Cellular Microbiology (2011) **13**(5), 717–730
doi:10.1111/j.1462-5822.2010.01567.x
First published online 10 January 2011

Dual roles of NOD2 in TLR4-mediated signal transduction and –induced inflammatory gene expression in macrophages

Wen-Hsiang Tsai,[1] Duen-Yi Huang,[1] Yang-Hao Yu,[2] Chih-Yi Chen[3] and Wan-Wan Lin[1]*

[1]*Department of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan.*
[2]*Divisions of Pulmonary and Critical Care Medicine, Department of Medicine, China Medical University Hospital, Taichung, Taiwan.*
[3]*Cancer Center, China Medical University Hospital, Taichung, Taiwan.*

Summary

NOD2 of the NLRs and TLR4 of the TLRs are major pattern-recognition receptors, which sense different microbial pathogens and have important roles in innate immunity. Herein, we investigated the roles of NOD2 in TLR4-mediated signalling and gene regulation in RAW264.7 macrophages. We found that MDP (a NOD2 ligand) increased LPS-induced expressions of TNF-α, IL-1β, IL-6, iNOS and COX-2. MDP did not affect LPS-induced activation of MAPKs or IKK, while it potentiated LPS-induced NF-κB activation. Meanwhile TLR4 activation increased NOD2 mRNA expression, and upregulated NOD2 upon MDP treatment is a positive regulator of TLR4-mediated signalling. Intriguingly we found that NOD2 silencing led to increases in LPS-induced signal transduction and inflammatory responses, and a decrease in LPS-elicited homologous tolerance. We thus propose that NOD2 in the absence of MDP treatment might also play a negative regulatory role in the action of TLR4. Further, we demonstrated that both CARD and LRR domains of the NOD2 protein were responsible for the negative regulatory action on TLR4. In summary, it is the first time to demonstrate that NOD2 have dual effects on TLR4 signalling and exert a novel ligand-independent action. Elucidating molecular mechanisms by which NOD2 exerts its ligand-independent action on TLR4 requires further investigation.

Received 26 August, 2010; revised 6 December, 2010; accepted 12 December, 2010. *For correspondence. E-mail wwllaura1119@ntu.edu.tw; Tel. (+886) 2 23123456; Fax (+886) 2 23915297.

Introduction

Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are two major pattern-recognition receptors (PRRs) in the early host defence against pathogen invasion (Akira *et al*., 2006). TLRs are expressed on plasma membranes, lysosomal and/or endosomal vesicles, whereas cytosolic NLRs detect microbial components in the cytosol (Strober *et al*., 2006). Activation of TLRs can elicit conserved inflammatory pathways such as NF-κB and AP-1 (Akira *et al*., 2006; Kawai and Akira, 2006). In immune cells, NF-κB regulates many kinds of cytokines and mediators such as TNF-α, IL-6, IL-12β, inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2. AP-1 activation through stimulation by TLRs is commonly mediated by the three MAPKs, including p38, ERK and JNK. In co-ordination with NF-κB's actions, MAPKs upregulate some proinflammatory gene expressions under TLR stimulation.

NOD2 is composed of two N-terminal caspase recruitment domains (CARDs) responsible for protein–protein interactions, a central NOD domain required for nucleotide binding, self-oligomerization, and possession of ATPase activity, and a C-terminal leucine-rich repeat (LRR) domain required for ligand recognition (Fritz *et al*., 2006; Strober *et al*., 2006; Kanneganti *et al*., 2007). NOD2 can sense the components of peptidoglycan derived from bacteria in the host cytosol, e.g. muramyl dipeptide (MDP). Stimulation of NOD2 by ligand recognition triggers the recruitment of receptor-interacting protein (RIP)2, which stimulates the NF-κB and MAPK pathways through TRAF6, TAK-1-binding protein 2 – transforming growth factor-β-activated kinase 1 (TAK1), and IκB kinase (IKK) (Abbott *et al*., 2007; Hasegawa *et al*., 2008). Baseline expression of NOD2 in epithelial and myelomonocytic cells is low, and stimulation of the inflammatory cytokines and lipopolysaccharide (LPS) upregulates NOD2 expression via NF-κB (Gutierrez *et al*., 2002; King *et al*., 2007). In RAW264.7 macrophages, two phases of expressional changes of NOD2 mRNAs, occurring at 2 and 8~12 h after endotoxin treatment were, respectively, observed. The first rise results from NF-κB activation via a TLR4-mediated pathway, whereas the second rise possibly results from TNF-α production (Takahashi *et al*., 2006).

NOD2 might play a role as a host-defence factor in the pathogenesis of human diseases, particularly Crohn's disease and inflammatory bowel disease (Eckmann and Karin, 2005). According to genetic studies, mutations of the NOD2 gene occur in a subpopulation (10%~15%) of patients with Crohn's disease (Hugot *et al*., 2001; Ogura *et al*., 2001). These mutations are all localized to the LRR domain of NOD2 and are thought to interfere with recognition of its ligand, giving rise to a reduced capacity to induce NF-κB activation and a host defence mechanism. In agreement with this, NOD2-deficient mice are viable and susceptive to bacterial infection (Watanabe *et al*., 2004; Kobayashi *et al*., 2005).

Based on the distinct microbial components sensed by receptors, and the marked different downstream signalling pathways involved in the PRR systems, the integrated and cross-regulating cellular events of TLRs and NLRs after pathogen infection are of great pathological significance and interest. In the case of the TLR2-mediated response, the function of NOD2 acting as a negative regulator of TLR2 signalling and thus contributing to Crohn's pathology is also proposed (Watanabe *et al*., 2004). However, this negative regulatory model is still controversial because a study showed that MDP had a synergistic effect on TLR2 agonist (Netea *et al*., 2005). Apart from TLR2, synergistic activation of NF-κB and MAPKs by NOD2 and other TLRs, and requirement of pretreatment with TLR ligands for innate immune responses induced by NOD2 were demonstrated (Strober *et al*., 2006; Kim *et al*., 2008a). In this respect, a study performed in human dendritic cells suggested that the NOD2 ligand in combination with TLR3, TLR4 and TLR9 ligands, but not with the TLR2 ligand, synergistically induced IL-12 and IFN-γ production to induce Th1-lineage immune responses (Tada *et al*., 2005). Our current knowledge of NOD2-mediated signalling pathways in terms of the interaction with TLRs is still limited and controversial. Therefore, the cross-interaction between membrane-bound TLR4 and cytosolic NOD2 was explored in this study using murine RAW264.7 macrophages as a cell model. Here we not only confirm the synergistic actions of NOD2 and TLR4 co-stimulation, but also for the first time suggest a ligand-independent action of NOD2 to inhibit TLR4-mediated responses.

Results

MDP enhances LPS-induced inflammatory gene expressions in RAW264.7 macrophages without effects on early signalling activation

To assess the role of NOD2 in LPS-induced inflammatory responses, we first determined the effects of MDP and LPS, either alone or in combination, in murine RAW264.7 macrophages. After treatment with LPS (0.1, 1 and 10 μg ml^{-1}) and/or MDP (0.1 and 0.5 μM) for 24 h, we found that MDP alone failed to induce nitrite production, but enhanced LPS-induced nitrite production (Fig. 1A). In line with the results of product formation, LPS-elicited iNOS protein expression dramatically increased with co-treatment with MDP (0.1 μM). In addition to iNOS, the expression of the proinflammatory mediators, COX-2, TNF-α and IL-6, also were increased (Fig. 1B and D). Results of the qualitative PCR analysis confirmed this action of MDP (0.1 μM) in potentiating LPS-induced TNF-α, IL-1β, and iNOS mRNA expressions (Fig. 1C).

To explore the action mechanisms underlying the effects of MDP, we determined the NF-κB and MAPK signalling pathways, which are two co-ordinated signalling branches of TLR4. First we conducted a κB reporter assay to reflect NF-κB activity. As a result, MDP co-treatment was able to increase LPS (1 μg ml^{-1})-induced NF-κB activation after 6 h of incubation, even though MDP itself barely affected NF-κB activity (upper panel, Fig. 2A). Intriguingly, MDP treatment did not change LPS-induced IKK phosphorylation or IκBα degradation, which rapidly occurred within 15 min (lower panel, Fig. 2A). Using protein phosphorylation as an index of MAPK activities, we found that LPS (0.01~1 μg ml^{-1})-elicited ERK, p38 and JNK activations at 30 min were not altered by MDP (0.1 and 0.5 μM) (Fig. 2B). Likewise, the time-dependent activation responses of MAPKs by LPS (1 μg ml^{-1}) within 3 h were also unchanged by MDP (Fig. 2C). These results suggest that the enhancement effect of MDP on LPS-induced inflammatory gene expression might, at least partially, be ascribed to increased NF-κB activation, but not to the effects on activating upstream signals, i.e. IKK and MAPKs.

