

# Nucleotide-binding oligomerization domain-containing protein 2 regulates suppressor of cytokine signaling 3 expression in *Burkholderia pseudomallei*-infected mouse macrophage cell line RAW 264.7

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## Abstract

*Burkholderia pseudomallei*, a causative agent of melioidosis, is a facultative intracellular Gram-negative bacterium that can survive and multiply inside the macrophages. Toll-like receptors are one class of pattern recognition receptors (PRRs) that have been documented to play significant role in *B. pseudomallei* infection. In the present study, we investigated a potential role of nucleotide-binding oligomerization domain-containing protein 1 and 2 (NOD1 and NOD2), cytoplasmic pattern recognition receptors, in *B. pseudomallei*-infected mouse macrophage cell line RAW 264.7. Both live and heat-killed *B. pseudomallei* were able to up-regulate NOD1 and NOD2 expression in a time-dependent manner. Marked reduction of a negative regulator, suppressor of cytokine signaling 3 (SOCS3), expression was observed only in *B. pseudomallei*-infected NOD2-depleted macrophages and not in NOD1-depleted macrophages. The decrease in SOCS3 expression also led to an increase in IFN- $\gamma$  responsiveness as judged by an enhanced STAT-1 phosphorylation on tyrosine 701 in the *B. pseudomallei*-infected macrophages. Together, these results suggested that, in addition to using other PRRs to evade macrophage defense, *B. pseudomallei* may also use NOD2 to regulate a negative regulator like SOCS3.

## Keywords

*Burkholderia pseudomallei*, melioidosis, NOD2, pattern recognition receptors, SOCS3

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## Introduction

*Burkholderia pseudomallei* is a causative agent of melioidosis, an infectious disease endemic in certain tropical regions especially in Southeast Asia and Northern Australia.<sup>1,2</sup> The clinical signs of melioidosis range from subacute and chronic suppurative infections to acute pneumonias and fulminating septicemias.<sup>3,4</sup> Systemic infections are associated with high mortality rate and high rate of relapse despite prolonged treatment.<sup>1,5</sup> Due to the severity of infection and its potential as a biothreat agent, *B. pseudomallei* is now listed as a category B agent by the US Centers for Disease Control.<sup>6,7</sup> At the cellular level, *B. pseudomallei* can survive and multiply inside both phagocytic and non-phagocytic cells.<sup>8</sup> After being internalized, this

bacterium can escape from a membrane-bound phagosome into the cytoplasm and induce a cell-to-cell fusion, resulting in multinucleated giant cell (MNGC) formation.<sup>8–10</sup> This unique phenomenon has also been observed in tissues of patients with melioidosis.<sup>11</sup> Multinucleated giant cell formation may facilitate the spreading of *B. pseudomallei* from one cell to another.<sup>10</sup> Recent studies demonstrated that

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both type III secretion systems (T3SSs) and global regulatory factor RpoS are involved in this process.<sup>12,13</sup> The macrophage is known to play an essential role in innate immune response against many bacterial infections. However, *B. pseudomallei* can escape macrophage killing, resulting in its ability to survive and replicate intracellularly.<sup>14</sup> Previously, we demonstrated that this bacterium failed to stimulate IFN- $\beta$  production, thus resulting in a decreased expression of a key antimicrobial enzyme, inducible nitric oxide synthase (iNOS).<sup>15</sup> The inability to induce iNOS expression may be due to the fact that, unlike most other Gram-negative bacteria, *B. pseudomallei* fails to activate MyD88-independent pathway which is an essential signaling pathway of TLR4.<sup>16</sup> These observations suggest that signaling through TLR can regulate the fate of *B. pseudomallei* inside macrophages. Pretreatment with exogenous IFN- $\beta$  or IFN- $\gamma$  prior to infection could restore the ability of macrophages to activate iNOS expression and resulted in enhanced killing of intracellular *B. pseudomallei*.<sup>17</sup> Although these results suggest that both type I and II IFN could promote host cell ability to eliminate intracellular *B. pseudomallei*, previous findings by our group demonstrated that this bacterium could also up-regulate expression of suppressor of cytokine signaling 3 (SOCS3) and cytokine-inducible Src homology 2-containing protein (CIS), both of which are negative regulators that suppress IFN- $\gamma$  signaling pathway.<sup>18</sup> Up-regulation of these two molecules may thus facilitate *B. pseudomallei* to survive macrophage killing in the presence of IFN- $\gamma$ .

Besides TLRs, recent studies by several groups of investigators have identified nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) as an additional innate immune receptor family.<sup>19,20</sup> These intracellular receptors have also been implicated in the pathogenesis of many bacterial infections including *Mycobacterium tuberculosis*, *Salmonella enterica* sv. Typhimurium, and *Shigella flexneri*, all of which can activate NLRs.<sup>20</sup> The best characterized NLRs are NOD1 and NOD2, both of which have been shown to play a critical role in regulating host innate immune response against bacterial infections by sensing the cytosolic presence of the molecules containing meso-diaminopimelic acid (DAP) and muramyl dipeptide (MDP), respectively.<sup>21–23</sup> While NOD1 is widely expressed in many cell types, NOD2 expression is more restricted and so far has been described only in macrophages, dendritic cells, keratinocytes, lung and Paneth cells.<sup>20</sup> Stimulation of NOD1 or NOD2 results in the activation of NF- $\kappa$ B and MAPKs pathways which can turn on the transcription of numerous genes needed for host defense, particularly those involved in cytokine and chemokine production.<sup>24</sup> NOD2-deficient mice exhibited impaired clearance of *Listeria monocytogenes*, thus demonstrating the significance of NOD2 in this bacterial infection.<sup>25</sup> The aim of the present study was to investigate the possible involvement of NOD1 and NOD2 in *B. pseudomallei* infection in mouse macrophage cell line RAW 264.7.

