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BRIEF COMMUNICATION

Expression of Nucleotide-oligomerization Domain (NOD) and Related Genes in Mouse Tissues Infected with *Mycobacterium leprae*

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The nucleotide-oligomerization domain (NOD) is an important molecule involved in host defense against bacterial infection. To study the role of NODs in the host response to *Mycobacterium leprae*, we measured the mRNA levels of NODs and related genes in infected mouse tissues. The mRNA expression of NOD1, NOD2, caspase-1 and ASC was increased in mouse footpads. Whereas NOD2 expression in macrophages was increased at 2 and 24 h post-infection with *M. leprae*, there was no expression of NOD1 at these time points. An increase in caspase-1 expression was observed at 2 h and continued at 24 h. However, the expression of ASC was increased only at the early time point. The expression of caspase-1 is regulated by NOD2-dependent pathway in established HEK 293. Our results suggest NOD2, rather than NOD1, may be associated with the host response to *M. leprae* and that caspase-1 activation is essential for the host response.
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Keywords: *Mycobacterium leprae*, NOD2, Caspase-1, ASC

INTRODUCTION

Mycobacterium leprae is an intracellular pathogen that resides and replicates in phagocytes such as macrophages. *M. leprae* can induce phagocytes to produce inflammatory cytokines, such as TNF-α and IL-12, which are involved in the control of bacterial replication and the coordination of adaptive immune responses (1,2).

Pattern recognition receptors (PRRs) are essential components for probing pathogen infection and evoking production of pro-inflammatory cytokines in the innate immune system. PRRs include toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) (3). NLRs are cytosolic receptors that are involved in manifold biological processes, including host defense against pathogens (4,5).

NOD1 and NOD2 are a subfamily of NLRs and their stimulation leads to activation of the nuclear transcription factor (NF)-κB, extracellular signal-regulated kinase (ERK) and Jun N-terminal kinase (JNK), which are known to be triggered by mycobacteria (6,7). This activation results in the expression of pro-inflammatory molecules that induce both innate and adaptive immune responses. NOD2 also induces caspase-1 activation (8), which mediates the maturation of pro-IL-1β to its active form, IL-1β (9).

NODs recognize bacterial molecules produced during the synthesis and degradation of peptidoglycan. In partic-

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Abbreviations: NOD, Nucleotide-oligomerization domain; ASC, apoptosis speck protein containing a caspase recruitment domain

ular, NOD2 is activated by muramyl dipeptide (MDP), a component of peptidoglycan (PGN) (10-12). NOD2 has also been implicated in sensing intracellular pathogens such as *Listeria monocytogenes* (6) and *M. tuberculosis* (13). However, despite their importance, the role of NODs in *M. leprae* infection has not been elucidated.

The inflammasome is a protein complex consisting of a nucleotide-binding domain, a leucine-rich repeats-containing family, a pyrin domain-containing (NLRP), apoptotic speck protein containing a caspase recruitment domain (ASC) PYCARD and caspase-1, and is a component of the innate immune system. The inflammasome promotes maturation of the inflammatory cytokine IL-1β and is responsible for activation of an inflammatory reaction (14,15).

To date, many studies of NODs have focused on the secretion of pro-inflammatory cytokine such as IL-1β through binding of their ligands, but there is no such evidence for their role in mycobacterial disease, particularly in leprosy. In this study, we assess the role of NODs in the host response to *M. leprae* infection by measuring the mRNA levels of NODs and related genes in infected mouse tissues.

