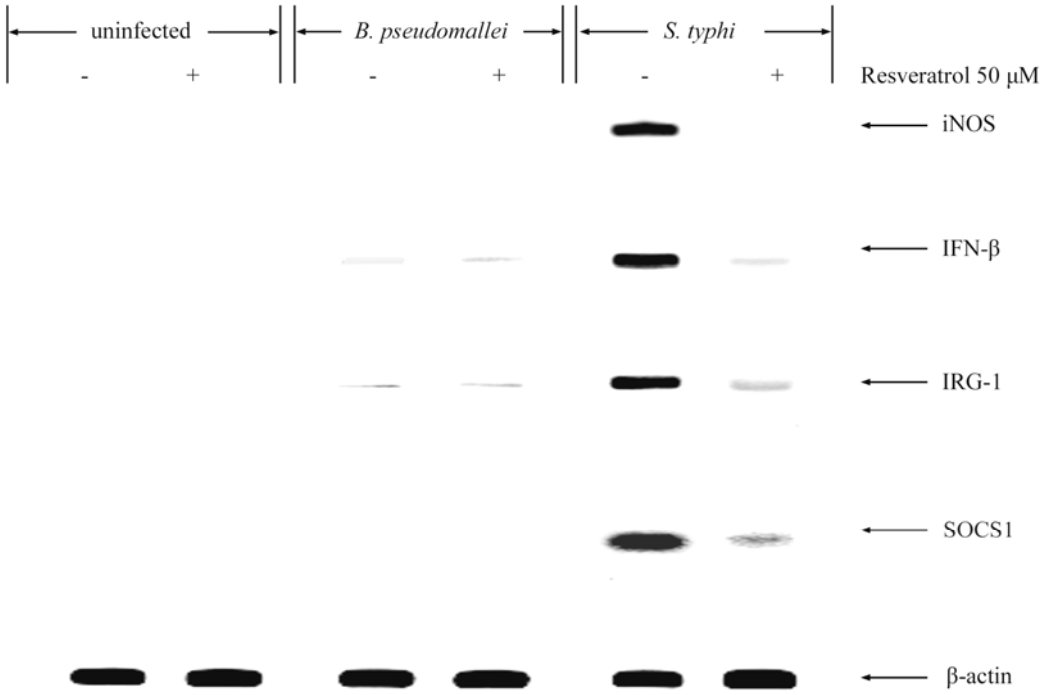

Figure 21 Expression of mRNAs via MyD88-independent pathway in bacterial infected macrophages. Mouse macrophages were treated with or without resveratrol (50μM) for 1 h before exposed with *B. pseudomallei* or *S. typhi* at MOI of 2:1. After 4 h of infection, the infected cells were harvested and mRNA expression was determined by RT-PCR. β-actin mRNA expression served as an internal control.

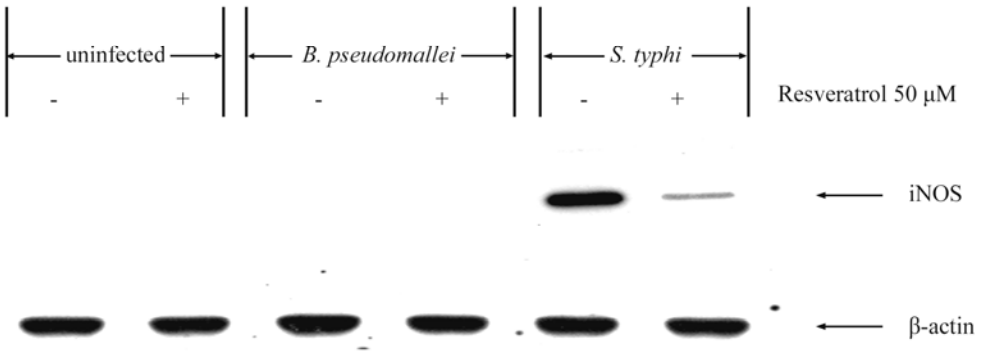

Figure 22 Ability of resveratrol to suppress iNOS protein in *S. typhi*-infected mouse macrophages. Mouse macrophages were pretreated with resveratrol (50μM) for 1 h before exposed with either *B. pseudomallei* or *S. typhi* at MOI of 2:1. After 8 h of infection, cell lysates were analyzed for iNOS and β-actin immunoblots.

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Exogenous IFN-γ synergistically enhances B. pseudomallei-infected macrophages gene expression and suppresses intracellular survival of the bacteria.

The macrophages were pretreated with IFN-γ (10 U/ml) overnight before being infected with *B. pseudomallei* at MOI of 2:1. Four hours after the infection, the gene expression of the infected cells were determined by RT-PCR. As shown in Figure 24, IFN-γ was able to restore ability of the *B. pseudomallei*-infected macrophages to express the genes downstream of the MyD88-independent pathway. The expression of these genes also correlated with the inhibition of intracellular replication (Figure 25).

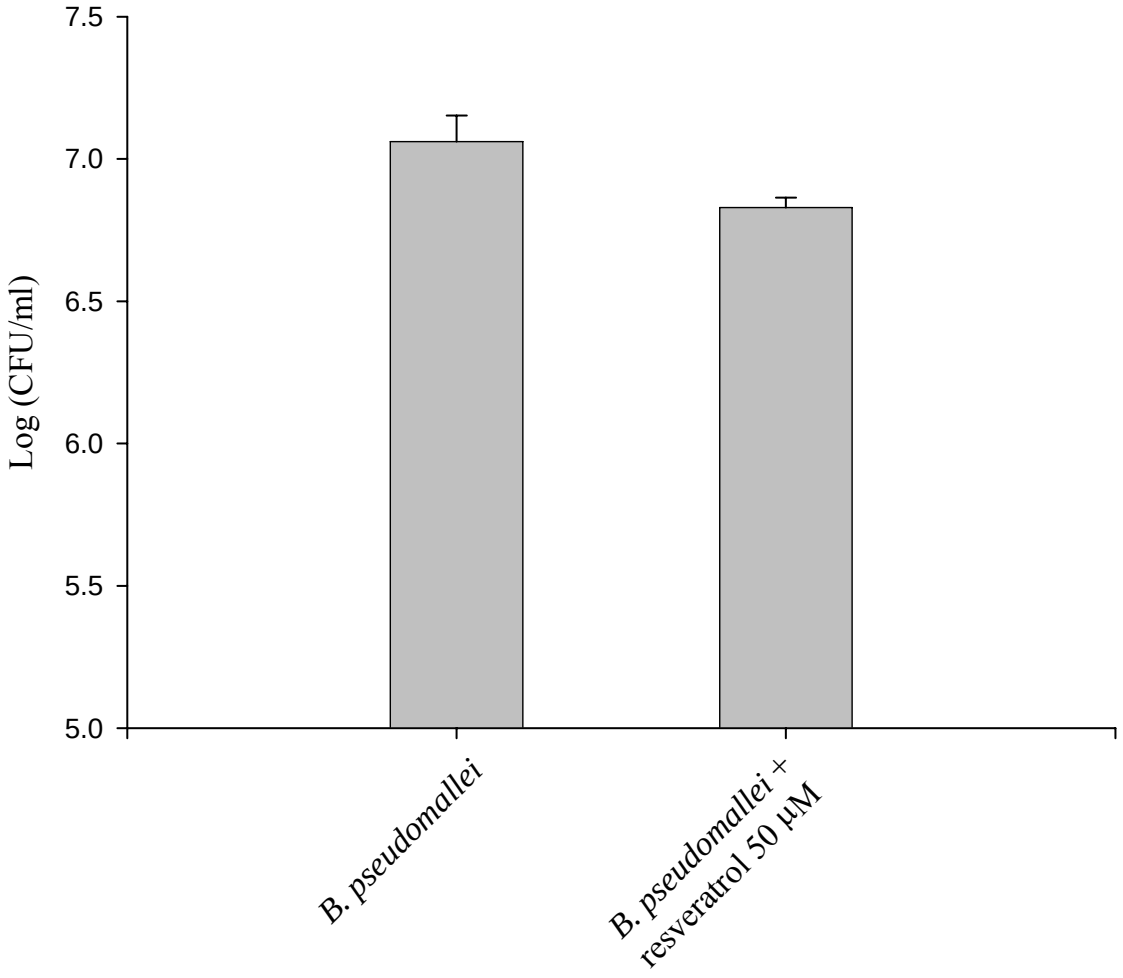

Figure 23 Resveratrol does not interfere with intracellular survival of *B. pseudomallei* inside the mouse macrophages. Mouse macrophages were treated with resveratrol (50µM) for 1 h before infecting with *B. pseudomallei* at MOI of 2:1. At 8 h after infection, the cells were lyzed with 1 ml 0.1% TritonX-100 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.

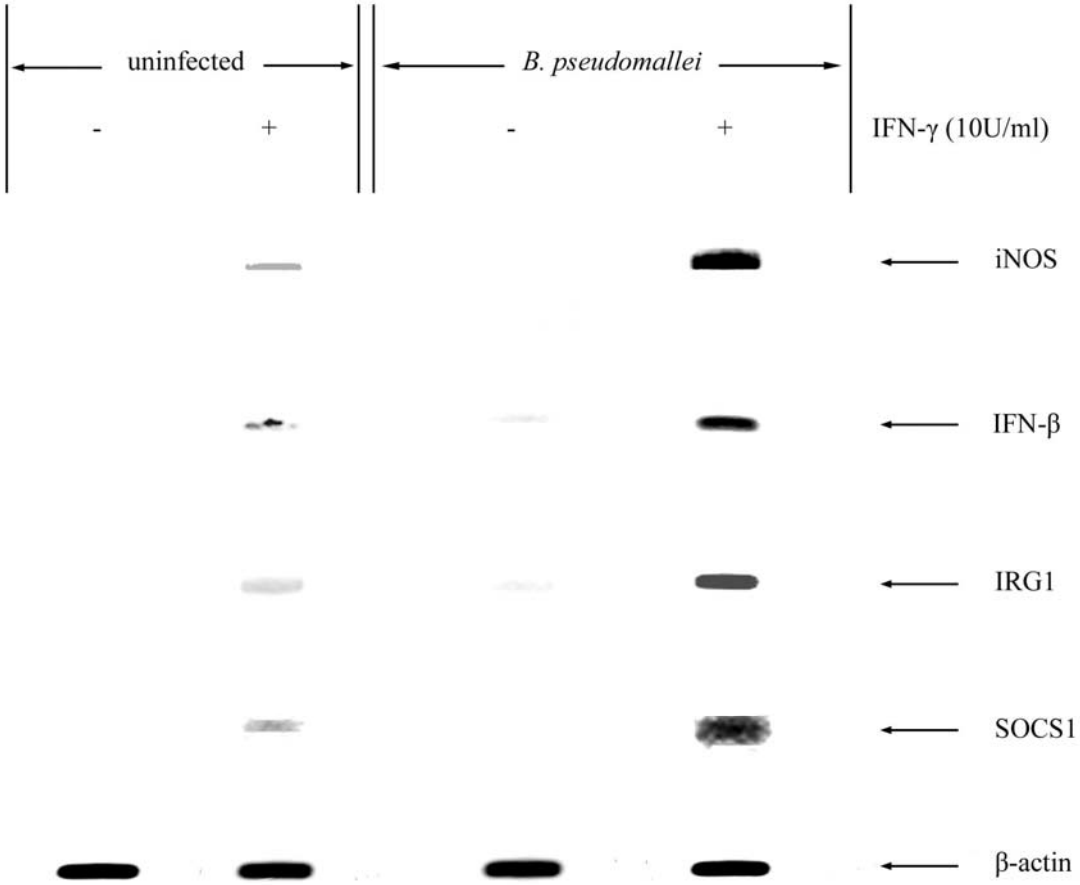

Figure 24 IFN-γ synergistically enhances gene expression downstream of the MyD88-independent pathway. The macrophages were pretreated with IFN-γ (10 U/ml) overnight before being infected with *B. pseudomallei* at MOI of 2:1. After 4 h of infection, the infected cells were lysed and the gene expression was determined by RT-PCR

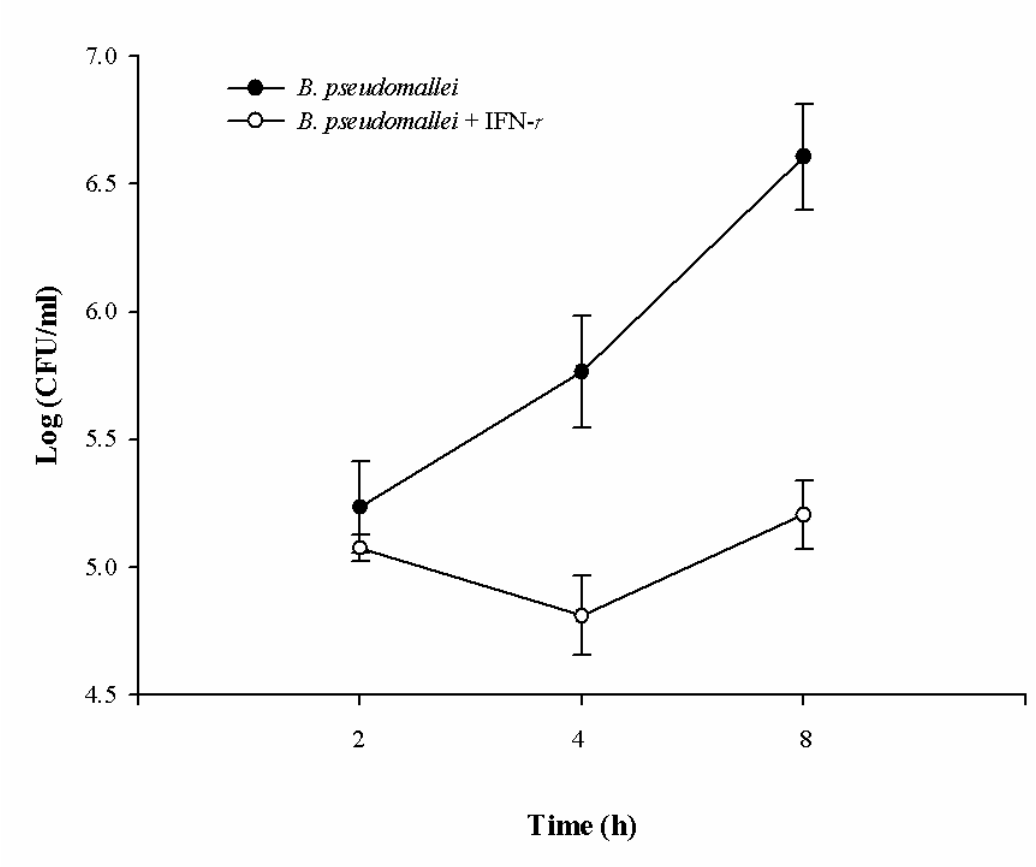

Figure 25 IFN-γ synergistically suppresses intracellular replication of *B. pseudomallei*. The macrophages were pretreated with IFN-γ (10 U/ml) overnight before being infected with *B. pseudomallei* at MOI of 2:1. After 2, 4 and 8 h of infection, the number of intracellular bacteria was determined by standard antibiotic protection assay.

Discussion

Induction of SOCS expression and its significance has been extensively studied in recent years. For example, *Leishmania donovani*-infected macrophages were reported to induce SOCS3 expression that resulted in suppression of macrophage activation (6). Activation of SOCS3 expression has also been reported in macrophages infected with gram-positive bacteria (e.g. *L. monocytogenes* and *Staphylococcus aureus*) (6, 42). Induction of SOCS proteins by isolated bacterial components like LPS has also been demonstrated; however, very limited information on the regulation of these proteins in response to infection with gram-negative bacteria is currently available (9). This study clearly demonstrated that there is a novel association between the intracellular gram-negative bacterium *B. pseudomallei* and the induction of SOCS3 and CIS expression in an *in vitro* mouse macrophage model (Fig. 3, 4). However, it was found that unlike other gram-negative bacteria, such as *S. enterica* serovar Typhi, *B. pseudomallei* failed to induce SOCS1 expression (Fig. 3, 4), suggesting that the mechanisms employed by these different gram-negative bacteria to modulate the macrophage response is different.

The pivotal role of the transcription factor STAT-1 in the innate immune response against microbial infection is well documented (13). STAT-1 deficient mice are highly sensitive to stimulation by intact organisms or isolated individual components (33). Increased susceptibility to intracellular bacteria, such as *Mycobacterium*, has also been observed in STAT-1 deficient human macrophages (14). Purified LPS has also been shown to activate phosphorylation of Y701-STAT-1 through TLR, and this correlated with IFN-β synthesis, suggesting that the activation of pY701-STAT-1 is mediated through type I IFN synthesized by LPS-activated cells (28, 31). The data presented in this study which demonstrated that the gram-negative bacterium *S. enterica* serovar Typhi was able to activate Y701-STAT-1 phosphorylation after only 2 h of infection and that the process could be inhibited by neutralizing antibody against IFN-β (Fig. 16) lend support to this idea. In contrast to *S. enterica*

serovar Typhi, *B. pseudomallei* failed to stimulate pY701-STAT-1 (Fig. 15), a result that was consistent with the organism's inability to activate IFN-β production (48). In addition to IFN-β, IFN-γ could also activate phosphorylation of STAT-1. Activation of this transcription factor is known to regulate SOCS1 expression (17). By contrast, STAT-1 deficient mice could still activate SOCS3 expression in response to IFN-γ stimulation, suggesting that SOCS3 gene regulation does not involve activation of STAT-1 (17). It is possible that the inability of *B. pseudomallei* to activate SOCS1 expression in our study was also related to its inability to activate phosphorylation of STAT-1 (Figs. 3 and 15, 16).