## Materials and methods

### Cell line and culture condition

Mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Unless otherwise indicated, the cells were cultured in Advanced Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone) and 1% L-glutamine (Gibco Labs, Grand Island, NY, USA) at 37°C under a 5% CO<sub>2</sub> atmosphere.

### Bacterial strain

*Burkholderia 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 and cultured as described.<sup>14,26</sup> For the experiments, bacteria were cultured in Trypticase soy broth at 37°C with shaking at 150 rpm. The overnight (18 h) cultures were washed twice in phosphate-buffered saline (PBS) and adjusted to a desired concentration by measuring the optical density at 650 nm and the colony-forming unit (CFU) was calculated from the pre-calibrated standard curve.

### Heat-killed bacteria

Non-viable *B. pseudomallei* were prepared by heating a tube of bacteria suspended in PBS at 10<sup>8</sup> CFU/ml in a boiling water bath for 15 min. The heat-treated bacteria were washed 3 times with PBS and complete killing was confirmed by inoculating the suspension on tryptic soy agar and observe growth after 48 h.

### NOD1 and NOD2 depletion in macrophage cell line RAW 264.7

RAW 264.7 macrophages were transfected with siRNAs against NOD1 and NOD2 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, which was modified as follows. Macrophages (1.5 × 10<sup>5</sup> cells) were cultured overnight in 6-well plates before the siRNAs were transiently transfected using lipofectamine 2000 (Invitrogen). After 24 h of incubation, the expression of NOD1 and NOD2 were determined by both RT-PCR and immunoblotting. The siRNA sequences used were as follows: NOD1 sense 5'-UUG UCC ACC AGA CAC UGA GUG UUC C-3' and antisense 5'-GGA ACA CUC AGU GUC UGG UGG ACA A-3' NOD2 sense 5'-UAU GGU GUC GGC AUC UCU GUU CAG G-3' and antisense 5'-CCU GAA CAG AGA UGC CGA CAC CAU A-3'. As a control, we used AllStars Negative Control siRNA (Qiagen, Hilden, Germany).

### Infection of mouse macrophage cell line RAW 264.7

An overnight culture of mouse macrophages in a 6-well plate was infected with bacteria at a multiplicity of infection (MOI) of 2 for 1 h. To remove extracellular bacteria, the cells were washed twice with 1 ml of PBS, and residual bacteria were killed by incubating in 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.<sup>17</sup>

### Quantification of intracellular bacteria

To determine intracellular survival and replication of the bacteria, a standard antibiotic protection assay was performed as previously described.<sup>17</sup> In brief, at times indicated, the infected cells were washed twice with PBS, and 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 colony forming unit (CFU), was determined by bacterial colony counting.

### Reverse transcriptase PCR

Total RNA was extracted from infected cells according to the manufacturer's instruction (Roche Diagnostics, Mannheim, Germany) before it was used for cDNA synthesis using AMV RT (Promega, Madison, WI, USA). PCR was performed using cDNAs as template, and the primer pairs specific for *Nod1*, *Nod2*, *Socs3*, *Cis*, *Tnf-α*, *Il-1β*, *Il-6* and *β-actin* (Table 1) were amplified by *Taq* DNA polymerase (Invitrogen). The amplified products

were electrophoresed on 1.5% agarose gel and stained with ethidium bromide before being visualised under an ultraviolet lamp.

### Immunoblotting

The cells were lysed in lysis buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates were separated in 8% SDS-PAGE gel by electrophoresis in SDS-PAGE buffer. The protein on SDS-PAGE gel was transferred onto a nitrocellulose membrane (Amersham Bioscience, Dassel, Germany). Non-specific binding site on the membrane was blocked in 5% blocking solution (Roche Diagnostics) in PBS for 1 h before allowing to react overnight at 4°C with specific primary antibody against NOD1 (Cell Signaling Technology, Beverly, MA, USA) or NOD2 (eBioscience, CA, USA). The membrane was washed 3 times with 0.1% PBST and incubated with horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) for 1 h at 25°C. Thereafter, the membrane was washed 4 times with 0.1% PBST before a chemiluminescence substrate (Roche Diagnostics) was added, and protein bands were detected by enhanced chemiluminescence.

