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Nucleotide-Binding Oligomerization Domain 2 (Nod2) Is Dispensable for the Innate Immune Responses of Macrophages against *Yersinia enterocolitica*

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Nucleotide-binding oligomerization domain 2 (Nod2) is a cytosolic sensor for muramyl dipeptide, a component of bacterial peptidoglycan. In this study, we have examined whether Nod2 mediates the immune response of macrophages against *Yersinia enterocolitica*. Bone-marrow-derived macrophages (BMDMs) were isolated from WT and Nod2-deficient mice and were infected with various strains of *Y. enterocolitica*. ELISA showed that the production of IL-6 and TNF-α in BMDMs infected with *Y. enterocolitica* was not affected by the Nod2 deficiency. iNOS mRNA expression was induced in both WT and Nod2-deficienct BMDMs in response to *Y. enterocolitica*, beginning 2 h after infection. Nitric oxide (NO) production by *Y. enterocolitica* did not differ between WT and Nod2-deficient BMDMs. Western blot analysis revealed that *Y. enterocolitica* induces activation of NF-κB, p38, and ERK MAPK through a Nod2-independent pathway. Neither LDH release by *Y. enterocolitica* nor the phagocytic activity of the macrophages was altered by Nod2 deficiency. An *in vivo* experiment showed that bacterial clearance ability and production of IL-6 and KC in serum were comparable in WT and Nod2-deficient mice infected with *Y. enterocolitica*. These findings suggest that Nod2 may not be critical for initiating the innate immune response of macrophages against *Yersinia* infection.

Keywords: Nod2, *Yersinia*, innate immunity, macrophages

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Introduction

The innate immune system is the first line in host defense against invading pathogens and is mediated by phagocytes, including macrophages and dendritic cells (DCs). The phagocytes recognize invariant molecular structures called "pathogen-associated molecular patterns" (PAMPs) by using a limited number of pattern-recognition receptors (PRRs). PRRs can be grouped into Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (Nod)-like receptors (NLRs), and retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) (Akira *et al*., 2006; Creagh and O'Neill, 2006).

NLRs mediate cytosolic recognition of microbial molecules. Nucleotide-binding oligomerization domain 2 (Nod2) is a member of the NLR family that regulates the intracellular recognition of bacterial components by immune cells (Franchi *et al*., 2009b). It consists of two N-terminal caspase recruitment domains (CARDs), an intermediate Nod domain, and a C-terminal leucine-rich repeat (LRR) domain, which is responsible for sensing muramyl dipeptide (MDP), a small molecule derived from bacterial cell wall peptidoglycan (Inohara *et al*., 2005). After MDP recognition, Nod2 associates with the serine/threonine kinase RICK/Rip2 via a CARD-CARD interaction, subsequently leading to nuclear factor kappa-B (NF-κB) activation and the production of inflammatory mediators (Hasegawa *et al*., 2008). Nod2 activation also recruits a platform of proteins called the inflammasome, resulting in the activation of caspase-1 and the release of IL-1β and IL-18 (Mariathasan and Monack, 2007; Franchi *et al*., 2009a).

The genus *Yersinia*, Gram-negative rod shaped bacteria, contains about ten species. Among those, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* are pathogenic for humans and for rodents. *Y. pestis* is the causative agent of plague. It causes bubonic plague when transmitted by flea bite and pneumonic plague when acquired through aerosol transmission (Stenseth *et al*., 2008; Williamson, 2009). *Y. pseudotuberculosis* and *Y. enterocolitica* are enteropathogens that cause a wide range of symptoms and pathologies–including diarrhea, enteritis, and mesenteric lymphadenitis–in humans and in rodents (Bottone, 1999; Naktin and Beavis, 1999). These pathogens are a major concern for public health organizations, because infections are usually acquired by the ingestion of contaminated food or water. Following oral uptake, they invade the underlying Peyer's patches of the small intestine, entering through specialized epithelial cells called "M cells". Subsequently, the bacteria disseminate to

the lymph nodes, spleen, and liver. Systemic infection can cause abscesses, granulomatosis lesions, and immune pathological lesions, such as reactive arthritis (Lamps *et al.*, 2001; Oellerich *et al.*, 2007; Townes, 2010). In patients with compromised or underdeveloped immune systems, enteric *Yersinia* disseminates systemically and can develop into fatal septicemia (Abbott *et al.*, 1986; Bockemuhl and Roggentin, 2004).