MATERIALS AND METHODS

Mycobacterium leprae isolation

The use of *M. leprae*-infected mice for the preparation of *M. leprae* was approved by the Department of Laboratory Animal IACUC in Songeui Campus. *M. leprae* was prepared from the foot-pads of *M. leprae*-infected BALB/c *nude* mice. Foot-pads were treated with Potadine solution and washed with ice-cold DPBS to remove exogenous contamination. To isolate *M. leprae*, the foot-pads were excised, cut into small pieces, and ground with a MACs isolator (Miltenyl biotec. Germany). The extract was filtered using a Cell strainer (BD Falcon, Durham, NC, USA) to remove tissue debris and centrifuged for 20 min at 4°C. The pellet was resuspended in 1 ml of ice-cold DPBS, and treated with 2N sodium hydroxide for 5 min to remove tissue-derived cells. The reaction was neutralized by adding 13 ml of ice-cold DPBS. After centrifugation and resuspension, acid-fast staining was performed, and bacteria were counted under light microscopy (1,000×oil-lens) using the procedure of Shepard and McRae (16).

Cell culture and treatment with *M. leprae*

The human embryonic kidney HEK 293 and murine RAW 264.7 cell lines were purchased from the American Type Culture Collection. The cell lines were cultured in DMEM (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (Gibco, Grand Island, NY, USA). The cells were infected with *M. leprae* at a multiplicity-of-infection (MOI) of 10. RAW cells were also stimulated with LPS (derived from *E. coli* O111:B4, Sigma-Aldrich, St. Louis, USA). In some experiments, the cells were incubated with caspase-1 inhibitor (Ac-YVAD-CMK, Calbiochem, Darmstadt, Germany) 1 h before infection.

ELISA for IL-1β and caspase-1 assay

Culture supernatants were assayed for mouse IL-1β by ELISA (DuoSet, R&D, Minneapolis, MN, USA) according to manufacturer protocols. Using this assay, the lower limit of detection of IL-1β was 3.9 pg/ml. Cell lysates were centrifuged at 10,000 *g* for 5 min at 4°C and caspase-1 activity assay was performed using a caspase-1 assay kit (Calbiochem) as previously described (17,18). The total increase in the optical density at 405 nm versus that of the sample alone was calculated. Caspase-1 activity was expressed as: (maximum OD_{405}/microgram protein)×10,000.

Transfection

Human embryonic kidney (HEK) 293T cells were plated into 12-well plates (BD Falcon) at 2×10^5 cells/well and incubated overnight in a CO_2 incubator. The cells were co-transfected with varying amounts of pcDNA3-NOD1, pcDNA3-NOD2, or pcDNA3-NOD1-NOD2. The transfection was performed with transfection reagent (FuGENE HD, Roche, Switzerland) according to the manufacturer's instruction. The transfected cells were washed and placed in serum-free DMEM and stimulated with *M. leprae* (MOI of 10).

Quantitative RT-PCR

Total RNA from *M. leprae*-infected or non-infected tissues and cells was prepared using TRIzol reagent (Invitrogen, Waltham, MA, USA) and treated with DNase I (Qiagen, Valencia, CA, USA) to remove any contaminating genomic DNA. The amount of total RNA was quantified with spectrophotometer. cDNA was synthesized using a SuperScript cDNA synthesis III kit (Invitrogen) according

to manufacturer instructions.

Quantitative RT-PCR was used to detect *Caspase-1*, *Nod1*, *Nod2* and *ASC* transcripts in mouse footpads and RAW264.7 cells. β2M was used as an endogenous control. PCR amplification was performed with 2×QantiTect SYBR Green PCR Master mix (Qiagen) with validated primers (Qiagen) according to manufacturer protocols. The PCR conditions were as follows: 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 55°C (*Caspase1*, *Nod1*, *Nod2*, *ASC*, and β2M) for 30 s and 72°C for 30 s. Levels of mRNA were measured using Chromo 4 (MJ Research, Waltham, MA, USA). For relative quantification, the expression of each gene was normalized to the expression of β2M in the cells relative to a calibrator. The amount of target was represented as $2^{-\Delta\Delta Ct}$. The primers used in this study are shown in Table I.

Statistical Analysis

Statistical analyses were carried out using SigmaStat, version 3.1, by one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA, depending on the data. The significance was further confirmed by the Tukey test. Differences were considered significant when p was less than 0.05.

