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ORIGINAL ARTICLE

NLRP12 attenuates tumor necrosis factor-α production in *Burkholderia pseudomallei*-infected RAW264.7 macrophages

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Abstract

Background: NLRP12 has been shown to play an essential role as a negative regulator in several bacterial infection.

Objective: The purpose of this study is to elucidate the role of NLRP12 in *B. pseudomallei*-infected RAW264.7 macrophages.

Methods: The protein expression and the level of TNF-α production were determined by immunoblotting and ELISA assay, respectively.

Results: The results demonstrated that unlike the LPS-mutant strain which lacks O antigenic polysaccharide, the wild-type *B. pseudomallei* was able to upregulate NLRP12 protein expression in RAW264.7 macrophages. NLRP12 expression also correlated with the suppression of TNF-α production as demonstrated in wild-type *B. pseudomallei*-infected *Nlrp12*-depleted macrophages when compared to that of the control siRNA-transfected cells. The expression of NLRP12 was also inhibited in cytochalasin D treated cells.

Conclusion: Our findings showed that wild-type *B. pseudomallei* can activate NLRP12 expression leading to the suppression of TNF-α production. It is possible that the regulation of NLRP12 may contribute to the pathogenesis of *B. pseudomallei* infection in melioidosis patients.

Key words: *Burkholderia pseudomallei*, mouse macrophage, NLRP12, RAW264.7, TNF-α

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Introduction

Melioidosis is a life-threatening infectious disease caused by a facultative intracellular bacterium, *Burkholderia pseudomallei*.[1] This disease is mainly endemic in Southeast Asia and Northern Australia.[2] The clinical manifestations of the disease can range from acute septicemia to chronic infection. However, the severity of this disease varies depending on the route of bacterial entry, virulence factors and the capacity of *B. pseudomallei* to manipulate host immune signaling.[1]

Innate immune response has been demonstrated to play an essential role in *B. pseudomallei* infection, particularly in suppressing intracellular bacterial replication. Previously, our group demonstrated that wild-type *B. pseudomallei* could regulate several negative regulators of Toll-like receptors (TLRs) signaling pathways such as sterile-α and armadillo motif (SARM)-containing protein, suppressor of cytokine signaling 3 (SOCS3).[3,4] The upregulation of these negative regulators subverts macrophage killing, enhancing the wild-type *B. pseudomallei* intracellular burden. On the contrary, the LPS mutant *B. pseudomallei* lacking O antigenic polysaccharide was unable to survive and multiply

in mouse macrophage cell line (RAW264.7) and human alveolar epithelial cell line (A549).[5,6]

Besides TLRs, NOD-like receptors (NLRs) are classified as cytosolic pattern recognition receptors (PRRs) that trigger by pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs).[7] These molecules have been shown to play a pivotal role in many bacterial infections. Among the NLRs family, NLRP12 is identified as a negative regulator of the inflammatory signaling pathway. Several studies demonstrate that NLRP12 is a crucial suppressor of innate immune signaling during bacterial infection. Macrophages deficient in NLRP12 produce high levels of proinflammatory cytokines and antimicrobial molecules upon *Salmonella typhimurium* infection.[8] Moreover, the potential role of NLRP12 as a negative regulator of proinflammatory cytokines was also investigated in response to *Escherichia coli* LPS, *Klebsiella pneumoniae*, *Porphyromonas gingivalis* and *Mycobacterium tuberculosis* TDB.[9,10] However, the functional role of NLRP12 in *B. pseudomallei* infection has not been elucidated. Herein, this study aims to elucidate the regulation of NLRP12 in response to *B. pseudomallei*-infected mouse macrophage cell line (RAW264.7).

Methods

Cell line and culture condition.

RAW 264.7 macrophages (ATCC) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco Labs, Grand Island, NY, USA) at 37°C under a 5% CO2 atmosphere.

Bacterial strain

The wild-type *B. pseudomallei* (1026b strain) and its lipopolysaccharide (LPS) mutant (SRM117 strain) were used in this study. The bacteria were cultured in Luria-Bertani (LB) at 37°C with agitation at 150 rpm. Overnight cultures were adjusted to the desired concentration, as previously described.[3] In brief, the log-phase bacterial cultures were washed twice with phosphate-buffered saline (PBS) and measured the optical density at 650 nm. CFU were calculated from the precalibrated standard curve.

