European Journal of Immunology

# Engagement of NOD2 has a dual effect on proIL-1β mRNA transcription and secretion of bioactive IL-1β

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Synthesis and release of pro-inflammatory cytokines, such as IL-1β, play a crucial role in the intestinal inflammation that characterizes Crohn's disease. Mutations in the nucleotide oligomerization domain 2 (NOD2) gene are associated with an increased risk of Crohn's disease. Although it is known that NOD2 mediates cytokine responses to muramyl dipeptide (MDP), it is yet unclear whether NOD2 stimulation mediates only transcription of pro-IL-1β mRNA, or whether NOD2 is also involved in the activation of caspase-1 and release of active IL-1β. By investigating the response of MNC from Crohn's disease patients homozygous for the 3020insC NOD2 mutation, we were able to show that NOD2 signaling after stimulation with MDP has a dual effect by activating proIL-1β mRNA transcription and inducing release of bioactive IL-1β. Because NOD2 engagement amplifies TLR stimulation, we investigated whether activation of caspase-1 by MDP is involved in the NOD2/TLR synergism. The synergy in IL-1β production between NOD2 and TLR is mediated at post-translational level in a caspase-1-dependent manner, which indirectly suggests that NOD2 also induces caspase-1 activation. In contrast, the synergy in TNF-α production after stimulation with MDP and LPS is induced at transcriptional level. This demonstrates that both caspase-1-dependent and -independent mechanisms are involved in the synergy between NOD2 and TLR.

Received 21/1/07
Revised 1/10/07
Accepted 9/11/07

[DOI 10.1002/eji.200737103]

**Key words:** Cytokines · Gene expression · Immune regulation · Monocytes · TLR

## Introduction

NOD-like receptors (NLR) are intracellular receptors for bacterial peptidoglycans, which complement the recognition of pathogen-associated molecular patterns (PAMP) by membrane-bound TLR [1, 2]. Nucleotide oligomerization domain 2 (NOD2) is a member of the NACHT-LRR (NLR) receptor family, which recognizes muramyl dipeptide (MDP), the minimal motif of peptidoglycan of both Gram-positive and Gram-negative bacteria [3]. Mutations in the NOD2 gene are associated with Crohn's disease [4, 5], but how NOD2 exactly acts

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**Abbreviations:** **CARD:** caspase-recruitment domain · **fs:** frameshift · **ICE-i:** caspase-1 inhibitor · **MDP:** muramyl dipeptide · **MNC:** mononuclear cells · **NLR:** NOD-like receptors · **NOD2:** nucleotide oligomerization domain 2 · **PAMP:** pathogen-associated molecular patterns · **YVAD:** Ac-Tyr-Val-Ala-Asp-2,6-dimethylbezoyloxymethylketone

in the pathogenesis of this auto-inflammatory disease is unclear [6–8]. Therefore, a better understanding of the intracellular events induced by the interaction between NOD2 and peptidoglycan is crucial for both the insight into recognition of Gram-positive pathogens by the innate immune system, and for the pathogenesis of the inflammatory reactions in Crohn's disease.

Activation of human mononuclear cells (MNC) by MDP leads to production of pro-inflammatory cytokines, especially IL-1β [7, 9]. IL-1β is produced as pro-IL-1β a 31–34-kDa inactive form of the cytokine, which is later cleaved by caspase-1 to the bioactive 17-kDa IL-1β [10]. This is followed by IL-1β excretion in microvesicles into the extracellular environment [11]. Apparently, MDP is capable of inducing all three steps, but it is unclear whether NOD2 alone or other receptors are involved in one or more of these steps of IL-1β production. It has been proposed that several of the NLR family members are able to recognize MDP, most notably NOD2, NALP3, and NALP1, and that they execute different functions necessary for cytokine production. In this concept, recognition of MDP by NOD2 would mainly activate NF-κB and thereby gene transcription of pro-IL-1β, whereas NALP3/NALP1-mediated recognition of MDP leads to caspase-1 activation and the subsequent release of the active IL-1β form [12–14]. However, there are controversies regarding this mechanism of IL-1β production by MDP. The caspase-recruitment domain (CARD) of NOD2 interacts with the serine/tyrosine kinase RIP2, leading to NF-κB translocation and transcription of mRNA for pro-inflammatory cytokines [15–17]. However, it is also known that RIP2 interacts with the CARD domain of caspase-1 [18, 19]. Thus, one could envisage that NOD2 engagement by MDP could activate caspase-1 and lead to the release of mature IL-1β, without the need for a secondary interaction with NALP3/NALP1.