MDP-induced enhancement of the LPS response is through upregulation of NOD2 by TLR4

Previous studies showed that *NOD2* is an NF-κB target gene, and the mRNA level of NOD2 can be increased in a biphasic manner after LPS treatment. The early phase of the rise that occurs at 2 h is directly due to TLR4-triggered NF-κB signalling, while the late phase of the rise that occurs at 8~12 h is due to subsequent activation of NF-κB coming from the released TNF-α and ILs, and/or upregulation of NOD2 itself (Takahashi *et al*., 2006). Since our results showed that MDP enhancement of LPS-induced NF-κB activity was not due to the stimulation of IKK activation within 1 h, we wondered if MDP's action was associated with NOD2 protein expression. To this end, we determined NOD2 mRNA levels before and after LPS stimulation. We found that a moderate mRNA level of

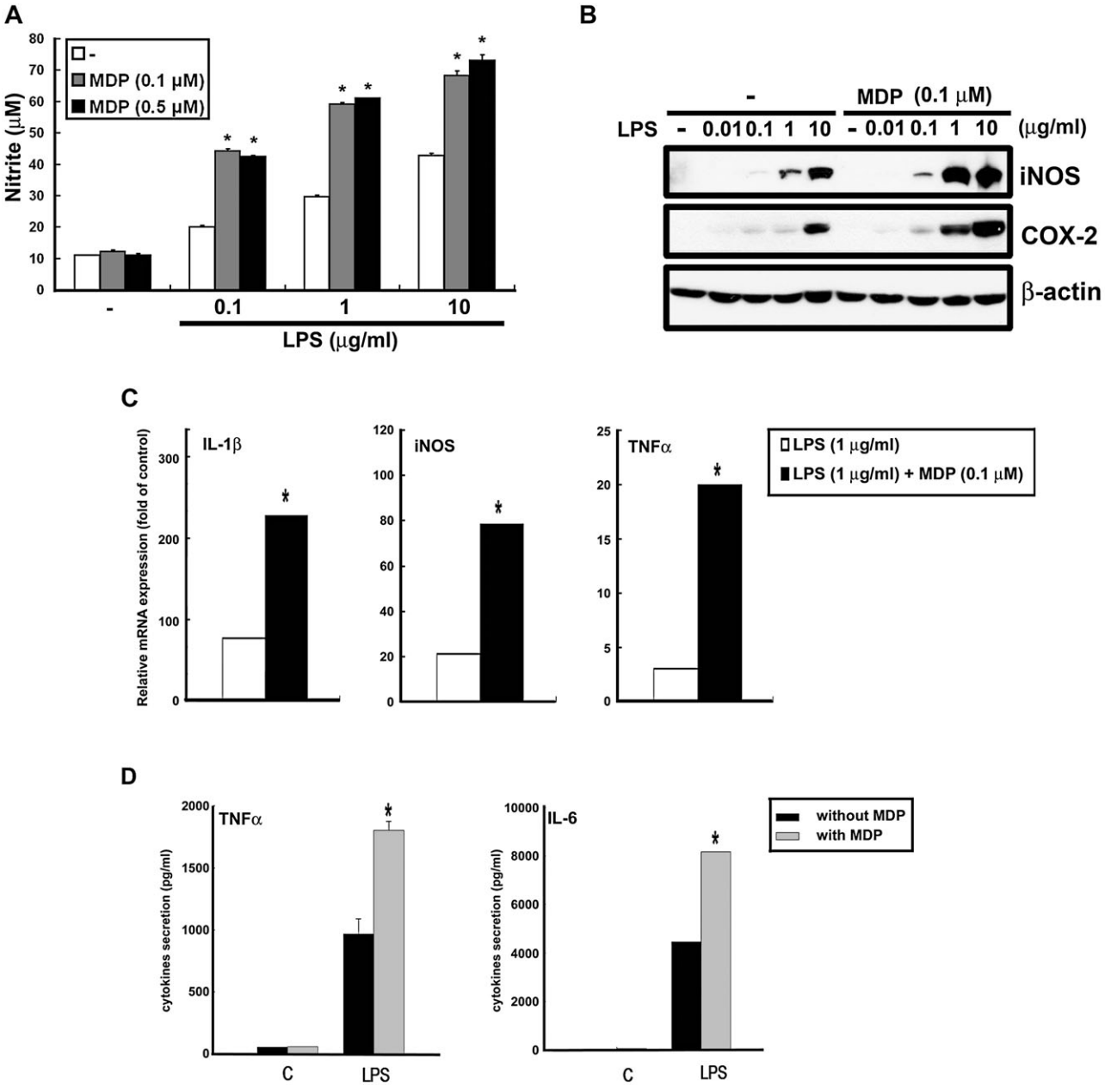

Fig. 1. MDP enhances LPS-induced inflammatory responses in RAW264.7 macrophages.
A, B. RAW264.7 macrophages were treated with LPS (0.1~10 μg ml^{-1}) and/or MDP (0.1 and 0.5 μM) as indicated. After incubation for 24 h, the nitrite production of the supernatant was measured (A), and cell lysates were analysed by immunoblotting for iNOS and COX-2 (B). The protein level of β-actin was used as an internal control to reflect equal protein loading.
C. Cells were treated with LPS (1 μg ml^{-1}) and/or MDP (0.1 μM). After incubation for 6 h, total RNA was extracted to measure iNOS, IL-1β and TNF-α mRNA levels by a real-time PCR. Values were normalized to β-actin gene expression and expressed relative to the control group.
D. After treatment with LPS (1 μg ml^{-1}) and/or MDP (0.1 μM) for 24 h, TNF-α and IL-6 levels in the culture medium were determined by ELISA. *$P < 0.05$, indicates a significant increase of the LPS response by MDP.

NOD2 was expressed in resting RAW264.7 macrophages, while LPS (1 μg ml^{-1}) treatment for 6 h increased NOD2 mRNA expression by around threefold (Fig. 3A). Next to delineate whether the enhancing effect of MDP results from the upregulation of NOD2 at a later time, we administered MDP at several time intervals after LPS, which were beyond the time window required for inducing upstream signal cascades (< 1 h). After treatment with LPS (1 μg ml^{-1}) for 3 or 6 h, RAW264.7 cells were additionally treated with MDP (0.1 μM), followed by a total 24 h of incubation with LPS. Figure 3B shows that the enhanced extents of iNOS and COX-2 expressions by

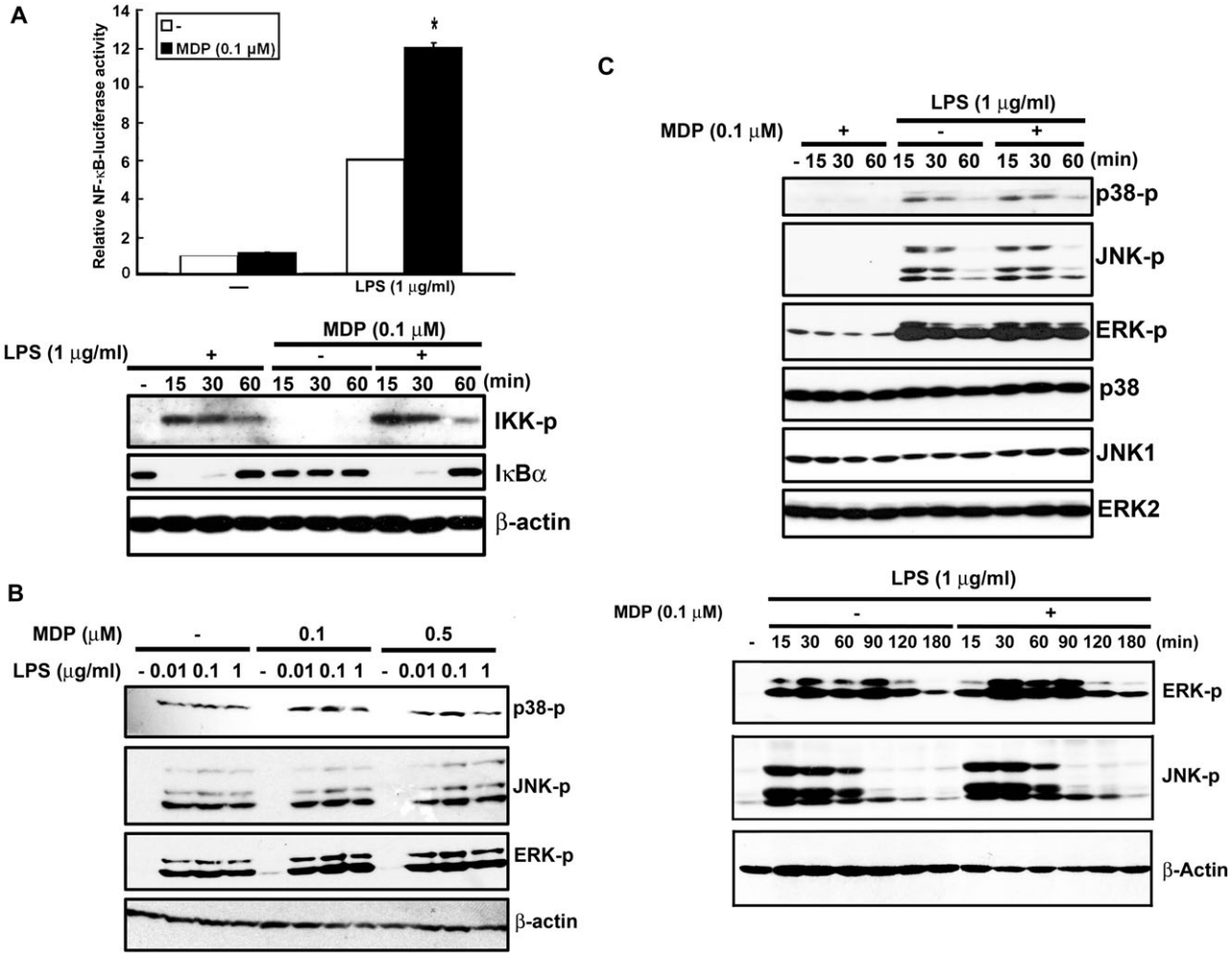

Fig. 2. MDP enhances LPS-induced NF-κB activation, but does not affect upstream signalling of IKK or MAPKs.
A. RAW264.7 macrophages were transfected with a κB reporter construct followed by treatment with LPS (1 μg ml^{-1}) and/or MDP (0.1 μM) for 6 h. Luciferase activity normalized to β-galactosidase was expressed as a percentage of the control group (upper panel). Data are the mean ± SEM from three independent experiments. *$P < 0.05$, indicates a significant increase in the LPS response by MDP. In some experiments, cells were treated with LPS (1 μg ml^{-1}) and/or MDP (0.1 μM) for different periods. Cell lysates were analysed by immunoblotting for phospho-IKK and IκBα (lower panel).
B, C. RAW264.7 macrophages were treated with LPS (0.01, 0.1, and 1 μg ml^{-1}) and/or MDP (0.1 and 0.5 μM) as indicated. After incubation for 30 min (B) or for different periods (C), cell lysates were analysed by immunoblotting for p38, JNK and ERK.