## Results

### *Burkholderia pseudomallei* stimulates NOD1 and NOD2 expression in mouse macrophage cell line RAW 264.7

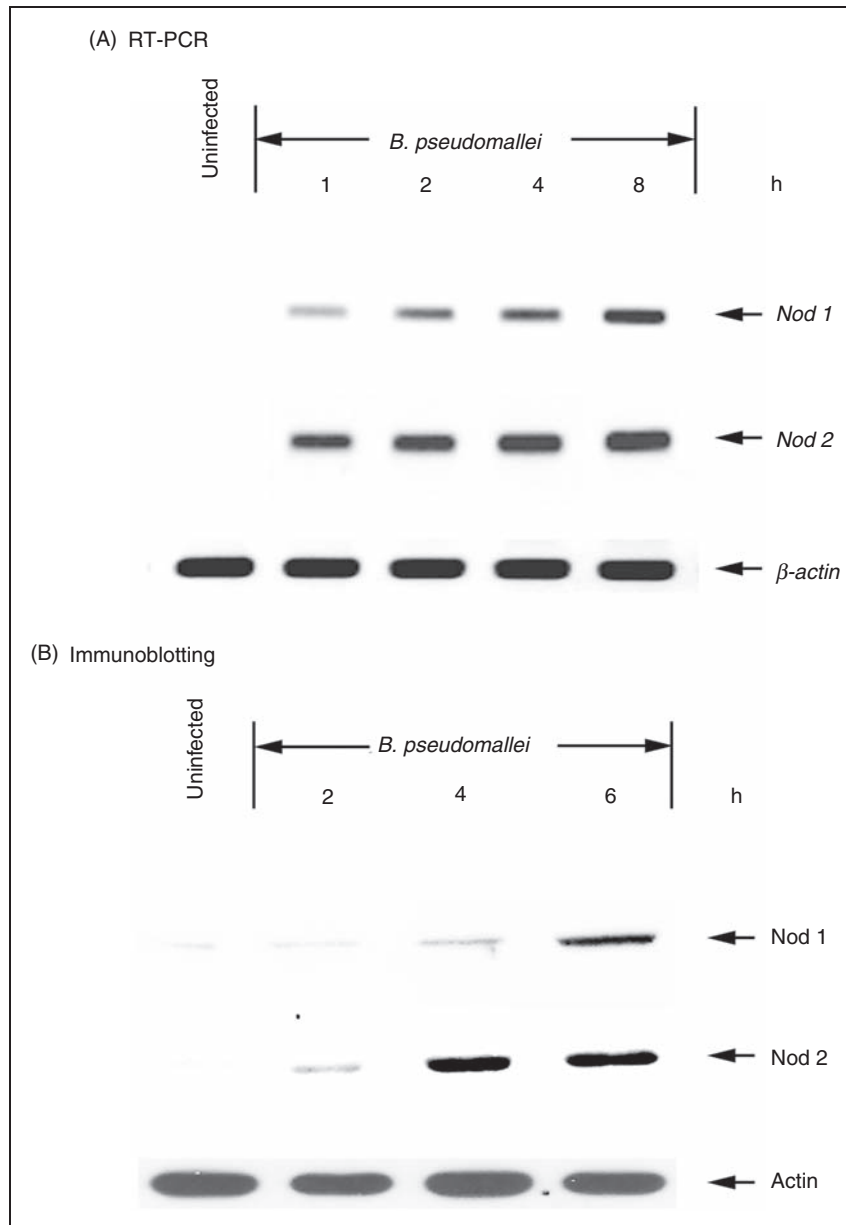
To detect NOD1 and NOD2 expression, mouse macrophages were infected with *B. pseudomallei* at a MOI