In the present study, we aimed to assess whether recognition of MDP by NOD2 is important for both the induction of pro-inflammatory cytokines gene transcription, as well as for the activation of caspase-1 and IL-1β release, by comparing the response of MNC from patients with Crohn's disease homozygous for the 3020insC NOD2 mutation (NOD2fs) with the response of MNC isolated from individuals with wild-type NOD2 allele (NOD2wt). In addition, because MDP is able to amplify the TLR stimulation through a NOD2-dependent pathway [9, 20], we also investigated whether activation of caspase-1 by MDP is involved in the NOD2/TLR synergism.

## Results

### MDP induces NOD2-dependent transcription of cytokine genes

In MNC of Crohn's disease patients with a homozygous 3020insC mutation (NOD2fs), the cytokine production after stimulation with MDP is completely abolished, whereas the cytokine production after stimulation with the TLR4 ligand LPS is normal compared to healthy volunteers (HC) and Crohn's disease patients bearing the wild-type allele (NOD2wt) (Fig. 1A). This impaired cytokine response to MDP is caused by a transcriptional defect, since MDP was found to increase mRNA in MNC of healthy volunteers and Crohn's disease patients without NOD2 mutations of IL-1β and TNF-α, but not in patients homozygous for the 3020insC mutation (Fig. 1B).

### Induction of IL-1β by MDP is caspase-1 dependent

Various bacterial stimuli can induce mature IL-1β production, which requires both transcription of pro-IL-1β mRNA and post-translational processing by

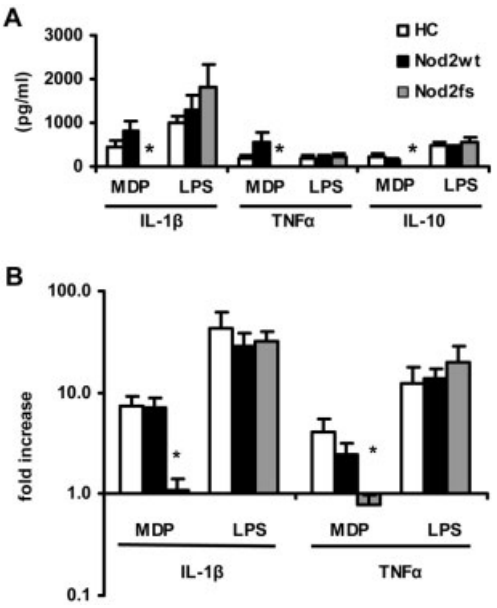


**Figure 1.** MDP induces NOD2 dependent cytokine production and transcription of pro-IL-1β and TNF-α in MNC. MNC of five healthy controls (HC), five Crohn's patients without NOD2 mutations (NOD2wt) and four patients homozygous for the 3020insC mutation (NOD2fs) were stimulated with LPS (10 ng/mL) or MDP (100 nM). Cytokines were measured by ELISA in the supernatant after 24-h incubation at 37°C (A). Quantitative measurement of mRNA levels of IL-1β and TNF-α was performed by real-time PCR and expressed as fold increase compared to unstimulated cells. Cells were lysed in RNAzolB after 4-h incubation at 37°C. (B). Data are presented as means ± SEM and compared by Mann-Whitney U test (*$p < 0.05$).

 

caspase-1. The IL-1β production in MNC after stimulation with MDP was blocked with the caspase-1 inhibitor (ICE-i) Ac-Tyr-Val-Ala-Asp-2,6-dimethylbezoyloxymethylketone (YVAD) (Fig. 2A). This indicates that the post-translational processing of pro-IL-1β by caspase-1 is important for the IL-1β production by MDP. Inhibition of the IL-1R with IL-1Ra did not block the IL-1β production (Fig. 2A). Thus, an autocrine feedback of IL-1β inducing IL-1β, as shown previously using LPS as a stimulus [21], does not seem to play a major role in MDP-induced IL-1β.