There is a controversy regarding the function of Nod2 in the innate immune response against *Yersinia* infection. Previous studies have suggested that Nod2 may play an important role in the innate immune response against *Yersinia* infection (Ferwerda *et al.*, 2009; Netea *et al.*, 2010). Peripheral blood mononuclear cells (PBMCs) from individuals with a homozygous *NOD2* 3020incC mutation have been shown to display a tendency toward reduced TNF-α production and a significantly lower IL-1β production in response to three pathogenic *Yersinia* spp. (Ferwerda *et al.*, 2009). Moreover, IL-6 production by *Y. enterocolitica* was impaired in PBMCs taken from a patient with chronic yersiniosis, who was also found to be homozygous for the *NOD2* 3020incC mutation (Netea *et al.*, 2010). In contrast, Nod2 deficiency did not affect IL-6 or TNF-α production by murine macrophages in response to *Y. pseudotuberculosis* (Kim *et al.*, 2008a). Meinzer *et al.* (2008) showed that Nod2 is not critical for immunity to systemic infection by *Y. pseudotuberculosis* via an intraperitoneal route, although it is involved in mediating local intestinal inflammation. However, it is unclear whether this discrepancy is due to the difference in *Yersinia* species (*Y. enterocolitica* vs. *Y. pseudotuberculosis*). In the present study, we investigated the role of Nod2 in the innate immune response of macrophages against *Y. enterocolitica* infection.

Materials and Methods

Mice

Nod2-deficient mice having the C57BL/6 background were purchased from the Jackson Laboratories (USA). Wild type C57BL/6 mice were obtained from Orient (Korea). The animal studies were approved by the Institutional Animal Care and Use Committee in Konyang University, and the regulations of the committee were followed.

Bacterial culture

Y. enterocolitica strains 8081, ATCC 23715, ATCC 700822, and ATCC 55075 were used. Single colonies were inoculated into 5 ml of Luria-Bertani (LB) broth and grown overnight at 26°C in a shaking incubator. A 1/5 dilution of the overnight culture was prepared and allowed to grow at 37°C with shaking to A600=0.6, which corresponds to ~10^9 CFU/ml. After the bacteria were washed twice with phosphate buffered saline (PBS; pH 7.4), they were diluted to desired concentrations with PBS or medium and were used in further experiments.

Preparation of murine macrophages and bacterial infection

Bone-marrow-derived macrophages (BMDMs) were prepared as previously described (Celada *et al.*, 1984). The cells were seeded on 48-well plates at a concentration of 2×10^5 cells/well and were placed in a 5% CO_2 incubator at 37°C overnight. Subsequently, the cells were infected with *Y. enterocolitica* at the indicated multiplicity of infection (MOI) for 60 min, and extracellular bacterial growth was inhibited by gentamicin treatment (50 μg/ml). Culture supernatant was collected 18 h after infection for further analysis.

Measurement of cytokines and nitrite

The concentrations of IL-6 and TNF-α in culture supernatants were determined using a commercial ELISA kit (R&D System, USA). Nitrite accumulation in the supernatant of cultured cells was assayed by the Griess reaction (Green *et al.*, 1982).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

BMDMs were infected with *Y. enterocolitica* strain 8081 at MOI 1/10, and extracellular bacteria were removed by the addition of gentamicin 90 min after infection. Total RNA was extracted from the cells using easy-BLUE (Intron Biotechnology, Korea) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed into cDNA by using the Power cDNA Synthesis Kit (Intron Biotechnology, Korea), and PCR was performed using AccuPower® HotStart PCR PreMix (Bioneer, Korea). The following primer sets were used:

mouse iNOS (expected size: 550 bp),
F: 5′-GAGATTGGAGTTCGAGACTTCTGTG-3′
and R: 5′-TGGCTAGTGCTTCAGACTTC-3′;
mouse GAPDH (expected size: 550 bp),
F: 5′-GTGGAGATTGTTGCCATCAACG-3′ and
R: 5′-CAGTGGATGCAGGGATGATGTTCTG-3′.