RESULTS AND DISCUSSION

Our previous report showed that *M. leprae* induced caspase-1 activation and IL-1β production in peritoneal cells from C57BL6 mice (19). In the present study, RAW 264.7 cells (a macrophage cell line) were exposed to *M. leprae* overnight, and the levels of IL-1β production and caspase-1 activity were measured. Consistent with our previous results, *M. leprae* bacilli induced caspase-1 activation and a low level of IL-1β production in RAW cells (Fig. 1). In contrast, LPS induced higher levels of IL-1β production under the same conditions, suggesting that the low level of IL-1β production was due to the doubling time of *M. leprae*. To determine whether caspase-1 is essential for IL-1β secretion in *M. leprae* infection, RAW cells were incubated with the caspase-1 inhibitor Ac-YVAD-CMK for 1 h before infection, and then caspase-1 activity and IL-1β secretion were measured. The *M. leprae*-induced IL-1β secretion was blocked in cells that lacked caspase-1 (Fig. 1A and B), suggesting that caspase-1 activity is necessary for IL-1β secretion in response to *M. leprae* infection.

Previous reports have shown that NOD2 induces caspase-1 activation, and that NOD signaling has a dual effect

Table I. Primers used in this study

Gene	Primer sequence
NOD1 (mouse)	Sense: 5'-AGCTGCAGCCTTGCTTTAGCC-3' Anti-sense: 5'-TCAGCCATAAATGCCGTAGCG-3'
NOD2 (mouse)	Sense: 5'-CCGAAGCCCTAGCACTGATGC-3' Anti-sense: 5'-CAACCATCACGACTCCTCGGG-3'
ASC (mouse)	Sense: 5'-AGACATGGGCTTACAGGA-3' Anti-sense: 5'-CTCCCTCATCTTGTCTTGG-3'
Caspase-1 (mouse)	Sense: 5'-TGAAAGAGGTGAAAGAATT-3' Anti-sense: 5'-TCTCCAAGACACATTATCT-3'
Caspase-1 (human)	Sense: 5'-GAAGAGAAAGCTGTTTATCCG-3' Anti-sense: 5'-AATCGAACCTTGCGGAAA-3'

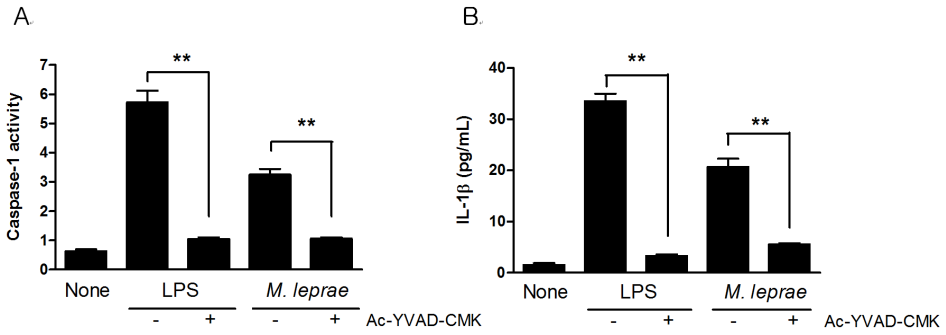

Figure 1. Caspase-1 activation and IL-1β production in RAW264.7 cells infected with *M. leprae*. Macrophages (10^6) were incubated with caspase-1 inhibitor (Ac-YVAD-CMK) for 1 h and then treated with LPS (100 ng/ml) and *M. leprae* (MOI of 10) for 18 h, and the supernatants and cell lysates were assayed for caspase-1 activity (A) and IL-1β (B), respectively. Data are representative of at least three independent experiments, each performed in triplicate. **$p < 0.01$.

by activating proIL-1 β mRNA transcription and inducing the release of bioactive IL-1 β (8). To assess the role of NODs in the host response to *M. leprae* infection, we examined the expression of NODs and related genes in mouse tissues infected with *M. leprae*, and compared that to non-infected tissues. We first measured the mRNA expression of NOD1, NOD2, caspase-1 and ASC in footpads from *nude* mice infected with *M. leprae* for 18 months. *M. leprae* infection increased the expression of both NOD1 and NOD2 mRNA. The mRNA expression of caspase-1 and ASC, which are components of the inflammasome, was also induced by *M. leprae* infection (Fig. 2).