During the exposure to MDP, caspase-1 mRNA levels did not increase compared to unstimulated cells, as shown in Fig. 2B. Since the caspase-1 activity is not regulated at transcriptional level, the regulation of caspase-1 activation is likely post-translational by enzymatic cleavage of pro-caspase-1 [22].

## The synergism between NOD2 and TLR for IL-1β is caspase-1 dependent

Stimulation of cells with MDP and the TLR4 ligand LPS induces cytokines in a synergistic fashion [9, 20]. Because MDP can activate caspase-1 and induce mature IL-1β, we investigated whether caspase-1 is important for this synergy. The induction of IL-1β mRNA in MNC of HC, NOD2wt and NOD2fs does not increase after stimulation with MDP and LPS compared to LPS alone (Fig. 3A). In addition, there is no synergy in the production of proIL-1β production, as assessed both by a specific pro-IL-1β ELISA (Fig. 3B) or Western blots (Fig. 3C). However, mature IL-1β is secreted in a synergistic way (Fig. 3E). The increased ratio between intracellular proIL-1β and secreted IL-1β indicates that the synergistic production of IL-1β after stimulation with MDP and LPS is established either by extra cleaving of proIL-1β or/and by increased release of bioactive IL-1β (Fig. 3D).

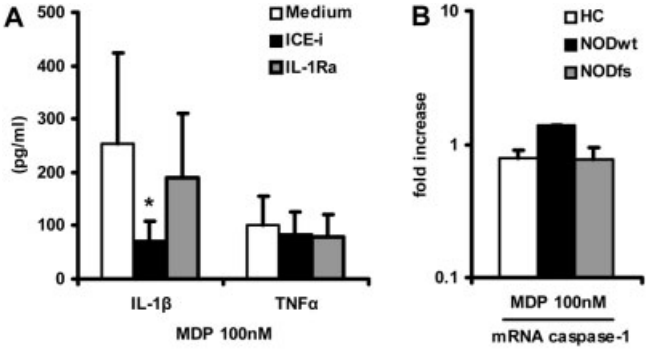


**Figure 2.** MDP activates caspase-1 but does not enhance transcription of caspase-1 mRNA. Stimulation of MNC of five healthy volunteers with MDP (100 nM) during inhibition of caspase-1 with ICE-i (20 μM) or the IL-1-receptor with IL-1Ra (10 μg/mL). Cytokines were measured by ELISA in the supernatant after 24-h incubation at 37°C (A). MNC of four healthy controls (HC), four Crohn's patients without NOD2 mutations (NOD2wt) and four patients homozygous for the 3020insC mutation (NOD2fs) were stimulated with MDP (100 nM). After 4-h incubation at 37°C, cells were lysed in RNAzolB. Quantitative measurement of mRNA levels of caspase-1 was performed by real-time PCR and expressed as fold increase compared to unstimulated cells (B).

In support of the notion that processing of pro-IL-1β is the level at which the synergism between NOD2 and TLR takes place, when caspase-1 was inhibited with YVAD (ICE-i) the synergy declined, showing that caspase-1 is responsible for the synergy of IL-1β production after stimulation with MDP and LPS (Fig. 3F). In contrast to IL-1β, the induction of TNF-α mRNA in MNC of HC and NOD2wt increases after stimulation with MDP and LPS compared to LPS alone in the same ratio as TNF-α in the supernatant. In MNC of NOD2fs no increase of TNF-α mRNA and TNF-α is found (Fig. 3A). Inhibition of caspase-1 does not influence the TNF-α production or synergy (Fig. 3F). Blocking the IL-1R with IL-1Ra has no effect on the synergistic production of IL-1β and TNF-α, indicating that autocrine stimulation of IL-1R cannot explain the synergy (Fig. 3F). These data show that the synergy in TNF-α production after stimulation with MDP and LPS is induced at transcriptional level, whereas the synergy in IL-1β production is regulated at post-translational level by activation of caspase-1.