**Table 1.** Primer specifications for RT-PCR

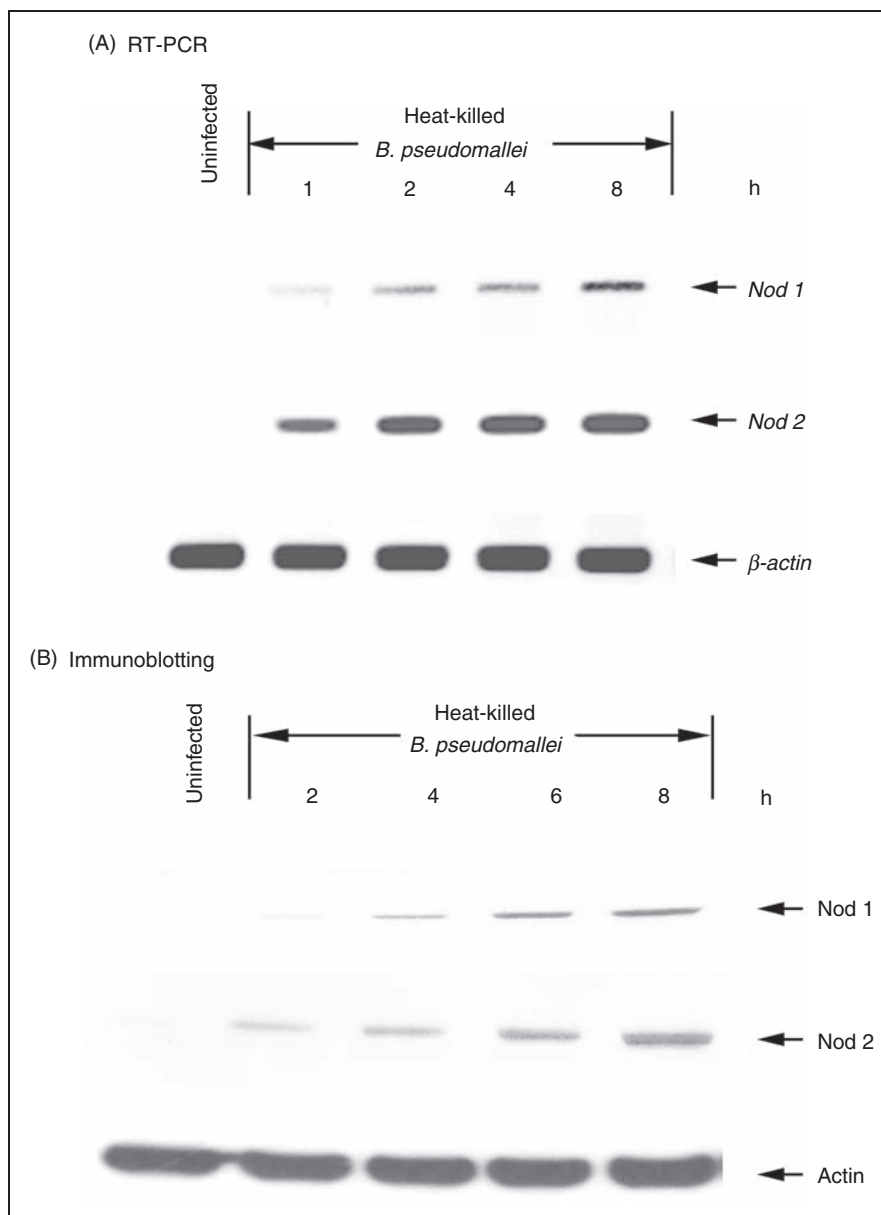
Gene	Primer	Sequence (5'→3')
<i>Nod1</i>	Forward	TCT CAG TCT TGC ATT CAA TGG C
	Reverse	TGT CAG TGT GGT GTT CTG CTT
<i>Nod2</i>	Forward	CTC CTC AGG AAG TTC GTT CG
	Reverse	GTA CAT GTC CGT GCT GGT TG
<i>Socs3</i>	Forward	ATG GTC ACC CAC AGC AAG TT
	Reverse	AAT CCG CTC TCC TGC AGC TT
<i>Cis</i>	Forward	GAA CCG AAG GTG CTA GAC CCT
	Reverse	TGT ACC CTC CGG CAT CTT CT
<i>Tnf-α</i>	Forward	GTA GCC CAC GTC GTA GCA AA
	Reverse	CCC TTC TCC AGC TGG GAG AC
<i>Il-6</i>	Forward	GGT CTC TGG GAA ATC GTG GA
	Reverse	GCT GAC CCT AGA GCA TCC TG
<i>Il-1β</i>	Forward	TCA TGG GAT GAT GAT GAT AAC CTG CT
	Reverse	CCC ATA CTT TAG GAA GAC ACG GAT T
<i>β-actin</i>	Forward	CCA GAG CAA GAG AGG TAT CC
	Reverse	CTG TGG TGG TGA AGC TGT AG

of 2. At specific times after infection, the levels of NOD1 and NOD2 mRNAs were determined by RT-PCR. With the condition used, NOD1 and NOD2 mRNAs could not be detected in the uninfected macrophages (Figure 1A). In contrast, *B. pseudomallei* was able to activate the transcription of both receptors within 1 h of infection and the levels gradually increase with time of incubation. Trace quantities of NOD1 and NOD2 proteins could be detected as early as 4 h and 2 h after the infection, respectively (Figure 1B). In order to determine if the expression of NOD1 or NOD2 by the macrophages requires viability of the

bacteria, the cells were exposed to heat-killed *B. pseudomallei* at concentration equivalent to MOI of 10. At 1, 2, 4 and 8 h of infection, the infected cells were harvested and mRNA and protein of NOD1 or NOD2 were assessed as described above. The results from Figure 2 indicated that exposure of macrophages to non-viable *B. pseudomallei* yielded NOD1 and NOD2 expression patterns that were similar to those obtained using viable bacteria (Figure 1), thus suggesting that the expression of both receptors does not require active invasion of the macrophages by live *B. pseudomallei*.



**Figure 1.** Up-regulation of NOD1 and NOD2 expression in *B. pseudomallei*-infected mouse macrophages. The macrophages were infected with the bacteria at MOI of 2. At different time intervals, the infected cells were lysed and the kinetics of NOD1 and NOD2 mRNA (A) and protein expressions (B) were determined by RT-PCR and immunoblotting, respectively.  $\beta$ -Actin mRNA and protein were served as an internal control.