Of the different SOCS members that have been studied, SOCS1 is probably the most potent inhibitor of IFN signaling, and it acts by suppressing the phosphorylation of STAT-1 (7, 26). SOCS3, on the other hand, is known to play an essential role as a negative inhibitor of IL-6, possibly by interfering with the activation of another transcription factor, STAT-3 (10, 29, 53). Based on the data from the STAT-1 phosphorylation studies presented here, it appeared that SOCS3 and CIS could also inhibit IFN-γ signaling, albeit to a lesser extent than they could with STAT-3 (5, 11). Macrophages infected with *L. monocytogenes* also exhibited diminishing IFN-γ-induced Y701-STAT-1 phosphorylation (42). A decreased response to IFN-γ signaling in *L. monocytogenes* and *L. donovani*-infected macrophages correlated with the induction of SOCS3 expression (6, 42). In the present study, we demonstrated that the gram-negative bacterium *B. pseudomallei* interfered with IFN-γ-induced Y701-STAT-1 phosphorylation in a mouse macrophage system (Fig. 17). The suppression of STAT-1 phosphorylation also directly correlated with the expression of SOCS3 and CIS (Fig. 3). On the basis of all of the results, it is tempting to conclude that activation of SOCS3 and CIS expression in response to *B. pseudomallei* infection can interfere with IFN-γ signaling, thus resulting in suppression of IFN-γ inducing proteins (e.g., IRF-1 and iNOS expression) (Fig. 18). Both IRF-1 and iNOS are known to play a crucial role in controlling the fate of intracellular pathogens (31, 45). In the present study,

B. pseudomallei-infected macrophages not only failed to express both IRF-1 and iNOS, but also failed to eliminate intracellular *B. pseudomallei* (Fig. 19).

During the last few years, our group has been working on elucidating the mechanism by which *B. pseudomallei* modulates the innate host defense, thus allowing it to survive inside macrophages (47, 48). This intracellular bacterium can invade macrophages without stimulating IFN-β production, which is an essential factor for IRF-1 and iNOS expression (48). Failure to activate IRF-1 and iNOS provides the bacteria with the means to survive the antimicrobial activity of macrophages (47, 48). However, exogenous IFN-γ or IFN-β added prior to or at the time of infection could enhance both IRF-1 and iNOS expression and restore antimicrobial activity of these macrophages (49). The protective role of IFN-γ in the host defense against *B. pseudomallei* has been observed previously in animal models (37). Administration of neutralizing antibody against IFN-γ could significantly lower the 50% lethal dose and also increase bacterial burdens in the liver and spleen of these animals (37). More recently, we demonstrated that the IFN-γ level in the serum of infected BALB/c mice was significantly higher than that in the serum of uninfected mice, yet the high level of IFN-γ failed to eliminate the bacteria from the blood, spleen, liver, and lungs and these animals subsequently died over the next 7 to 10 days (50). The data for this animal model are in accord with the elevated level of IFN-γ noted in patients with severe melioidosis (30). Together, these results suggest that the presence of high IFN-γ levels during ongoing *B. pseudomallei* infection is not sufficient to control the infection or to eliminate the bacteria from the infected host. The failure of IFN-γ to protect the animals from fatal sepsis (50) could be explained by the ability of *B. pseudomallei* to activate negative regulator proteins, such as SOCS3 and CIS, as demonstrated in the present study. The expression of these proteins can interfere with IFN-γ signaling via a STAT-1 pathway, making the infected macrophages unable to respond to IFN-γ. This, in turn, results in an inability to activate IFN-γ - responsive protein expression (i.e., IRF-1 and iNOS). Therefore, in addition to interference with proinflammatory cytokine production and antimicrobial activity, the results obtained in the present study provided evidence for another possible

mechanism by which *B. pseudomallei* could modulate the macrophage bactericidal response, thus facilitating its intracellular survival, and may also explain the persistence of the organism in the human host and the high rate of relapse in melioidosis.

In the innate immunity, Toll-like receptors (TLRs) is a skillful system that detects invasion of microbial pathogens (43). 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 (32, 46). 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 (1, 44). 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 (51). 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 (1, 44).

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 (48). These results indirectly implied that *B. pseudomallei* may not activate MyD88-independent pathway of macrophages. The result from the present study demonstrate that *B. pseudomallei* is capable to stimulate the gene expression only through MyD88-dependent pathway (IκBζ, IL-6, TNF-α, SOCS3) (Figure 20) and failed to stimulate a number of gene known to require MyD88-independent pathway (IFN-β, IRG1, iNOS, SOCS1)

(Figure 21). This is in contrast to *S. typhi* which is capable of activating the gene expressions utilizing both MyD88-dependent and -independent pathways (Figure 20, 21). 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 20, 21). It also did not interfere with the intracellular survivor of *B. pseudomallei* (Figure 23), 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 20, 21, 22). These results suggested that MyD88-independent pathway is essential to control intracellular survival of *B. pseudomallei* in macrophage. Failure to stimulate this pathway facilitate *B. pseudomallei* to survive and multiply inside the macrophage.

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 (4, 24, 27, 36). Later on, the activated Jaks phosphorylate a tyrosine-containing sequence, thereby forming paired binding sites for STAT1 that interact through their SH2 domains (18, 21). 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 (31, 40, 45). As mentioned earlier, *B. pseudomallei*-infected macrophages failed to activate IRF-1, iNOS causing a failure to eliminate intracellular *B. pseudomallei* (15, 47, 48). However, exogenous IFN-γ could enhance IRF-1, iNOS expression and TNF-α release from the cells infected with *B. pseudomallei* (15, 47). Previously we demonstrated that IFN-γ was able to enhance macrophage ability to suppress intracellular replication of *B.*

pseudomallei. In the present study we demonstrated that addition of IFN-γ could restore the macrophage ability, thus resulting in enhanced killing of intracellular survival of *B. pseudomallei* inside macrophage (Figure 25). In the presence of IFN-γ, this cytokine alone induce only low level of gene expression of both MyD88-dependent and -independent pathway (Figure 24). Nevertheless, IFN-γ synergistically enhances gene expression of both pathway in *B. pseudomallei*-infected macrophages. Altogether these results suggested that 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.

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References

1. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, *et al*. 1998. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. Immunity. 9:143-150.
2. Alexander W, and Hilton D. 2004. The role of suppressor of cytokine signaling (SOCS) proteins in regulation of the immune response. Ann Rev Immunol. 22:503-529.
3. Akira S, Takeda K. 2004. Toll-like receptor signalling. Nat Rev Immunol. 4:499-511.
4. Bach EA, Tanner JW, Marsters S, Ashkenazi A, Aguet M, Shaw AS, *et al*. 1996. Ligand-induced assembly and activation of the gamma interferon receptor in intact cells. Mol Cell Biol. 16:3214-3221.
5. Baetz A, Frey M, Heeg K, and Dalpke A. 2004. Suppressor of cytokine signaling (SOCS) proteins indirectly regulate Toll-like receptor signaling in innate immune cells. J Biol Chem. 279:54708-54715.
6. Bertholet S, Dickensheets H.L, Sheikh F, Gam A.A, Donnelly R.P, and Kenny R.T. 2003. *Leishmania donovani*-induced expression of suppressor of cytokine signaling 3 in human macrophages: a novel mechanism for intracellular parasite suppression of activation. Infect Immune. 71:2095-2101.
7. Brysha M, Zhang J.G, Bertolino P, Corbin J.E, Alexander W.S, Nicola N.A, Hilton D.J, and Starr R. 2001. Suppressor of cytokine signaling-1 attenuates the duration of interferon γ signal transduction *in Vitro* and *in Vivo*. J Biol Chem. 276:22086-22089.
8. Cooney R.N. 2002. Suppressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. Shock. 17:83-90.
9. Crespo A, Filla M.B, and Murphy W.J. 2002. Low responsiveness to IFN-γ, after pretreatment of mouse macrophages with lipopolysaccharides, develops via diverse regulatory pathways. Eur J Immunol. 32:710-719.
10. Croker B.A., Krebs D.L, Zhang J.G, Wormald S, Willson T.A, Stanley E.G, Robb L, Greenhalgh C.J, Forster R, Clausen B.E, Nicola N.A, Metcalf D,

.

Hilton D.J, Roberts A.W, and Alexander W.S. 2003. SOCS3 negatively regulates IL-6 signaling *in vivo*. Nat Immunol. 4:540-545

11. Dalpke A, Eckerle S, Frey M, and Heeg K. 2003. Triggers of Toll-like receptor modulates IFN-γ signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signal. Eur J Immunol. 33:1776-1787.
12. Decker T, Kovarik P, and Meinke A. 1997. Gas elements: a few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res. 17:121-134.
13. Decker T, Stockinger S, Karaghiosoff M, Muller M, and Kovarik P. 2002. IFNs and STATs in innate immunity to microorganism. J Clin Invest. 109:1271-1277.
14. Dupuis S, Dargemont C, Fieschi C, Thomassin N, Rosenzweig S, Harris J, Holland S.M, Schrieber R.D, Casanova J.L. 2001. Impairment of mycobacterial but not viral immunity by a germline human STAT-1 mutation. Science. 293:300-303.
15. Ekchariyawat P, Pudla S, Limposuwan K, Arjcharoen S, Sirisinha S and Utaisincharoen P. 2005. *Burkholderia pseudomallei*-induced expression of suppressor of cytokine signaling 3 and cytokine-inducible src homology 2-containing protein in mouse macrophages: a possible mechanism for suppression of the response to gamma interferon stimulation. Infect Immun. 73:7332-7339.
16. Gao J, Morrison D.C, Parmely T.J, Russell S.W, Murphy W.J. 1997. An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. J Biol Chem. 272:1226-1230.
17. Gil M. P, Bohn E, O'Guin A.K, Ramana C.V, Levine B, Stark G. R, Virgin H. W, and Schreiber R. D. 2001. Biologic consequences of Stat1-independent IFN signaling. PNAS. 98:6680-6685.
18. Greenlund AC, Farrar MA, Viviano BL, Schreiber RD. 1994. Ligand-induced IFN gamma receptor tyrosine phosphorylation couples the receptor to its signal transduction system (p91). Embo J. 13:1591-1600.

.

19. Harada H, Takahashi E, Itoh S, Harada K, Hori T, Taniguchi T. 1994. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implication for a gene network in the interferon system. Mol Cell Biol. 14:1500-1509.
20. Harley VS, Dance DA, Drasar BS, and Tovey G. 1998. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. Microbios. 96:71-93.
21. Heim MH, Kerr IM, Stark GR, and Darnell JE Jr. 1995. Contribution of STAT SH2 groups to specific interferon signaling by the Jak-STAT pathway. Science. 267:1347-1349.
22. Hoshino K, Kaisho T, Iwabe T, Takeuchi O, and Akira S. 2002. Differential involvement of IFN-beta in Toll-like receptor-stimulated dendritic cell activation. Int Immunol. 14:1225-1231.
23. Jones AL, Beveridge TJ, and Woods DE. 1996. Intracellular survival of *Burkholderia pseudomallei*. Infect Immun. 64:782-790.
24. Kaplan DH, Greenlund AC, Tanner JW, Shaw AS, and Schreiber RD. 1996. Identification of an interferon-gamma receptor alpha chain sequence required for JAK-1 binding. J Biol Chem. 271:9-12.
25. Kespichayawattana W, Rattanachetkul S, Wanun T, Utaisincharoen P, and Sirisinha S. 2000. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect Immun. 68:5377-5384.
26. Kinjyo I, Hanada T, Inagabi-Ohara K, Mori H, Aki D, et al. 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity. 17:583-591.
27. Kotenko SV, Izotova LS, Pollack BP, Mariano TM, Donnelly RJ, Muthukumaran G, *et al*. 1995. Interaction between the components of the interferon gamma receptor complex. J Biol Chem. 270:20915-20921.
28. Kovarik P, Stoiber D, Novy M, and Decker T. 1998. Stat1 combines signals derived from IFN-γ and LPS receptors during macrophage activation. EMBO J. 17:3660-3668.

.

29. Lang R, Pauleau A.L, Parganas E, Takahashi Y, Mages J, Ihle J.N, Rutschman R, and Murray P.J. 2003. SOCS3 regulates the plasticity of gp130 signaling. Nat Immunol. 4:546-550.
30. Lauw F.N, Simpson A.J, Prins J.M, Smith M.D, Kurimoto M, van Deventer S.J, Speelman P, Chaowagul W, White N.J, and van der Poll T. 1999. Elevated plasma concentrations of interferon (IFN)-gamma and the IFN-gamma-inducing cytokines interleukin (IL)-18, IL-12, and IL-15 in severe melioidosis. J Infect Dis. 180:1878-1885.
31. Martin E, Nathan C, and Xie Q.W. 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. J Exp Med. 180:977-984.
32. Medzhitov R. 2001. Toll-like receptors and innate immunity. Nat Rev Immunol. 1:135-145.
33. Meraz M.A, White J.M, Sheehan K.C, Bach E.A, Rodig S.J, Dighe A.S, Kaplan D.H, Riley J.K, Greenlund A.C, Campbell D, Carver-Moore K, DuBois R.N, Clark R, Aguet M, and Schreiber R.D. 1996. Targeted disruption of the Stat 1 gene in mice reveals unexprected physiologic specificity in the JAK-STAT signaling pathway. Cell. 84:431-442.
34. Miyaki K, Kawakami K, and Saito A. 1997. Role of reactive nitrogen and oxygen intermediates in gamma interferon stimulated murine macrophage bactericidal activity against *Burkholderia pseudomallei*. Infect Immun 67:4108-4113.
35. Nakagawa R, Naka T, Tsutshui H, Fujimoto M, Kimura A, et al. 2002. Socs-1 participate in negative regulation of LPS responses. Immunity. 17:677-687.
36. Sakatsume M, Igarashi K, Winestock KD, Garotta G, Larner AC, Finbloom DS. 1995. The Jak kinases differentially associate with the alpha and beta (accessory factor) chains of the interferon gamma receptor to form a functional receptor unit capable of activating STAT transcription factors. J Biol Chem. 270:17528-17534.
37. Santanirand P, Harley V.S, Dance D.A.B, Drasar B.S, and Bancroft G.J. 1999. Obligatory role of gamma interferon for host survival in murine model of infection with *Burkholderia pseudomallei*. Infect Immun**.** 67**:**3593-3600.

.

38. Seki E, Tsutsui H, Iimuro Y, Naka T, Son G, Akira S, *et al*. 2005. Contribution of Toll-like receptor/myeloid differentiation factor 88 signaling to murine liver regeneration. Hepatology. 41:443-450.
39. Song M, and Shuai K. 1998. The suppressor of cytokine signaling (SOCS)1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferation activity. J Biol Chem. 273:35056-35062.
40. Stark GR, Kerr IM, Williams BR, Silverman RH, and Schreiber RD. 1998. How cells respond to interferons. Annu Rev Biochem. 67:227-264.
41. Stoiber D, Kovarik P, Cohney S, Johnston J, Steinlein P, and Decker T. 1999. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. J Immunol. 163:2640-2647.
42. Stoiber D, Stockinger S, Steinlein P, Kovarik J, and Decker T. 2001. *Listeria monocytogenes* modulates macrophage cytokine responses through STAT serine phosphorylation and the induction of suppressor of cytokine signaling 3. J Immunol. 166:466-472.
43. Takeda K, and Akira S. 2005. Toll-like receptors in innate immunity. Int Immunol. 17:1-14.
44. Takeda K, Kaisho T, and Akira S. 2003. Toll-like receptors. Annu Rev Immunol. 21:335-376.
45. Taniguchi T, Ogasawara K, Takaoka A, and Tanaka N. 2001. IRF family of transcription factors as regulators of host defense. Annu Rev Immunol. 19:623-655.
46. Toshchakov V, Jones BW, Perera PY, Thomas K, Cody MJ, Zhang S, *et al*. 2002. TLR4, but not TLR2, mediates IFN-beta-induced STAT1alpha/beta-dependent gene expression in macrophages. Nat Immunol.3:392-398.
47. Utaisincharoen P, Tangthawornchaikul N, Kespichayawattana W, Chaisuriya P, Sirisinha S. 2001. *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. Microbiol Immunol. 45:307-313.
48. Utaisincharoen P, Anuntagool N, Limposuwan K, Chaisuriya P, and Sirisinha S. 2003. Involvement of beta interferon in enhancing inducible

.

nitric oxide synthase production and antimicrobial activity of *Burkholderia pseudomallei*-infected macrophages. Infect Immun. 71:3053-3057.

49. Utaisincharoen P, Anuntagool N, Arjcharoen S, Limposuwan K, Chaisuriya P, and Sirisinha S. 2004. Induction of iNOS expression and antimicrobial activity by interferon (IFN)-β is distinct from IFN-γ in *Burkholderia pseudomallei*-infected mouse macrophages. Clin Exp Immunol. 136:277-283.
50. Wongratanacheewin S, Kespichayawattana W, Intachote P, Pichyangkul P, Sermswan R.W, Krieg A.M, and Sirisinha S. 2004. Immunostimulatory CpG oligodeoxynucleotide confers protection in a murine model of infection with *Burkholderial pseudomallei*. Infect Immun. 72**:**4494-4502.
51. Yamamoto M, Yamazaki S, Uematsu S, Sato S, Hemmi H, Hoshino K, *et al*. 2004. Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaB-zeta. Nature. 430:218-222.
52. Yamazaki S, Muta T, Matsuo S, and Takeshige K. 2005. Stimulus-specific induction of a novel nuclear factor-kappaB regulator, IkappaB-zeta, via Toll/Interleukin-1 receptor is mediated by mRNA stabilization. J Biol Chem. 280:1678-1687.
53. Yasukawa H, Ohishi M, Mori H, Murakami M, Chinen T, Aki D, Hanada T, Takeda K, Akira S, Hoshijima M, Hirano T, Chien K.R, and Yoshimura A. 2003. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nat Immunol. 4:551-556.

Publications

1. *Burkholderia pseudomallei*-induced expression of suppressor of cytokine signaling 3 and cytokine-inducible Src homology 2-containing protein in mouse macrophages: a possible mechanism for suppression of the response to gamma interferon stimulation.
   Infection and Immunity 2005;73(11):7332-39.

2. *Burkholderia pseudomallei* RpoS regulates multinucleated giant cell formation and inducible nitric oxide synthase expression in mouse macrophage cell line (RAW 264.7).
   Microbial Pathogenesis 2006;40(4):184-9.

3. Expression of suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) induced in *Burkholderia pseudomallei*-infected mouse macrophages requires bacterial internalization.
   Microbial Pathogenesis 2007;42(2-3):104-10.