The PCR conditions consisted of 1 cycle of 94°C for 5 min; 35 cycles of 94°C for 30 sec, 56–60°C for 30 sec, and 72°C for 30 sec; followed by 1 cycle of 72°C for 10 min. PCR products were then electrophoresed on a 1.5% agarose gel and visualized using a gel-documentation system.

Immunoblotting

The cells were lysed in a buffer containing 1% Nonidet-P40 supplemented with a complete protease inhibitor "cocktail" (Roche, Germany) and 2 mM dithiothreitol. Lysates were separated by 10% SDS-PAGE and were transferred to polyvinylidene fluoride (PVDF) membranes by electroblotting. The membranes were immunoblotted with primary antibodies, such as regular- and phospho-IκB-α, p38, and ERK (Cell Signaling Technology, USA). After immunoblotting with secondary antibodies, proteins were detected with enhanced chemiluminescence (ECL) reagent (Intron Biotechnology).

Lactate dehydrogenase measurement

Lactate dehydrogenase (LDH) in culture supernatants was measured with a commercial cytotoxicity assay kit (Promega, USA).

Phagocytosis analysis

For a gentamicin protection assay, live *Y. enterocolitica* (1×10^8 CFU/ml in serum-free cell culture medium; 1 ml/reaction) were incubated with BMDMs (1×10^8/well) under antibiotic-free conditions. After 90-min incubation, the cell-membrane-impermeable antibiotic gentamicin (10 μg/ml) was added to the medium for 30 min to eliminate extracellular bacteria. The macrophage cells, containing engulfed bacteria, were washed with 1× PBS and were subsequently lysed with 1% Triton X-100 in PBS. The cell lysate was plated on LB agar to determine the number of living bacteria engulfed by macrophages.

In vivo experiment

Y. enterocolitica strain 8081 was cultured as described above. Mice were injected i.p. with PBS (for uninfected mice) or with 2×10^6 CFU of bacteria (in 200 μl). Blood was collected by orbital venous plexus bleeding with heparinized capillary tubes (Scientific Glass Inc., USA) at 3 and 24 h after infection. Serum was isolated by centrifugation at 3,000 rpm for 20 min and was stored in a deep freezer before use. Bacterial clearance was determined in liver and spleen samples of mice infected with *Y. enterocolitica* at 24 h after infection.

Results

The production of IL-6 and TNF-α by BMDMs in response to *Y. enterocolitica*

To confirm the phenotype of Nod2-deficient BMDMs, the ability of the cells to produce cytokines in response to LPS and MDP was determined. Compared with WT BMDMs, Nod2-deficient BMDMs produced a similar level of IL-6 and TNF-α in response to LPS (100 ng/ml; Figs. 1A and 1B). Combination treatment with LPS and MDP led to increased production of IL-6 and TNF-α in WT BMDMs but not in Nod2-deficient BMDMs (Figs. 1A and 1B), indicating that Nod2 is essential for the recognition of MDP in BMDMs. To determine whether Nod2 in macrophages mediates cytokine production in response to *Y. enterocolitica*, BMDMs were infected with *Y. enterocolitica* for 18 h, followed by measurement of cytokines in the culture supernatant. *Y. enterocolitica* strain 8081 increased the production of IL-6,