## Activation of caspase-1 and release of bioactive IL-1β by MDP is NOD2 dependent

To test whether MDP uses another receptor than NOD2 for the activation of caspase-1 (*e.g.* NALP3), and in the absence of a reliable caspase-1 p10 Western-blot methodology in human primary cells, we designed an indirect functional assay to evaluate this hypothesis. If MDP would activate capsase-1 through NALP3 or NALP1, one would expect that although MDP is unable by itself to induce IL-1β in cells from NOD2fs patients, due to its inability to activate transcription, it would still be able to amplify IL-1β production induced by a TLR ligand (Fig. 4). This would happen because of the intracellular proIL-1β induction by the TLR agonist, while processing of IL-1β at the level of caspase-1 activation would be amplified by the MDP-NALP3/NALP1 interaction (Fig. 4A). However, the data presented in Fig. 4B strongly argue against this hypothesis. These data show clearly that MDP was unable to amplify IL-1β production when cells of Crohn's disease patients with NOD2fs were stimulated with LPS, suggesting that NOD2 is necessary for the caspase-1 activation by MDP.

In order to investigate the role of NOD2 for the release of active IL-1β from the cells, we have tested the effects of the NOD2-deficiency in an ATP/LPS stimula-

tion assay. When ATP was added to LPS-primed cells, a significant increase in the IL-1β secreted from the cells bearing only the wild-type NOD2 allele was observed. In contrast, the ATP-dependent IL-1β release was severely impaired in the cells isolated from individuals homozygous for the NOD2fs mutation (Fig. 4C). IL-1β production after LPS stimulation alone was not different in NOD2wt and NOD2fs individuals (Fig. 4C).

## Discussion

In this study, we show that NOD2 has dual effects on both proIL-1β mRNA transcription and the release of bioactive IL-1β from the cells. In addition, indirect functional tests on the role of caspase-1 for the synergistic effects between NOD2 and TLR also suggest a role for NOD2 for the activation of caspase-1 by MDP, the active component of bacterial peptidoglycan.

The regulation and activation of caspase-1 and thereby the regulation of the production of mature IL-1β, followed by its secretion from the cells, is a rapid evolving field of research. The importance of understanding these crucial steps in IL-1β processing and release is stressed by the role of IL-1β in virtual all forms of inflammation and especially in auto-inflammatory diseases, such as familial Mediterranean fever (FMF), hyperimmunoglobulinemia D syndrome (HIDS) and