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Burkholderia pseudomallei-Induced Expression of Suppressor of Cytokine Signaling 3 and Cytokine-Inducible Src Homology 2-Containing Protein in Mouse Macrophages: a Possible Mechanism for Suppression of the Response to Gamma Interferon Stimulation

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***Burkholderia pseudomallei*, the causative agent of melioidosis, is a facultative intracellular gram-negative bacterium that is able to survive and multiply in macrophages. Previously, we reported that *B. pseudomallei* was able to escape macrophage killing by interfering with the expression of inducible nitric oxide synthase (iNOS). In the present study, we extended this finding and demonstrated that *B. pseudomallei* was able to activate the expression of suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) but not SOCS1 in a mouse macrophage cell line (RAW 264.7). The expression of SOCS3 and CIS in *B. pseudomallei*-infected macrophages directly correlated with a decreased gamma interferon (IFN-γ) signaling response, as indicated by a reduction in Y701–STAT-1 phosphorylation (pY701–STAT-1). Moreover, a reduction in the expression of IFN-γ-induced proteins, such as interferon regulatory factor 1 (IRF-1), was observed in *B. pseudomallei*-infected macrophages that were treated with IFN-γ. Since pY701–STAT-1 and IRF-1 are essential transcription factors for regulating iNOS expression, the failure to activate these factors could also result in depression of iNOS expression and a loss of macrophage killing capacity. Taken together, the data indicate that the activation of SOCS3 and CIS expression in *B. pseudomallei*-infected macrophages interfered with IFN-γ signaling, thus allowing the bacteria to escape killing by these phagocytic cells.**

Burkholderia pseudomallei is a gram-negative bacterium that is responsible for a large proportion of community-acquired septicemia in several tropical areas, including Southeast Asia and northern Australia (41, 13). This organism is able to survive and multiply inside both phagocytic and nonphagocytic cells (19). After internalization, the bacterium can escape from a membrane-bound phagosome to the cytoplasm, thus facilitating actin-associated membrane protrusion, which may facilitate its spread from one cell to another (18, 19, 21).

Although macrophages are known to play a major role in innate immunity to a number of bacterial infections, *B. pseudomallei* can survive and multiply inside macrophages (38). Unlike macrophages infected with other gram-negative bacteria (e.g., *Salmonella enterica* serovar Typhi), *B. pseudomallei*-infected macrophages are not able to activate inducible nitric oxide synthase (iNOS) expression (38). We previously reported evidence suggesting that the failure to induce iNOS expression may result from an inability to activate beta interferon (IFN-β) production, leading to reduced interferon regulatory factor 1 (IRF-1) and iNOS expression (39). Addition of exogenous IFN-β or IFN-γ at the time of bacterial challenge restored the macrophages' ability to activate both IRF-1 and iNOS expression, which resulted in enhanced killing of intracellular *B. pseudomallei* (29, 39, 40). These results demonstrated that under the appropriate conditions, IFNs play an essential role in stimulating the antimicrobial activity of macrophages in response to *B. pseudomallei* infection.

One common property of IFNs is to induce a transcriptional response through a JAK-STAT signal transduction pathway (12). Once STAT-1 is phosphorylated, it is translocated into the nucleus to regulate transcription of promoters containing an IFN-γ-activated site (GAS) element (11, 15). Several IFN-γ-responsive genes, including IRF-1 and iNOS genes, are known to function in macrophage antimicrobial activity (12, 17). However, the macrophage response to IFNs can be influenced by the expression of suppressor of cytokine signaling (SOCS) proteins. Eight members of the SOCS protein family have been identified that serve as negative effectors of cytokine signaling via the JAK/STAT pathway, thus attenuating the macrophage response (1, 6). SOCS1, SOCS3, and cytokine-inducible Src homology 2-containing protein (CIS) have been shown to inhibit tyrosine 701–STAT-1 (Y701–STAT-1) phosphorylation (pY701–STAT-1) in response to IFN stimulation (3, 33). In addition to cytokines, pathogens and their products can induce SOCS expression. For example, lipopolysaccharide (LPS) could induce SOCS1, SOCS3, and CIS expression in macrophages (10, 22, 30, 34). Some pathogens could interfere with the host immune response by activating SOCS expression, leading to suppression of macrophage activation. For example, persistent *Listeria monocytogenes* infection of macrophages diminished IFN-γ-stimulated transcription and phosphorylation

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of STAT-1 (35). The decreased IFN-γ signaling also correlated with the induction of SOCS3 expression in the *L. monocytogenes*-infected macrophages. In the present study, we demonstrated that *B. pseudomallei* is able to stimulate SOCS3 and CIS expression in macrophages. The expression of these negative regulators in *B. pseudomallei*-infected macrophages correlated with a decrease in Y701–STAT-1 phosphorylation, which is normally stimulated by exogenous IFN-γ and is essential for IRF-1 and iNOS production, thereby allowing *B. pseudomallei* to escape killing by the macrophages.

MATERIALS AND METHODS

Cell line and culture conditions. Mouse macrophage cell line RAW 264.7 was obtained from the American Type Culture Collection (Rockville, MD). Unless indicated otherwise, the cells were cultured in Dulbecco's modified Eagle's medium (Gibco Labs, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT) at 37°C under a 5% CO_2 atmosphere.

Bacterial strains. *B. pseudomallei* strain 844 used in this study was originally isolated from a patient admitted to Srinagarind Hospital in the Khon Kaen province of Thailand, where melioidosis is endemic. This bacterium was originally identified as *B. pseudomallei* based on its biochemical characteristics, colonial morphology on selective media, antibiotic sensitivity profiles, and reactivity with polyclonal and monoclonal antibodies (2, 20, 43). *S. enterica* serovar Typhi, which was used for comparison throughout the experiments, was originally isolated from a patient at Ramathibodi Hospital (Mahidol University, Bangkok, Thailand).

Nonviable *B. pseudomallei* was prepared by heating the bacteria (10^8 CFU/ml) in a boiling water bath for 15 min. The heat-treated bacteria were washed three times with phosphate-buffered saline (pH 7.2) (PBS), and complete killing was confirmed by inoculating the suspension onto tryptic soy agar and observing growth after 48 h.

Infection of mouse macrophage cell line RAW 264.7. An overnight culture of mouse macrophages (1×10^6 cells) in a six-well plate was cocultured with bacteria at a multiplicity of infection (MOI) of 2:1 for 1 h. To remove extracellular bacteria, the cells were washed three times with 2 ml of PBS, and residual bacteria were killed by incubating the preparations in Dulbecco's modified Eagle's medium containing 250 μg/ml kanamycin (Gibco Labs) for 2 h. After this, the infection was allowed to continue in medium containing 20 μg/ml of kanamycin until the experiment was terminated. The viability of the infected macrophages was determined by trypan blue staining and was found to be more than 90% during the time course employed in this study.

To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed as previously described (21). 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.

Immunoblotting. Mouse macrophage preparations were lysed in buffer containing 20 mM Tris, 100 mM NaCl, and 1% NP-40. Lysate samples containing 30 μg of protein were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and then electrotransferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with 5% milk for 1 h before incubation overnight with appropriate specific polyclonal rabbit antibodies to mouse SOCS1, SOCS3, CIS, pY701–STAT-1, STAT-1, iNOS, IRF-1, or actin (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were then allowed to react with horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin G (Pierce, Rockford, IL). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany).

Reverse transcriptase PCR. Total RNA was extracted from infected cells according to the manufacturer's instructions (Eppendorf, Hamburg, Germany) before it was used for cDNA synthesis with cMaster reverse transcriptase (RT) (Eppendorf). The PCR was performed using cDNA as the template and primer pairs specific for SOCS1, SOCS2, SOCS3, CIS, and actin in amplification reactions with *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). The following primers were used to amplify the genes: for SOCS1, sense primer 5′-CACCTT CTTGGTGCGCG-3′ and antisense primer 5′-AAGCCATCTTCACGCTGAG C-3′ (9); for SOCS2, sense primer 5′-AGGCCCAGAAGCCCCAC-3′ and antisense primer 5′-TTGGTAAAGGCAGTCCCCA-3′ (10); for SOCS3, sense primer 5′-ATGGTCACCCACAGCAAGTT-3′ and antisense primer 5′-AATC CGCTCTCCTGCAGCTT-3′; for CIS, sense primer 5′-GAACCGAAGGTGC TAGACCCT-3′ and antisense primer 5′-TGTACCCTCCGGCATCTTCT-3′ (10); and for actin, sense primer 5′-CCAGAGCAAGAGAGGTATCC-3′ and antisense primer 5′-CTGTGGTGGTGAAGCTGTAG-3′. The amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide before they were visualized under a UV lamp.

RESULTS

***B. pseudomallei* induces SOCS3 and CIS expression in mouse macrophage cell line RAW 264.7.** Mouse macrophages were cocultured with bacteria at an MOI of 2:1 and incubated for 1, 2, and 4 h after infection. The levels of SOCS1, SOCS2, SOCS3, and CIS mRNA expression in the infected cells were determined by RT-PCR. Figure 1A shows that *B. pseudomallei* was able to activate the transcription of SOCS3 and CIS but not the transcription of SOCS1 and SOCS2. Expression of both SOCS3 and CIS was observed within 1 h of infection, and the levels gradually increased with time of incubation. By contrast, macrophages similarly infected with *S. enterica* serovar Typhi, which was used as a reference for comparison, could activate not only SOCS3 and CIS expression but also SOCS1 mRNA expression within 1 h after infection. SOCS protein expression as determined by immunoblotting (Fig. 1B) of the infected cells was consistent with the mRNA results shown in Fig. 1A.

In order to determine if the expression of SOCSs by macrophages required viable bacteria, the cells were cocultured with heat-killed *B. pseudomallei* or *S. enterica* serovar Typhi at MOIs of 1:1, 10:1, and 100:1. After 8 h, the cells were harvested, and SOCS protein expression was assessed as described above. Figure 2 shows that exposure of macrophages to nonviable bacteria yielded SOCS and CIS expression patterns that were similar to those obtained with viable bacteria (Fig. 1B), suggesting that SOCS expression does not require active invasion. The level of SOCS3 protein expression directly correlated with the degree of stimulation (MOI). It should be noted that, unlike macrophages cocultured with *S. enterica* serovar Typhi, macrophages cocultured with killed *B. pseudomallei* showed no SOCS1 expression even at an MOI as high as 100:1.

Cytokine activation is not a prerequisite for *B. pseudomallei*-induced SOCS3 and CIS expression in macrophages. A number of cytokines, including interleukin-10 and tumor necrosis factor alpha (TNF-α), have been reported to have the ability to induce SOCS expression (23). We reported previously that TNF-α could be produced by macrophages infected with *B. pseudomallei* (38). In order to rule out the possibility that the observed SOCS expression was not related to prior cytokine production, similar experiments were performed in the presence of an inhibitor of protein synthesis, cycloheximide. Macrophages were pretreated with cycloheximide (5 μg/ml) for 1 h before infection, and the levels of SOCS3 and CIS mRNA were determined after 4 h of infection. It was found that the induction of SOCS3 and CIS mRNA expression was not affected by cycloheximide (Fig. 3A). However, the levels of SOCS proteins were markedly reduced in the presence of the inhibitor (Fig. 3B). This was not unexpected in view of the fact that cycloheximide is known to block protein synthesis in cells at the level of translation. It should be mentioned that cycloheximide at the concentration used in this experiment also

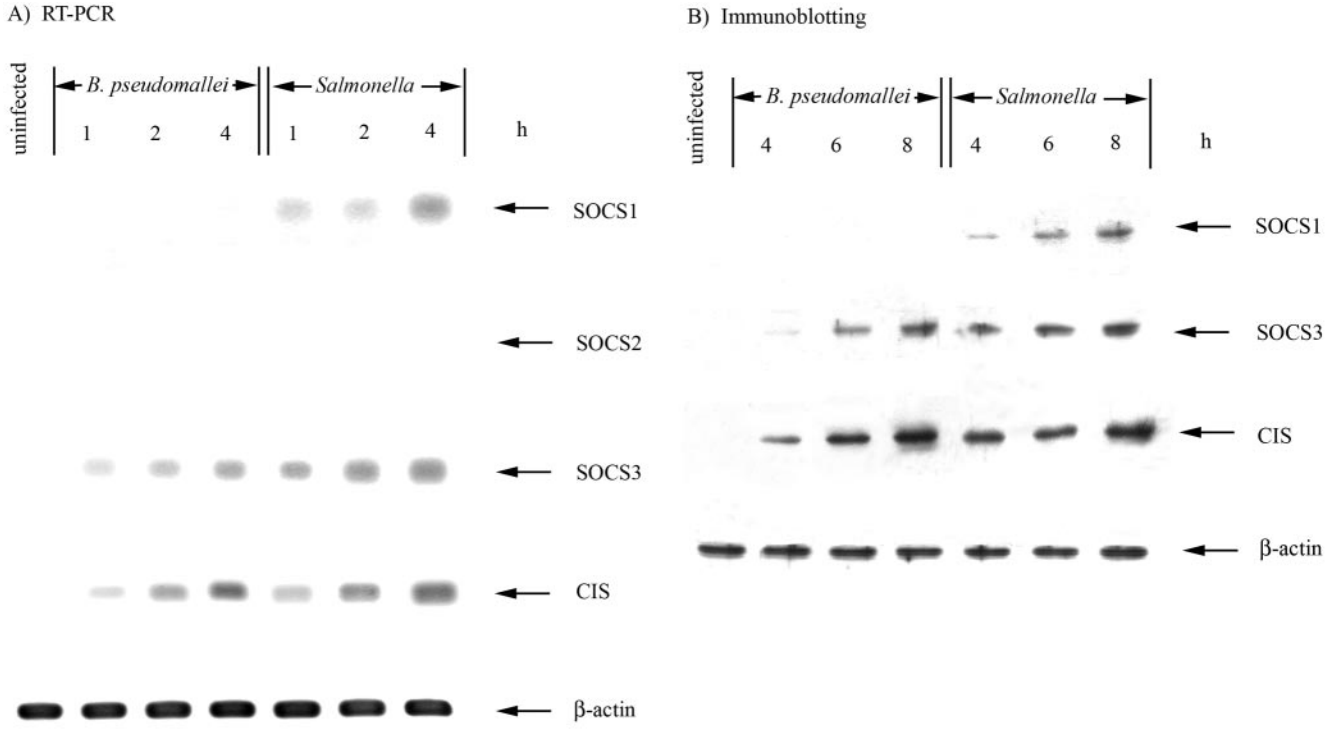

FIG. 1. *B. pseudomallei*-induced SOCS3 and CIS expression in macrophages. Mouse macrophages were infected with either *B. pseudomallei* or *S. enterica* serovar Typhi (used as reference) at an MOI of 2:1. At different times, the infected cells were lysed and the levels of SOCS mRNAs (A) and proteins (B) in the cell lysates were determined by RT-PCR and immunoblotting, respectively.

inhibited TNF-α secretion by the *B. pseudomallei*-infected macrophages (data not shown). This raises the possibility that the expression of SOCS3 and CIS in *B. pseudomallei*-infected macrophages is not mediated through the cytokines produced prior to the time of SOCS induction and that *B. pseudomallei* may be able to directly activate SOCS3 and CIS expression in macrophages.

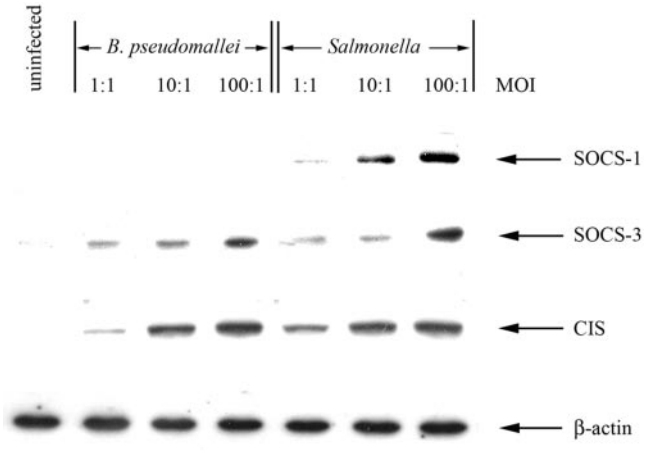

FIG. 2. Expression of SOCS protein in macrophages activated with killed bacteria. Mouse macrophages were treated with either killed *B. pseudomallei* or *S. enterica* serovar Typhi at MOIs of 1:1, 10:1, and 100:1. Eight hours later the treated cells were lysed, and the levels of SOCS proteins in the lysate were determined by immunoblotting.

***B. pseudomallei* infection interferes with activation of Y701–STAT-1 phosphorylation.** In order to determine whether *B. pseudomallei* could stimulate Y701–STAT-1 phosphorylation, macrophages were first infected with *B. pseudomallei* at an MOI of 2:1 and incubated for different times following the initial infection, and pY701–STAT-1 was then detected by immunoblotting. No phosphorylation of Y701–STAT-1 was observed in macrophages infected with *B. pseudomallei*, while pY701–STAT-1 was detected within 2 h in *S. enterica* serovar Typhi-infected macrophages (Fig. 4A). However, the presence of neutralizing antibody against IFN-β markedly reduced the level of pY701–STAT-1 in *S. enterica* serovar Typhi-infected macrophages (Fig. 4B). These results suggest that in *S. enterica* serovar Typhi-infected macrophages, the phosphorylation of Y701–STAT-1 was mediated through the production of IFN-β. Because *B. pseudomallei* is unable to induce IFN-β production (39), it is possible that the absence of an IFN-β signal is responsible for the inability to activate phosphorylation of Y701–STAT-1 in *B. pseudomallei*-infected cells. In fact, we have limited data for exogenous IFN-β that support the possibility described above (data not shown).