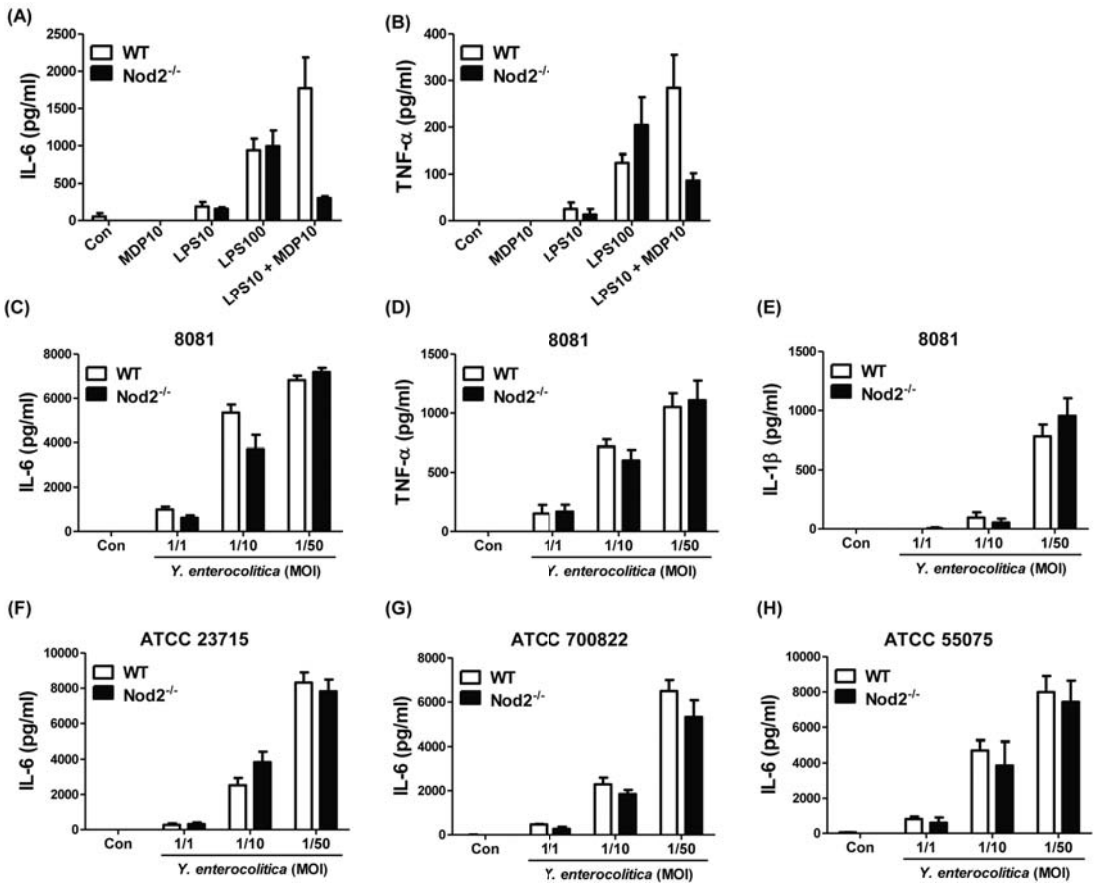

Fig. 1. Cytokine production by BMDMs in response to *Y. enterocolitica*. BMDMs were stimulated with different doses of MDP (10 μg/ml) and LPS (10 and 100 ng/ml) or the combination of LPS (10 ng/ml) and MDP (10 μg/ml) for 18 h (A and B). For the *in vitro* infection experiment, BMDMs were infected with *Y. enterocolitica* strain 8081 (C–E), ATCC 23715 (F), ATCC 700822 (G), or ATCC 55075 (H) at the indicated MOI and were treated with gentamicin 90 min after infection. After 18 h, the culture supernatant was collected and IL-6 and TNF-α production was measured by ELISA. The results are from a single representative experiment of three independent experiments.