Next, we investigated NOD expression in RAW 264.7 cells after *M. leprae* infection. Compared to uninfected cells, mRNA levels for NOD1 and NOD2 were increased at 1 h after infection with *M. leprae*. Whereas NOD2 ex-

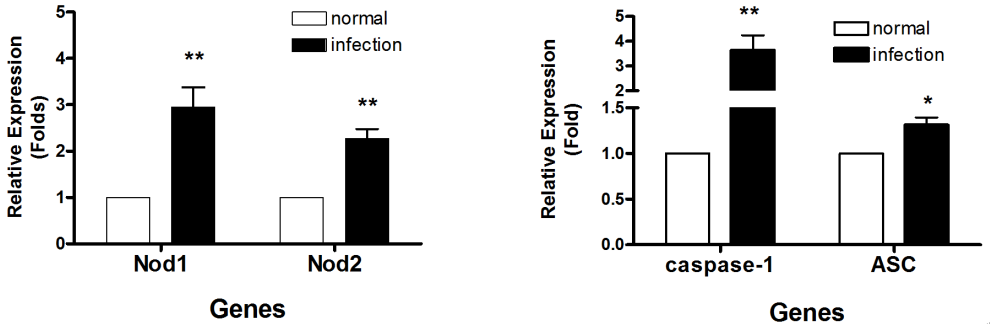

Figure 2. The expression of NOD- related genes in mouse footpads infected with *M. leprae* by determined by real-time PCR. Data are representative of at least three independent experiments, each performed in triplicate. *p < 0.05; **p < 0.01 vs. no stimulation.

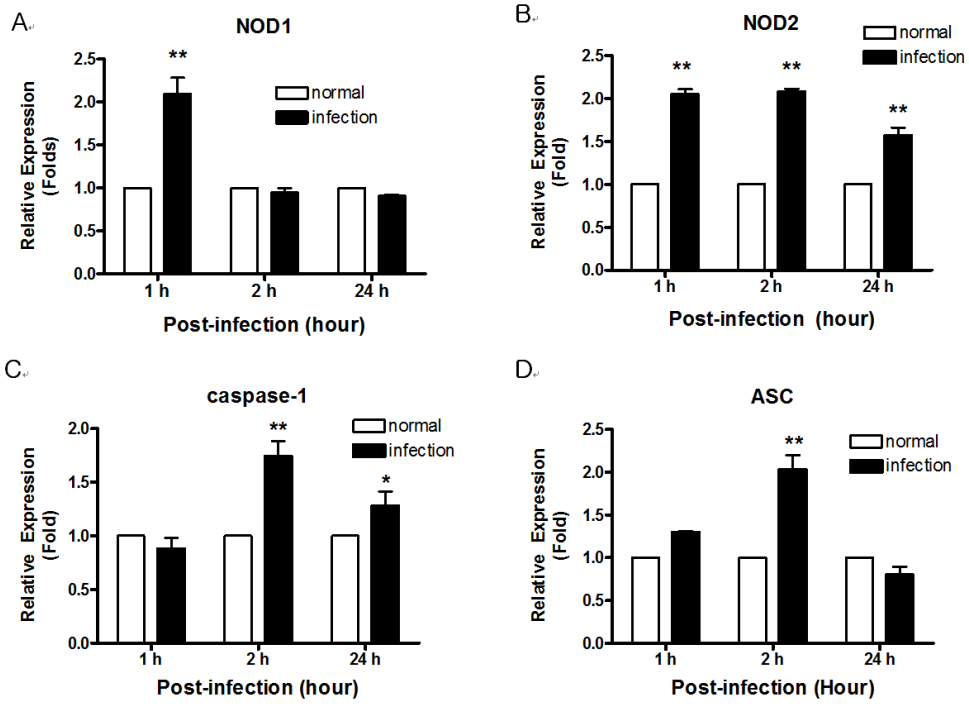

Figure 3. The expression of NOD-related genes (A; NOD1, B; NOD2, C; caspase-1, D; ASC) in RAW 264.7 cells infected with *M. leprae* determined by real-time PCR. Data are representative of at least three independent experiments, each performed in triplicate. *p < 0.05; **p < 0.01 vs. no stimulation.