Exogenous IFN-γ added after *B. pseudomallei* infection fails to induce phosphorylation of Y701–STAT-1. IFN-γ is known to play an essential role in macrophage activation by signaling through STAT-1 (32), and both SOCS3 and CIS have been reported to interfere with IFN-γ signaling by inhibiting Y701–STAT-1 phosphorylation (3). In order to determine whether induction of SOCS3 and CIS in *B.*

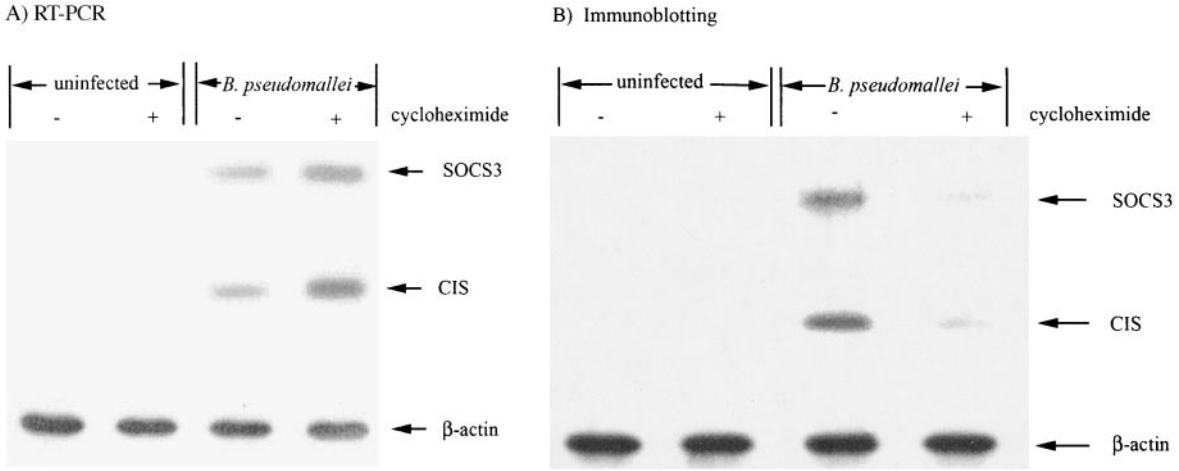

FIG. 3. *B. pseudomallei*-induced SOCS3 and CIS mRNA expression in cycloheximide-treated macrophages. Cycloheximide (5 μg/ml) was added to a macrophage culture 1 h prior to infection. The pretreated macrophage culture was then infected with *B. pseudomallei* at an MOI of 2:1. The levels of expression of SOCS3 and CIS in the lysates of cycloheximide-treated and untreated infected macrophages were determined and compared by RT-PCR (A) at 4 h after infection and by immunoblotting (B) at 8 h after infection.

pseudomallei-infected macrophages adversely affected their ability to respond to subsequent IFN-γ activation, the level of pY701–STAT-1 was monitored in *B. pseudomallei*-infected macrophages that were exposed to IFN-γ at 1, 2, and 4 h postinfection (Fig. 5). IFN-γ exposure at 1 h postinfection reduced the level of Y701–STAT-1 phosphorylation only slightly relative to the level in uninfected cells, while exposure at 2 and 4 h postinfection resulted in drastic reductions in the levels of pY701–STAT-1. It should be noted that the observed decrease in IFN-γ-induced Y701–STAT-1 phosphorylation in infected cells directly correlated with increased levels of SOCS3 and CIS, as shown in Fig. 1.

***B. pseudomallei* infection interferes with IRF-1 and iNOS expression in macrophages stimulated with IFN-γ.** We previously demonstrated that IRF-1 and iNOS played an important role in regulating the bactericidal activity of macrophages against *B. pseudomallei* (38, 39). Induction of both IRF-1 and iNOS expression in uninfected cells is known to require prior phosphorylation of Y701–STAT-1 (15, 34). Therefore, it was of interest to determine if the depressed pY701–STAT-1 level in *B. pseudomallei*-infected macrophages (Fig. 4) influenced the expression of IRF-1 and iNOS. In order to address this question, IFN-γ was added to macrophage cultures either at the time of infection (costimulation) or 4 h after infection (poststimulation). IRF-1 and iNOS expression was then determined 4 h after the addition of IFN-γ. Consistent with our previous report (40), IFN-γ exposure alone was able to activate IRF-1 expression but not iNOS expression in uninfected cells (Fig. 6). While *B. pseudomallei* infection alone in the absence of IFN-γ failed to activate expression of IRF-1 and iNOS, the expression was observed when IFN-γ was added to the cells at the time of infection (Fig. 6). However, the levels of both IRF-1 and iNOS were noticeably reduced when IFN-γ was added 4 h after the cells were infected. These results suggested that the host cell response to IFN-γ was suppressed in *B. pseudomallei*-infected macrophages and that the suppression required time to become established. The suppression of both IRF-1 and iNOS correlated with both the decrease in the pY701–STAT-1 level shown in Fig. 5 and the increased expression of SOCS3 and CIS shown in Fig. 1.

Inability of IFN-γ to enhance killing of intracellular *B. pseudomallei* in infected macrophages. Previously, we demonstrated that exogenous IFN-γ, when added to macrophages

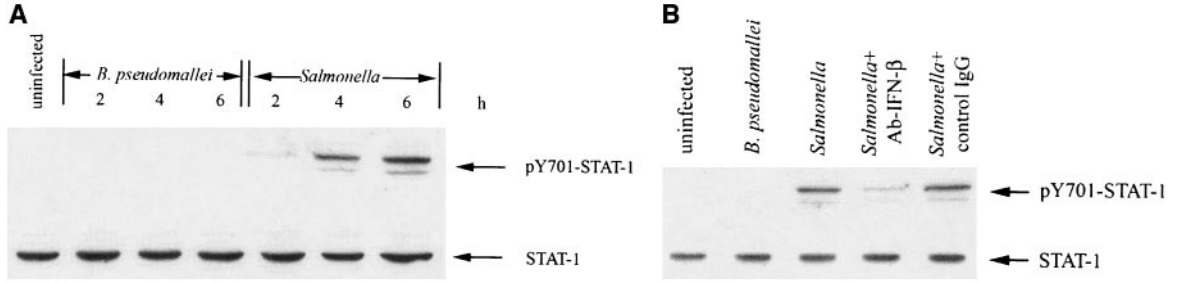

FIG. 4. Failure of *B. pseudomallei* to induce Y701–STAT-1 phosphorylation. (A) Mouse macrophages were infected with either *B. pseudomallei* or *S. enterica* serovar Typhi at an MOI of 2:1. After various times, the levels of phosphorylation of Y701–STAT-1 in the infected cell lysates were determined by immunoblotting. pY701–STAT-1 could be detected only in the *S. enterica* serovar Typhi-infected macrophages. (B) Phosphorylation of Y701–STAT-1 in *S. enterica* serovar Typhi-infected macrophages depends on IFN-β production. Mouse macrophages were infected with *S. enterica* serovar Typhi at an MOI of 2:1 in the presence of neutralizing antibody against IFN-β (10 μg/ml). After 4 h of incubation, the infected cells were lysed, and the level of pY701–STAT-1 was analyzed by immunoblotting. IgG, immunoglobulin G.

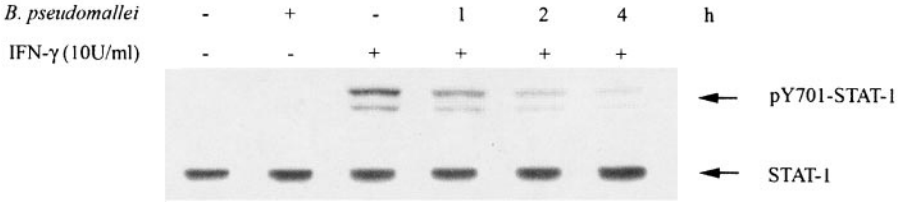

FIG. 5. Failure of *B. pseudomallei*-infected macrophages to phosphorylate Y701–STAT-1 in response to IFN-γ stimulation. Mouse macrophages were infected with *B. pseudomallei* at an MOI of 2:1 and incubated for 1, 2, and 4 h before IFN-γ (10 U/ml) was added. The cells were then incubated for 1 h before the level of pY701–STAT-1 was determined by immunoblotting. Compared with the uninfected control culture, *B. pseudomallei* infection interfered with the macrophage response, particularly when the time between bacterial infections was prolonged (4 h).

either prior to or at the time of *B. pseudomallei* infection, could significantly enhance killing of intracellular *B. pseudomallei*, suggesting that this effect was likely attributable to the enhanced production of iNOS (40). It was of interest to determine if postinfection incubation had any effect on the observed IFN-γ-induced enhanced killing. Costimulation and poststimulation of infected macrophages with IFN-γ were performed as described previously (Fig. 6), except that the number of viable intracellular bacteria was determined 4 h after IFN-γ addition. Addition of IFN-γ 4 h after infection failed to enhance the intracellular killing of *B. pseudomallei*. Viable intracellular *B. pseudomallei* levels were significantly higher in cells that were subjected to poststimulation with IFN-γ than in cells that were costimulated at the time of infection (Fig. 7). The failure of IFN-γ to stimulate killing of intracellular *B. pseudomallei* when it was added to macrophages at 4 h postinfection correlated well with the reduced IRF-1 and iNOS expression shown in Fig. 6.

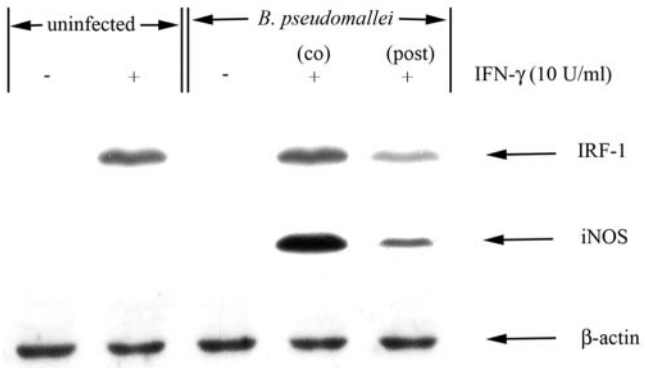

FIG. 6. Failure of *B. pseudomallei*-infected macrophages to activate IRF-1 and iNOS expression in response to postinfection IFN-γ stimulation. Mouse macrophages were infected with *B. pseudomallei* at an MOI of 2:1 as described in Materials and Methods. In the postinfection stimulation (post) experiment, IFN-γ (10 U/ml) was added 4 h after the cells were infected with *B. pseudomallei*. The infected cell cultures were incubated for one more hour before excess IFN-γ was removed. The cell cultures were then incubated for a further 3 h, the macrophages were lysed, and the levels IRF-1 and iNOS in the lysates were determined by immunoblotting. For the costimulation (co) experiment, the macrophages were infected with *B. pseudomallei* in the presence of IFN-γ (10 U/ml). After 1 h of incubation, the macrophages were washed three times with PBS and cultured for three more hours before the levels of IRF-1 and iNOS in the cell lysate were determined by immunoblotting.

DISCUSSION

The induction of SOCS expression and its significance have been extensively studied in recent years. For example, *Leishmania donovani*-infected macrophages were reported to induce SOCS3 expression that resulted in suppression of macrophage activation (4). Activation of SOCS3 expression has also been reported in macrophages infected with gram-positive bacteria (e.g., *L. monocytogenes* and *Staphylococcus aureus*) (4, 35). Induction of SOCS proteins by isolated bacterial components like LPS has also been demonstrated; however, very limited information on the regulation of these proteins in response to infection with gram-negative bacteria is currently available (7). This study clearly demonstrated that there is a novel association between the intracellular gram-negative bacterium *B. pseudomallei* and the induction of SOCS3 and CIS expression in an in vitro mouse macrophage model (Fig. 1). Expression of both SOCS3 and CIS mRNA was not affected in the presence of cycloheximide, suggesting that *B. pseudomallei* itself, and not the cytokines that it induced, was able to directly induce mRNA expression of these negative regulators (Fig. 3). This finding is reinforced by the fact that cycloheximide at the

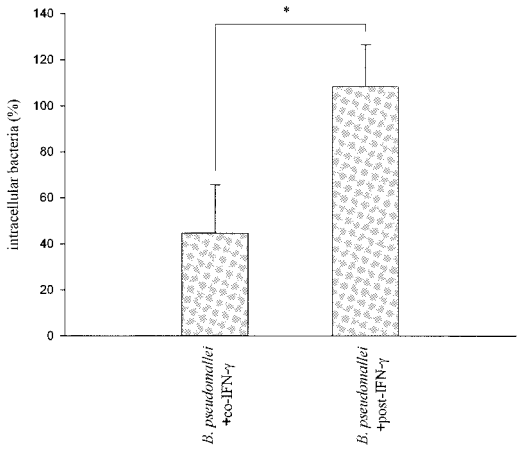

FIG. 7. Inability of IFN-γ to enhance antimicrobial activity of *B. pseudomallei*-infected macrophages. The experimental protocol for this experiment was similar to that described in the legend to Fig. 6, except that the viability of intracellular bacteria was determined and the results of poststimulation and costimulation conditions were compared. The data indicate the means and standard deviations of three separate experiments, each carried out in duplicate. The asterisk indicates that the P value is <0.01, as determined by Student's t test.

concentration used was found to interfere with neither invasion nor intracellular replication of *B. pseudomallei* (data not shown). However, it was found that unlike other gram-negative bacteria, such as *S. enterica* serovar Typhi, *B. pseudomallei* failed to induce SOCS1 expression (Fig. 1), suggesting that the mechanisms employed by these different gram-negative bacteria to modulate the macrophage response are different. Moreover, it appears that the induction of these negative regulators, at least in the *B. pseudomallei* system, did not require active invasion since the macrophage responses to viable and nonviable bacteria were similar (Fig. 2). This finding is in accord with the results from other studies showing that purified LPS from other bacteria could also activate SOCS expression (3, 7, 34). It is possible that the type of SOCS response to different gram-negative bacteria, as demonstrated for *S. enterica* serovar Typhi and *B. pseudomallei* in the present study, is related to differences in LPS structure. For example, 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 (20). This unusual LPS structure has also been shown to be a weak macrophage activator compared with the LPS isolated from other gram-negative bacteria, such as *S. enterica* serovar Typhi and *Escherichia coli* (20, 37). It is tempting, therefore, to speculate that the cellular response associated with the expression of different SOCS proteins depends partly on signals generated from different pattern recognition receptors. The participation of a Toll-like receptor (TLR) in SOCS expression has received considerable attention recently. For example, it was reported that SOCS1-deficient mice exhibited increased LPS sensitivity and decreased endotoxin tolerance (22), suggesting that SOCS protein might be involved in TLR signaling. It was subsequently demonstrated that the induction of SOCS3 and CIS, but not the induction of SOCS1, was MyD88 dependent (3). The ability of *B. pseudomallei* to induce SOCS3 and CIS but not SOCS1, together with its failure to stimulate IFN-β production (39), is consistent with the possibility that *B. pseudomallei* may also activate macrophages via a MyD88-dependent pathway. The type(s) of TLR and/or non-TLR involved in the activation of macrophages has not been defined yet and is currently being investigated in our laboratory.

The pivotal role of the transcription factor STAT-1 in the innate immune response against microbial infection is well documented (12). STAT-1-deficient mice are highly sensitive to stimulation by intact organisms or isolated individual components (28). Increased susceptibility to intracellular bacteria, such as *Mycobacterium*, has also been observed in STAT-1-deficient human macrophages (14). Purified LPS has also been shown to activate phosphorylation of Y701–STAT-1 through TLR, and this correlated with IFN-β synthesis, suggesting that the activation of pY701–STAT-1 is mediated through type I IFN synthesized by LPS-activated cells (10, 24). The data presented in this study which demonstrated that the gram-negative bacterium *S. enterica* serovar Typhi was able to activate Y701–STAT-1 phosphorylation after only 2 h of infection and that the process could be inhibited by neutralizing antibody against IFN-β (Fig. 4) lend support to this idea. In contrast to *S. enterica* serovar Typhi, *B. pseudomallei* failed to stimulate pY701–STAT-1 (Fig. 4), a result that was consistent with the organism's inability to activate IFN-β production (39). In addition to IFN-β, IFN-γ could also activate phosphorylation of STAT-1. Activation of this transcription factor is known to regulate SOCS1 expression (16). By contrast, STAT-1-deficient mice could still activate SOCS3 expression in response to IFN-γ stimulation, suggesting that SOCS3 gene regulation does not involve activation of STAT-1 (16). It is possible that the inability of *B. pseudomallei* to activate SOCS1 expression in our study was also related to its inability to activate phosphorylation of STAT-1 (Fig. 1 and 4).

Of the different SOCS members that have been studied, SOCS1 is probably the most potent inhibitor of IFN signaling, and it acts by suppressing the phosphorylation of STAT-1 (5, 22). SOCS3, on the other hand, is known to play an essential role as a negative inhibitor of interleukin-6, possibly by interfering with the activation of another transcription factor, STAT-3 (8, 25, 44). Based on the data from the STAT-1 phosphorylation studies presented here, it appeared that SOCS3 and CIS could also inhibit IFN-γ signaling, albeit to a lesser extent than they could with STAT-3 (3, 10). Macrophages infected with *L. monocytogenes* also exhibited diminishing IFN-γ-induced Y701–STAT-1 phosphorylation (35). A decreased response to IFN-γ signaling in *L. monocytogenes*- and *L. donovani*-infected macrophages correlated with the induction of SOCS3 expression (4, 35). In the present study, we demonstrated that the gram-negative bacterium *B. pseudomallei* interfered with IFN-γ-induced Y701–STAT-1 phosphorylation in a mouse macrophage system (Fig. 5). The suppression of STAT-1 phosphorylation also directly correlated with the expression of SOCS3 and CIS (Fig. 1). On the basis of all of the results, it is tempting to conclude that activation of SOCS3 and CIS expression in response to *B. pseudomallei* infection can interfere with IFN-γ signaling, thus resulting in suppression of IFN-γ-inducing proteins (e.g., IRF-1 and iNOS expression) (Fig. 6). Both IRF-1 and iNOS are known to play a crucial role in controlling the fate of intracellular pathogens (27, 36). In the present study, *B. pseudomallei*-infected macrophages not only failed to express both IRF-1 and iNOS but also failed to eliminate intracellular *B. pseudomallei* (Fig. 7).