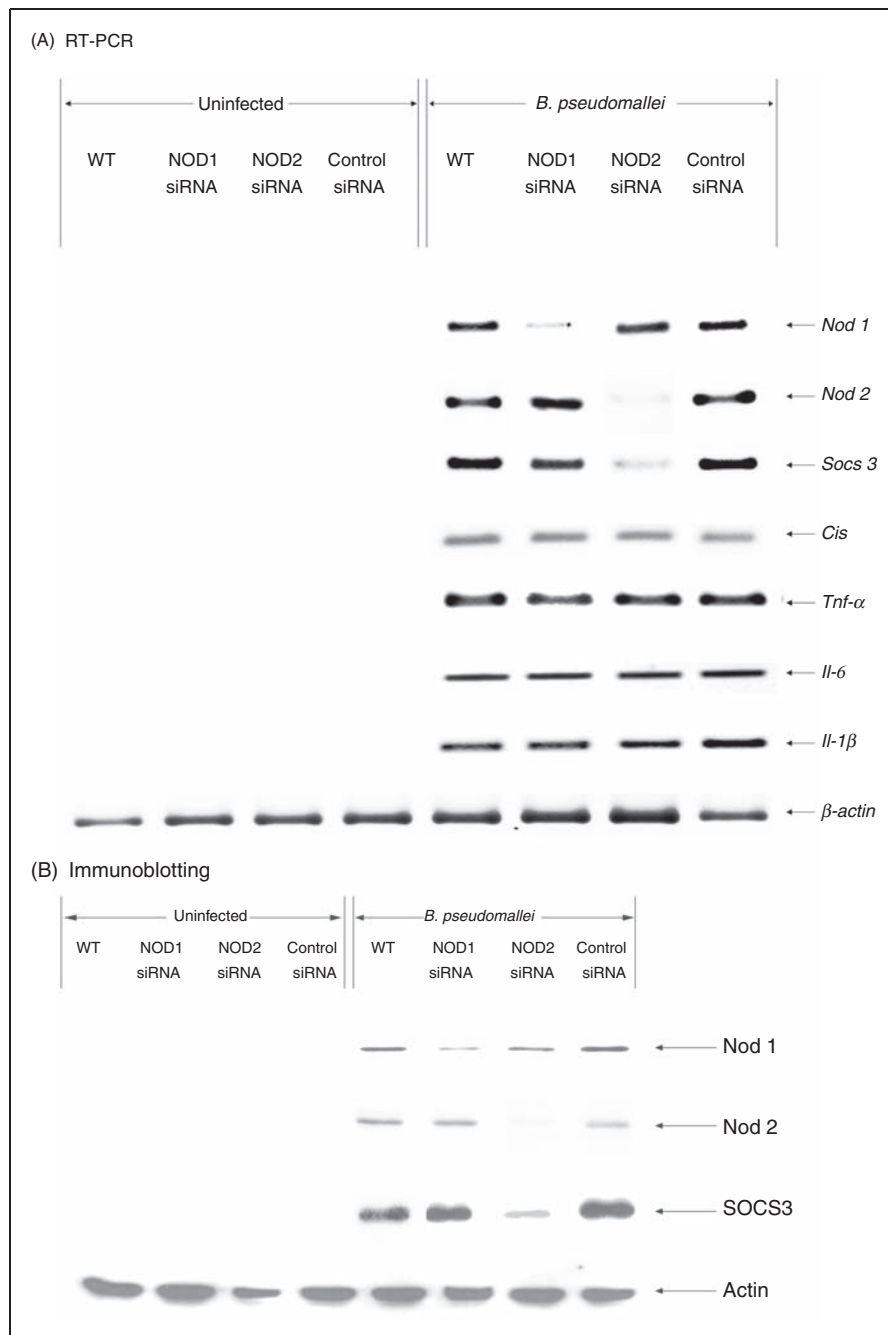


**Figure 2.** Kinetics of NOD1 or NOD2 expression in mouse macrophages treated with heat-killed *B. pseudomallei*. The macrophages were exposed to heat-killed *B. pseudomallei* at concentration equivalent to MOI of 10. Expression of mRNA (A) and protein (B) was determined as described in Figure 1.

### NOD2 is required for *B. pseudomallei*-induced SOCS3 expression

In order to determine the possible involvement of NOD1 and/or NOD2 on the intracellular fate of *B. pseudomallei*, the macrophages were first treated with NOD1 or NOD2 siRNAs before infecting with *B. pseudomallei*. The mRNA level and protein expression of NOD1 or NOD2 of uninfected and *B. pseudomallei*-infected macrophages were assayed 24 h after transfection. Results presented in Figure 3 showed that when the cells were treated with siRNAs, both mRNA and protein of NOD1 and NOD2 were diminished.

We previously demonstrated that *B. pseudomallei*-infected macrophages were able to activate the expression of SOCS3 and CIS.<sup>18</sup> It was suspected that the expression of these negative regulators might be initiated intracellularly rather than at the surface of the macrophages.<sup>27</sup> Extrapolating this further, we now hypothesize that stimulation of SOCS3 and CIS expression involves activation of NOD proteins. In order to demonstrate this relationship, the expression of SOCS3 and CIS in *B. pseudomallei*-infected NOD1 or NOD2 knockdown macrophages were analyzed by RT-PCR. As shown in Figure 3A, the level of SOCS3 mRNA was markedly reduced in *B. pseudomallei*-infected NOD2 knockdown macrophages.



**Figure 3.** Alteration of mRNA and protein expression profiles in NOD1 and NOD2 knockdown mouse macrophages infected with *B. pseudomallei*. The wild-type and NOD knockdown macrophages were infected with *B. pseudomallei* as described in Figure 1. The infected cells were lysed after 4 h and 6 h of infection, respectively, for mRNA (A) and protein (B) analysis. The expression of uninfected cells serves as control.

In contrast, NOD1 depletion did not interfere with SOCS3 expression. It should be noted, however, that the mRNA expression of CIS, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 was not affected in both *B. pseudomallei*-infected NOD1 and NOD2 knockdown macrophages. The interfering of SOCS3 protein expression in NOD2 knockdown macrophages was also demonstrated by immunoblotting as shown in Figure 3B. These results clearly demonstrated that only NOD2 is involved in SOCS3 up-regulation.