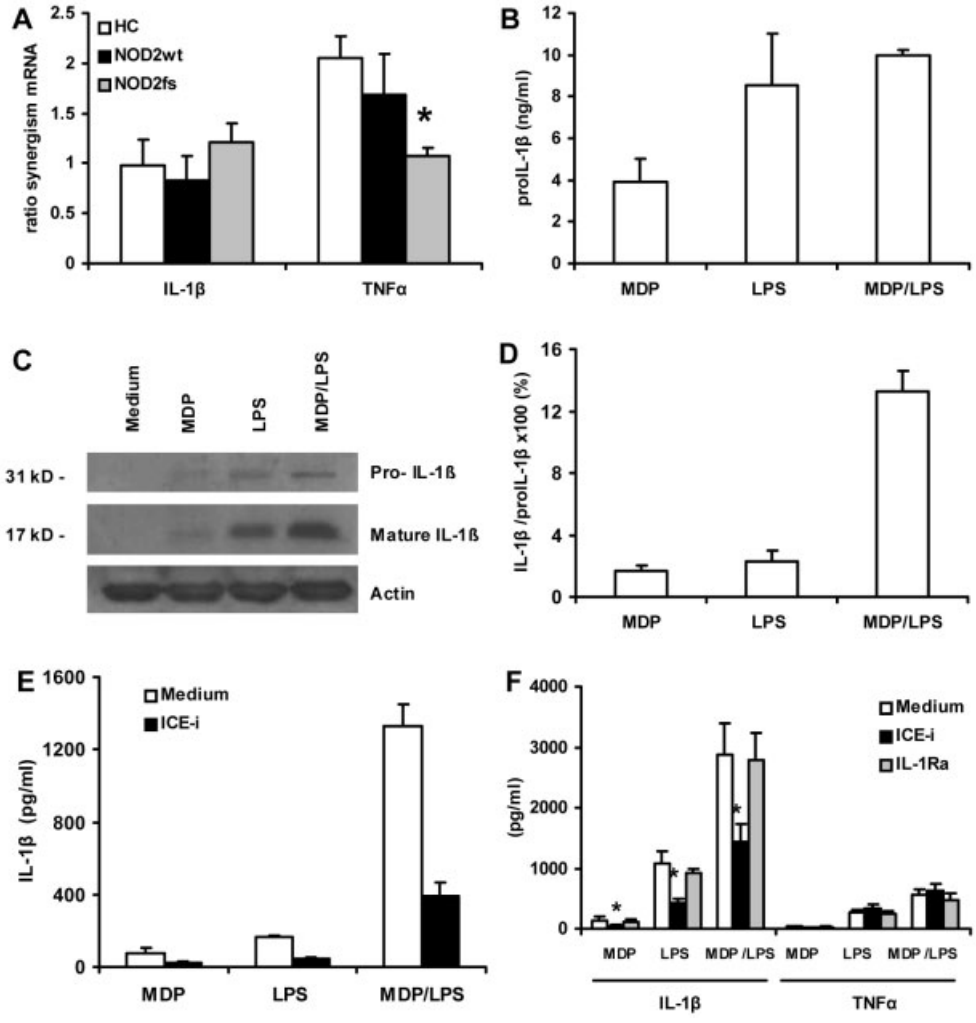


**Figure 3.** The synergism between TLR and NOD2 for the release of IL-1β is exerted at post-transcriptional level. MNC from four healthy volunteers were stimulated with MDP (100 nM), LPS (10 ng/mL) or a combination of MDP and LPS. After 4-h incubation at 37°C, cells were lysed in RNAzolB. Quantitative measurement of mRNA levels of IL-1β and TNF-α was performed by real-time PCR and expressed as fold increase compared to LPS-treated cells (A). The proIL-1β production after 4-h stimulation was assessed by a specific ELISA (B) or Western blots (C). Similarly, IL-1β concentrations were assessed by an ELISA (E). The percentages of mature IL-1 secreted from the cells when they were stimulated with MDP, LPS or the combination MDP/LPS are presented in (D). Stimulation of MNC of nine healthy volunteers with LPS (10 ng/mL) in combination with MDP (100 nM) during inhibition of caspase-1 with ICE-i (20 μM) or the IL-1R with IL-1Ra (10 μg/mL). Cytokines were measured by ELISA in the supernatant after 24-h incubation at 37°C (F). Data presented as means ± SEM and compared by Mann-Whitney U test (*$p < 0.05$).

 

Muckle-Wells syndrome [22]. Targeting IL-1β in these disorders has shown spectacular results [23–27], and targeting caspase-1 could lead to new treatment strategies. Some reports indicate that caspase-1 activity is partially regulated at transcriptional level [28]. However, our results show that stimulation with MDP did not result in an increase expression of caspase-1 mRNA. This is in line with the current opinion that caspase-1 activity is mainly regulated at post-translational level [29, 30]. In this view, caspase-1 is activated by close proximity mechanism in a multimeric protein platform, called the inflammasome. It is unclear which pathway directly activates the inflammasome and how many different of these platforms exists. The most studied inflammasomes consist of NALP3/ASC/cardinal/caspase-1 and NALP1/ASC/caspase-1/caspase-5.