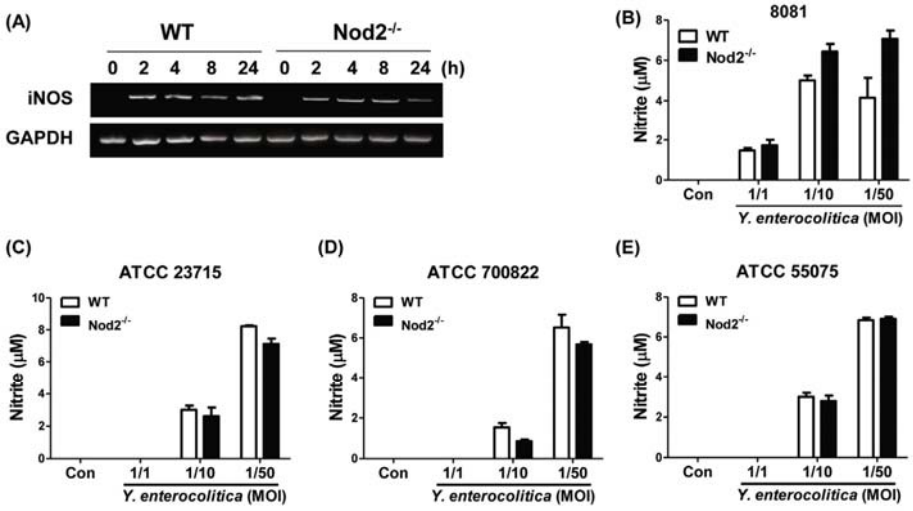

Fig. 2. iNOS expression and NO production in *Y. enterocolitica*-infected BMDMs. BMDMs were infected with *Y. enterocolitica* strain 8081 (MOI 1/10) for the indicated times. mRNA expression of iNOS was determined by RT-PCR (A). For the experiment to measure NO, the cells were infected with different strains of *Y. enterocolitica* for 18 h, and nitrite production was measured as described in 'Materials and Methods' (B–D). The results are from one representative experiment of two independent experiments.

TNF-α, and IL-1β in BMDMs in an infection dose-dependent manner, and the measured cytokine levels were comparable between WT and Nod2-deficient BMDMs (Figs. 1C–1E). Further, there was no difference in IL-6 production between WT and Nod2-deficient BMDMs in response to 3 other *Y. enterocolitica* strains (ATCC 23715, 700822, 55075) (Figs. 1F–1H). These findings indicate that Nod2 is dispensable with respect to *Y. enterocolitica*-induced cytokine production in macrophages.

mRNA expression for iNOS and NO production by *Y. enterocolitica* in BMDMs

RT-PCR was performed to determine whether Nod2 mediates the gene expression of iNOS in BMDMs in response to *Y. enterocolitica*. There was no difference between WT and Nod2-deficient BMDMs in the mRNA expression of iNOS in response to *Y. enterocolitica* strain 8081 (Fig. 2A). Moreover, Nod2-deficient BMDMs produced a level of NO after *Yersinia* infection comparable to that in WT BMDMs (Fig. 2B). Other strains of *Y. enterocolitica* (ATCC 23715, 700822, and 55075) also induced a similar level of NO production in both WT and Nod2-deficient BMDMs in a bacterial dose-dependent manner (Figs. 2C–2E).

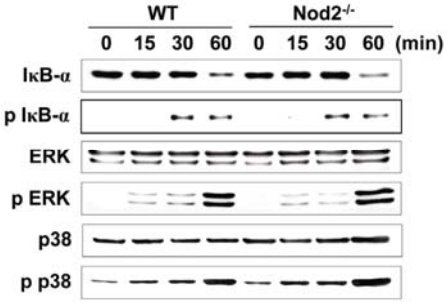

Fig. 3. NF-κB and MAPK activation in BMDMs in response to *Y. enterocolitica*. The cells were infected with *Y. enterocolitica* strain 8081, and protein was extracted at the indicated time points. IκB-α degradation and the phosphorylation of IκB-α, p38, and ERK were examined by western blotting. Primary antibodies against the regular forms of p38 and ERK were used to confirm the loaded protein doses. The results are from one representative experiment of two independent experiments.

NF-κB and MAPK activation in BMDMs in response to *Y. enterocolitica*

NF-κB and MAPK play a crucial role in initiating the immune response of immune cells against microbial infections. To determine whether Nod2 deficiency is associated with an alteration in the activation of these signaling pathways, we infected BMDMs with *Y. enterocolitica* strain 8081 at MOI 1/10. Protein was extracted at specific times after infection, and the activation of IκB-α, p38, and ERK was analyzed using western blot. The results show that in WT BMDMs, *Y. enterocolitica* led to the phosphorylation of IκB-α at 30 and 60 min after infection (Fig. 3). Degradation of IκB-α was detected at 60 min after infection. In the analysis of MAPK, phosphorylation of p38 and ERK was enhanced, beginning 15 min after infection (Fig. 3). All of these changes were similarly observed in Nod2-deficient BMDMs (Fig. 3). These findings indicate that Nod2 is not involved in mediating the activation of NF-κB and MAPK in macrophages infected with *Y. enterocolitica*.