MDP remained the same regardless of the addition time point being the same as or 3~6 h after LPS treatment (Fig. 3B).

The above results suggested that the increased NOD2 expression after LPS might account for the potentiation effect of MDP on the TLR4-mediated inflammatory response. In order to verify this point, we adapted a siRNA knock-down approach for NOD2. Because of the low quality of the commercial NOD2 antibody, we could not reliably determine changes in NOD2 protein levels after LPS and MDP treatment. However, when siRNA targeted against NOD2 was used, the basal and LPS-increased NOD2 mRNA levels were significantly reduced (Fig. 3A). Therefore in the following study, we conducted siRNA experiments to address if NOD2 upregulation is required for MDP's action.

NOD2 knockdown without ligand stimulation enhances the LPS response

In NOD2 siRNA-treated cells, we unexpectedly found that NOD2 silencing rendered cells more sensitive to LPS in terms of iNOS and COX-2 protein expressions (lanes 11~15 vs. lanes 1~5, left panel, Fig. 4A). LPS (0.01~10 μg ml^{-1})-induced concentration-dependent iNOS and COX-2 expressions dramatically increased in low NOD2-expressing cells. The threshold concentration required for iNOS expression by LPS decreased from 0.1 μg ml^{-1} to

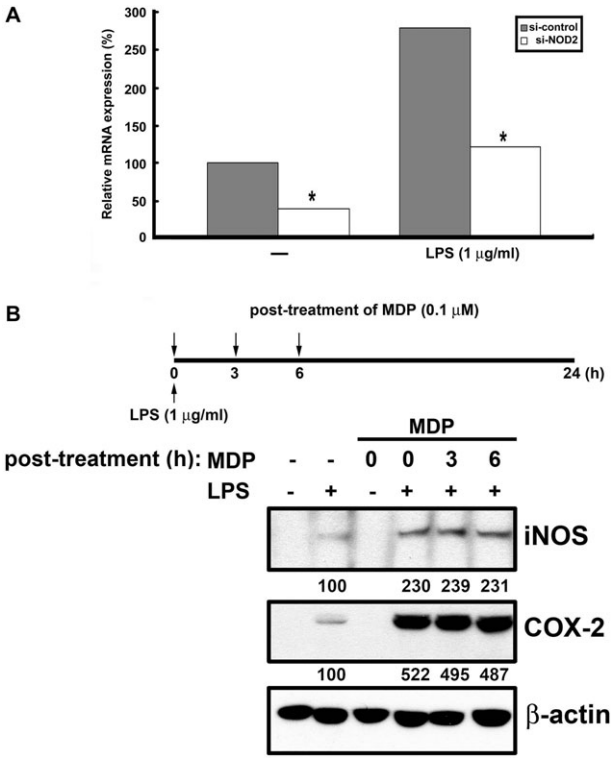

Fig. 3. NOD2 upregulation by TLR4 contributes to the enhancement of the LPS response by MDP.
A. RAW264.7 macrophages were transiently transfected with control (100 nM) or NOD2 (100 nM) siRNA for 48 h, and then were treated with LPS (1 μg ml^{-1}) for 6 h. Real-time PCR analyses of NOD2 and β-actin mRNA were conducted. Values were normalized to β-actin gene expression and were expressed relative to the control group. *$P < 0.05$, indicates a significant decrease in NOD2 mRNA.
B. After RAW264.7 macrophages were treated with LPS (1 μg ml^{-1}) for 3 and 6 h, cells were additionally treated with MDP (0.1 μM). Total cell lysates were analysed by immunoblotting for iNOS and COX-2 following 24 h of incubation with LPS.

10 ng ml^{-1} (lane 12 vs. lane 2), and the iNOS and COX-2 expressions induced by 1 μg ml^{-1} LPS further increased by 2.6- and 2.3-fold, respectively, upon NOD2 silencing (lane 14 vs. lane 4, left panel, Fig. 4A). Under such a sensitization situation, MDP treatment no longer increased iNOS protein expression as it did in the control group (lanes 16~20 vs. lanes 6~10, left panel, Fig. 4A). Meanwhile, enhancement of LPS (1 μg ml^{-1})-induced COX-2 expression by MDP was diminished in the NOD2-knock-down group (1.6-fold) compared with the control group (3.3-fold). Accordingly TLR4-mediated nitrite production in NOD2-knock-down cells was much higher than that seen in cells transfected with control siRNA (right panel, Fig. 4A). Concomitantly, LPS-increased mRNA levels of iNOS, COX-2, IL-6, IL-1β and MIP2 were all significantly enhanced upon NOD2 silencing (Fig. 4B). Taken together, these results not only confirm the dependence of MDP's action on NOD2, but also suggest the existence of ligand-independent, negative regulatory actions of NOD2 towards the TLR4 response.

In order to understand how NOD2 silencing affects TLR4 responses, we examined the TLR4-mediated NF-κB and MAPK signalling pathways. Results indicated that the increased levels of IKK phosphorylation, p65 nuclear translocation (left panel) and NF-κB reporter activity (right panel) caused by LPS were higher in NOD2-knock-down cells (Fig. 5A). Moreover, LPS-induced JNK and ERK phosphorylation levels were also more prominent in NOD2-knock-down cells (Fig. 5B). On the other hand, p38 phosphorylation induced by LPS was minimally enhanced. As reported, LPS can induce STAT1 phosphorylation with a more-delayed onset than IKK and MAPKs, and contributing to amplifying iNOS gene transcription. Thus, we also treated siRNA-manipulated cells with LPS (0.01, 0.1 and 1 μg ml^{-1}) for 4 h. We found that in NOD2-knock-down cells, the LPS-stimulated effect was more apparent than in control cells with NOD2 expression (Fig. 5C). These results suggest that the basal state of constitutively expressed NOD2 without ligand stimulation plays a negative role in TLR4-mediated signalling pathways.

Both the CARD and LRR domains of NOD2 are required to achieve negative regulation of TLR4 signalling

Following observation of the unexpected results suggesting that NOD2 is a negative regulator of TLR4, we further verified this notion by overexpressing NOD2 in RAW264.7 macrophages. In order to dissect which domains of NOD2 are critical in this respect, we constructed different Myc-tagged NOD2-deletion domains (NOD2, NOD2ΔCARD, NOD2ΔLRR and LRR) and individually transfected them in RAW264.7 macrophages. The expressions of truncated NOD2 proteins encoded by these plasmids were confirmed by immunoblotting with the Myc antibody (Fig. 6A).

Results revealed that LPS-induced iNOS and COX-2 expressions and nitrite production were attenuated in cells transfected with Myc-tagged NOD2, but not in cells transfected with different NOD2-deletion domains (NOD2ΔCARD, NOD2ΔLRR and LRR) (Fig. 6B). Likewise, LPS-induced IKK phosphorylation, p65 nuclear translocation and NF-κB promoter activation were suppressed in cells transfected with Myc-tagged NOD2, while the same activation extents were remained in cells transfected with other NOD2-deletion domains (Fig. 6C). In addition, LPS-induced MAPK activation was suppressed in cells transfected with Myc-tagged NOD2 but not in other NOD2-deletion domains (Fig. 6D). These results suggest that both the CARD and LRR domains of NOD2 are required for the negative regulation of TLR4-induced inflammatory responses.