During the last few years, our group has been working on elucidating the mechanism by which *B. pseudomallei* modulates the innate host defense, thus allowing it to survive inside macrophages (38, 39). This intracellular bacterium can invade macrophages without stimulating IFN-β production, which is an essential factor for IRF-1 and iNOS expression (39). Failure to activate IRF-1 and iNOS provides the bacteria with the means to survive the antimicrobial activity of macrophages (38, 39). However, exogenous IFN-γ or IFN-β added prior to or at the time of infection could enhance both IRF-1 and iNOS expression and restore antimicrobial activity of these macrophages (40). The protective role of IFN-γ in the host defense against *B. pseudomallei* has been observed previously in animal models (31). Administration of neutralizing antibody against IFN-γ could significantly lower the 50% lethal dose and also increase bacterial burdens in the liver and spleen of these animals (31). More recently, we demonstrated that the IFN-γ level in the serum of infected BALB/c mice was significantly higher than that in the serum of uninfected mice, yet the high level of IFN-γ failed to eliminate the bacteria from the blood, spleen, liver, and lungs and these animals subsequently died over the next 7 to 10 days (42). The data for this animal model are in

accord with the elevated level of IFN-γ noted in patients with severe melioidosis (26). Together, these results suggest that the presence of high IFN-γ levels during ongoing *B. pseudomallei* infection is not sufficient to control the infection or to eliminate the bacteria from the infected host. The failure of IFN-γ to protect the animals from fatal sepsis (42) could be explained by the ability of *B. pseudomallei* to activate negative regulator proteins, such as SOCS3 and CIS, as demonstrated in the present study. The expression of these proteins can interfere with IFN-γ signaling via a STAT-1 pathway, making the infected macrophages unable to respond to IFN-γ. This, in turn, results in an inability to activate IFN-γ-responsive protein expression (i.e., IRF-1 and iNOS). Therefore, in addition to interference with proinflammatory cytokine production and antimicrobial activity, the results obtained in the present study provided evidence for another possible mechanism by which *B. pseudomallei* could modulate the macrophage bactericidal response, thus facilitating its intracellular survival, and may also explain the persistence of the organism in the human host and the high rate of relapse in melioidosis.

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REFERENCES

1. **Alexander, W., and D. Hilton.** 2004. The role of suppressor of cytokine signaling (SOCS) proteins in regulation of the immune response. Annu. Rev. Immunol. **22:**503–529.
2. **Anuntagool, N., P. Intachote, V. Wuthiekanun, N. J. White, and S. Sirisinha.** 1998. Lipopolysaccharide from nonvirulent Ara^+ *Burkholderia pseudomallei* isolates is immunologically indistinguishable from lipopolysaccharide from virulent Ara^- clinical isolate. Clin. Diagn. Lab. Immunol. **5:**225–229.
3. **Baetz, A., M. Frey, K. Heeg, and A. Dalpke.** 2004. Suppressor of cytokine signaling (SOCS) proteins indirectly regulate Toll-like receptor signaling in innate immune cells. J. Biol. Chem. **279:**54708–54715.
4. **Bertholet, S., H. L. Dickensheets, F. Sheikh, A. A. Gam, R. P. Donnelly, and R. T. Kenny.** 2003. *Leishmania donovani*-induced expression of suppressor of cytokine signaling 3 in human macrophages: a novel mechanism for intracellular parasite suppression of activation. Infect. Immun. **71:**2095–2101.
5. **Brysha, M., J. G. Zhang, P. Bertolino, J. E. Corbin, W. S. Alexander, N. A. Nicola, D. J. Hilton, and R. Starr.** 2001. Suppressor of cytokine signaling-1 attenuates the duration of interferon γ signal transduction *in vitro* and *in vivo*. J. Biol. Chem. **276:**22086–22089.
6. **Cooney, R. N.** 2002. Suppressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. Shock **17:**83–90.
7. **Crespo, A., M. B., Filla, and W. J. Murphy.** 2002. Low responsiveness to IFN-γ, after pretreatment of mouse macrophages with lipopolysaccharides, develops via diverse regulatory pathways. Eur. J. Immunol. **32:**710–719.
8. **Croker, B. A., D. L. Krebs, J. G. Zhang, S. Wormald, T. A. Willson, E. G. Stanley, L. Robb, C. J. Greenhalgh, R. Forster, B. E. Clausen, N. A. Nicola, D. Metcalf, D. J. Hilton, A. W. Roberts, and W. S. Alexander.** 2003. SOCS3 negatively regulates IL-6 signaling *in vivo*. Nat. Immunol. **4:**540–545.
9. **Dalpke, A. H., S. Opper, S. Zimmermann, and K. Heeg.** 2001. Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. J. Immunol. **166:**7082–7089.
10. **Dalpke, A., S. Eckerle, M. Frey, and K. Heeg.** 2003. Triggers of Toll-like receptor modulate IFN-γ signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signal. Eur. J. Immunol. **33:**1776–1787.
11. **Decker, T., P. Kovarik, and A. Meinke.** 1997. Gas elements: a few nucleotides with a major impact on cytokine-induced gene expression. J. Interferon Cytokine Res. **17:**121–134.
12. **Decker, T., S. Stockinger, M. Karaghiosoff, M. Muller, and P. Kovarik.** 2002. IFNs and STATs in innate immunity to microorganism. J. Clin. Investig. **109:**1271–1277.
13. **Dhrarakul, T., and S. Songsivilai.** 1999. The many facets of melioidosis. Trends Microbiol. **7:**138–146.
14. **Dupuis, S., C. Dargemont, C. Fieschi, N. Thomassin, S. Rosenzweig, J. Harris, S. M. Holland, R. D. Schrieber, and J. L. Casanova.** 2001. Impairment of mycobacterial but not viral immunity by a germline human STAT-1 mutation. Science **293:**300–303.
15. **Gao, J., D. C. Morrison, T. J. Parmely, S. W. Russell, and W. J. Murphy.** 1997. An interferon-gamma-activated site (GAS) is necessary for full expression of the mouse iNOS gene in response to interferon-gamma and lipopolysaccharide. J. Biol. Chem. **272:**1226–1230.
16. **Gil, M. P., E. Bohn, A. K. O'Guin, C. V. Ramana, B. Levine, G. R. Stark, H. W. Virgin, and R. D. Schreiber.** 2001. Biologic consequences of Stat1-independent IFN signaling. Proc. Natl. Acad. Sci. USA **98:**6680–6685.
17. **Harada, H., E. Takahashi, S. Itoh, K. Harada, T. Hori, and T. Taniguchi.** 1994. Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and IRF-2 genes: implication for a gene network in the interferon system. Mol. Cell. Biol. **14:**1500–1509.
18. **Harley, V. S., D. A. B. Dance, B. J. Drasar, and G. Tovey.** 1998. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. Microbios **96:**71–93.
19. **Jones, A. L., T. J. Beveridge, and D. E. Woods.** 1996. Intracellular survival of *Burkholderia pseudomallei*. Infect. Immun. **64:**782–790.
20. **Kawahara, K., S. Dejsirilert, H. Danbara, and T. Ezaki.** 1992. Extraction and characterization of lipopolysaccharide from *Pseudomonas pseudomallei*. FEMS Microbiol. Lett. **96:**129–134.
21. **Kespichayawattana, W., S. Rattanachetkul, T. Wanun, P. Utaisincharoen, and S. Sirisinha.** 2000. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect. Immun. **68:**5377–5384.
22. **Kinjyo, I., T. Hanada, K. Inagabi-Ohara, H. Mori, D. Aki, et al.** 2002. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity **17:**583–591.
23. **Kovanen, P. E., and W. J. Leonard.** 1999. Inhibitors keep cytokines in check. Curr. Biol. **9:**R899–R902.
24. **Kovarik, P., D. Stoiber, M. Novy, and T. Decker.** 1998. Stat1 combines signals derived from IFN-γ and LPS receptors during macrophage activation. EMBO J. **17:**3660–3668.
25. **Lang, R. A. L. Pauleau, E. Parganas, Y. Takahashi, J. Mages, J. N. Ihle, R. Rutschman, and P. J. Murray.** 2003. SOCS3 regulates the plasticity of gp130 signaling. Nat. Immunol. **4:**546–550.
26. **Lauw, F. N., A. J. Simpson, J. M. Prins, M. D. Smith, M. Kurimoto, S. J. van Deventer, P. Speelman, W. Chaowagul, N. J. White, and T. van der Poll.** 1999. Elevated plasma concentrations of interferon (IFN)-gamma and the IFN-gamma-inducing cytokines interleukin (IL)-18, IL-12, and IL-15 in severe melioidosis. J. Infect. Dis. **180:**1878–1885.
27. **Martin, E., C. Nathan, and Q. W. Xie.** 1994. Role of interferon regulatory factor 1 in induction of nitric oxide synthase. J. Exp. Med. **180:**977–984.
28. **Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet, and R. D. Schreiber.** 1996. Targeted disruption of the Stat 1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway. Cell **84:**431–442.
29. **Miyaki, K., K. Kawakami, and A. Saito.** 1997. Role of reactive nitrogen and oxygen intermediates in gamma interferon-stimulated murine macrophage bactericidal activity against *Burkholderia pseudomallei*. Infect. Immun. **67:**4108–4113.
30. **Nakagawa, R., T. Naka, H. Tsutshui, M. Fujimoto, A. Kimura, et al.** 2002. Socs-1 participate in negative regulation of LPS responses. Immunity **17:**677–687.
31. **Santanirand, P., V. S. Harley, D. A. B. Dance, B. S. Drasar, and G. J. Bancroft.** 1999. Obligatory role of gamma interferon for host survival in murine model of infection with *Burkholderia pseudomallei*. Infect. Immun. **67:**3593–3600.
32. **Schindler, C., and J. E. Darnell, Jr.** 1995. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu. Rev. Biochem. **64:**621–651.
33. **Song, M., and K. Shuai.** 1998. The suppressor of cytokine signaling (SOCS)1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferation activity. J. Biol. Chem. **273:**35056–35062.
34. **Stoiber, D., P. Kovarik, S. Cohney, J. Johnston, P. Steinlein, and T. Decker.** 1999. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. J. Immunol. **163:**2640–2647.
35. **Stoiber, D., S. Stockinger, P. Steinlein, J. Kovarik, and T. Decker.** 2001. *Listeria monocytogenes* modulates macrophage cytokine responses through STAT serine phosphorylation and the induction of suppressor of cytokine signaling 3. J. Immunol. **166:**466–472.
36. **Taniguchi, T., K. Ogasawara, A. Takaoka, and N. Tanaka.** 2001. IRF family of transcription factors as regulators of host defense. Annu. Rev. Immunol. **19:**623–655.
37. **Utaisincharoen, P., N. Tangthawornchaikul, W. Kespichayawattana, N. Anuntagool, P. Chaisuriya, and S. Sirisinha.** 2000. Kinetic studies of the production of nitric oxide (NO) and tumor necrosis factor alpha (TNF-α) in macrophages stimulated with *Burkholderia pseudomallei* endotoxin. Clin. Exp. Immunol. **122:**324–329.

38. **Utaisincharoen, P., N. Tangthawornchaikul, W. Kespichayawattana, P. Chaisuriya, and S. Sirisinha.** 2001. *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. Microbiol. Immunol. **45:**307–313.
39. **Utaisincharoen, P., N. Anuntagool, K. Limposuwan, P. Chaisuriya, and S. Sirisinha.** 2003. Involvement of beta interferon in enhancing inducible nitric oxide synthase production and antimicrobial activity of *Burkholderia pseudomallei*-infected macrophages. Infect. Immun. **71:**3053–3057.
40. **Utaisincharoen, P., N. Anuntagool, S. Arjcharoen, K. Limposuwan, P. Chaisuriya, and S. Sirisinha.** 2004. Induction of iNOS expression and antimicrobial activity by interferon (IFN)-β is distinct from IFN-γ in *Burkholderia pseudomallei*-infected mouse macrophages. Clin. Exp. Immunol. **136:**277–283.
41. **White, N.** 2003. Melioidosis. Lancet **361:**1715–1722.
42. **Wongratanacheewin, S., W. Kespichayawattana, P. Intachote, S. Pichyangkul, R. W. Sermswan, A. M. Krieg, and S. Sirisinha.** 2004. Immunostimulatory CpG oligodeoxynucleotide confers protection in a murine model of infection with *Burkholderia pseudomallei*. Infect. Immun. **72:**4494–4502.
43. **Wuthiekanun, V., M. D. Smith, D. A. B. Dance, A. L. Walsh, T. L. Pitt, and N. J. White.** 1996. Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*. J. Med. Microbiol. **45:**408–412.
44. **Yasukawa, H., M. Ohishi, H. Mori, M. Murakami, T. Chinen, D. Aki, T. Hanada, K. Takeda, S. Akira, M. Hoshijima, T. Hirano, K. R. Chien, and A. Yoshimura.** 2003. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nat. Immunol. **4:**551–556.

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Burkholderia pseudomallei RpoS regulates multinucleated giant cell formation and inducible nitric oxide synthase expression in mouse macrophage cell line (RAW 264.7)

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Abstract

Burkholderia pseudomallei is the causative agent of melioidosis. This bacterium can invade and survive inside the phagocytic and nonphagocytic cells. After internalization, the bacteria can escape from the membrane-bound phagosome into the cytoplasm. Internalised *B. pseudomallei* can also induce a cell-to-cell fusion, resulting in a multinucleated giant cell (MNGC) formation. In the present study, we demonstrated that *B. pseudomallei rpoS* null mutant was similar to its wild type parent in its ability to survive and multiply inside the mouse macrophages, but it failed to stimulate MNGC formation. The *rpoS* mutant was also unable to activate inducible Nitric Oxide Synthase (iNOS) in resting mouse macrophages but in gamma interferon (IFN-γ)-activated macrophages, the mutant was able to induce significantly higher levels of iNOS and NO when compared with its wild-type counterpart, resulting in a significantly lower number of bacteria inside the infected host cells.

Keywords: *Burkholderia pseudomallei*; Melioidosis; RpoS; Multinucleated giant cell formation; iNOS

1. Introduction

Burkholderia pseudomallei is the causative agent of melioidosis, a disease of man and animals in southeast Asia and northern Australia [1,2]. The clinical features of melioidosis vary greatly from acute fatal sepsis to localised chronic infections [1]. Systemic infections are associated with high mortality rate and high rate of relapse despite prolonged treatment [1,2]. At a cellular level, this gram-negative bacterium can survive and multiply in both phagocytic and non-phagocytic cells [3]. After internalisation, it can readily escape from the membrane bound phagosome into the cytoplasm [3]. The internalised *B. pseudomallei* can also induce a cell-to-cell fusion, resulting in a multinucleated giant cell (MNGC) formation and cell death [4,5]. The MNGC has also been observed in the tissues of patients with melioidosis [6]. It was hypothesised that this unique phenomenon, which has never been observed in any other bacteria, may facilitate the spreading of the bacterium from one cell to another [5].

The mechanism by which *B. pseudomallei* is able to escape host defense is not fully understood. However, we have demonstrated previously that the macrophages infected with this microorganism failed to activate inducible nitric oxide synthase (iNOS) [7]. The failure to stimulate iNOS expression may facilitate this bacterium to survive and multiply inside the macrophages [7,8]. However, preactivation of the macrophages with exogenous IFN-γ or IFN-β could enhance iNOS expression and facilitate intracellular killing of *B. pseudomallei* [7–9].

RpoS is a global regulatory factor known to control the expression of a large number of chromosomal genes involved in resistance to stress conditions and prolonged nutrient deprivation [10,11]. It has also been reported to regulate virulence gene expression in a number of pathogenic bacteria. In *Salmonella enterica* serovar Typhimurium, the RpoS controls the expression of *Salmonella* plasmid virulence (*spv*) genes required to initiate systemic spread [12–14]. The *S. typhimurium rpoS* mutant was significantly less lethal for mice, thus suggesting its role in disease-producing potential [14,15]. However, the ability of the mutant to survive inside the

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macrophages was unaltered [14]. Although the possible involvements of RpoS in the pathogenesis of melioidosis have never been investigated, our group demonstrated previously that the *B. pseudomallei rpoS* null mutant of this bacterium exhibited an increased sensitivity to oxidative stress [16]. In this communication, we extend our finding to demonstrate the involvement of *rpoS* null mutant in modulating the host cell response, particularly with regard to the macrophage antimicrobial capacity to control the intracellular fate of *B. pseudomallei* itself.

2. Results

2.1. Internalisation and intracellular multiplication of rpoS knockout mutant in mouse macrophage cell line (RAW 264.7)

In order to evaluate a possible involvement of RpoS in internalisation and intracellular multiplication of *B. pseudomallei*, mouse macrophage cell monolayers were infected with *rpoS* mutant and wild type *B. pseudomallei* at MOI of 2:1 and the number of intracellular bacteria was then analysed by standard antibiotic protection assay. The results presented in Fig. 1 showed that *rpoS* mutant appeared to have significantly lower invasive potential comparing with wild-type, judging from the number of intracellular bacteria 3 h after the infection was initiated. In contrast, once internalised, both *rpoS* and wild-type *B. pseudomallei* could similarly survive and multiply intracellularly inside the macrophages. The doubling time of the *rpoS* mutant and the wild-type calculated was 45 and 47 min, respectively, suggesting that the rate of replication of the *rpoS* mutant inside the macrophages was similar to that of the wild-type (data not shown). These results are consistent with the possibility that RpoS plays a role in cellular invasion but not in controlling the rate of replication inside the macrophages.

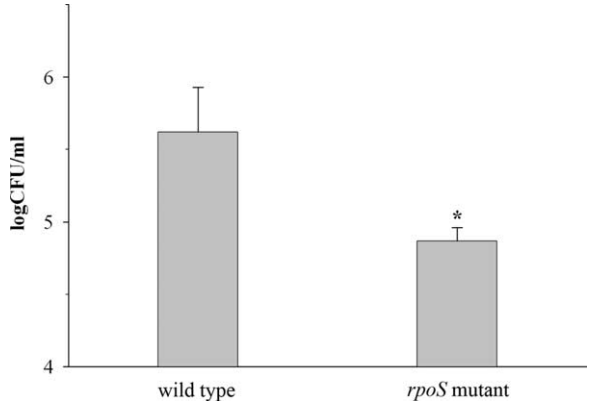

Fig. 1. Internalisation of *rpoS* mutant. Mouse macrophage cell line, (RAW 264.7) was infected with either the *rpoS* mutant or the wild type *B. pseudomallei* at MOI of 2:1 for 1 h. Internalisation of the bacteria was determined after 2 more hours of incubation. The number of viable intracellular bacteria was determined by standard antibiotic protection assay as described in Materials and Methods. Data shown represent means and standard errors of 3 separate experiments, each carried out in duplicate. $*P<0.01$ by Students's *t*-test.