LDH release and phagocytic activity

Yersinia infection is known to lead to death of naïve macrophages (Haase *et al.*, 2003; Zhang and Bliska, 2003). To examine whether Nod2 mediates death of *Yersinia*-infected macrophages, LDH release was measured. LDH release was elevated in *Y. enterocolitica*-infected macrophages in a dose-dependent manner, independent of Nod2 deficiency (Fig. 4A). In addition, a gentamicin protection assay revealed that Nod2 deficiency did not affect the phagocytic activity of macrophages against *Y. enterocolitica* (Fig. 4B).

In vivo bacterial clearance and cytokine/chemokine production in serum

Finally, we investigated whether Nod2 contributes to innate immunity against *Y. enterocolitica in vivo*. Mice were in-

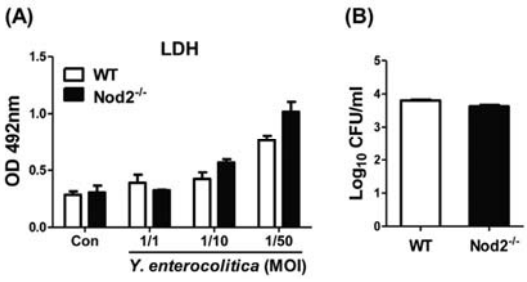

Fig. 4. BMDM LDH release and phagocytic activity. BMDM cells were infected with *Y. enterocolitica* strain 8081 at the indicated doses and were treated with gentamicin 90 min after infection. LDH release was measured from culture supernatant collected 18 h after infection (A). For the experiment to measure phagocytic activity, BMDMs in triplicate were infected with the bacteria at MOI 1/10. After 30 min of gentamicin treatment, the cell lysates were plated onto LB agar and incubated overnight. The phagocytosed bacteria were then counted and expressed as mean±SD (B).

fected i.p. with *Y. enterocolitica* strain 8081 (2×10^6 CFU/200 μl per mouse). At 24 h after infection, the bacterial load in the liver and the spleen did not differ between WT and Nod2-deficient mice (Figs. 5A and 5B). Moreover, systemic infection with *Y. enterocolitica* led to an increase in IL-6 and KC levels in the serum at 3 and 24 h after infection, and this increase was not altered in Nod2-deficient mice (Figs. 5C–5F). These results indicate that Nod2 deficiency may not affect innate immunity against systemic *Y. enterocolitica* infection.

Discussion

Nod1 is ubiquitously expressed in various cell types, whereas Nod2 is mainly expressed in immune cells, such as macrophages (Inohara *et al.*, 2005). Further, all Gram-negative (G-) and some Gram-positive (G+) bacteria, such as *Listeria* and *Bacillus* spp., posses *meso*-diaminopimelic acid (*meso*-DAP), whereas MDP exists in all G- and G+ bacteria (Inohara *et al.*, 2005). These findings imply that Nod2 may play a role in triggering the immune response of immune cells against bacterial infections.

Nod2 cooperates with TLRs with respect to the production of cytokines in macrophages (van Heel *et al.*, 2005a; Park *et al.*, 2007). MDP is known to enhance cytokine production induced by TLR agonists, such as LPS, in macrophages (Park *et al.*, 2007; Kim *et al.*, 2008b), suggesting that Nod2 deficiency may hinder the optimal production of cytokines in immune cells in response to bacteria. In fact, macrophages from Nod2-deficient mice showed lower production of proinflammatory cytokines and NO following infection with *M. tuberculosis* (Gandotra *et al.*, 2007; Brooks *et al.*, 2010). Moreover, inflammatory cytokine responses to bacterial CNS pathogens such as *B. burgdorferi* and *N. meningitidis* were partially impaired in Nod2-deficient glial cells and astrocytes (Chauhan *et al.*, 2009).