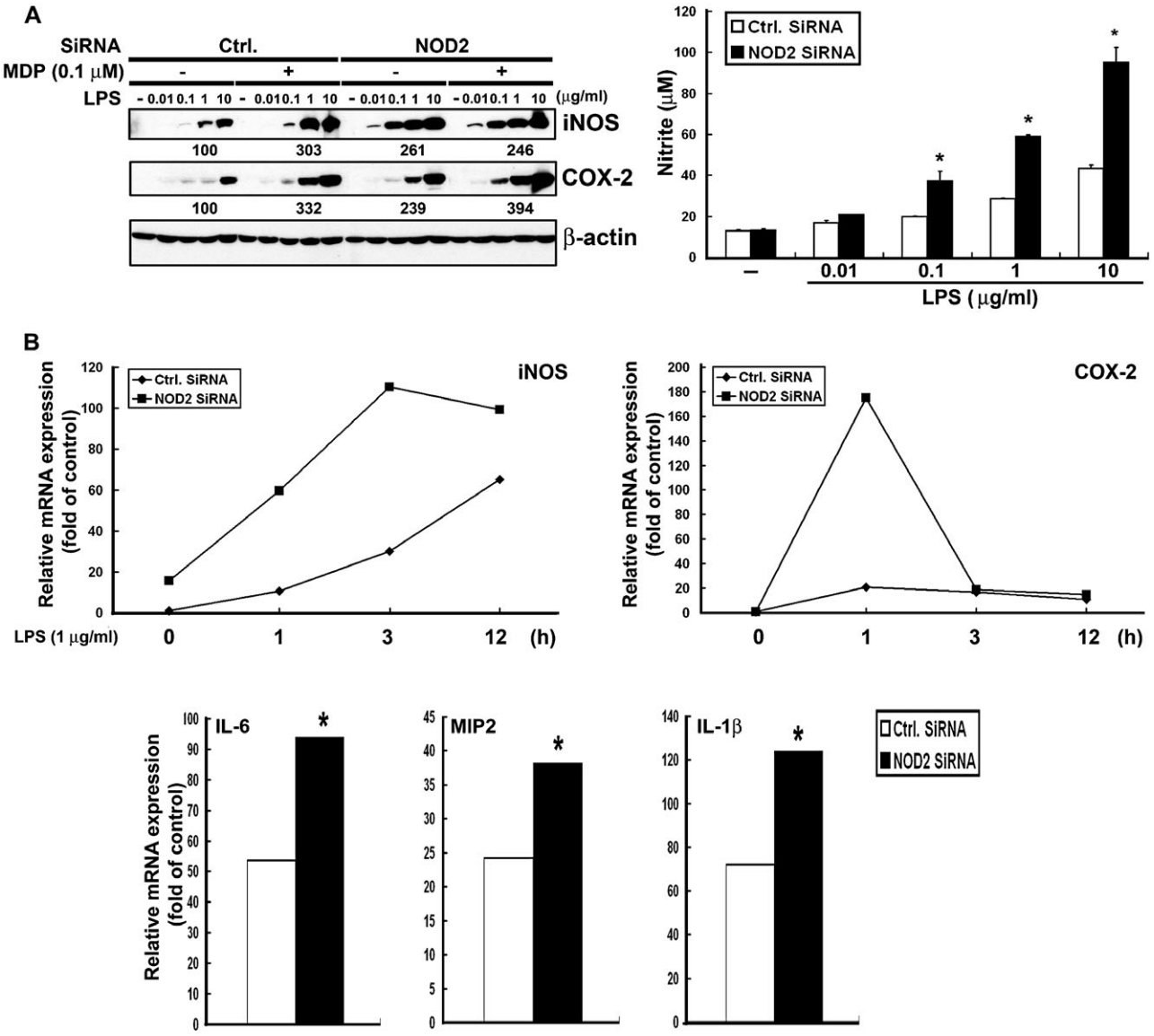

Fig. 4. Silencing NOD2 enhanced TLR4-induced inflammatory responses.
A. RAW264.7 macrophages were transiently transfected with control (100 nM) or NOD2 (100 nM) siRNA for 48 h. Cells were treated with LPS (0.01, 0.1, 1, and 10 μg ml^{-1}) for 24 h, and iNOS, COX-2 (left panel) and nitrite (right panel) were measured.
B. In the NOD2-knock-down condition, RAW264.7 macrophages were treated with LPS (1 μg ml^{-1}) for different time periods (1, 3, and 12 h). Total RNA was extracted for real-time PCR analyses of iNOS and COX-2 mRNA levels. In some cases, mRNA levels of IL-6, IL-1β and MIP2 after 6 h of LPS (1 μg ml^{-1}) treatment were determined. *$P < 0.05$, indicates a significant increase in the LPS responses in NOD2-silenced cells.

MDP-activated NOD2 decreased LPS tolerance, while ligand-independent NOD2 enhanced homologous desensitization of TLR4

Homologous desensitization of TLR4 provides a self-protective mechanism to avoid the occurrence of severe systemic inflammatory disorders (Akira and Takeda, 2004). Because NOD2 might exert opposite effects in regulating TLR4-mediated inflammatory response whether based on ligand stimulation or not, we tried to understand whether both roles played by NOD2 also affect the desensitization event of TLR4. First, to induce LPS tolerance, cells were treated with LPS (1 μg ml^{-1}) for 24 h, followed by washing and then re-stimulating cells with LPS for an additional 24 h. Our results indicated that LPS-stimulated iNOS and COX-2 expressions were prominently abated in LPS-primed cells (lanes 8 and 9 vs. lanes 2 and 3, Fig. 7A). Moreover, such homologous desensitizing phenomena of TLR4 were partially counteracted by treating MDP (0.1 μM) at the same time upon

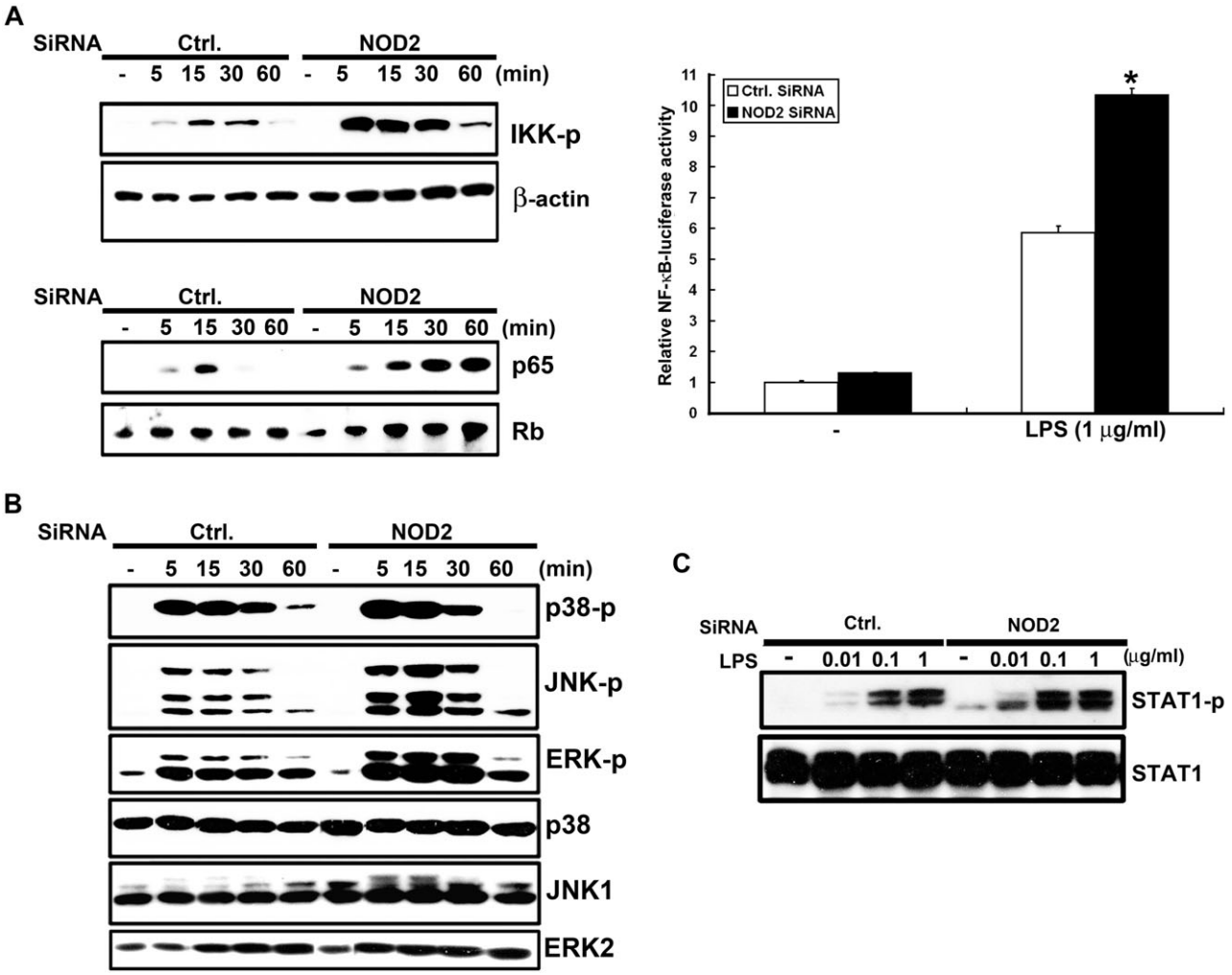

Fig. 5. Silencing of NOD2 enhanced TLR4-induced upstream signalling. In the NOD2-knock-down condition, RAW264.7 cells were treated with LPS (1 μg ml^{-1}) for the indicated time periods. Total cell lysates were subjected to SDS-PAGE to determine IKK (left panel, A), p38, JNK and ERK (B). Nuclear fraction was prepared to determine p65 (left panel, A) and protein level of Rb was regarded as an internal control. In (C), cells were treated with LPS (0.01, 0.1, and 1 μg ml^{-1}) for 4 h. Whole lysates were analysed for STAT1 by immunoblotting. In some experiments, NOD2-knock-down RAW264.7 macrophages were transiently transfected with the NF-κB luciferase reporter plasmid. Cells were treated with LPS (1 μg ml^{-1}) for 6 h before harvesting for the luciferase activity assay (right panel, A). *$P < 0.05$, indicates significant enhancement of the LPS response in NOD2 siRNA-treated cells.

LPS re-challenge (lanes 11 and 12 vs. lanes 5 and 6, Fig. 7A). Intriguingly, when desensitization to LPS was induced, MDP treatment alone was sufficient to induce iNOS and COX-2 expressions (lane 10 vs. lane 4, Fig. 7A).