#### *Interferon- $\gamma$ induces STAT-1 phosphorylation on tyrosine 701 (Y701-STAT-1) in B. pseudomallei-infected NOD2 knockdown macrophages*

It should be noted that the results shown in Figure 3 in the present study are in accordance with our previous report which showed that *B. pseudomallei* could enhance SOCS3 expression in mouse macrophage



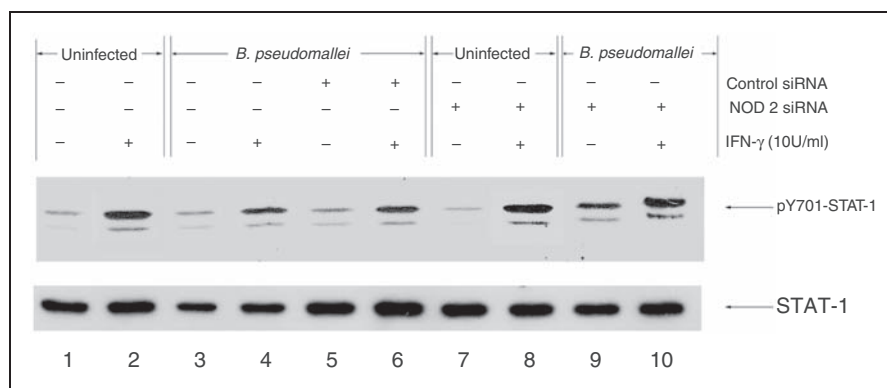
cell line.<sup>27</sup> It was further shown in that report that the expression of SOCS3 in *B. pseudomallei*-infected macrophages correlated with a reduction of Y701-STAT-1 phosphorylation following activation with IFN- $\gamma$ .<sup>27</sup> Therefore, in the present study, we predicted that the inhibition of SOCS3 in *B. pseudomallei*-infected NOD2 knockdown macrophages (Figure 3) may adversely affect their ability to respond to subsequent IFN- $\gamma$  activation. In this experiment, the NOD2-depleted macrophages were infected with *B. pseudomallei* for 4 h and then treated with IFN- $\gamma$  for 1 h before the level of pY701-STAT-1 was determined by immunoblotting. It was clearly shown in Figure 4 lanes 2 and 8 that while IFN- $\gamma$  was able to activate Y701-STAT-1 phosphorylation in the uninfected macrophages in the presence or absence of NOD2 siRNA, the level of pY701-STAT-1 was decreased in *B. pseudomallei*-infected wild-type macrophages (Figure 4, lane 4), thus suggesting that the expression of SOCS3 by *B. pseudomallei* reduces the levels of pY701-STAT-1. However, the level of pY701-STAT-1 was increased in *B. pseudomallei*-infected NOD2 knockdown macrophages (Figure 4, lane 10). The increase of pY701-STAT-1 level directly correlated with the decrease in SOCS3 expression as demonstrated in Figure 3. It should be noted that, in contrast to NOD2, NOD1 depletion did not interfere with phosphorylation of Y701-STAT-1 in the infected macrophages (data not shown).

## Discussion

Pattern recognition receptors (PRRs) are known to be the first to detect microbial invasion and to initiate subsequent adaptive immune response. These immune sensors include TLRs which initiate signals from the cell surface or endosome upon binding with appropriate ligands. In contrast, NLRs detect microbial components in the cytosol. *B. pseudomallei* expresses several potential ligands, including lipid A, peptidoglycan, flagellin and DNA that may activate these PRRs.

A number of reports have demonstrated that *B. pseudomallei* could activate NF- $\kappa$ B via TLR2, TLR4 and TLR5.<sup>28,29</sup> Patients with septic melioidosis had increased expression of CD14, TLR1, TLR2 and TLR4 on the surface of monocytes.<sup>29</sup> Although this Gram-negative bacterium exhibits LPS on the surface, it remains controversial whether the LPS of *B. pseudomallei* can signal through TLR2 or TLR4. It was previously demonstrated that the LPS of this bacillus exhibits an unusual structure which may be associated with its relatively poor ability to activate macrophage compared with that of other Gram-negative bacteria.<sup>30–32</sup> *In vivo* study demonstrated that TLR2-deficient mice displayed enhanced immunity against *B. pseudomallei* infection, as judged by an increase of host survival together with a decrease in bacterial load. The response of TLR4-deficient mice, on the other hand, was indistinguishable from that of the wild-type animals.<sup>29</sup> Therefore, these results suggested that TLR2 and not TLR4 might be a major contributor to host defense against *B. pseudomallei* infection. This conclusion is consistent with our recent finding which demonstrated that *B. pseudomallei* activated only MyD88-dependent pathway and not MyD88-independent pathway which is more specifically associated with the TLR4 activation.<sup>16</sup>

In contrast to the TLRs, the NLRs are activated by microbial components in the cytosol. Several NLRs have been identified but only a few ligands for NLRs have been identified. The most well characterized NLRs are NOD1 and NOD2. NOD1 senses peptidoglycan-derived DAP-containing muramyl peptides which are a structural component of cell wall of Gram-negative bacteria, whereas NOD2 senses MDP present in both Gram-positive and Gram-negative bacteria.<sup>21–23</sup> In the present study, we showed that the activation processes of NOD1 and NOD2 are associated with *B. pseudomallei* components as shown by the up-regulation of both NOD1 and NOD2 expression in the infected macrophages (Figure 1). The expression of