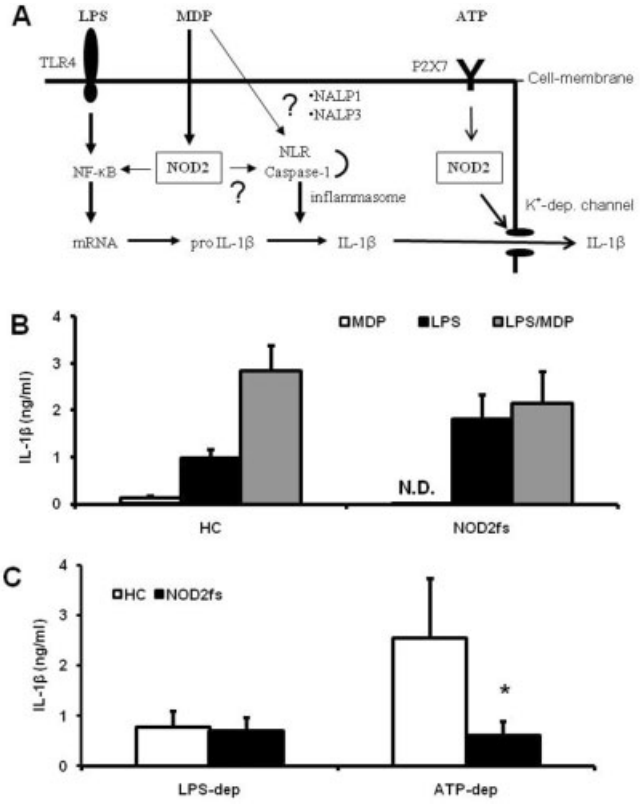


**Figure 4.** Activation of caspase-1 by MDP is NOD2 dependent. Putative scheme of activation of the inflammasome by MDP, in which the MDP/NOD2 interaction activates transcription, processing and release of IL-1β. A potential interaction of NOD2 with NALP3/NALP1 for the activation of the inflammasome is still unclear (A). Stimulation of MNC of four healthy controls (HC) and 4 patients homozygous for the 3020insC mutation (NOD2fs) with MDP (100 nM), LPS (10 ng/mL) or a combination of MDP and LPS. Cells were incubated for 24 h at 37°C and IL-1β was measured in the supernatant by ELISA (B). The role of NOD2 for the release of IL-1β by MNC primed for 4 h with LPS (1 μg/mL), and after an additional incubation with ATP (1 mM) for 15 min (C). Data are presented as means ± SEM and compared by Mann-Whitney U test (*$p < 0.05$).

NALP belong to the same protein family as NOD and CIITA, which is named the NLR (NACHT-LRR receptors or NOD-like receptors) family. All NLR have a NACHT domain that is involved in forming multimers. In addition, some members have a CARD used for interaction with other CARD-containing proteins, such as caspases. Finally, the LRR domain can interact with PAMP (*e.g.* MDP interaction with LRR of NOD2). An interesting hypothesis is that the interaction of a PAMP with the LRR of an NLR in the inflammasome can activate caspase-1. Indeed, Martinon *et al.* [12] proposed that MDP interacts with the LRR of NALP3 in cell lines and can activate the inflammasome in human macrophages. Similarly, a recent study has suggested that NALP-1 is also an MDP receptor activating caspase-1 [14].

In human MNC, we confirm the activation of the inflammasome by MDP, indicated by the release of mature IL-1β after stimulation with MDP. Importantly, indirect proof based on a NOD2/TLR stimulation assay suggests that activation of the caspase-1 seems to occur in a NOD2-dependent manner. If MDP would activate caspase-1 independently of NOD2, stimulation of cells lacking a functional NOD2, such as NOD2fs MNC, with LPS and MDP should result in a amplification of LPS-induced IL-1β production through MDP-NALP3/NALP1-dependent mechanisms. In NOD2-deficient cells, LPS induces pro-IL-1β and activation of caspase-1, and MDP should amplify caspase-1 activation and IL-1β release in a NOD2-independent, NALP3- or NALP1-dependent manner. However, the data presented here clearly show that this is not the case: no increase in IL-1β production was documented when NOD2fs cells are stimulated with LPS and MDP at the same time. This suggests that NOD2 has a non-redundant function for caspase-1 activation by MDP (Fig. 4A). However, it cannot be excluded that NOD2 also requires the presence of NALP3 and/or NALP1 and collaborate with one or both of these molecules for the activation of caspase-1. A recent study by Pan and colleagues [31] showing that both NOD2 and NALP3 are necessary for IL-1β secretion by MDP in murine macrophages gives weight to this hypothesis. Unfortunately, the lack of a reliable Western blot assay for the activated caspase-1 p10 in human primary monocytes has precluded us to obtain direct evidence of the role of NOD2 for the caspase-1 activation. However, by using a well-established model of IL-1β release after LPS priming of cells, followed by stimulation with the $K^+$-channel activator ATP, we were able to demonstrate an important role for NOD2 in the release of active IL-1β. This is in line with a recent study showing that NALP3, another member of the NLR family, binds ATP [32].