A recent report indicated that prolonged exposure of primary human monocyte-derived macrophages to MDP (100 μg ml^{-1}, approximately equal to 200 μM) can inhibit proinflammatory responses of TLR4, such as TNF-α production (Hedl *et al.*, 2007). Therefore, it was suggested that MDP treatment might induce heterologous desensitization of the TLR4-mediated pathway. To address this event in a murine species, we incubated RAW264.7 cells with MDP (0.1 or 200 μM) for 24 h, followed by treatment with LPS (1 μg ml^{-1}) for an additional 24 h. Moreover, to determine if MDP can induce homologous desensitization of NOD2 itself as is the case with TLR4, we also re-challenged cells with MDP. Results showed that after MDP pretreatment and washout, MDP re-addition still failed to upregulate iNOS or COX-2 (lane 7 vs. lane 3, Fig. 7B). However, in MDP (0.1 or 200 μM)-pretreated cells, LPS-induced iNOS and COX-2 expressions greatly increased (lane 6 vs. lane 2, Fig. 7B). Upon re-challenge with MDP together with LPS in low-concentration MDP-pretreated cells, both inducible proteins were further increased compared with LPS re-challenge alone (lane 8 vs. lane 6, left panel, Fig. 7B). Accordingly, LPS-induced nitrite formation increased to a similar extent in

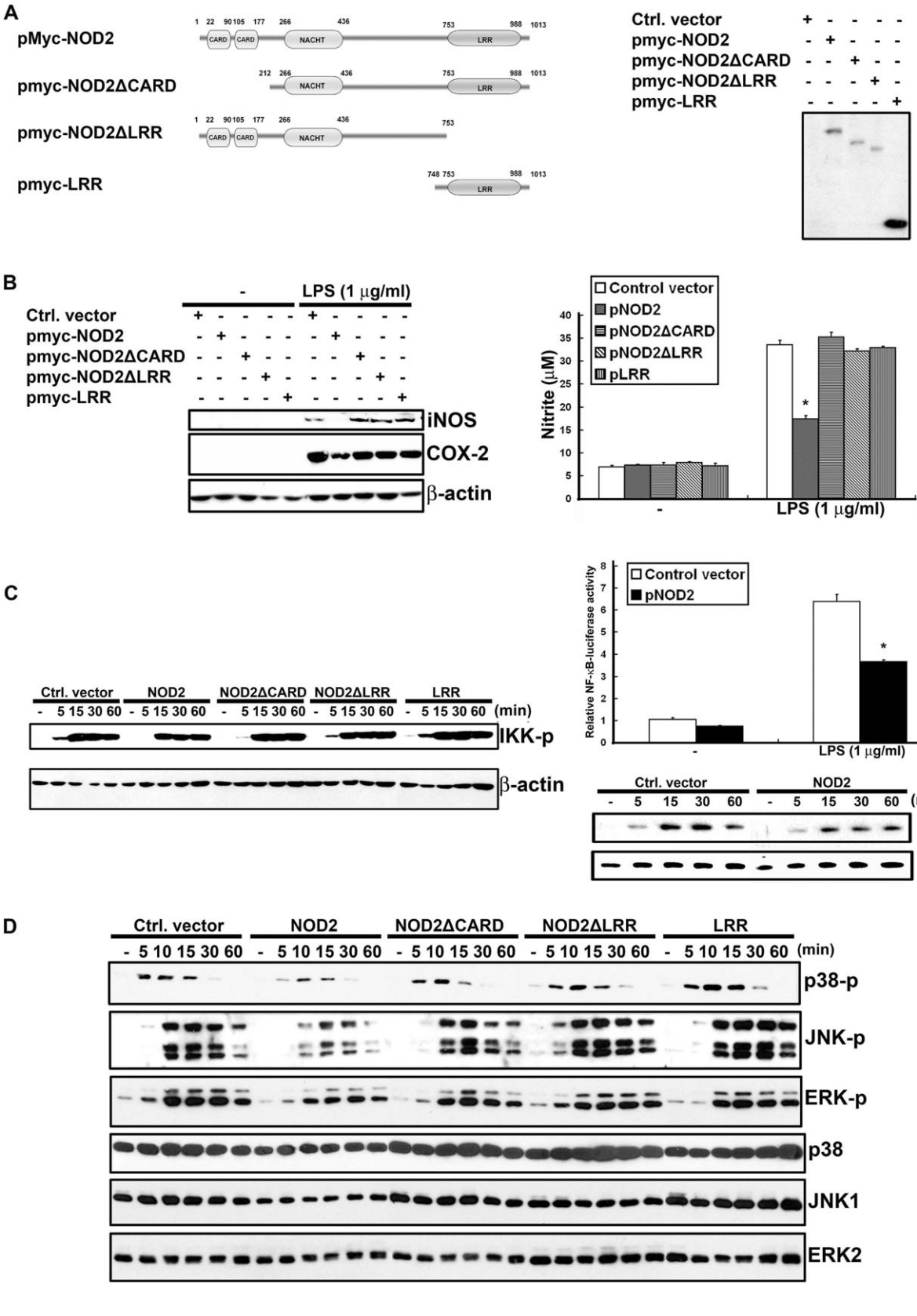
A
pMyc-NOD2
pmyc-NOD2ΔCARD
pmyc-NOD2ΔLRR
pmyc-LRR
CARD
NACHT
LRR
Ctrl. vector
pmyc-NOD2
pmyc-NOD2ΔCARD
pmyc-NOD2ΔLRR
pmyc-LRR
B
LPS (1 μg/ml)
iNOS
COX-2
β-actin
Control vector
pNOD2
pNOD2ΔCARD
pNOD2ΔLRR
pLRR
Nitrite (μM)
C
Ctrl. vector
NOD2
NOD2ΔCARD
NOD2ΔLRR
LRR
(min)
IKK-p
β-actin
Relative NF-κB-luciferase activity
p65
Rb
D
p38-p
JNK-p
ERK-p
p38
JNK1
ERK2

Fig. 6. The CARD and LRR domains of NOD2 are required for the uncoupling of TLR4-induced signalling cascades and inflammatory responses. RAW264.7 cells were transiently transfected with different Myc-tagged NOD2-deletion domains.
A. Cell lysates were analysed by immunoblotting for Myc-tagged proteins with a Myc antibody.
B. Cells were treated with LPS (1 μg ml^{-1}) for 24 h. Whole cell lysates were analysed by immunoblotting for iNOS and COX-2, and the supernatant was measured for nitrite production.
C. Cells were treated with LPS (1 μg ml^{-1}) for the indicated time periods. Cell lysates were subjected to SDS-PAGE to determine phospho-IKK (left panel). In some experiments, NF-κB luciferase activity was measured after 6 h of treatment with LPS (1 μg ml^{-1}), and p65 nuclear translocation was determined within 5–60 min (right panel).
D. Cells were treated with LPS (1 μg ml^{-1}) for the indicated time periods. Protein expression was determined by a Western blot analysis using specific antibodies. *$P < 0.05$, indicates significant inhibition of the LPS response in wild-type NOD2-expressing cells.

MDP-pretreated or co-treated conditions (Fig. 7B). These results indicate that MDP-activated NOD2 cannot induce homologous desensitization or heterologous desensitization to TLR4-mediated responses; instead a sensitizing effect as that seen in a co-treatment condition still existed.

To further understand if NOD2-silencing affected the process of TLR4-mediated response tolerance, we conducted LPS-induced homologous desensitization of NOD2-knock-down cells. Results in Fig. 7C showed that in NOD2 siRNA-treated cells, homologous desensitization induced by LPS became less apparent compared with the control cell group (lanes 10~12 vs. lanes 4~6). This result suggests that endogenous NOD2 might positively regulate LPS-induced desensitization via a ligand-binding-independent manner.

Discussion

It is believed that functional and regulatory integrations between PRRs determine the immune processes and outcomes of pathogen infection. After bacterial infection, macrophages are activated by multiple bacterial cell wall components, such as LPS derived from Gram-negative bacteria, and MDP, which is a hydrolysed by-product of proteoglycan produced after lysosomal digestion. Compared with long-term extensive studies and much knowledge gained on TLR4-mediated responses, the cellular functions and pathological roles of NOD2, especially its integrative interactions with other PRRs, are far from well understood. Therefore, in this study using murine RAW264.7 macrophages as a cell model, we explored the role of NOD2 itself in innate immunity and its coordination of TLR4-mediated responses.

Current findings of MDP-induced inflammatory responses and signals still remain controversial. Some studies working in bone marrow-derived macrophages (BMDM), peritoneal macrophages, THP-1 monocytes and mouse primary keratinocytes, showed the ability of MDP alone (≥ 10 μg ml^{-1}) to activate NF-κB, MAPKs, and induce inflammatory cytokines (Kobayashi *et al*., 2005; Uehara *et al*., 2005; Hsu *et al*., 2007, 2008; Kim *et al*., 2008b). However, some studies in BMDM cannot detect inflammatory response upon MDP treatment (Fritz *et al*., 2005; Park *et al*., 2007). In human CD14^{+} monocytes, there was also no significant production of inflammatory cytokines by MDP (100 nM) stimulation (Fritz *et al*., 2005). Our current study performed in murine RAW264.7 macrophages indicated that no matter lower (0.1 and 0.5 μM) or higher (200 μM) concentrations of MDP was used, MDP alone cannot trigger the upstream signalling pathways, such as IKK and MAPKs, nor induce iNOS or COX-2 expression. Thus we suggest the existence of a cell-type specific sensitivity to MDP, which might be associated with the constitutive expression levels of NOD2 and/or adaptor molecules required for signal propagation.

