

The Role of Intracellular Receptor NODs for Cytokine Production by Macrophages Infected with *Mycobacterium leprae*

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The nucleotide-oligomerization domain (NOD) proteins are members of the NOD-like receptor (NLR) family, which are intracellular and cytoplasmic receptors. We analyzed the role of NODs for cytokine production by macrophages infected with intracellular pathogen *M. leprae*, the causative agent of leprosy. Production of pro-inflammatory cytokines such as IL-1 β and TNF- α was inhibited in the presence of cytochalasin D, an agent blocking phagocytosis, suggesting that intracellular signaling was, partially, required for macrophage activation to *M. leprae* infection. Next, we investigated the role of NOD1 and NOD2 proteins on NF- κ B activation and cytokine expression. Treatment with *M. leprae* significantly increased NF- κ B activation and expression of TNF- α and IL-1 β in NOD1- and NOD2-transfected cells. Interestingly, their activation and expression were inhibited by cytochalasin D, suggesting that stimulation of NOD proteins may be associated with the enhancement of cytokine production in host to *M. leprae*.

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Mycobacterium leprae, the causative agent of leprosy, is an intracellular pathogen that often resides within specialized compartments and replicates in macrophages. This pathogen can induce macrophages to release inflammatory cytokines, such as IL-1 β , IL-12, and TNF- α which are involved in the innate immune response for bacterial elimination and the coordination of adaptive immune responses (1-3).

Pattern recognition receptors (PRRs) are essential components for recognizing conserved microbial structure known as pathogen-associated molecular patterns (PAMPs) and stimulat-

ing production of pro-inflammatory cytokines in the innate immune system. There are three major classes of PRRs; toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) (4). Among these, NLRs are intracellular and cytoplasmic sensors that involved in several biological processes, which include host defense against pathogen and inflammation (5,6).

Stimulation of NOD1 and NOD2, which are a subfamily of NLRs, leads to activation of the nuclear transcription factor (NF)- κ B and AP-1, p38, extracellular signal-regulated kinase (ERK), and Jun N-terminal kinase (JNK), which are known to be triggered by mycobacteria (7,8). These activations of intracellular pathways result in expression of pro-inflammatory molecules that arouse both innate and adaptive immune responses. Both NOD1 and NOD2 sense bacterial molecules produced during the synthesis and degradation of peptidoglycan, NOD2 is especially activated by muramyl dipeptide (MDP), a component of peptidoglycan (PGN) (9-11). NOD2 has also been implicated in sensing intracellular pathogens such as *Listeria monocytogenes* (7) and *Mycobacterium tuberculosis* (12). However, despite their importance, the roles of NOD 1 and NOD 2 have not been elucidated in *M. leprae* infection. The role of NOD1 and NOD2 on the activation of NF- κ B in response to *M. leprae* was determined in this study.

M. leprae was prepared from the foot-pads of *M. leprae*-infected nude mice as previously described (3) and bacteria were counted by the procedure of Shepard and McRae (13).

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The human embryonic kidney (HEK) 293 T cells and mouse RAW 264.7 cell lines were purchased from American Type Culture Collection. The cell lines were cultivated in DMEM (Hyclone) media supplemented with 10% fetal bovine serum (Hyclone) and 1% penicillin-streptomycin (Gibco).

Raw274.7 cells were infected for 8 h with *M. leprae* at a multiplicity-of-infection (MOI) of 1 or 10. In some experiments, the cells were incubated for 30 min with cytochalasin D, a compound that inhibits phagocytosis by interacting with microfilament. Culture supernatants were assayed for mouse IL-1 β , IL-6, IL-12, and TNF- α by ELISA (DuoSet, R & D) according to the manufacturer's protocol.

HEK293T cells were seeded into 12-well Falcon plates (BD Falcon) at 2×10^5 cells/well, and then incubated overnight in a CO₂ incubator. Cells were washed with DPBS and co-transfected for 3 hr with varying amounts of pcDNA3-NOD1 and pcDNA3-NOD2. The final DNA quantity was adjusted to 1.5 μ g/well with empty pcDNA3 vector (Invitrogen, Carlsbad, CA). The transfection was carried out with FuGENE HD trans-

fection reagent (Roche) according to the manufacturer's protocol. Cells were washed with DPBS and left overnight in a CO₂ incubator in DMEM. The transfected cells were washed with DPBS placed in serum-free DMEM and stimulated with *M. leprae* at an M.O.I. of 10.

Cytosolic and nuclear extracts were isolated and NF- κ B activity assay was performed by the colorimetric assay kit (NF- κ B EZ-TFA Transcription Factor Assay, Upstate) according to the manufacturer's instructions.