Some of the differences between our findings and the study of Martinon *et al.* [12] in terms of IL-1β induction by purified LPS could be explained by the use of

different cell types: freshly MNC produce mature IL-1β after stimulation with LPS, whereas monocytes-derived macrophages do not. Furthermore, it has to be realized that data from cell lines and overexpression models are not always compatible with data obtained from freshly isolated human cells. In addition, differences exist between NALP3$^{-/-}$ mice and human cells [12, 33, 34], and between NOD2$^{-/-}$ mice and cells obtained from Crohn's disease patients homozygous for the NOD2fs allele [7, 35].

The unique role of NOD2 as key receptor of MDP for the induction of NF-κB-dependent transcription of mRNA of cytokines is underlined by showing that mRNA of IL-1β and TNF-α in NOD2fs cells is not increased after stimulation with MDP. Subsequently, the production of the cytokines is abolished. Our quantitative data by real-time PCR, obtained in primary cells, are supported by the results obtained by other groups [36–38].

When MNC are stimulated with low dose MDP and the TLR4 ligand LPS, a remarkable synergy in cytokine production is apparent in MNC from individuals with a intact NOD2 [9, 20]. A potential role for caspase-1 in the synergy between TLR4 and NOD2 could be envisaged, since MDP can activate caspase-1 and induce IL-1β, which is known to induce IL-1β production in an autocrine fashion through stimulation of the IL-1R [21]. In addition, IL-1β can induce the production of other cytokines, like IL-10 and IL-6 [39]. Indeed, the synergistic production of IL-1β by MDP and LPS is caspase-1dependent, and not regulated at transcriptional level. However, in line with other studies, we show that the mechanism of synergy for TNF-α is exerted at a transcriptional level [40].

In conclusion, we demonstrate that NOD2 is non-redundant for both the production of IL-1β mRNA and the IL-1β release from the activated cells. In addition, indirect data supports a role for NOD2 in the activation of caspase-1 in human MNC after stimulation with MDP. Furthermore, caspase-1-dependent mechanisms are responsible for the synergistic effect on IL-1β production between NOD2 and TLR. Currently, it is unclear whether NOD2 is part of an inflammasome protein complex or it directly activates caspase-1. Further investigation is needed to unravel the complete extension of the role of NOD2, but the picture emerges that NOD2 is the key receptor for MDP and executes its function at different levels in the pathways of cytokine production.

## Materials and methods

### Reagents

Synthetic Pam3Cys was purchased from EMC Microcollections (Tubingen, Germany). LPS (*Escherichia coli* serotype 055:B5) was purchased from Sigma (St. Louis, MO) and an extra purification step was performed as previously described [41]. The purified LPS was tested in TLR4$^{-/-}$ mice for the presence of contaminants and it did not have any TLR4-independent activity. Synthetic MDP was obtained from Sigma. The reversible caspase-1 inhibitor (ICE-i) Ac-Tyr-Val-Ala-Asp-2,6-dimethylbezoyloxymethylketone (YVAD) was purchased from Alexis Biochemicals (San Diego, CA) and solubilized in DMSO at 10 mg/mL. The ICE-i was diluted to the desired concentration in RPMI.

### Genotyping of *NOD2* variants

Blood was collected from 154 patients with Crohn's disease and 10 healthy volunteers. PCR amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50-μL reaction volumes containing 100–200 ng genomic DNA as previously described [2]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 Genetic Analyzer according to the protocol of the manufacturer (Applied Biosystems).

Seven patients with Crohn's disease were found homozygous for the 3020insC mutation, and four of them were further investigated in the cytokine studies. As control groups, four patients with Crohn's disease and four healthy volunteers homozygous for the wild-type *NOD2* allele were included.

### Isolation of MNC and stimulation of cytokine production

After obtaining informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10 mL EDTA tubes (Monoject). Isolation of MNC was performed as described elsewhere [3], with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). Cells were washed twice in saline and suspended in culture medium (RPMI 1640) supplemented with gentamicin 10 μg/mL, L-glutamine 10 mM and pyruvate 10 mM. The cells were counted in a Coulter counter (Coulter Electronics) and the number was adjusted to $5 \times 10^6$ cells/mL. MNC ($5 \times 10^5$) in a 100-μL volume were added to round-bottom 96-wells plates (Greiner) and incubated with either 100 μL of culture medium (negative control), or the various stimuli: LPS (1 ng/mL), MDP (10 nM), or combinations of MDP and LPS at various concentrations. In separate experiments, inhibitors (ICE-i, 20 μM or IL-1Ra, 10 μg/mL) were added 10 min before stimulation. The stimuli were checked for the contamination with LPS in the LAL assay and found to be negative. After 24 h, the supernatants were collected and stored at –70°C until assayed.