In this study, we examined the role of Nod2 in the innate immune response of macrophages against *Y. enterocolitica*. The production of cytokines and NO in *Yersinia*-infected BMDMs was not affected by Nod2 deficiency. Activation of NF-κB and MAPK, LDH release, and phagocytic activity in response to *Y. enterocolitica* were also not altered in BMDMs from Nod2-deficient mice as compared to WT BMDMs. These results are in accordance with the results of a study by Meinzer *et al.* (2008) showing immune functions of macrophages and dendritic cells from Nod2-deficient mice after *Y. pseudotuberculosis* infection. In contrast, Ferwerda *et al.* (2009) showed involvement of the NOD2 pathway in recognition of three pathogenic Yersinia species. They performed stimulations of human peripheral blood mononuclear cells (PBMCs) isolated from Caucasian individuals with *Y. enterocolitica*, *Y. pseudotuberculosis*, or *Y. pestis*. The PBMCs from individuals homozygous for the 3020insC *NOD2* mutation displayed reduced IL-1β production after stimulation with all three *Yersinia* spp. Because the pathogenesis and clinical signs of yersiniosis are very similar in humans and

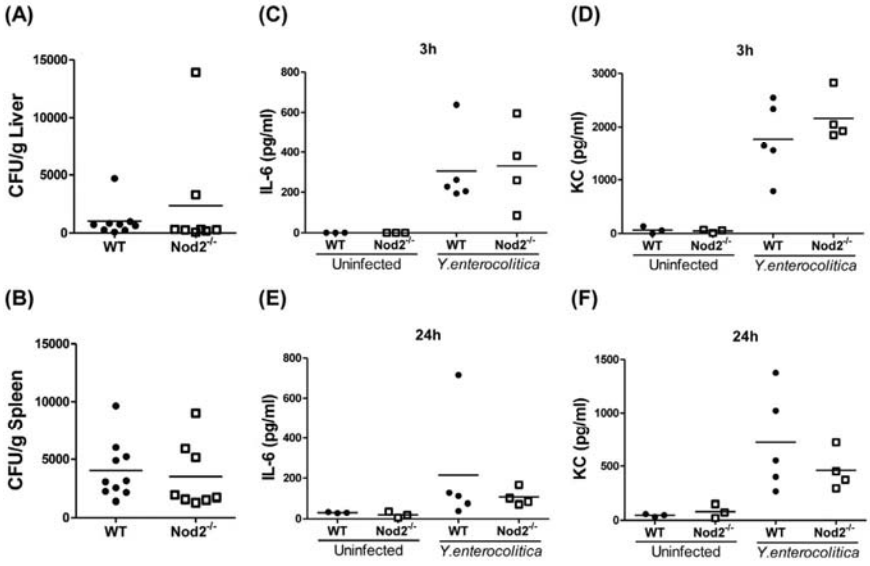

Fig. 5. Bacterial clearance and the measurement of serum cytokines. Mice were infected i.p. with *Y. enterocolitica* strain 8081 (2×10^6 CFU in 200 μl). At 24 h after infection, bacterial clearance was determined in the liver (A) and spleen (B) from WT and Nod2-deficient mice infected with the bacteria. Serum was collected at 3 and 24 h after infection, and IL-6 (C and E) and KC (D and F) were measured using a commercial ELISA kit.