2.2. RpoS interferes with MNGC formation in mouse macrophage cell line (RAW 264.7)

Mouse macrophages were infected with either the *rpoS* mutant or the wild-type *B. pseudomallei* at MOI of 2:1 for a total of 8 h before the host cell morphological changes were analysed under a microscope. As is to be expected from our previous reports [5,9,17], the wild-type *B. pseudomallei* could readily induce cell fusion and MNGC formation (arrows in Fig. 2A). The *rpoS* mutant, on the other hand, failed to initiate these changes. These results suggested that the RpoS of *B. pseudomallei* is involved in this process. This prediction is supported by the results showing that in the presence of chloramphenicol, the MNGC formation induced by the wild-type was inhibited. In this experiment, the macrophages were infected with the wild-type bacteria for 1 h before the chloramphenicol was added at a concentration known to inhibit bacterial protein synthesis. In the presence of this inhibitor, no MNGC formation could be observed, suggesting that the process leading to MNGC formation in the wild type-infected macrophages was initiated after bacterial internalisation (Fig. 2A). It should be mentioned that the concentration of chloramphenicol used in these experiments was able to significantly inhibit bacterial growth (data not shown). The number of MNGC induced by the bacteria was also enumerated by Giemsa staining. Results presented in Fig. 2B showed that the percentage of MNGC induced by *rpoS* mutant and the chloramphenicol-treated wild-type infection was less than 3% while that of the wild-type infection in the absence of the inhibitor was 17%.

2.3. Involvement of RpoS in iNOS expression and nitric oxide production

To investigate the possible association of RpoS with iNOS activation, the mouse macrophages were infected with *rpoS* mutant and wild-type *B. pseudomallei* or *Salmonella enterica* serovar Typhi (used as positive control) at MOI 2:1. Eight hours after the infection was initiated, the iNOS expression of infected macrophages was determined by immunoblotting. Unlike the *Salmonella* control, both *rpoS* mutant and wild-type *B. pseudomallei* failed to stimulate iNOS expression. We previously demonstrated that IFN-γ could enhance iNOS expression and killing capacity of the macrophages if it was added to the cell culture prior to the time of infection [7,9]. In the present study, a similar experiment using *rpoS* mutant-infected macrophages was performed. Briefly, the macrophages were preactivated overnight with IFN-γ prior to *B. pseudomallei* exposure and at 4, 6 and 8 h post-infection, the expression of iNOS was determined by immunoblotting. The results showed that although the IFN-γ could enhance iNOS expression of the macrophages, the level of iNOS expression in the cells infected with *rpoS* mutant was noticeably higher than in those infected with the wild type (Fig. 3B). Consistent with this observation, the level of NO, determined by Griess reaction, in the IFN-γ activated macrophages infected with the *rpoS* mutant was also

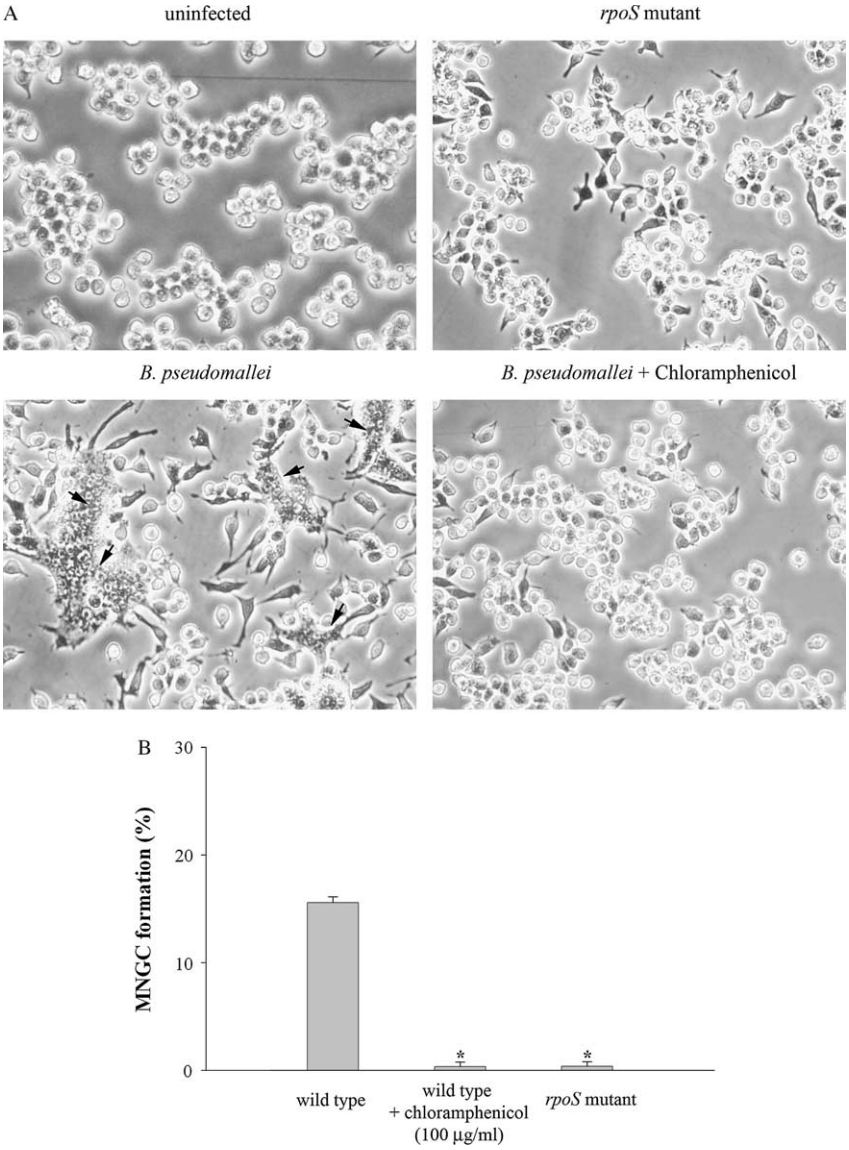

Fig. 2. Inability of *rpoS* mutant to induce MNGC formation. Mouse macrophage cell line (RAW 264.7) was infected with either the *rpoS* mutant or the wild type *B. pseudomallei* at MOI of 2:1 for 1 h. The infected cells were subsequently cultured in the medium with or without chloramphenicol added (100 μg/ml). The MNGC formation (arrows) after 8 h of infection was observed by microscopic examination (100x) (A). To enumerate MNGC formation, the infected cells were fixed, stained with Giemsa and the number of MNGC was determined under microscope (400x) (B). Data shown represent means and standard errors of 3 separate experiments, each carried out in duplicate. $*P < 0.01$ by Students's *t*-test.

significantly higher than that of the wild-type control (Fig. 3C). These results suggested the involvement of RpoS in regulating antimicrobial activity of the IFN-γ activated macrophages.

2.4. Bacterial RpoS modulates intracellular killing capacity of IFN-γ activated macrophages

It was shown previously that the expression of iNOS in macrophages infected with the wild type *B. pseudomallei* directly correlated with the macrophage ability to kill intracellular bacteria [7–9]. To determine whether or not a similar phenomenon would be observed with the macrophages infected with the *rpoS* mutant, the number of viable intracellular bacteria in the IFN-γ activated macrophages was analysed 8 h post infection. The results presented in Fig. 4 clearly demonstrated that although IFN-γ could enhance the macrophage ability to suppress the intracellular growth of both wild-type *B. pseudomallei* and *rpoS* mutant, the level of suppression was higher in the *rpoS* mutant-infected macrophages (a decrease by 2 orders of magnitude for the *rpoS* mutant compared with a decrease of only 1 order of magnitude for the wild-type).

3. Discussion and conclusion

RpoS is recognized as a global stationary-phase sigma factor that controls the expression of several genes including those encoding for virulence factors [12–14,18,19]. The *S. typhimurium rpoS* mutant was shown to be less virulent than its wild-type counterpart [20,21], even though it could still

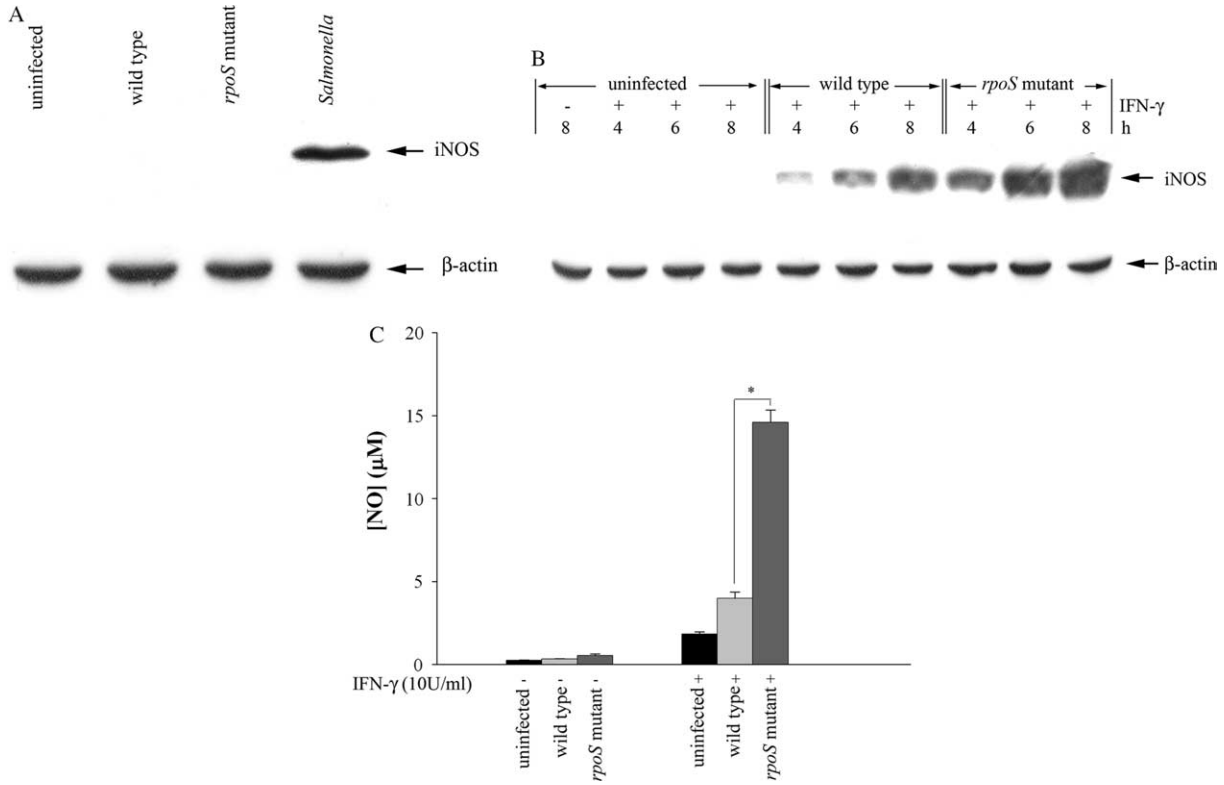

Fig. 3. Influence of RpoS on iNOS expression and NO production. Mouse macrophage cell line (RAW 264.7) was infected with either *rpoS* mutant or wild type *B. pseudomallei* or *Salmonella* at MOI of 2:1. At 8 h time point, the iNOS expression of the infected cells was determined by immunoblotting (A). The effect of IFN-γ on iNOS expression and NO production by the macrophages infected with either the *rpoS* mutant or the wild type *B. pseudomallei* was also investigated (B and C). The macrophages were pretreated with IFN-γ (10 U/ml) overnight before being infected with either the *rpoS* mutant or the wild type *B. pseudomallei* at MOI of 2:1. The expression of iNOS was determined after 4, 6 and 8 h of infection by immunoblotting (B). The level of NO in the supernatant of 8 h macrophage culture was determined by Griess reaction (C). Data represent means and standard errors of 3 separate experiments, each carried out in duplicate. $*P < 0.01$ by Students's *t*-test.

survive and multiply inside the macrophages [18]. The modulation of human activity by *S. typhi rpoS* mutant had also been investigated. For instance, the mutant exhibited lower degree of cytoxicity for human macrophages, judging from a decreased ability to induce host cell death compared with the wild type counterpart [22]. As with the *rpoS* mutant of *Salmonella*, *rpoS* deficient *B. pseudomallei* in the present study could also survive and multiply inside the mouse macrophages in a similar way to the wild type parent (Fig. 1).

One of the unique characteristics of *B. pseudomallei* is their ability to induce a cell-to-cell fusion, resulting in MNGC formation which may facilitate the bacteria to spread from one cell to the others [4,5]. The mechanism of *B. pseudomallei*-induced cell-to-cell fusion is yet to be determined. However, we demonstrated in this communication that the wild-type *B. pseudomallei* failed to induce MNGC when the bacterial protein synthesis was inhibited after the bacteria had been internalised (Fig. 2), suggesting that it is the bacterial factor(s) expressed after being internalised that plays a role in the induction of MNGC formation. This conclusion is based on the data that in the presence of chloramphenicol, no MNGC formation was observed in the macrophages that were infected with the wild-type *B. pseudomallei* even at a very high MOI ratio of 100:1 (data not shown). In contrast to the wild-type, the *B. pseudomallei* lacking *rpoS* at the same MOI could not

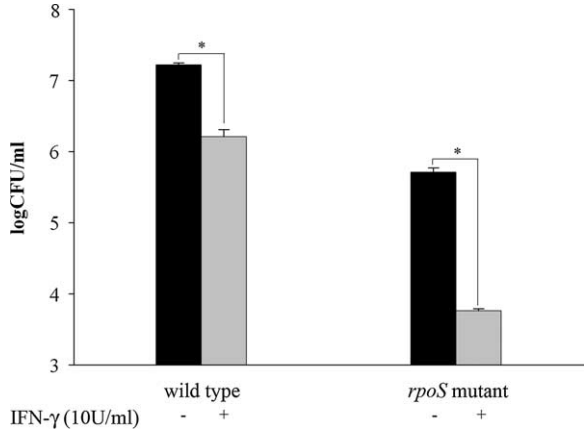

Fig. 4. Effects of IFN-γ in suppression of intracellular bacteria in the infected-macrophages. Mouse macrophage cell line (RAW 264.7) was preactivated with or without IFN-γ (10 U/ml) overnight before being infected with either *rpoS* mutant or wild type *B. pseudomallei* at MOI of 2:1. Eight hours after the infection, the number of intracellular viable bacteria was determined by standard antibiotic protection assay as described in Materials and Methods. A highly significant reduction was noted with the *rpoS* mutant. Data shown represent means and standard errors of 3 separate experiments, each carried out in duplicate. $*P < 0.01$ by Students's *t*-test.

induce MNGC formation, suggesting a possible involvement of RpoS in the induction of MNGC formation (Fig. 2).

Activation of iNOS and NO production in macrophages is known to play an essential role in inhibiting growth and killing of intracellular bacteria including *B. pseudomallei* [7–9]. Previously we demonstrated that *B. pseudomallei* could interfere with iNOS expression, thus allowing it to survive macrophage killing [7,8]. However, exogenous IFN-γ or IFN-β added to resting macrophage could enhance and restore its ability to kill intracellular *B. pseudomallei* by activating iNOS expression [7–9]. In the present study, we demonstrated that by itself the *rpoS* mutant, like its wild-type counterpart, also failed to activate iNOS expression (Fig. 3). However, in the presence of IFN-γ, the level of iNOS in the *rpoS* mutant-infected macrophages was markedly elevated compared with its wild-type control. The increase of iNOS expression and NO production level also correlated to the decrease of intracellular survive of the bacteria (Fig. 4). However, it could also be argued that the lower number of intracellular *rpoS* mutant may be due to inherently higher susceptibility of the mutant to killing by reactive nitrogen intermediate than the wild-type. This problem is now under investigation by our group.

RpoS is known to play an important role in response of bacteria to a number of environmental stresses. *Escherichia coli* produces KatE (hydroperoxidase I) which has both catalase and peroxidase activity, allowing it to overcome the antimicrobial activity of hydrogen peroxide produced by the host [23]. This enzyme is also known to be under the control of RpoS [24]. In the host tissue, RpoS could also regulate virulence gene expression, such as the *spv* of *S. typhimurium* [25]. In our study, we demonstrated that RpoS of *B. pseudomallei* could regulate not only the expression of a putative bacterial factor(s) which may be involved in MNGC formation, but also of the iNOS. However, the virulence factors of *B. pseudomallei* that are regulated by RpoS have never been identified but needed to be investigated if we are to more fully understand the pathogenesis of melioidosis and to develop novel approaches for the management of the disease.

4. Materials and Methods

4.1. Cell line and culture condition

Mouse macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC, Rockville, MD). If not indicated otherwise, the cells were cultured in Dulbecco's modified Eagles' medium (DMEM) (Gibco Labs, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) at 37 °C under a 5% CO_2 atmosphere.

4.2. Bacterial strains

B. pseudomallei strain 844 used in this study was originally isolated from a patient admitted to Srinagarind Hospital in the melioidosis endemic Khon Kaen province of Thailand as previously described [7–9]. *Salmonella enterica* serovar Typhi (*Salmonella*) used for comparison throughout this study was originally isolated from a patient at Ramathibodi Hospital (Mahidol University, Bangkok, Thailand).

rpoS mutant of *B. pseudomallei* was constructed and had been characterized as previously described [16]. It should be mentioned that the mutant was still reactive with polyclonal anti-*B. pseudomallei* and monoclonal antibodies against the lipopolysaccharide and exopolysaccharide components of *B. pseudomallei* [26].

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

An overnight culture of mouse macrophages (1×10^6 cells) in a six-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 three times with 2 ml of PBS and residual bacteria were killed by incubating in DMEM 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 [5,8]. To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed as previously described [5]. The number of intracellular bacteria expressed as colony forming unit (CFU) was determined by bacterial colony counting

4.4. Immunoblotting

Mouse macrophage preparations were lysed in buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates containing 30 μg of protein were electrophoresed on 10% SDS-PAGE and then electrotransferred to nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany). The membrane was blocked with 5% skim milk for 1 h before incubating overnight with appropriate specific polyclonal rabbit antibodies to mouse iNOS (Santa Cruz, Santa Cruz, CA). The blots were then allowed to react with horseradish peroxidase-conjugated swine anti-rabbit IgG (Pierce, Rockford, IL). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany).

4.5. Enumeration of multinucleated giant cells (MNGCs) in B. pseudomallei-infected macrophages

In order to quantitate the degree of MNGC formation, the macrophages (1×10^6) were first cultured overnight on a coverslip as previously described [5]. For the chloramphenicol experiment, the drug (100 μg/ml) was added into the culture medium after the bacteria were internalised for 1 h. Eight hours after the infection, the coverslips were washed with PBS, fixed for 15 min with 1% paraformaldehyde and then washed sequentially with 50 and 90% ethanol for 5 min each. The coverslips were air dried before staining with Giemsa [5]. For enumeration of the MNGC formation, at least 1000 nuclei per coverslip were counted using light microscope at

a magnification of 40× and the percentage of multinucleated cells was calculated [5]. The MNGC was defined as the cell possessing more than one nuclei within the same cell boundary.