Despite the inability of MDP alone to trigger inflammatory signals and cytokine response in RAW264.7 macrophages, our data are consistent with previous findings regarding the potentiation effect of MDP on LPS-induced inflammatory response. In line with studies in BMDM (Kobayashi *et al*., 2005; Park *et al*., 2007; Kim *et al*., 2008a) and human monocytes (Yang *et al*., 2001; Fritz *et al*., 2005), we demonstrated that in RAW264.7 macrophages, MDP at concentrations as low as 0.1 μM indeed could increase LPS-induced nitrite, IL-6 and TNF-α production, iNOS and COX-2 protein expression, and mRNA levels of IL-1β, iNOS and TNF-α. Our data further suggest that LPS-induced NOD2 gene expression, rather than the direct enhancement of upstream signals by MDP, is necessary for this potentiation event in RAW264.7 macrophages. Regardless no effect on the rapid occurring IKK and MAPKs activation induced by LPS within 1 h, MDP significantly enhances NF-κB activation caused by LPS, as previously documented in BMDM (Maeda *et al*., 2005). In this study, evidence from two strategies supports the note that NOD2 upregulation accounted for the sensitization effects upon MDP treatment. First, we stimulated cells with MDP after LPS treatment for 3 and 6 h. As a result, the enhanced iNOS and COX-2 effects of MDP remained the same, implying that MDP-triggered molecular mechanisms participate in the late stage of TLR4-mediated events. Second, to prove the necessity of NOD2 rather than the nonspecific action of MDP, we knocked-down NOD2 by a siRNA approach. Our data indicated that MDP no longer increased LPS-induced iNOS expression in NOD2-knock-down cells. Because LPS can induce NF-κB-dependent NOD2 gene expression (Takahashi *et al*., 2006; Kim *et al*., 2008a) and MDP

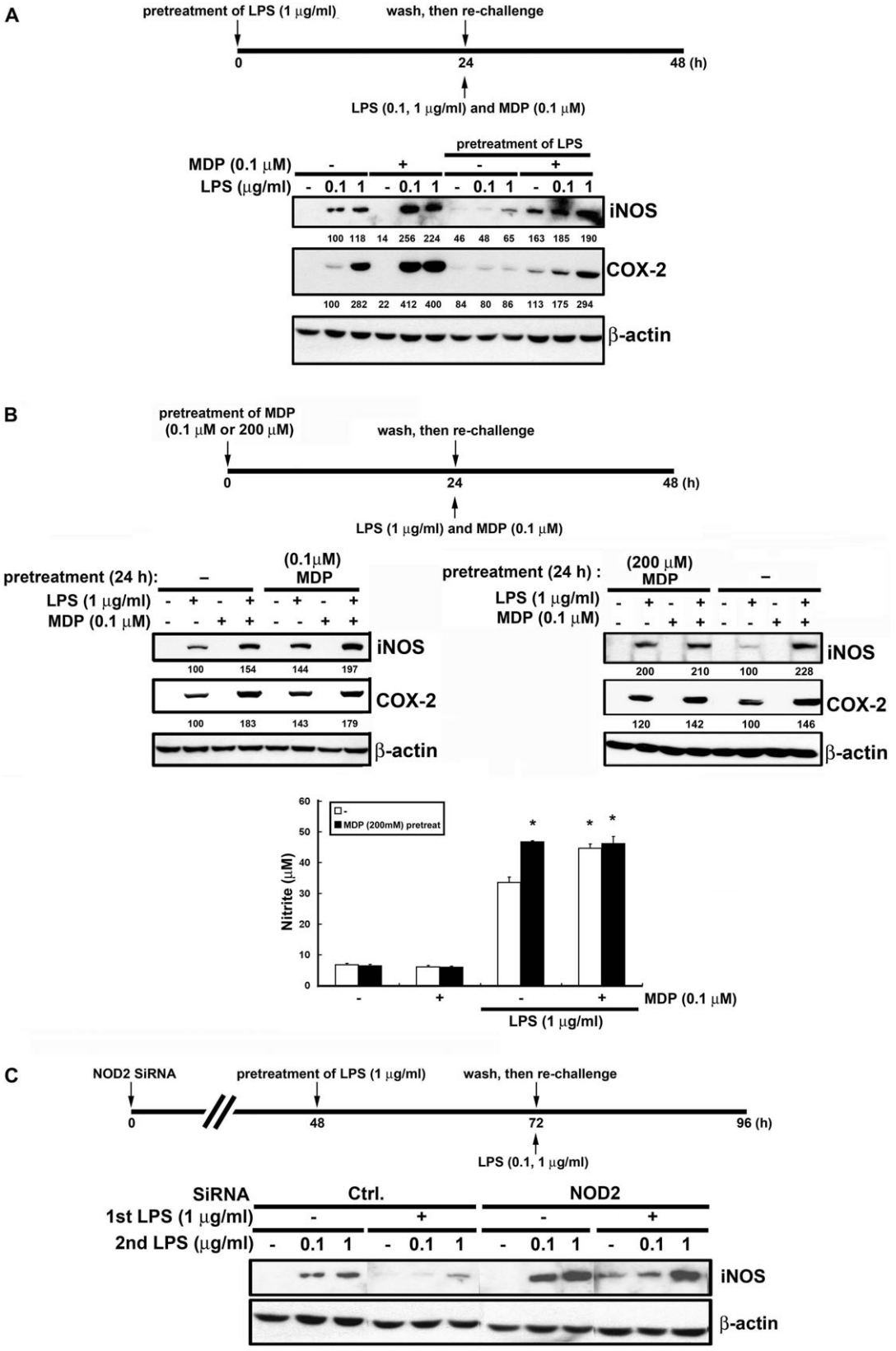
A
pretreatment of LPS (1 μg/ml)
wash, then re-challenge
0
24
48 (h)
LPS (0.1, 1 μg/ml) and MDP (0.1 μM)
pretreatment of LPS
MDP (0.1 μM) - + - +
LPS (μg/ml) - 0.1 1 - 0.1 1 - 0.1 1 - 0.1 1
iNOS
100 118 14 256 224 46 48 65 163 185 190
COX-2
100 282 22 412 400 84 80 86 113 175 294
β-actin
B
pretreatment of MDP
(0.1 μM or 200 μM)
wash, then re-challenge
0
24
48 (h)
LPS (1 μg/ml) and MDP (0.1 μM)
pretreatment (24 h): – (0.1μM) MDP
LPS (1 μg/ml) - + - + - + - +
MDP (0.1 μM) - - + + - - + +
iNOS
100 154 144 197
COX-2
100 183 143 179
β-actin
pretreatment (24 h) : (200 μM) MDP –
LPS (1 μg/ml) - + - + - + - +
MDP (0.1 μM) - - + + - - + +
iNOS
200 210 100 228
COX-2
120 142 100 146
β-actin
Nitrite (μM)
□-
■MDP (200mM) pretreat
MDP (0.1 μM)
LPS (1 μg/ml)
C
NOD2 SiRNA
pretreatment of LPS (1 μg/ml)
wash, then re-challenge
0
48
72
96 (h)
LPS (0.1, 1 μg/ml)
SiRNA Ctrl. NOD2
1st LPS (1 μg/ml) - + - +
2nd LPS (μg/ml) - 0.1 1 - 0.1 1 - 0.1 1 - 0.1 1
iNOS
β-actin

Fig. 7. Ligand-dependent and -independent effects of NOD2 on TLR4-mediated desensitization of the LPS response.
A. RAW264.7 macrophages were treated with LPS (1 μg ml^{-1}) for 24 h, and then cells were washed with PBS three times and further treated with LPS (0.1 and 1 μg ml^{-1}) and/or MDP (0.1 μM) for 24 h.
B. RAW264.7 macrophages were pretreated with MDP (0.1 and 200 μM) for 24 h, and then cells were washed with PBS three times and re-challenged with LPS (1 μg ml^{-1}) and/or MDP (0.1 μM) for 24 h.
C. NOD2 siRNA-treated cells were stimulated with LPS (1 μg ml^{-1}) for 24 h, washed, and re-challenged with LPS (0.1 and 1 μg ml^{-1}) for 24 h. Whole lysates were analysed by immunoblotting for iNOS and COX-2, and the supernatant was measured for nitrite production. $*P < 0.05$, indicates significant enhancement of the control LPS response by MDP.

is a stable ligand, co-treatment of MDP with LPS is able to enhance NF-κB activity at late phase upon more NOD2 is induced, as reflected in the 6 h luciferase activity. These results suggest that NOD2 upregulation is responsible for the MDP-induced potentiation of inflammatory responses caused by LPS.

When studying the homologous desensitization of TLR4 and NOD2, our results confirmed previous findings (Foster *et al*., 2007), indicating the existence of homologous desensitization (or self-tolerance) of LPS-induced iNOS and COX-2 expressions. In contrast, even though a high concentration of MDP (100 μg ml^{-1}) was reported to possess homologous desensitization in human monocyte-derived macrophages where MDP itself exerts a significant inflammatory response (Hedl *et al*., 2007), we did not detect such phenomenon in RAW264.7 macrophages. Intriguingly, when studying MDP's action in cells under TLR4 tolerance, we found that MDP alone became efficient at inducing iNOS and COX-2 expressions. These results suggest that NOD2 may be a critical and potent pathogen sensor when TLR4's function is desensitized. Alternatively, the desensitized innate immunity function of TLR4 can be substituted by NOD2. Again, we speculate that upregulation of NOD2 might be the key factor for this event as mentioned above. Moreover, MDP (0.1 μM) pretreatment for 24 h was still able to enhance the subsequent LPS response, suggesting the bioactive stability of MDP. Such a long-lasting enhancing effect on TLR4's response was also observed in MDP-pretreated BMDM (Kim *et al*., 2008a).