**Figure 4.** Enhancement of IFN- $\gamma$  responsiveness in *B. pseudomallei*-infected NOD2-depleted macrophages. NOD2 knockdown and wild-type mouse macrophages ( $1.5 \times 10^5$  cells) were infected with *B. pseudomallei* at MOI of 2 for 4 h before IFN- $\gamma$  (10 U/ml) was added. After 1 h of incubation with IFN- $\gamma$ , cells were lysed and the levels of pY701-STAT-1 were determined by immunoblotting.

these cytosolic receptors could also be induced by heat-killed *B. pseudomallei*, suggesting the involvement of heat-stable components most likely present on the surface of *B. pseudomallei* (Figure 2). Upon interacting with their respective ligands, both NOD1 and NOD2 activate NF- $\kappa$ B, resulting in the induction of pro-inflammatory cytokines such as TNF- $\alpha$ .<sup>24</sup> However, the levels of TNF- $\alpha$ , IL-1 $\beta$  or IL-6 (Figure 3) were not noticeably altered in the *B. pseudomallei*-infected macrophages whose NOD1 or NOD2 were depleted. It should be noted that NOD1 or NOD2 knockdown did not interfere with internalization and intracellular replication of *B. pseudomallei* suggesting that NOD1 or NOD2 was not directly involved in bactericidal activity of macrophages (data not presented).

The SOCS protein family is known to function as negative effectors of cytokine signaling via the JAK/STAT pathway and its activation by pathogens leads to suppression of macrophage activation.<sup>33</sup> For example, *L. monocytogenes* induced SOCS3 expression in macrophages, resulting in diminishing IFN- $\gamma$  stimulating phosphorylation of STAT-1 which facilitated their intracellular survival.<sup>34</sup> Previously, our group reported that *B. pseudomallei* was capable of suppressing IFN- $\gamma$  signaling by activating expression of SOCS3 and CIS.<sup>18</sup> Activating these negative effectors markedly reduced the level of Y701-STAT-1 phosphorylation, which is one of the transcription factors for iNOS expression, resulting in a loss of macrophage killing capacity. We subsequently demonstrated that the induction of SOCS3 and CIS expression required *B. pseudomallei* internalization, thus implying that a cytosolic receptor is needed.<sup>27</sup> In the present study, we attempted to identify cytosolic receptor(s) involved in SOCS3 and CIS expression and the results presented in Figures 3 and 4 showed the involvement of NOD2 and not NOD1 in SOCS3 expression. It is possible that other NLRs may be responsible for CIS expression. Although activating NOD1 expression was observed in *B. pseudomallei*-infected macrophages (Figs 1 and 2), the biological significance of this cytosolic receptor remains to be investigated. It has been established recently that NOD1 was involved in autophagic response to several invasive bacteria.<sup>35</sup> Although autophagic response to *B. pseudomallei* was demonstrated recently,<sup>36</sup> it is not certain if this involves NOD1 and this is a question that is currently being investigated by our group. Together, the results presented here identify another host molecule which *B. pseudomallei* may use to modulate the macrophage response in favor of their intracellular survival.

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## References

1. Cheng AC and Currie BJ. Melioidosis: epidemiology, pathophysiology, and management. *Clin Microbiol Rev* 2005; 18: 383–416.
2. Peacock SJ. Melioidosis. *Curr Opin Infect Dis* 2006; 19: 421–428.
3. Brett PJ and Woods DE. Pathogenesis of and immunity to melioidosis. *Acta Trop* 2000; 74: 201–210.
4. Wiersinga WJ, Van Der Poll T, White NJ, Day NP and Peacock S. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. *Nat Rev Microbiol* 2006; 4: 272–282.
5. Leelarasamee A. Recent development in melioidosis. *Curr Opin Infect Dis* 2004; 17: 131–136.
6. Rotz LD, Khan AS, Lillibridge SR, Ostroff SM and Hughes JM. Public health assessment of potential biological terrorism agents. *Emerg Infect Dis* 2002; 8: 225–230.
7. Voskuhl GW, Cornea P, Bronze MS and Greenfield RA. Other bacterial diseases as a potential consequence of bioterrorism: Q fever, brucellosis, glanders, and melioidosis. *J Okla State Med Assoc* 2003; 96: 214–217.
8. Jone A, Beveridge TJ and Woods DE. Intracellular survival of *Burkholderia pseudomallei*. *Infect Immun* 1996; 64: 782–790.
9. Harley VS, Dance DAB, Drasar BJ and Tovey G. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. *Microbios* 1998; 96: 71–93.
10. Kespichayawattana W, Rattanachetkul S, Wanun T, Utaisincharoen P and Sirisinha S. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. *Infect Immun* 2000; 68: 5377–5384.
11. Wong KT, Puthucheary SD and Vadivelu J. The histopathology of human melioidosis. *Histopathology* 1995; 26: 51–55.
12. Suparak S, Kespichayawattana W, Haque A, et al. Multinucleated giant cell formation and apoptosis in infected host cells is mediated by *Burkholderia pseudomallei* type III secretion protein BipB. *J Bacteriol* 2005; 187: 6556–6560.
13. Utaisincharoen P, Arjcharoen S, Limposuwan K, Tungpradabkul S and Sirisinha S. *Burkholderia pseudomallei* RpoS regulates multinucleated giant cell formation and inducible nitric oxide synthase expression in mouse macrophage cell line (RAW 264.7). *Microb Pathog* 2006; 40: 184–189.
14. Utaisincharoen P, Tangthawornchaikul N, Kespichayawattana W, Chaisuriya P and Sirisinha S. *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. *Microbiol Immunol* 2001; 45: 307–313.
15. Utaisincharoen P, Anuntagool N, Limposuwan K, Chaisuriya P and Sirisinha S. Involvement of IFN- $\beta$  in enhancing inducible nitric oxide synthase (iNOS) production and antimicrobial activity of *B. pseudomallei*-infected macrophages. *Infect Immun* 2003; 71: 3053–3057.
16. Tangsudjai S, Pudla M, Limposuwan K, Sirisinha S and Utaisincharoen P. Involvement of the MyD88-independent pathway in controlling the intracellular fate of *Burkholderia pseudomallei* infection in the mouse macrophage cell line RAW 264.7. *Microbiol Immunol* 2010; 54: 282–290.
17. Utaisincharoen P, Anuntagool N, Arjcharoen S, Limposuwan K, Chaisuriya P and Sirisinha S. Induction of iNOS expression and antimicrobial activity by interferon (IFN)- $\beta$  is distinct from IFN- $\gamma$  in *Burkholderia pseudomallei*-infected mouse macrophages. *Clin Exp Immunol* 2004; 136: 277–283.