4.6. NO assay

The production of NO in the form of nitrite in the supernatant was determined by the Griess reaction [27]. The nitrite was used as standard ranged from 0 to 40 μM.

4.7. Statistical Analysis

If not otherwise indicated, all experiments in this study were conducted at least three times. Experimental values were expressed as means ± standard errors. Statistical significance of differences between the two means was evaluated by Student's *t* test, and *P* value <0.01 was considered significant.

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References

[1] Cheng AC, Currie BJ. Melioidosis: epidemiology, pathophysiology, and management. Clin Microbiol Rev 2005;18:383–416.
[2] Dharakul T, Songsivilai S. The many facets of melioidosis. Trends Microbiol 1999;7:138–46.
[3] Jone A, Beveridge TJ, Woods DE. Intracellular survival of *Burkholderia pseudomallei*. Infect Immun 1996;64:782–90.
[4] Harley VS, Dance DAB, Drasar BJ, Tovey G. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. Microbios 1998;96:71–93.
[5] Kespichayawattana W, Rattanachetkul S, Wanun T, Utaisincharoen P, Sirisinha S. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protusion: a possible mechanism for cell-to-cell spreading. Infect Immun 2000;68:5377–84.
[6] Wong KT, Puthucheary SD, Vadievelu J. The histopathology of human melioidosis. Histopathology 1995;26:51–5.
[7] Utaisincharoen P, Tangthawornchaikul N, Kespichayawattana W, Chaisuriya P, Sirisinha S. *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. Microbiol Immunol 2001;45:307–13.
[8] Utaisincharoen P, Anuntagool N, Limposuwan K, Chaisuriya P, Sirisinha S. Involvement of IFN-β in enhancing inducible nitric oxide synthase (iNOS) production and antimicrobial activity of *B. pseudomallei*-infected macrophages. Infect Immun 2003;71:3053–7.
[9] Utaisincharoen P, Anuntagool N, Arjcharoen S, Limposuwan K, Chaisuriya P, Sirisinha S. Induction of iNOS expression and antimicrobial activity by interferon (IFN)-β is distinct from IFN-γ in *Burkholderia pseudomallei*-infected mouse macrophages. Clin Exp Immunol 2004;136:277–83.
[10] Muffler A, Traulsen DD, Lange R, Hengge-Aronis R. Posttranscriptional osmotic regulation of the σ^S subunit of RNA polymerase in *Escherichia coli*. J Bacteriol 1996;178:1607–13.
[11] Hengge-Aronis R. Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. Microbiol Mol Biol Rev 2002;66:373–95.
[12] Kowarz L, Coynault C, Robbe-Saule V, Norel F. The *Salmonella typhimurium katF (rpoS)* gene: cloning, nucleotide sequence, and regulation of *spvR* and *spvABCD* virulence plasmid genes. J Bacteriol 1994;176:6852–60.
[13] Norel F, Robbe-Saule V, Popoff MY, Coynault C. The putative sigma factor KatF (RpoS) is required for the transcription of the *Salmonella typhimurium* virulence gene *spvB* in *Escherichia coli*. FEMS Microbiol Lett 1992;99:271–6.
[14] Nickerson CA, Curtiss III R. Role of sigma factor RpoS in initial stages of *Salmonella typhimurium* infection. Infect Immun 1997;65:1814–23.
[15] Fang FC, Libby SJ, Buchmeier NA, Loewen PC, Switala J, Harwood J, et al. The alternative sigma factor KatF (RpoS) regulates *Salmonella* virulence. Proc Natl Acad Sci USA 1992;89:11978–82.
[16] Subsin B, Thomas MS, Katzenmeier G, Shaw JG, Tungpradabkul S, Kunakorn M. Role of the stationary growth phase sigma factor RpoS of *Burkholderia pseudomallei* in response to physiological stress conditions. J Bacteriol 2003;185:7008–14.
[17] Utaisincharoen P, Kespichayawattana W, Anuntagool N, Chaisuriya P, Pichyangkul S, Krieg AM, et al. CpG ODN enhances uptake of bacteria by mouse macrophages. Clin Exp Immunol 2003;132:70–5.
[18] Foster JW, Spector MP. How *Salmonella* survive against the odds. Annu Rev Microbiol 1995;49:145–74.
[19] Kolter R, Siegele DA, Tormo A. The stationary phase of bacterial life cycle. Annu Rev Microbiol 1993;47:855–74.
[20] Wilmes-Riesenberg MR, Foster JW, Curtiss III R. An altered rpoS allele contributes to the avilulence of *Salmonella typhimurium* LT12. Infect Immun 1997;65:203–10.
[21] Coynault C, Robbe-Saule V, Norel F. Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS (ss) regulon. Mol Microbiol 1996;22:149–60.
[22] Khan AQ, Zhao L, Hirose K, Miyake M, Li T, Hashimoto Y, et al. *Salmonella typhi rpoS* mutant is less cytotoxic than the parent strain but survives inside resting THP-1 macrophages. FEMS Microbiol Lett 1998;161:201–8.
[23] Gonzalez-Flecha B, Demple B. Homeostatic regulation of intracellular hydrogen peroxide concentration in aerobically growing *Escherichia coli*. J Bacteriol 1997;179:382–8.
[24] Schellhorn HE, Stones VL. Regulation of *katF* and *katE* in *Escherichia coli* K-12 by weak acids. J Bacteriol 1992;170:3903–9.
[25] Chen CY, Eckmann L, Libby SJ, Fang FC, Okamoto S, Kagnoff MF, et al. Expression of *Salmonella typhimurium rpoS* and *rpoS*-dependent genes in the intracellular environment of eukaryotic cells. Infect Immun 1996;64:4739–43.
[26] Anuntagool N, Intachote P, Wuthiekanun V, White NJ, Sirisinha S. Lipopolysaccharide from nonvirulent Ara^+ *Burkholderia pseudomallei* isolates is immunologically indistinguishable from lipopolysaccharide from virulent Ara^- clinical isolate. Clin Diagn Lab Immunol 1998;5:225–9.
[27] Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok S, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Anal Biochem 1982;126:131–8.

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Short communication

Expression of suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) induced in *Burkholderia pseudomallei*—infected mouse macrophages requires bacterial internalization

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Abstract

We recently reported that *Burkholderia pseudomallei* was able to activate the expression of suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS). In the present study, we presented evidence showing that the induction of these negative regulators was most probably triggered from within rather than at the cell surface of mouse macrophage cell line (RAW264.7) suggesting that macrophage activation most likely requires the interaction of bacteria with a putative host cell cytoplasmic component(s).

Keywords: *B. pseudomallei*; Melioidosis; SOCS; SOCS3; CIS

1. Introduction

Burkholderia pseudomallei is the causative agent of melioidosis which is an infectious disease in endemic areas such as southeast Asia, northern Australia and temperate regions bordering the equator [1,2]. This gram-negative bacterium could survive and multiply in both phagocytic and non-phagocytic cells [3,4]. The mechanisms which *B. pseudomallei* uses to escape macrophage killing include interfering with beta interferon (IFN-β) production by the infected macrophages and with a subsequent failure to activate inducible nitric oxide synthase (iNOS) [5]. Addition of exogenous IFN-β or IFN-γ at the time of bacterial challenge could enhance the ability of the macrophages to kill intracellular *B. pseudomallei* by restoring their ability to activate iNOS expression [6]. However, addition of exogenous IFN-γ after the macrophages had been infected with *B. pseudomallei* resulted in a failure of the infected cells to eliminate intracellular *B. pseudomallei* [7]. These results suggested that a precise timing is needed for the IFNs to exert their antimicrobial activity in relation to the time of *B. pseudomallei* infection.

IFN-γ is known to exert its effects by interacting with a specific surface receptor which then activates the receptor-associated Janus tyrosine kinases JAK1 and JAK2 required to phosphorylate STAT-1 at Y701 before its translocation to the IFN-γ activation site (GAS) in the nucleus [8,9]. This process is required for a transcription of IFN-γ responsive genes, including IRF-1 and iNOS genes [10]. However, the macrophage response to IFNs can be influenced by several negative regulators including suppressor of cytokine signaling (SOCS) proteins. Eight members of the SOCS protein family (SOCS1 to 7 and CIS) have been identified. They all serve as negative regulators for the cytokine signaling via JAK/STAT pathway, thus attenuating the macrophage response including antimicrobial activity [11]. Microbes also can induce SOCS expression. For example, *Listeria monocytogenes* was able to diminish IFN-γ-stimulated transcription and phosphorylation of STAT-1

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in the infected macrophages and this correlated with the activation of SOCS3 expression [12].

We recently demonstrated that both living- and heat-killed *B. pseudomallei* was able to stimulate SOCS3 and CIS but not SOCS1 expression in mouse macrophage cell line (RAW 264.7) [7]. The level of SOCS3 and CIS expression was directly proportional with the number of bacterial infection. Moreover, the expression of these two negative regulators also directly correlated with a decrease of Y701-STAT-1 phosphorylation induced by IFN-γ. The decrease of IFN-γ signaling in this model also correlated with the failure of the *B. pseudomallei*-infected cells to activate iNOS expression, thus limiting its capacity to kill intracellular *B. pseudomallei* [7]. In the present communication, we extended our finding to demonstrate that the signal needed to initiate both SOCS3 and CIS expression in this model required bacterial internalization, suggesting that the triggering signal is initiated from within rather than at the surface of the macrophages.

2. Results and discussion

2.1. Cytochalasin D inhibits SOCS3 and CIS expression in *B. pseudomallei*-infected macrophages

In order to elucidate the possible mechanism of SOCS3 and CIS expression, experiments were set up essentially as described in our previous report [7] but the internalization of *B. pseudomallei* by the macrophages was inhibited by cytochalasin D. Mouse macrophage cell line (RAW 264.7) was pretreated with 2 μg/ml of cytochalasin D (Sigma, St. Louis, MO) for 2 h prior to the time of bacterial challenge (MOI of 2:1). The mRNA expression of SOCS3 and CIS was determined by RT-PCR. As is to be expected from our previous report, SOCS3 and CIS gene expression was observed in *B. pseudomallei*-infected macrophages within 1 h and gradually increased to 4 h when the experiment was terminated (Fig. 1). However, in the presence of cytochalasin D, the level of SOCS3 and CIS

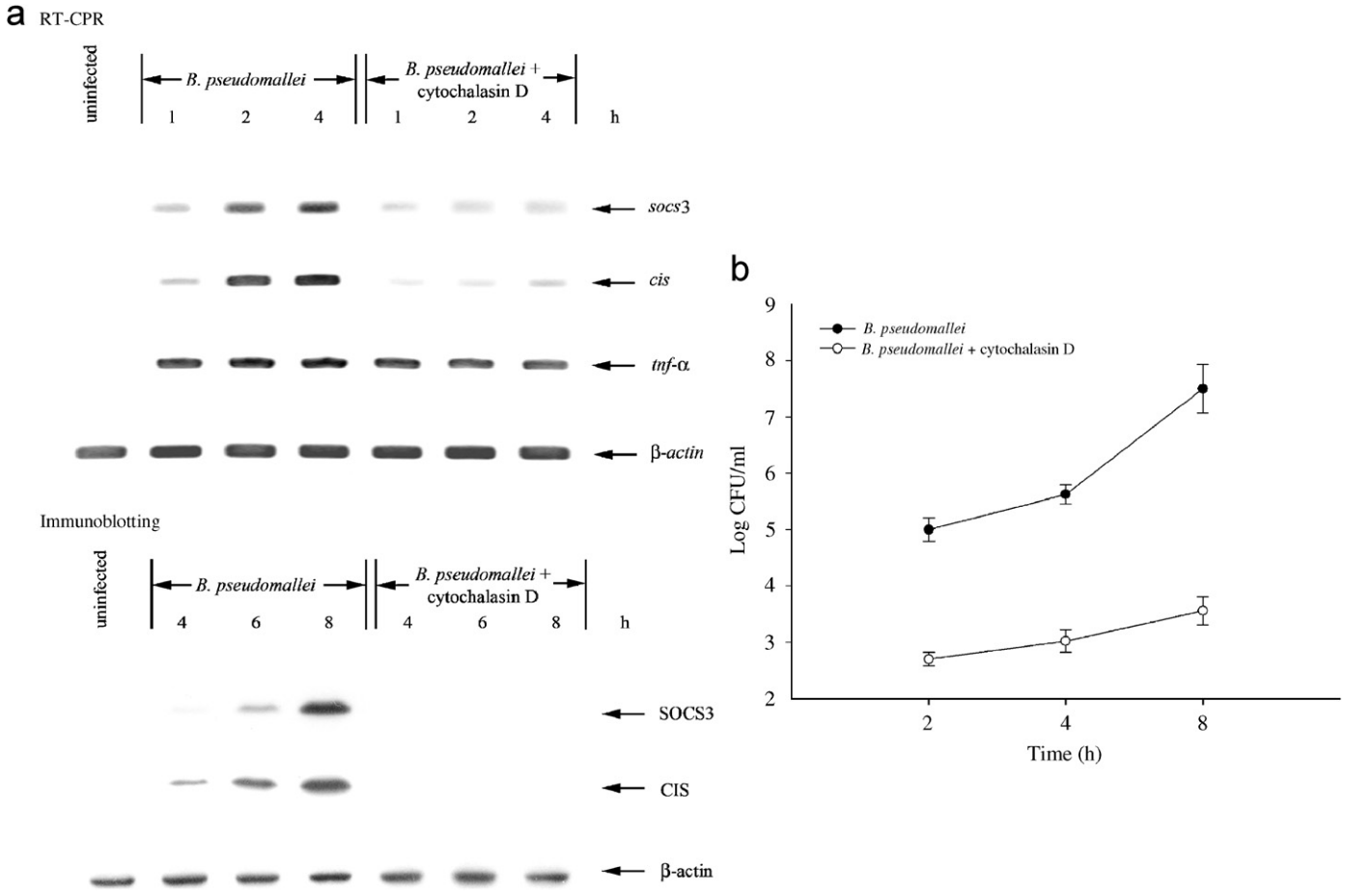

Fig. 1. Cytochlasin D inhibits activation of SOCS3 and CIS expression in *B. pseduomallei*-infected macrophages. Mouse macrophage cell line (RAW 264.7) (1×10^6 cells/ml) was exposed to *B. pseudomallei* at an MOI of 2:1. To inhibit internalization of *B. pseudomallei*, the macrophages were pretreated with cytochalasin D (2 μg/ml) for 1 h prior to the time of challenge. (a) At different time intervals, the infected cells were lyzed and the levels of SOCS mRNAs or proteins in the cell lysates were determined by RT-PCR and immunoblotting, respectively. Expressions of TNF-α and β-actin were determined and used as the control. (b) The number of intracellular *B. pseudomallei* in the infected macrophages pretreated/untreated with cytochalasin D was determined by standard antibiotic protection assay.

mRNA expression was markedly reduced. Similarly, the protein expression was also diminished in the presence of this inhibitor (Fig. 1). In contrast to the reduced SOCS3 and CIS expression, the level of TNF-α mRNA expression was unaltered by cytochalasin D treatment, suggesting different pathways are used for the induction of these 2 negative regulators and TNF-α. These results also suggested that the signal generated by the interaction of *B. pseudomallei* with appropriate receptor(s) at the surface of macrophages was sufficient to activate TNF-α expression, but insufficient to activate SOCS3 and CIS expression. It should be mentioned that, at the concentration used, the cytochalasin D was able to significantly reduce the number of intracellular *B. pseudomallei* in mouse macrophages (Fig. 1b).