In this study, we unexpectedly found that LPS-induced inflammatory responses, including the expressions of iNOS, COX-2, IL-6, IL-1β, and MIP2 and NO production, were dramatically upregulated in NOD2-knock-down cells. These unexpected results imply that NOD2 might play a role in the negative regulation of LPS-induced inflammatory responses. This implication apparently contrasts to our mentioned point that NOD2 activation by MDP is a positive regulator of the LPS response. Evidence supporting NOD2 being a negative regulator comes from the increased IKK, MAPK, STAT1 and NF-κB activation in LPS-stimulated NOD2-deficient macrophages. Moreover, overexpressing wild-type NOD2 in macrophages without MDP stimulation led to diminished LPS responses in inducing iNOS, COX-2, NO, and the activation of IKK, MAPKs and NF-κB. Furthermore, this ligand-independent inhibitory action of NOD2 identified in RAW264.7 macrophages is also contrast to previous findings in NOD2-/- BMDM. No significant changes of LPS-induced inflammatory gene expression in NOD2-/- BMDM as compared with control cells have been shown (Hsu *et al*., 2008; Kim *et al*., 2008b). In this respect, we predict at least two possible reasons contributing to the discrepancies. First, the NOD2 protein level in the absence of NOD2 ligand might tune the effect caused by LPS. In our study, NOD2 level upon applying siRNA approach in RAW264.7 macrophages is attenuated, while in NOD2-/- BMDM it is completely knockdown. The molecular events behind this issue might involve the unidentified protein interaction to non-activated NOD2, while this situation does not exist in NOD2-/- BMDM. Second, the heterogeneity of macrophages might be another explanation, which has also been applied to the distinct sensitivity to MDP as discussed above.

According to our signalling data that many signalling cascades induced by LPS (NF-κB, IKK, MAPKs and STAT1) all increased in NOD2-knock-down cells, we speculated that constitutive or LPS-induced NOD2 might exert a negative effect to counteract or balance TLR4 signalling. The immune system needs to constantly strike a balance between activation and inhibition to avoid detrimental and inappropriate inflammatory responses. For this purpose, timely control of TLR4-mediated desensitization is an important issue to limit excessive inflammatory responses (Liew *et al*., 2005; Medvedev *et al*., 2007). For this aspect, we also propose that ligand-independent NOD2 might be involved in this event. In NOD2-knock-down cells, the degree of LPS-elicited iNOS tolerance was reduced, suggesting that NOD2 is involved in controlling LPS tolerance in a ligand-independent manner.

In studying which structural domain of NOD2 is required for negative regulation of the TLR4 response, we found that only wild-type NOD2 could achieve this action. Cells with NOD2 overexpression, but not with other NOD2-deletion domains, suppressed LPS-induced IKK and MAPK phosphorylation and NF-κB-binding activity. Thus we suggest that NOD2-induced negative regulation of LPS responses might require not only the CARD or LRR domain of NOD2, but the full structure of NOD2 may be essential. Currently we still don't know the mechanism responsible for the ligand-independent action of NOD2 in TLR4 signalling and tolerance. Previous study showed that NOD2 can interact with TAK1 (Chen *et al*., 2004; Kim

et al., 2008b), which is a crucial signalling molecule of TLR4 response. Thus, the possibility that ligand-independent NOD2/TAK1 interaction exerts the negative regulation on TLR4 needs to be investigated in the future. On the other hand, whether the downstream proteins of NOD2, RIP2 and MAVS, are involved in the negative effect requires further study.

In summary, our present study demonstrates dual roles played by NOD2 in the regulation of TLR4 activation and function. On the one hand, MDP-activated NOD2 provides a sustained mechanism to enhance TLR4-induced inflammatory responses, either upon co-activation of both receptors or upon a TLR4-desensitizing state. On the other hand, NOD2 can negatively regulate TLR4 signalling and increase TLR4 tolerance through an unidentified ligand-independent action.

Experimental procedures

Cell culture

RAW264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA, USA), and cultured in DMEM complete medium as we previously described (Lin *et al*., 2010).

Reagents

LPS, MDP and other chemicals were obtained from Sigma Aldrich (St Louis, MO, USA). Antibodies directed against p38, JNK1, ERK2, IκBα, COX-2, STAT1, p65, Rb, and horseradish peroxidase (HRP)-coupled anti-rabbit, anti-goat and anti-mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-iNOS polyclonal antibody was from BD Bioscience (Franklin Lakes, NJ, USA). Specific anti-phospho-p38, anti-phospho-JNK, anti-phospho-ERK, anti-phospho-IKK and anti-phospho-STAT1 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody against β-actin was from Upstate Biotechnology (Charlottesville, VA, USA). The protein concentration was determined by a Bio-Rad protein assay (Richmond, CA, USA). DMEM, trypsin-EDTA, lipofectamine 2000, and antibiotic-containing penicillin/streptomycin were from Invitrogen (Rockville, MD, USA). The RNA-Bee isolation reagent was purchased from Tel-Test (Friendswood, TX, USA). The enhanced chemiluminescence reagent (Western Lightening Chemiluminescence Reagent Plus) was from PerkinElmer (Wellesley, MA, USA). The FastStart SYBR Green Master and anti-HA antibody were from Roche Applied Science (Nutley, NJ, USA). The luciferase assay system kit and lysis buffer was purchased from Promega (Heidelberg, Germany). All the siRNAs were obtained from Dharmacon Research (Lafayette, CO, USA).

Immunoblotting analysis

After ligand treatment, cells were rinsed twice with cold PBS, and then lysed in lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% deoxycholate, 2 mM EDTA, 2 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and a protease inhibitor cocktail). After cell harvest and sonication, total cell lysates were centrifuged at 12 500 r.p.m. and 4°C for 10 min. The protein concentrations were determined using a Bio-Rad protein assay. Equal amounts of soluble protein were electrophoresed on SDS-PAGE, transferred to Immobilon-P, and probed with specific antibody as we previously described (Lin *et al*., 2010).

Nitrite and cytokine ELISA assays

After stimulation for 24 h, the accumulation of nitrite in the culture supernatant was determined by a colorimetric assay with the Griess reagent, as previously described (Lin *et al*., 2010). TNF-α and IL-6 in the culture supernatants were determined by R&D ELISA kit. Each experiment was performed in duplicate and repeated at least three times.

Plasmid construct and transient overexpression

The open reading frame of the mouse NOD2 gene was amplified by a one-step reverse-transcription (RT)-PCR with NOD2-specific primers: sense 5′-ATCGAAGAATTCAATGTGCTCA CAGGAAGAGTTCCA-3′ and antisense 5′-ATCGAACTC GAGTCACAACAAGAGTCTGGCG-3′. The amplified DNA fragment was digested with EcoRI and XhoI then ligated into the pcDNA3.1 vector. To generate Myc-tagged wild (Myc-NOD2) and deleted mutant (Myc-NOD2ΔCARD) expression vectors, the 1~1013 and 212~1013 amino-acid regions of NOD2 cDNA were subcloned into the EcoRI/XhoI and StuI/XhoI sites of the pCS2-MT plasmid respectively. pCS2-MT was kindly provided by Dr. Dave Turner of (University of Michigan, Ann Arbor, MI, USA). Moreover, to generate other deleted mutant (Myc-NOD2ΔLRR and Myc-LRR) expression vectors, the 1~753 and 748~1013 amino-acid regions of NOD2 cDNA were amplified by a PCR with specific primers: sense 5′-ATCGAAGAATTCAATGTGCTCA CAGGAAGAGTTCCA-3′ and antisense 5′-ATCGAACTCGA GTCAAGCACACTCTGCAG-3′ (the primer pair for Myc-NOD2ΔLRR construction); and sense 5′-ATCGAAGAATTCAGG CCCTGCAGAGTGTGCT-3′ and antisense 5′-ATCGAACTCGA GTCACAACAAGAGTCTGGCG-3′ (the primer pair for Myc-LRR construction). The amplified DNA fragments were digested with EcoRI and XhoI then ligated into the pCS2-MT vector.

For cell transfection, we used lipofectamine 2000 and followed a commercial standard protocol. Briefly, cells (5×10^5 cells/well) were transfected with 1 μg of plasmid for 6 h and then changed to complete medium. After 24 h of transfection, cells were treated with the indicated reagents, followed by collecting cell lysates for the Western blotting and other experiments.

NF-κB-dependent luciferase assay

The reporter gene containing NF-κB-binding sites (pGL2-ELMA-luciferase) and the β-galactosidase expression vector (pCR3lacZ) were prepared using endotoxin-free plasmid preparation kits. Cells were seeded on 12-well plates overnight before transfection with 0.25 μg of each plasmid using the lipofectamine 2000 reagent. After treatment with the indicated reagents, cells were lysed in reporter lysis buffer, and then the lysates were assayed with a luciferase assay system kit.

Table 1. Primers for the real-time PCR.