18. Ekchariyawat P, Pudla S, Limposuwan K, Arjcharoen S, Sirisinha S and Utaisinchaoen 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: 7332–7339.
19. Inohara N, Chamaillard M, McDonald C and Nunez G. NOD-LRR proteins; role in host-microbial interactions and inflammatory disease. *Annu Rev Biochem* 2005; 74: 355–383.
20. Franchi L, Warner N, Viani K and Nunez G. Function of NOD-like receptors in microbial recognition and host defense. *Immunol Rev* 2009; 227: 106–128.
21. McDonald C, Inohara N and Nunez G. Peptidoglycan signaling in innate immunity and inflammatory disease. *J Biol Chem* 2005; 280: 20177–20180.
22. Girardin SE, Boneca IG, Carneiro LA, et al. NOD1 detects a unique muropeptide from Gram-negative bacterial peptidoglycan. *Science* 2003; 300: 1584–1587.
23. Chamaillard M, Hashimoto M, Horie Y, et al. An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid. *Nat Immunol* 2003; 4: 702–707.
24. Hayden MS and Ghosh S. Signaling to NF-kappaB. *Genes Dev* 2004; 18: 2195–2224.
25. Kobayashi KS, Chamaillard M, Ogura Y, et al. NOD2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 2005; 307: 731–734.
26. Anuntagool N, Intachote P, Wuthiekanun V, White NJ and Sirisinha S. Lipopolysaccharide from nonvirulent Ara<sup>+</sup> *Burkholderia pseudomallei* isolates is immunologically indistinguishable from lipopolysaccharide from virulent Ara<sup>-</sup> clinical isolates. *Clin Diagn Lab Immunol* 1998; 5: 225–229.
27. Ekchariyawat P, Pudla S, Limposuwan K, Arjcharoen S, Sirisinha S and Utaisinchaoen P. 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. *Microb Pathog* 2007; 42: 104–110.
28. West TE, Ernst RK, Janson-Hutson MJ and Skerrett SJ. Activation of Toll-like receptors by *Burkholderia pseudomallei*. *BMC Immunol* 2008; 9: 46.
29. Wiersinga WJ, Wieland CW, Dessing MC, et al. Toll-like receptor 2 impairs host defense in Gram-negative sepsis caused by *B. pseudomallei* (melioidosis). *PLoS Med* 2007; 4e: 248.
30. Kawahara K, Dejsirilert S, Danbara H and Ezaki T. Extraction and characterization of lipopolysaccharide from *Pseudomonas pseudomallei*. *FEMS Microbiol Lett* 1992; 96: 129–134.
31. Utaisinchaoen P, Tangthawornchaikul N, Kespichayawattana W, Anuntagool N, Chaisuriya P and Sirisinha S. Kinetic studies of the production of nitric oxide (NO) and tumor necrosis factor alpha (TNF- $\alpha$ ) in macrophages stimulated with *Burkholderia pseudomallei* endotoxin. *Clin Exp Immunol* 2000; 122: 324–329.
32. Novem V, Shui G, Wang D, et al. Structure and biological diversity of lipopolysaccharides from *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *Clin Vaccine Immunol* 2009; 16: 1420–1428.
33. Alexander W and Hilton D. The role of suppressor of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu Rev Immunol* 2004; 22: 503–529.
34. Stoiber D, Stockinger S, Steinlein P, Kovarik J and 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: 466–472.
35. Travassos LH, Carneiro LA, Ramjeet M, et al. NOD1 and NOD2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry. *Nat Immunol* 2010; 11: 55–62.
36. Cullinane M, Gong L, Li X, et al. Stimulation of autophagy suppresses the intracellular survival of *B. pseudomallei* in mammalian cell lines. *Autophagy* 2008; 16: 744–753.