2.2. Chloramphenicol inhibits SOCS3 and CIS expression in *B. pseudomallei*-infected macrophages

One could argue that by some unknown mechanism, the cytochalasin D might also interfere with the interaction of *B. pseudomallei* at the surface of macrophages; therefore, another experiment was conducted to rule out this possibility. In this experiment, *B. pseudomallei* was allowed to interact with the macrophage at an MOI of 2:1 for 1 h before culturing in the medium containing 100 μg/ml of chloramphenicol (Sigma). At this concentration, the chloramphenicol inhibited intracellular bacterial replication and under this condition, the number of intracellular *B. pseudomallei* determined at 4 and 8 h after the infection were 3×10^4 and 3.1×10^4 CFU, respectively, compared

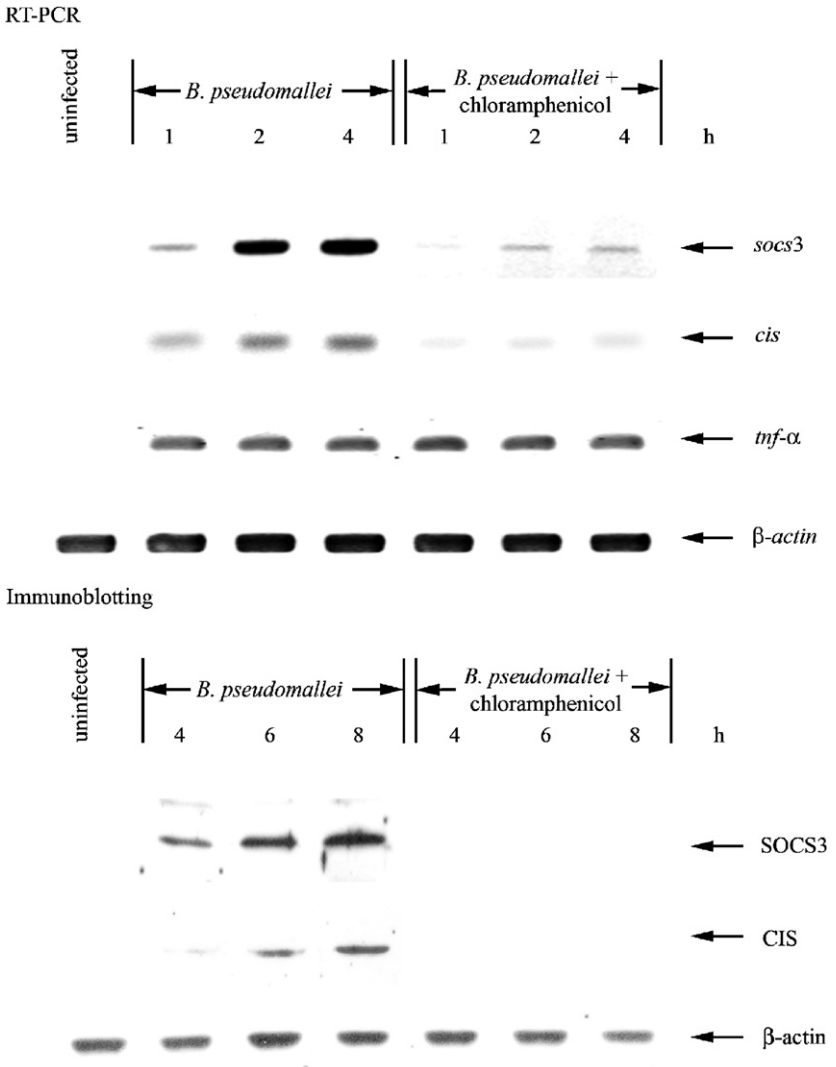

Fig. 2. Chloramphenicol inhibits activation of SOCS3 and CIS expression in *B. pseudomallei*-infected macrophages. To inhibit the replication of internalized bacteria, the macrophages were infected with *B. pseudomallei* as mentioned for 1 h before culturing in above culture medium containing in addition of 100 μg/ml of chloramphenicol. At different time intervals, the infected cells were lyzed and the levels of SOCS mRNAs or proteins in the cell lysates were determined by RT-PCR and immunoblotting, respectively. Expressions of TNF-α and β-actin were determined and used as control.

with 6×10^6 and 6×10^7 CFU in the untreated macrophages. The results presented in Fig. 2 showed that in the presence of chloramphenicol, there was no activation of SOCS3 and CIS expression. In contrast, the level of TNF-α mRNA expression was unaltered by the chloramphenicol treatment. This showed that the binding of the bacteria to surface receptors alone was not sufficient to initiate signaling that triggers SOCS3 and CIS expression. In order to rule out the possibility that the chloramphenicol itself did not interfere with the signaling pathway for SOCS3 and CIS activation, the chloramphenicol-treated macrophages were first exposed to heat-killed *B. pseudomallei* using a ratio equivalent to an MOI of 10:1 and 4 h later, the level of SOCS3 and CIS mRNA was determined. Previously, we demonstrated that both live and heat-killed *B. pseudomallei* were able to induce SOCS3 and CIS expression [7]. The results in the present communication showed that the level of SOCS3 and CIS mRNA expression in macrophages activated with heat-killed *B. pseudomallei* was unaffected by the presence of chloramphenicol (data not shown), indicating that at the concentration used, chloramphenicol did not interfere with SOCS3 and CIS activation of the macrophages. Altogether, these results suggested that the interaction of live *B. pseudomallei* on the surface of macrophages was not sufficient to trigger the activation of SOCS3 and CIS expression. It should be of great interest to identify the possible presence of intracytoplasmic receptor(s) that initiates the signaling process of these negative regulators.

2.3. Inhibition of SOCS3 and CIS expression lead to increase of Y701-STAT-1 phosphorylation induced by IFN-γ

Although SOCS1 is probably the most potent inhibitor of IFN signaling, acting by suppressing the phosphorylation of STAT-1 [13,14], to a lesser extent, SOCS3 and CIS, could also inhibit Y701-STAT-1 phosphorylation [15,16] and therefore, the level of phosphorylation could be taken as an indicator for the degree of cellular response to IFN-γ stimulation. In order to determine whether or not the diminished SOCS3 and CIS expression after either cytochalasin D or chloramphenicol treatments correlate with a possible increase of IFN-γ signaling, the phosphorylation of Y701-STAT-1 of the infected cells, in the presence of these inhibitors was determined and compared with that of the untreated infected cell control. In the cytochalasin D experiment, the mouse macrophages were infected with *B. pseudomallei* at an MOI of 2:1, with or without pretreatment with 2 μg/ml cytochalasin D, and thereafter the cultures were incubated further for 1, 2 and 4 h before adding IFN-γ (10 U/ml). One hour after the IFN-γ was added, the level of pY701-STAT-1 was determined by immunoblotting using antibody against pY701-STAT-1. For the chloramphenicol experiment, the macrophages were infected with the bacteria as above for 1 h before 100 μg/ml of chloramphenicol was added and the level of pY701-STAT-1 was then similarly determined. It should be noted that the level of pY701-STAT-1 in the untreated infected macrophages gradually declined as the time of infection was prolonged (Fig. 3a) and this correlated with the increase of SOCS3 and CIS expression shown earlier in Figs. 1 and 2. In contrast, the level of IFN-γ activated pY701-STAT-1 remained elevated in the macrophage cultures pretreated with cytochalasin D which interferes with bacterial internalization (Fig. 3b). Similarly, the macrophages that were treated with chloramphenicol 1 h after the infection was initiated exhibited the level of pY701-STAT-1 that was not different from the initial level (Fig. 3c). The level of IFN-γ induced pY701-STAT-1 in this experiment (Fig. 3) paralleled the diminishing of SOCS3 and CIS expression shown earlier in Figs. 1 and 2.

The activation of SOCS expression and its significance have attracted considerable attention from many groups of investigators in recent years. Although it has been demonstrated that different microbes including *B. pseudomallei* have been reported to induce SOCS expression in the macrophage, very limited information is available on the mechanism of induction of their expression [7,12,17,18]. It is well documented that purified microbial components like the lipopolysaccharide (LPS) from gram negative bacteria could also activate SOCS expression through interaction with different pattern recognition receptors [15,19,20]. The participation of the membrane-associated Toll-like receptor (TLR) in SOCS expression has thus received much attention [15]. It was previously reported that a typical LPS from *Escherichia coli* and several other gram-negative bacteria was able to stimulate macrophage to produce TNF-α production through TLR4 expressed on the cell surface [21]. With the pathogen models that have been reported (e.g., *L. monocytogenes* or *Leishmania donovani*), the signals for activating SOCS expression have not been elucidated. To our knowledge, the data in the present study is the first to show that the signaling for activating the SOCS expression is likely initiated via the interaction of intracellulary located bacteria with a yet to be defined intracellular pattern recognition receptor(s). Several intracellular pattern recognition receptors including NOD1 and NOD2 have been described [22]. However, the nature of the putative cytoplasmic receptors for intracellular *B. pseudomallei* that might participate in the activation of SOCS3 and CIS expression remains to be investigated.

3. Materials and methods

3.1. Cell line and culture condition

Mouse macrophage cell line (RAW 264.7) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). If not indicated otherwise, the cells were cultured in Dulbecco's modified Eagles' medium (DMEM) (Gibco Labs, Grand Island, NY) supplemented with 10%

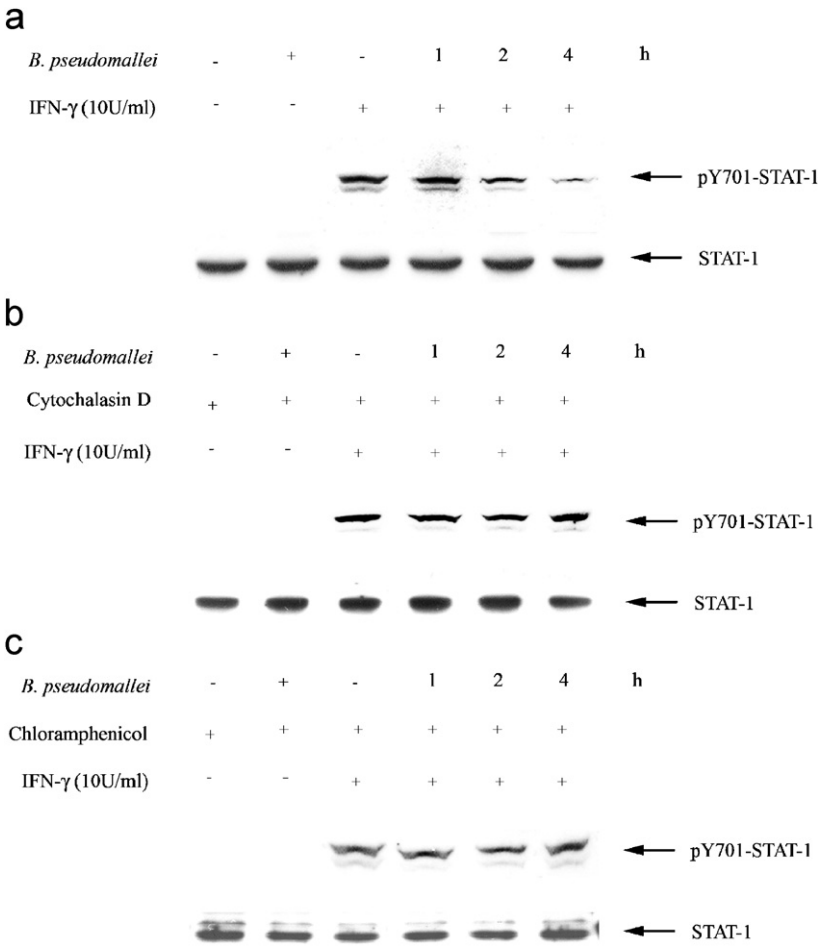

Fig. 3. Cytochalsin D and chloamphenicol interfere with IFN-γ-activated phosphorylation of Y701-STAT-1 in *B. pseudomallei*-infected macrophages. Mouse macrophages were infected with *B. pseudomallei* at an MOI of 2:1 and incubated for 1, 2 and 4 h before IFN-γ (10 U/ml) was added (a). To inhibit bacterial internalization, the macrophages were pretreated with 2 μg/ml of cytochalasin D for 1 h prior to the time of challenge. IFN-γ was then added 1, 2 and 4 h after the bacterial challenging (b). To inhibit intracellular bacterial replication, the macrophages were first infected with *B. pseudomallei* for 1 h before culturing in the medium containing 100 μg/ml of chloramphenicol and IFN-γ was added 1, 2 and 4 h after the bacterial challenge (c). The macrophages in all experiment were incubated further for one hour before the level of pY701-STAT-1 was determined by immunoblotting.

heat-inactivated fetal bovine serum (FBS) (HyClone, Logan, UT) at 37 °C under a 5% CO_2 atmosphere.

3.2. Bacterial strains

B. pseudomallei strain 844 used in this study was originally isolated from a patient admitted to Srinagarind Hospital in the melioidosis endemic Khon Kaen province of Thailand. The bacterium was originally identified as *B. pseudomallei* based on its biochemical characteristics, colonial morphology on selective media, antibiotic sensitivity profiles and reactivity with polyclonal and monoclonal antibodies [23–25].

Non-viable *B. pseudomallei* were prepared by heating the bacteria (10^8 CFU/ml) in a boiling water bath for 15 min. The heat-treated bacteria were washed 3 times with phosphate buffered saline pH 7.2 (PBS) and complete killing confirmed by inoculating the suspension on tryptic soy agar and observing growth after 48 h.

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

An overnight culture of mouse macrophages (1×10^6 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 3 times with 2 ml of PBS and residual bacteria were killed by incubating in DMEM containing 250 μg/ml kanamycin (Gibco Labs) for 2 h. Thereafter, the infection was allowed to continue in medium containing 20 μg/ml of kanamycin until the experiment was terminated. Viability of the infected macrophages was determined by tryphan blue staining and found to be higher than 90% throughout the time course employed in this study.

To determine intracellular survival and multiplication of the bacteria, a standard antibiotic protection assay was performed as previously described [7]. The intracellular bacteria were liberated by lyzing 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 colony forming units (CFU), was determined by bacterial colony counting.

3.4. Immunoblotting

Mouse macrophage preparations were lyzed in buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. Lysate samples containing 30 μg of protein were electrophoresed on 10% SDS-PAGE and then electrotransferred to nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany). The membrane was blocked with 5% milk for 1 h before incubating overnight with appropriate specific polyclonal rabbit antibodies to mouse SOCS3, CIS, pY701-STAT-1, STAT-1 or actin (Santa Cruz, Santa Cruz, CA). The blots were then allowed to react with horseradish peroxidase-conjugated swine anti-rabbit IgG (Pierce, Rockford, IL). Protein bands were detected by enhanced chemiluminescence as recommended by the manufacturer (Roche Diagnostics, Mannheim, Germany).

3.5. Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from infected cells according to the manufacturer's instructions (Eppendorf, Hamburg, Germany) before being used for cDNA synthesis by cMaster RT Enzyme (Eppendorf). The PCR reaction was performed using cDNA as the template and primer pairs specific for SOCS3, CIS and actin in amplification reactions with Taq DNA polymerase (Invitrogen, Carlsbad, CA). The primers used to amplify each gene were: SOCS3, sense: 5′-ATGGTCACCCACAGCAAGTT-3′, antisense: 5′-AATCCGCTCTCCTGCAGCTT-3′, CIS, sense: 5′-GAACCGAAGGTGCTAGACCCT-3′, antisense: 5′-TGTACCCTCCGGCATCTTCT-3′ and actin, sense: 5′-CCAGAGCAAGAGAGGTATCC-3′, antisense: 5′-CTGTGGTGGTGAAGCTGTAG-3′ [7]. The amplified products were electrophoresed on a 2% agarose gel and stained with ethidium bromide before being visualized under a UV lamp.

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References

[1] Wiersinga WJ, van der Poll T, White T, Day NP, Peacock SJ. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. Nat Rev Microbiol 2006;4:272–82.
[2] White NJ. Melioidosis. Lancet 2003;361:1715–22.
[3] Kespichayawattana W, Rattanachetkul S, Wanun T, Utaisincharoen P, Sirisinha S. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. Infect Immun 2000;68:5377–84.
[4] Utaisincharoen P, Tangthawornchaikul N, Kespichayawattana W, Chaisuriya P, Sirisinha S. *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. Microbiol Immunol 2001; 45:307–13.
[5] Utaisincharoen P, Anuntagool N, Limposuwan K, Chaisuriya P, Sirisinha S. Involvement of beta interferon in enhancing inducible nitric oxide synthase production and antimicrobial activity of *Burkholderia pseudomallei*-infected macrophages. Infect Immun 2003; 71:3053–7.
[6] Utaisincharoen P, Anuntagool N, Arjcharoen S, Limposuwan K, Chaisuriya P, Sirisinha S. Induction of iNOS expression and antimicrobial activity by interferon (IFN)-β is distinct from IFN-γ in *Burkholderia pseudomallei*-infected mouse macrophages. Clin Exp Immunol 2004;136:277–83.
[7] Ekchariyawat P, Pudla S, Limposuwan K, Arjcharoen S, Sirisinha S, Utaisincharoen P. *Burkholderia pseudomallei*-induced expression of suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS) in mouse macrophages: a possible mechanism for suppression of the response to gamma interferon stimulation. Infect Immun 2005;73:7332–9.
[8] Decker T, Kovarik P, Meinke A. GAS elements: a few nucleotides with a major impact on cytokine-induced gene expression. J Interferon Cytokine Res 1997;17:121–34.
[9] Schindler C, Darnell Jr. JE. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem 1995;64:621–51.
[10] Decker T, Stockinger S, Karaghiosoff M, Muller M, Kovarik P. IFNs and STATs in innate immunity to microorganism. J Clin Invest 2002;109:1271–7.
[11] Alexander W, Hilton D. The role of suppressor of cytokine signaling (SOCS) proteins in regulation of the immune response. Ann Rev Immunol 2004;22:503–29.
[12] Stoiber D, Stockinger S, Steinlein P, Kovarik J, Decker T. *Listeria monocytogenes* modulates macrophage cytokine responses through STAT serine phosphorylation and the induction of suppressor of cytokine signaling 3. J Immunol 2001;166:466–72.
[13] Brysha M, Zhang JG, Bertolino P, Corbin JE, Alexander WS, Nicola NA, et al. Suppressor of cytokine signaling-1 attenuates the duration of interferon γ signal transduction in *in vitro* and *in vivo*. J Biol Chem 2001;276:22086–9.
[14] Kinjyo I, Hanada T, Inagabi-Ohara K, Mori H, Aki D, Ohishi M, et al. SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. Immunity 2002;17:583–91.
[15] Baetz A, Frey M, Heeg K, Dalpke A. Suppressor of cytokine signaling (SOCS) proteins indirectly regulate. Toll-like receptor signaling in innate immune cells. J Biol Chem 2004;279:54708–15.
[16] Dalpke A, Eckerle S, Frey M, Heeg K. Triggers of Toll-like receptor modulates IFN-γ signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signal. Eur J Immunol 2003;33:1776–87.
[17] Bertholet S, Dickensheets HL, Sheikh F, Gam AA, Donnelly RP, Kenny RT. *Leishmania donovani*-induced expression of suppressor of cytokine signaling 3 in human macrophages: a novel mechanism for intracellular parasite suppression of activation. Infect Immun 2003;71:2095–101.
[18] Zimmermann S, Murray PJ, Heeg K, Dalpke AH. Induction of suppressor of cytokine signaling-1 by *Toxoplasma gondii* contributes to immune evasion in macrophages by blocking IFN-γ signaling. J Immunol 2006;176:1840–7.
[19] Crespo A, Filla MB, Murphy WJ. Low responsiveness to IFN-γ, after pretreatment of mouse macrophages with lipopolysaccharides, develops via diverse regulatory pathways. Eur J Immunol 2002; 32:710–9.
[20] Stoiber D, Kovarik P, Cohney S, Johnston J, Steinlein P, Decker T. Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction

in response to the activating factor IFN-gamma. J Immunol 1999; 163:2640–7.
[21] Akira S, Takeda K. Toll-like receptor signaling. Nat Rev Immunol 2004;4:499–511.
[22] Storber W, Murray JP, Kitani A, Watanabe T. Signaling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2006;6:9–20.
[23] Anuntagool N, Intachote P, Wuthiekanun P, White NJ, Sirisinha S. Lipopolysaccharide from nonvirulent Ara^{+} *Burkholderia pseudomallei* isolates is immunologically indistinguishable from lipopolysaccharide from virulent Ara^{-} clinical isolate. Clin Diagn Lab Immunol 1998;5:225–9.
[24] Kawahara K, Dejsirilert S, Danbara H, Ezaki T. Extraction and characterization of lipopolysaccharide from *Pseudomonas pseudomallei*. FEMS Microbiol Lett 1992;96:129–34.
[25] Wuthiekanun V, Smith MD, Dance DAB, Walsh AL, Pitt TL, White NJ. Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*. J Med Microbiol 1996;45:408–12.