Gene	Forward primer	Reverse primer
iNOS	CAGCTGGGCTGTACAAACCTT	CATTGGAAGTGAAGCGTTTCG
COX-2	GAGAGAAGGAAATGGCTGCAGAA	GGCTTCCAGTATTGAGGAGAACAGA
TNF-α	ATGAGAAGTTCCCAAATGGCC	TCCACTTGGTGGTTTGCTACG
MIP2	GGCTAACTGACCTGGAAAGG	GCACATCAGGTACGATCCAG
IL-6	AACCCAAGGGCATTTCAATC	CACCGCATCTATCACCACAG
IL-1β	GCTTCAGGCAGGCAGTATCAC	CGACAGCACGAGGCTTTTT
mNOD2	CCCTGGCTGAAGTTGTAGC	GAGTTCCTCTAGTGACTTG
β-Actin	CGGGGACCTGACTGACTACC	AGGAAGGCTGGAAGAGTGC

Silencing of gene expression with siRNA

RAW264.7 cells (3×10^6) were transfected with 100 nM siRNA targeting mRNA degradation of mouse NOD2 (catalogue No. L-052735–00, Dharmacon) with the DharmaFECT 1 Transfection Reagent. The control non-targeting pooled siRNA is a pool of four functional non-targeting siRNAs with guanine cytosine contents comparable with that of the functional siRNA but lacking specificity for known gene targets. After siRNA transfection for 48 h, cells were treated with the TLR4 ligand and/or MDP, and then the gene silencing effects were evaluated by RT-PCR.

Real-time PCR

The expression of NOD2 mRNA and proinflammatory cytokine mRNA were determined by a real-time RT-PCR analysis. Extraction of total RNA, construction of cDNA, real-time PCR amplification using FastStart SYBR Green Master, and data analysis were conducted as previously described (Lin *et al*., 2010). Specific primers for real-time PCR analysis were synthesized using ProTaq DNA polymerase (Protech Technology Enterprise) (Table 1).

Statistical evaluation

Values are expressed as the mean ± SEM of at least three independent experiments, which were performed in duplicate. An analysis of variance (ANOVA) was used to assess the statistical significance of the differences, and a *P*-value (*) of < 0.05 was considered statistically significant.

Acknowledgements

We thank Dr Michael Karin for helpful comments. This work was supported by the cooperative research program of NTUCM and CMUCM (98F008-203) and the National Science Council (NSC98-3112-B-002-024 and NSC99-3112-B-002-016) of Taiwan.

References

Abbott, D.W., Yang, Y., Hutti, J.E., Madhavarapu, S., Kelliher, M.A., and Cantley, L.C. (2007) Coordinated regulation of Toll-like receptor and NOD2 signaling by K63-linked polyubiquitin chains. *Mol Cell Biol* **27:** 6012–6025.

Akira, S., and Takeda, K. (2004) Toll-like receptor signalling. *Nat Rev Immunol* **4:** 499–511.

Akira, S., Uematsu, S., and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124:** 783–801.

Chen, C.M., Gong, Y., Zhang, M., and Chen, J.J. (2004) Reciprocal cross-talk between Nod2 and TAK1 signaling pathways. *J Biol Chem* **279:** 25876–25882.

Eckmann, L., and Karin, M. (2005) NOD2 and Crohn's disease: loss or gain of function? *Immunity* **22:** 661–667.

Foster, S.L., Hargreaves, D.C., and Medzhitov, R. (2007) Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447:** 972–978.

Fritz, J.H., Girardin, S.E., Fitting, C., Werts, C., Mengin-Lecreulx, D., Caroff, M., *et al*. (2005) Synergistic stimulation of human monocytes and dendritic cells by Toll-like receptor 4 and NOD1- and NOD2-activating agonists. *Eur J Immunol* **35:** 2459–2470.

Fritz, J.H., Ferrero, R.L., Philpott, D.J., and Girardin, S.E. (2006) Nod-like proteins in immunity, inflammation and disease. *Nat Immunol* **7:** 1250–1257.

Gutierrez, O., Pipaon, C., Inohara, N., Fontalba, A., Ogura, Y., Prosper, F., *et al*. (2002) Induction of Nod2 in myelomonocytic and intestinal epithelial cells via nuclear factor-kappa B activation. *J Biol Chem* **277:** 41701–41705.

Hasegawa, M., Fujimoto, Y., Lucas, P.C., Nakano, H., Fukase, K., Nunez, G., and Inohara, N. (2008) A critical role of RICK/RIP2 polyubiquitination in Nod-induced NF-kappaB activation. *EMBO J* **27:** 373–383.

Hedl, M., Li, J., Cho, J.H., and Abraham, C. (2007) Chronic stimulation of Nod2 mediates tolerance to bacterial products. *Proc Natl Acad Sci USA* **104:** 19440–19445.

Hsu, L.C., Ali, S.R., McGillivray, S., Tseng, P.H., Mariathasan, S., Humke, E.W., *et al*. (2008) NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to *Bacillus anthracis* infection and muramyl dipeptide. *Proc Natl Acad Sci USA* **105:** 7803–7808.

Hsu, Y.M., Zhang, Y., You, Y., Wang, D., Li, H., Duramad, O., *et al*. (2007) The adaptor protein CARD9 is required for innate immune responses to intracellular pathogens. *Nat Immunol* **8:** 198–205.

Hugot, J.P., Chamaillard, M., Zouali, H., Lesage, S., Cezard, J.P., Belaiche, J., *et al*. (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature* **411:** 599–603.

Kanneganti, T.D., Lamkanfi, M., and Nunez, G. (2007) Intracellular NOD-like receptors in host defense and disease. *Immunity* **27:** 549–559.

Kawai, T., and Akira, S. (2006) TLR signaling. *Cell Death Differ* **13:** 816–825.

Kim, J.Y., Omori, E., Matsumoto, K., Núñez, G., and Ninomiya-Tsuji, J. (2008b) TAK1 is a central mediator of NOD2 signaling in epidermal cells. *J Biol Chem* **283:** 137–144.

Kim, Y.G., Park, J.H., Shaw, M.H., Franchi, L., Inohara, N., and Nunez, G. (2008a) The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands. *Immunity* **28:** 246–257.

King, K., Bagnall, R., Fisher, S.A., Sheikh, F., Cuthbert, A., Tan, S., *et al*. (2007) Identification, evolution, and association study of a novel promoter and first exon of the human NOD2 (CARD15) gene. *Genomics* **90:** 493–501.

Kobayashi, K.S., Chamaillard, M., Ogura, Y., Henegariu, O., Inohara, N., Nunez, G., and Flavell, R.A. (2005) Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* **307:** 731–734.

Liew, F.Y., Xu, D., Brint, E.K., and O'Neill, L.A. (2005) Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* **5:** 446–458.

Lin, Y.C., Huang, D.Y., Chu, C.L., and Lin, W.W. (2010) Anti-inflammatory actions of Syk inhibitors in macrophages involve non-specific inhibition of Toll like receptors-mediated JNK signaling pathway. *Mol Immunol* **47:** 1569–1578.

Maeda, S., Hsu, L.C., Liu, H., Bankston, L.A., Iimura, M., Kagnoff, M.F., *et al*. (2005) Nod2 mutation in Crohn's disease potentiates NF-kappaB activity and IL-1beta processing. *Science* **307:** 734–738.

Medvedev, A.E., Piao, W., Shoenfelt, J., Rhee, S.H., Chen, H., Basu, S., *et al*. (2007) Role of TLR4 tyrosine phosphorylation in signal transduction and endotoxin tolerance. *J Biol Chem* **282:** 16042–16053.

Netea, M.G., Ferwerda, G., de Jong, D.J., Jansen, T., Jacobs, L., Kramer, M., *et al*. (2005) Nucleotide-binding oligomerization domain-2 modulates specific TLR pathways for the induction of cytokine release. *J Immunol* **174:** 6518–6523.

Ogura, Y., Bonen, D.K., Inohara, N., Nicolae, D.L., Chen, F.F., Ramos, R., *et al*. (2001) A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease. *Nature* **411:** 603–606.

Park, J.H., Kim, Y.G., McDonald, C., Kanneganti, T.D., Hasegawa, M., Body-Malapel, M., *et al*. (2007) RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs. *J Immunol* **178:** 2380–2386.

Strober, W., Murray, P.J., Kitani, A., and Watanabe, T. (2006) Signalling pathways and molecular interactions of NOD1 and NOD2. *Nat Rev Immunol* **6:** 9–20.

Tada, H., Aiba, S., Shibata, K., Ohteki, T., and Takada, H. (2005) Synergistic effect of Nod1 and Nod2 agonists with toll-like receptor agonists on human dendritic cells to generate interleukin-12 and T helper type 1 cells. *Infect Immun* **73:** 7967–7976.

Takahashi, Y., Isuzugawa, K., Murase, Y., Imai, M., Yamamoto, S., Iizuka, M., *et al*. (2006) Up-regulation of NOD1 and NOD2 through TLR4 and TNF-alpha in LPS-treated murine macrophages. *J Vet Med Sci* **68:** 471–478.

Uehara, A., Yang, S., Fujimoto, Y., Fukase, K., Kusumoto, S., Shibata, K., *et al*. (2005) Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. *Cell Microbiol* **7:** 53–61.

Watanabe, T., Kitani, A., Murray, P.J., and Strober, W. (2004) NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. *Nat Immunol* **5:** 800–808.

Yang, S., Tamai, R., Akashi, S., Takeuchi, O., Akira, S., Sugawara, S., and Takada, H. (2001) Synergistic effect of muramyldipeptide with lipopolysaccharide or lipoteichoic acid to induce inflammatory cytokines in human monocytic cells in culture. *Infect Immun* **69:** 2045–2053.