Infection of mouse macrophage cell line (RAW264.7)

An overnight culture of RAW264.7 macrophages in a 6-well plate was co-cultured with the bacteria at a multiplicity of infection (MOI) of 0.1 and the methods were performed as previously described.[3,11] For depletion of Nlrp12, RAW264.7 macrophages (2.5×10^5 cells/well) were transfected with scramble or *Nlrp12* siRNAs (Dharmacon, CO, USA) according to the manufacturer's protocol. In brief, macrophages were seeded overnight in a 6-well plate. The cells were then transfected with scramble or *Nlrp12* siRNAs (60 nM) using Lipofectamine 2000 reagent (Invitrogen) for 24 hours. At indicated time intervals, the infected cells were lyzed and the expression of protein was determined by immunoblotting.

Immunoblotting

The infected cells were lyzed in lysis buffer containing 20 mM Tris, 100 mM NaCl and 1% NP40. The lysates were separated on 8% SDS-PAGE gels. Proteins were transferred onto a nitrocellulose membrane (Amersham Biosciences Dassel, Germany). The non-specific binding sites on the membrane were blocked with 5% blocking solution (Roche Diagnostics) for 1 hour before proteins are allowed to react with specific primary antibodies against NLRP12 (Abcam), and ACTIN (Merck Millipore, NJ, USA) at 4°C overnight. The membrane was washed 3 times with 0.1% PBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (R&D Systems, Minneapolis, USA) for 1 hour at room temperature. Thereafter, the membrane was washed 4 times with 0.1% PBST before a chemiluminescence substrate (Roche Diagnostics, Mannheim, Germany) was added and proteins were detected by enhanced chemiluminescence.

Determination of TNF-α production

The supernatant from activated cells was collected and the level of TNF-α production was measured by mouse TNF-α ELISA kit (BD Bioscience, San Diego, USA) following manufacturer's instructions.

Statistical analysis

If not specified otherwise, all experiments were conducted at least three times. Experimental values are expressed as means ± the standard error of the mean (SEM). All data were analyzed by the Prism software (GraphPad) using one-way ANOVA with a Tukey's multiple comparison test. p value of < 0.05 was considered to be statistically significance.

Results

NLRP12 expression in wild-type B. pseudomallei-infected RAW264.7 macrophages

To investigate the expression of NLRP12 in *B. pseudomallei* infection, RAW264.7 macrophages were cocultured with either wild-type or LPS mutant *B. pseudomallei* at a multiplicity of infection (MOI) 0.1. After 1, 2 and 4 h of infection, the NLRP12 expression was determined by immunoblotting. The result presented in **Figure 1A** demonstrated that wild-type *B. pseudomallei* was able to upregulate NLRP12 protein expression at 1 h after infection before reaching the maximum level at 2 h. Moreover, to elucidate the involvement of NLRP12 in TNF-α production, the cells were infected with the bacteria at a multiplicity of infection (MOI) 0.1. The level of TNF-α production in wild-type *B. pseudomallei*-infected cells was significantly lower than that of the LPS mutant strain (**Figure 1B**) which was unable to upregulate NLRP12 expression (**Figure 1A**). These results suggested that the O antigenic polysaccharide plays an essential role in the upregulation of NLRP12.

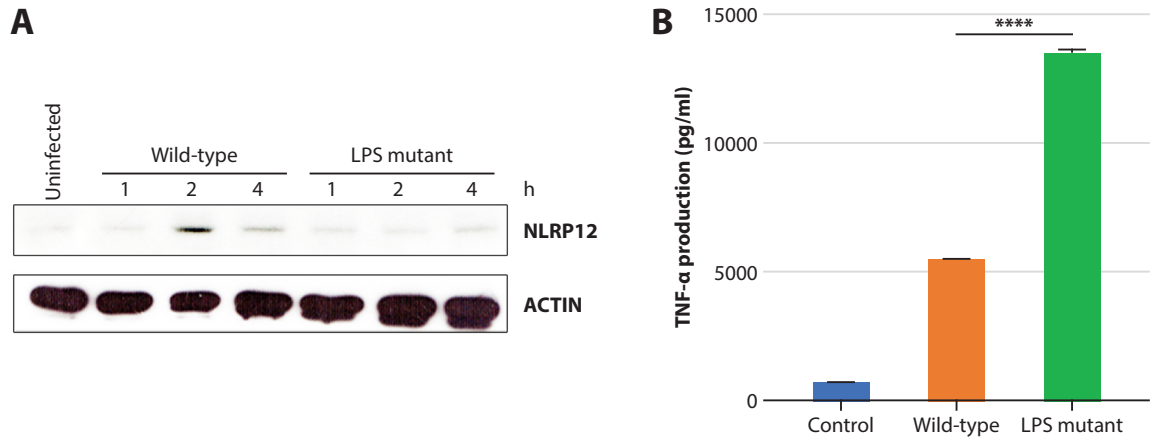

Figure 1. Wild-type *B. pseudomallei* upregulates NLRP12 protein expression. RAW264.7 macrophages were infected with either the wild-type or LPS-mutated *B. pseudomallei* at a MOI of 0.1. (A) At different time intervals, the infected cells were harvested and NLRP12 protein expression was determined by immunoblotting. Representative blot is shown from three independent experiments. (B) The supernatant was also collected and the level of TNF-α production was measured by ELISA assay at 6 h after infection. Data are expressed as mean ± SEM of three independent experiments. ****$P < 0.0001$.