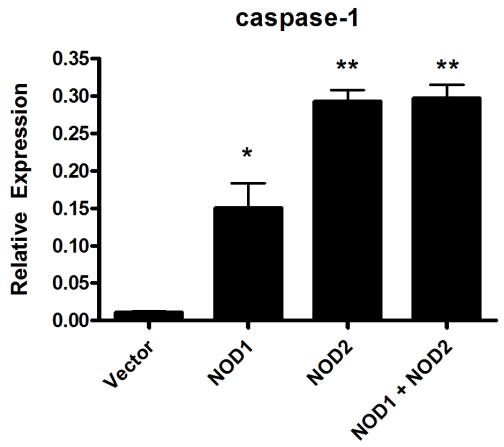

Figure 4. Caspase-1 expression in HEK cells transfected with NOD1 and NOD2. HEK cells were transfected with NOD1 or NOD2, or co-transfected with both NODs and stimulated for 24 hours with viable *M. leprae* at 10.0 MOI. Data are representative of at least three independent experiments, each performed in triplicate. *$p < 0.05$; **$p < 0.01$ vs. empty vector.

pression was increased at 2 and 24 h post-infection with *M. leprae*, while the expression of NOD1 was not affected at 2 and 24 h post-infection (Fig. 3A and B). Increased caspase-1 expression was observed at 2 h and continued at 24 h (Fig. 3C). However, Fig. 3D shows that the expression of ASC, a major adaptor protein involved in the inflammasome, was increased only at the early time points (1 and 2 h).

To investigate the role of NODs in the response of the host cell against *M. leprae*, the expression of caspase-1 in the HEK cells transfected with NOD1 or NOD2 expression plasmid was examined. Caspase-1 expression was increased in cells transfected with NOD2 (Fig. 4), not NOD1, suggesting that caspase-1-mediated IL-1β production was dependent on NOD2 signaling.

IL-1β is a pro-inflammatory cytokines that has a critical role in the prevention of intracellular pathogens, including *Bacillus anthracis* and *Francisella* (17,20). Our results from the current study showed that *M. leprae* also induced caspase-1-mediated IL-1 secretion in RAW 274.7 cells (Fig. 1). NLRs, one of the two major classes of PRR in the innate immune system, provide a crucial interface between invading bacterial pathogens and the host immune system. Activation of NLRs by bacterial products can stimulate the NF-κB pathway, a key regulator of the pro-inflammatory response, activating genes that are involved in immune responses to stimuli. Two NLRs, NOD1 and NOD2, induce caspase-1 activation and IL-1β expression via large protein complexes named inflammasomes.

In order to more clearly define the roles of NOD1 and NOD2, we transfected HEK 293T cells with NOD1, NOD2 or both NODs. There was low response to *M. leprae* in NOD1-transfected cells, but a higher response in NOD2-transfected cells in caspase-1 expression (Fig. 4). We expected that HEK 293T cells transfected with both receptors (NODs) to have a significantly higher response than cells transfected with either one alone. However, there was no synergistic effect between NOD1 and NOD2 in the response to *M. leprae* (Fig. 4). Therefore, our results suggest that NOD2, rather than NOD1, is associated with the host response to *M. leprae* infection. The future study will examine the role of NODs in host infected with *M. leprae* using siRNA and knock-out mice.

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CONFLICTS OF INTEREST

The authors have no financial conflict of interest.

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