Total RNA from *M. leprae*-infected HEK293T cells was prepared using Trizol reagent (Invitrogen) and treated with DNase I (Qiagen) to remove any contaminating genomic DNA. The amount of total RNA was quantified with the Hitachi DNA spectrophotometer and the quality was analyzed with 1% formaldehyde-agarose gel. cDNA was synthesized with SuperScript cDNA synthesis III kit (Invitrogen) in accordance with the manufacturer's instructions.

Quantitative RT-PCR was used to detect IL-1 β and TNF- α transcripts using β 2M as an endogenous control in the cells.

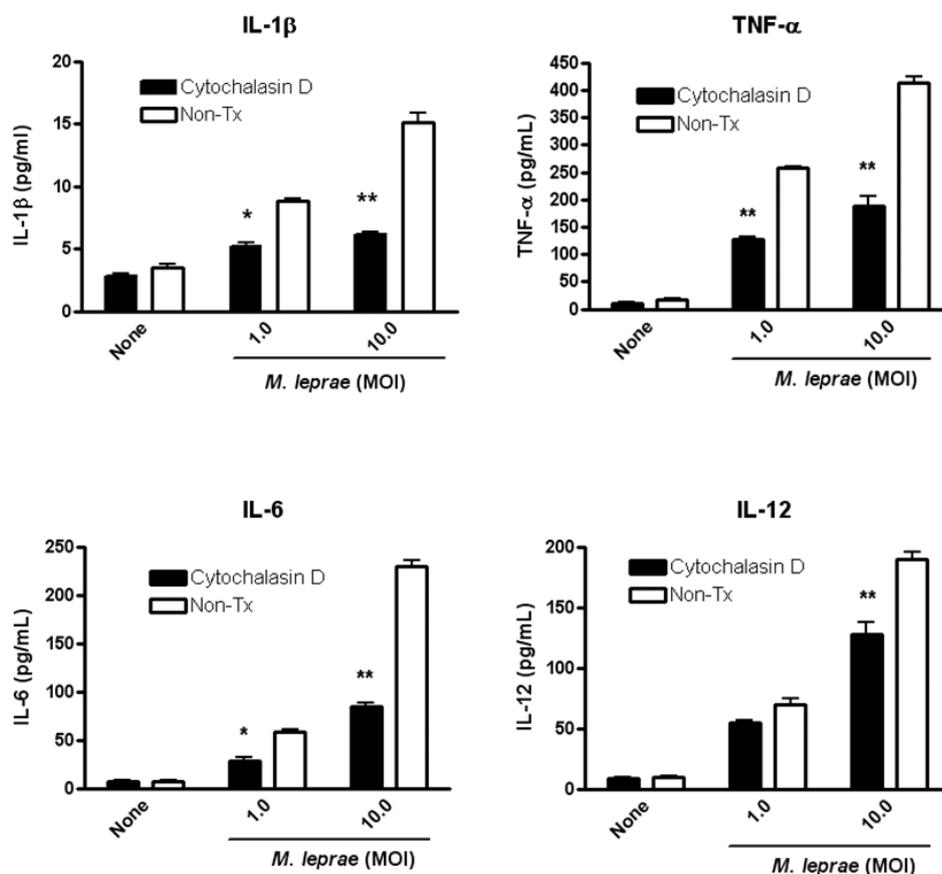


Figure 1. The role of phagocytosis in cytokine productions in RAW264.7 cells infected with *M. leprae*. The cells (10^6) were incubated with cytochalasin D (5 μ M) for 30 min and then treated *M. leprae* (MOI of 1 and 10) for 8 h, and supernatants were assayed for cytokines productions. Data are representative of at least three independent experiments, each performed in triplicates. * $p < 0.05$, ** $p < 0.01$ vs. non-treatment (Non-Tx).

PCR amplification was performed with 2×QantiTect SYBR Green PCR Master mix (Qiagen) with each validated primer (Qiagen) according to the manufacturer's protocol. Levels of mRNA were measured by a Chromo 4 (MJ Research). For relative quantification, the expression of each gene was normalized to the expression of $\beta 2M$ in the cells relative to a calibrator. The amount of target was represented by $2^{-\Delta\Delta Ct}$.

Macrophages have phagocytic properties that may contribute to the presentation of microbial structure to intracellular sensors. To examine the impact of phagocytosis on cytokine production, RAW264.7 cells were pre-treated with cytochalasin D before stimulation with *M. leprae*. We assumed that the inhibitory effect of cytochalasin D on phagocytosis may affect the cytokine production by macrophage infected with *M. leprae*. As we expected, treatment with cytochalasin D decreased the production of pro-inflammatory cytokines, such as IL-1 β , IL-6, and TNF- α , compared to non-treated group (Fig. 1). However, cytochalasin D had no effect on cytokine production after 24 h stimulation with *M. leprae* (data not shown). We concluded that the impact of phagocytosis is dependent on the early entry to the cells.