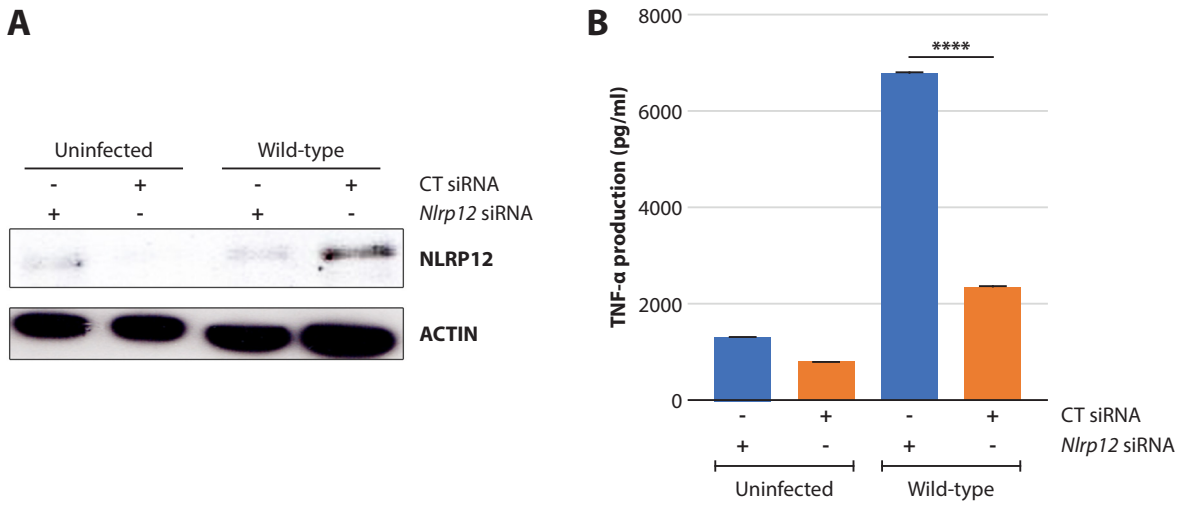

Figure 2. NLRP12 negatively regulates the production of TNF-α in wild-type *B. pseudomallei*-infected RAW264.7 macrophages. The depletion of NLRP12 was performed by transfecting *Nlrp12* siRNAs into RAW264.7 macrophages before infection with the wild-type *B. pseudomallei* at a MOI of 0.1. (A) Cell lysates collected at 2 h after infection were analyzed for NLRP12 expression by immunoblotting. Representative blot is shown from three independent experiments. (B) The level of TNF-α production was measured by ELISA assay at 6 h post infection. Data are expressed as mean ± SEM of three independent experiments. ****$P < 0.0001$. (CT siRNA = control siRNA)

Depletion of NLRP12 enhances the level of TNF-α production in wild-type B. pseudomallei-infected RAW264.7 macrophages.

To demonstrate the involvement of NLRP12 in the regulation of cytokine production, TNF-α was determined from *B. pseudomallei*-infected *Nlrp12*-depleted macrophages. As shown in **Figure 2A and 2B**, the increase in TNF-α production was observed in wild-type *B. pseudomallei*-infected *Nlrp12*-depleted macrophages compared to that of control siRNA-transfected cells, suggesting that NLRP12 negatively regulates TNF-α production in wild-type *B. pseudomallei*-infected cells. The viability of the infected macrophages was observed by trypan blue staining. The result showed that the viability of the infected cells transfected with siRNAs was 90% at 6 h after infection. However, unlike other NLRs such as NLRC4,[12] NLRP12 did not interfere with the bacterial intracellular survival (data not shown), suggesting that this molecule only interferes with the proinflammatory cytokine production.

Wild-type B. pseudomallei-induced NLRP12 expression requires intracellular receptor

Furthermore, we investigated whether the upregulation of NLRP12 requires extracellular receptor(s) or intracellular receptor(s), the cells were pretreated with cytochalasin D, an inhibitor for bacterial internalization,[3] prior to the infection.