rodents, this phenomenon does not seem to be caused by the difference in species but by a difference in the source of cells used in each experiment. MDP itself did not induce production of cytokines such as IL-6 and TNF-α in murine BMDMs, although it could induce the activation of NF-κB and MAPK (Park *et al.*, 2007; Kim *et al.*, 2008b). However, in human PBMCs, MDP alone could induce substantial IL-8 or TNF-α production (van Heel *et al.*, 2005a, 2005b). Moreover, MDP could induce IL-6 production in murine peritoneal macrophages, without any co-treatment (Hsu *et al.*, 2007). Taken together, these observations indicate that the strength of the Nod2 response may depend on the source of immune cells. A difference in the genetic mutation mechanism, namely, Nod2 knockout *versus* frameshift mutation, should also be considered as a possible reason for the discrepancy. In the study by Ferwerda *et al.* (2009), the authors obtained human PBMCs from patients with 3020insC frameshift mutations. There is a difference in the responsiveness to MDP between macrophages from Nod2-knockout mice and from mice with a frameshift mutation. Maeda *et al.* (2005) showed that MDP-induced NF-κB activation and cytokine production were increased in BMDMs from *Nod2*^{2939insC} frameshift mutant mice *versus* WT mice. However, many studies, including the present one, have revealed that Nod2-deficient BMDMs exhibit a loss of function for MDP response (Kobayashi *et al.*, 2005; Park *et al.*, 2007). The reason for this discrepancy remains unclear.

There is evidence showing redundancy of Nod1 and Nod2 in cytokine production of macrophages infected with bacteria. *Listeria*-induced IL-6 production was comparable between WT and Nod1 or Nod2 single-deficient BMDMs (Park *et al.*, 2007). However, it was partially impaired in Nod1/Nod2 double-deficient or RICK–(an adaptor molecule for Nod1 and Nod2)–deficient BMDMs, compared with WT macrophages. In addition, it is thought that TLR signaling masks Nod1 and Nod2 activity in macrophages in response to bacterial infection. Cytokine production was not different between naïve WT and RICK-deficient macrophages in response to G-bacteria, such as *Pseudomonas aeruginosa* and *Escherichia coli* (Park *et al.*, 2009). However, under TLRs-tolerized conditions (by LPS pre-stimulation), IL-1β and IL-6 production was significantly lower in RICK-deficient BMDMs compared with WT BMDMs (Park *et al.*, 2009). In addition, compared with *naïve* WT BMDMs, Nod1/Nod2-double-deficient cells exhibited an approximately 50% reduction in IL-6 and TNF-α production in response to *L. monocytogenes* infection (Kim *et al.*, 2008b). However, cytokine production and phosphorylation of IκB-α were almost abolished in LPS pre-stimulated Nod1/Nod2-double-deficient BMDMs (Kim *et al.*, 2008b). These findings indicate that Nod1 and Nod2 may play an important role in macrophage function in the absence of TLR activity.

A recent study revealed that TNF-α- and IFN-γ-mediated cell death is significantly decreased in MDP-stimulated macrophages, compared with unstimulated cells (Bansal and Balaji, 2011). Because *Y. enterocolitica* induces cell death in macrophages or dendritic cells (Bergsbaken and Cookson, 2007; Grobner *et al.*, 2007), we investigated whether Nod2 is involved in *Yersinia*-induced cell death in BMDMs. In this study, the release of cytosolic LDH was not different between *Yersinia*-infected WT and Nod2-deficient BMDMs, indicating that Nod2 may not be essential for *Yersinia*-induced cell death in macrophages. Richardson *et al.* (2010) showed that Nod2 activation reduced TLR4-induced apoptosis in enterocytes, whereas Nod2 did not inhibit TLR4 in macrophages. These findings indicate that negative regulation of Nod2 in TLR4-mediated cell death is restricted to enterocytes. In fact, when infected with *Y. pseudotuberculosis*, Nod2-deficient mice exhibited less apoptosis of intestinal epithelial cells surrounding Peyer's patch (Meinzer *et al.*, 2008).

Nod2 also contributes to the local immune response at enteric sites. Kobayashi *et al.* (2005) found that Nod2-deficient mice were more susceptible to infection with *Listeria monocytogenes* via the oral route because of a low expression of α-defensins in the ileum. However, in systemically infected Nod2-deficient mice, bacterial clearance was not affected (Kobayashi *et al.*, 2005), in accordance with the results of our study using *Y. enterocolitica*.

In conclusion, our results show that Nod2 is dispensable with respect to the innate immune response against *Y. enterocolitica* in naïve macrophages. It remains to be clarified whether Nod2 affects the immune response of macrophages in the absence of TLR. In addition, it would be valuable to determine the role of Nod2 in the adaptive immune response against *Yersinia* infection.

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