As bacterial entry into cells was important in cytokine production by macrophages, we were interested in the role of cytosolic receptor, NODs, for recognizing the molecular pattern from pathogen phagocytosed into cell. Therefore, the contribution of the NOD proteins to macrophage activation by *M. leprae* was examined by measuring the activation of NF- κ B and the expression of proinflammatory cytokines. To

investigate the role of NOD1 and NOD2 in the response of the host cell against *M. leprae* infection, HEK cells were transfected with NOD1 or NOD2 expression plasmid, and then the ability of *M. leprae* to activate these pathogen recognition receptors was assessed by monitoring the level of NOD-dependent expression of the pro-inflammatory cytokines, IL-1 β and TNF- α . Whereas *M. leprae* increased the activation of NF- κ B in the cell transfected with NOD1 or NOD2 expression plasmid, treatment with cytochalasin D decreased the NF- κ B activation in the cells (Fig. 2A). We also observed that NODs-transfected cells induced significantly IL-1 β (Fig. 2B) and TNF- α (Fig. 2C) expression to *M. leprae* in mRNA level. Inhibitory effect of cytochalasin D on cytokine expression in macrophages was also no longer observed after 24 h stimulation (data not shown).

As Ferwerda et al. (12) reported that the host response to *M. tuberculosis* was NOD2-dependent, we assessed whether the immune response to *M. leprae* was dependent on NOD1 or NOD2 (Fig. 2). There is no difference between NOD1 and NOD2 in response of cells to *M. leprae* infection. However, whereas there was minimal response to *M. leprae* in NOD1-transfected cells, a significantly higher response was in NOD2-transfected cells. Our future study will investigate the role of NOD2 in host infected with *M. leprae* using siRNA and knock-out mice. RICK (also called RIP2) is a caspase-recruitment domain (CARD)-containing kinase that is a critical downstream mediator of NOD1 and NOD2 signaling and plays a role in host defense against intracellular bacteria

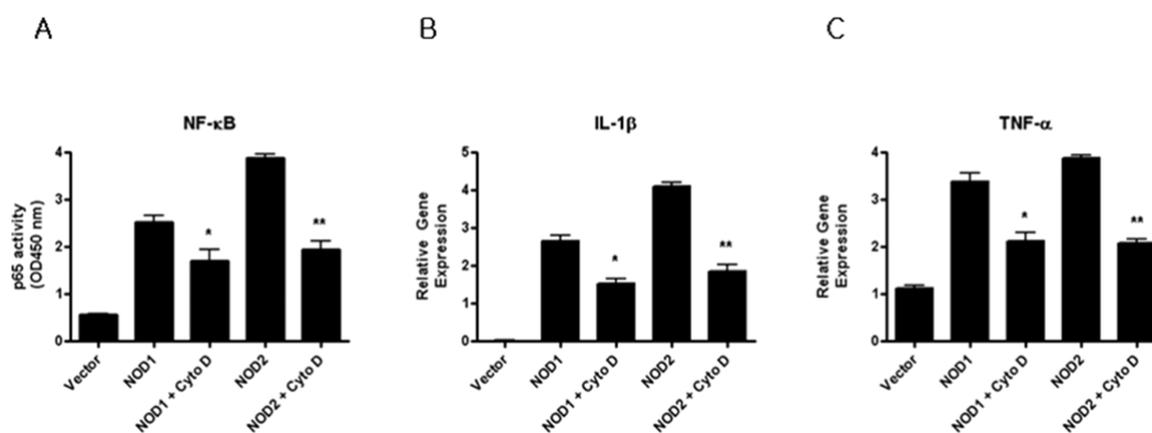


Figure 2. The role of phagocytosis and intracellular sensor, NOD proteins on the expression of pro-inflammatory cytokines in HEK cells infected with *M. leprae*. HEK cells were transfected with NOD1 or NOD2 for 8 hours with viable *M. leprae* at 10.0 MOI in absence or presence of cytochalasin D (cyto D). The cell extracts were used for measuring NF- κ B activation (A). The total RNA was isolated and the relative mRNA levels for IL-1 β (B) and TNF- α (C) were measured by real-time PCR. Data are representative of at least three independent experiments, each performed in triplicate. * $p < 0.05$, ** $p < 0.01$ vs. non-treatment with cytochalasin D.

(14-16). Therefore, the study about the relationship between NOD2 and RICK expression in host response against *M. leprae* infection will be also added in our future study.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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