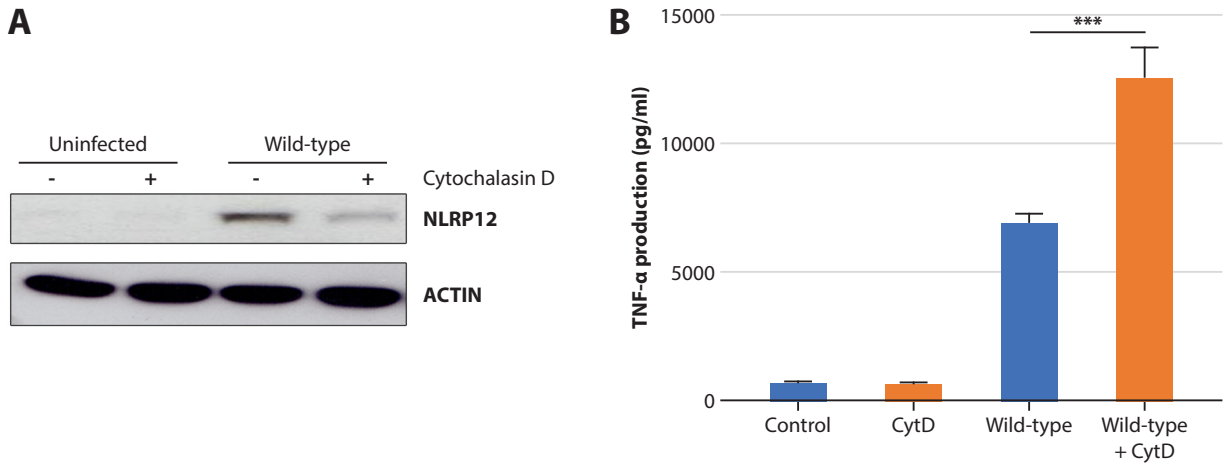

Figure 3. Expression of NLRP12 requires intracellular receptor. RAW264.7 macrophages were pretreated with cytochalasin D (CytD) (2 μg/ml) for 2 h before infection with the wild-type bacteria at a MOI of 0.1. The protein expression and TNF-α production level were determined by immunoblotting (A) and ELISA assay (B) at 6 h after infection. Representative blot is shown from three independent experiments. Data are expressed as mean ± SEM of three independent experiments. ***$p < 0.001$.

As shown in **Figure 3A**, the NLRP12 expression was drastically decreased in the presence of cytochalasin D, which is also consistent with the significantly increased TNF-α production (**Figure 3B**). These results implied that the intracellular receptor(s) may be involved in NLRP12 expression rather than the surface receptor(s).

Discussion

It has been well documented that proinflammatory cytokine production plays an essential factor during the early stage of host response following *B. pseudomallei* infection which then led to the pathogenesis of meleiodosis.[13] At present, our study demonstrated that *B. pseudomallei* can modulate macrophages by upregulating the negative regulator of proinflammatory cytokine, NLRP12, in wild-type *B. pseudomallei*-infected RAW264.7 macrophages. The upregulation of NLRP12 directly related to the decrease of TNF-α production. The significant role of this negative regulator has also been demonstrated in several bacterial infection. Another report also demonstrated that TDP from *M. tuberculosis* and LPS from *K. pneumoniae* induce increased levels of TNF-α and IL-6 in *Nlrp12*$^{-/-}$ bone marrow-derived DCs, suggesting that NLRP12 plays a role in suppressing of these proinflammatory cytokine production.[9] Consistently, *Nlrp12*$^{-/-}$ BMDMs produced a significantly increased level of TNF-α production in response to *S. typhimurium* infection.[8] In contrast, the level of TNF-α was attenuated in *Nlrp12*$^{-/-}$ alveolar macrophages infected with *K. pneumoniae*.[14] The contradiction in the biological relevance for NLRP12 activation may depend on the nature of bacteria. In our present study, we also demonstrated that the activation of NLRP12 was suppressed in the presence of cytochalasin D, suggesting that intracellular receptor(s) may mediate this regulation. However, the intracellular receptor(s) needs to be further investigated.

Conclusion

In summary, our findings showed that wild-type *B. pseudomallei* is able to activate NLRP12 expression leading to the suppression of TNF-α production. It is possible that the regulation of NLRP12 may contribute to the pathogenesis of *B. pseudomallei* infection in melioidosis patients.

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Author contributions

- MP and PU conceived and designed the experiments.
- MP performed most experiments, analyzed the data, prepared figure 1 and 2.
- PO prepared figure 3. PU edited the manuscript.
- MP and PU contributed to the writing of the manuscript.
- All authors read and approved the final version of the manuscript.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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