To investigate the role of NOD2 for the release of IL-1β, PBMC were initially stimulated for 4 h with LPS (1 μg/mL). After 4 h, supernatants were collected and medium containing

 

1 mM ATP was added to the cells for another 15 min. The LPS-dependent IL-1β production during the first 4 h and the ATP-dependent IL-1β secretion after the additional 15 min was assessed in the supernatant.

### Cytokine measurements

Human TNF-α concentrations were determined by specific ELISA [42]. IL-1β, proIL-1β and IL-10 were measured by commercial ELISA kits (R&D Systems, and Pelikine Compact, Sanquin), according to the instructions of the manufacturer.

### Quantitative PCR

MNC were stimulated as described above, after 4 h the supernatant was removed and the cells resuspended in 200 μL RNAzolB RNA isolation solvent (Campro Scientific) and frozen at –80°C for storage. mRNA was isolated following the manufacturer's protocol. The amount and quality of mRNA were determent by spectrophotometry and analyzed by agarose gel electrophoresis for DNA contamination. cDNA was synthesized from 1000 ng of total RNA using Super-Script$^{tm}$ Reverse Transcriptase (Invitrogen; 18064–014).

Relative mRNA levels were determined using the Bio-Rad iCycler and SYBR Green method (Invitrogen; S7563) [43]. The following primers were used: IL-1β forward (5′-TGGCCCAGG-CGTCAGA-3′), IL-1β reverse (5′-GGTTTGCTACAACATGGGCT-ACA-3′); TNF-α forward (5′-GCCCTAAACAGATGAAGTG-CTC-3′), TNF-α reverse (5′-GAACCAGCATCTTCCTCAG-3′); B2M forward (5′-ATGAGTATGCCTGCCGTGTG-3′), B2M reverse (5′-CCAAATGCGGCATCTTCAAAC-3′) (Biolegio, Malden, The Netherlands). Mean relative mRNA expression from at least two replicate measurements was calculated using Bio-Rad iCycler IQ software. Values are expressed as fold increase to mRNA levels of unstimulated cells.

### Western blots for pro-IL-1β and IL-1β

Human MNC were stimulated as described above. After 4-h stimulation, supernatant was removed and cells were lysed in 25 μL of ice-cold lysis buffer containing 150 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 0.1% SDS and protease inhibitors (Sigma*Fast*, Sigma). Lysates of $4 \times 10^6$ cells were pooled. Samples were 'taken up' in 25 μL sample buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 10% β-mercaptoethanol, 10% glycerol and 0.5 mg/mL of bromophenol blue), were separated by SDS-PAGE and were blotted onto polyvinyldifluoride membranes (Millipore). Membranes were blocked in 0.1% Tween-20 in Tris-buffered saline containing 5% nonfat dry milk and were incubated overnight with antibody against cleaved IL-1β (Cell Signaling) in 1% BSA and 0.1% Tween-20 in Tris-buffered saline. Horseradish peroxidase-conjungated secondary antibodies were visualized with Lumilite plus (Boehringer-Mannheim).

### Statistical analysis

All experiments were performed at least in duplicate with blood obtained from four Crohn's disease patients bearing the NOD2fs mutation, four Crohn's disease patients with the wild-type NOD2 genotype, as well as four healthy volunteers. Synergy was expressed as ratio of cytokine response of ligand in combination with MDP divided by the sum of cytokine responses obtained with each ligand alone. The differences in cytokine production between groups were analyzed by Mann-Whitney U test or Wilcoxon, and where appropriate by Kruskal-Wallis ANOVA test. For all other comparisons the Student's *t*-test was used. The level of significance between groups was set at $p < 0.05$. The data are given as means ± SEM.

**Acknowledgements:** M.G.N. was supported by a VDI grant from The Netherlands Organization for Scientific research.

**Conflict of interest**: The authors declare no financial or commercial conflict of interest.

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