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# *Mycobacterium paratuberculosis* is recognized by Toll-like receptors and NOD2

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**Abstract:** *Mycobacterium paratuberculosis* has been suggested to be involved in the pathogenesis of Crohn's disease (CD). The importance of microorganisms in CD is supported by the association of CD with mutations in the intracellular pathogen recognition receptor (PRR) nucleotide-binding oligomerization domain 2 (NOD2). The aim of this study is to investigate the PRR involved in the recognition of *M. paratuberculosis*. Methods used include in vitro stimulation of transfected cell lines, murine macrophages, and human PBMC. *M. paratuberculosis* stimulated human TLR2 (hTLR2)-Chinese hamster ovary (CHO) cells predominantly and hTLR4-CHO cells modestly. Macrophages from TLR2 and TLR4 knockout mice produced less cytokines compared with controls after stimulation with *M. paratuberculosis*. TLR4 inhibition in human PBMC reduced cytokine production only after stimulation with live *M. paratuberculosis*. TLR-induced TNF- $\alpha$ , IL-1 $\beta$ , and IL-10 production is mediated through MyD88, whereas Toll-IL-1R domain-containing adaptor inducing IFN- $\beta$  (TRIF) promoted the release of IL-1 $\beta$ . hNOD2-human embryo kidney (HEK) cells, but not hNOD1-HEK cells, responded to stimulation with *M. paratuberculosis*. PBMC of individuals homozygous for the 3020insC NOD2 mutation showed a 70% defective cytokine response after stimulation with *M. paratuberculosis*. These results demonstrate that TLR2, TLR4, and NOD2 are involved in the recognition of *M. paratuberculosis* by the innate immune system. *J. Leukoc. Biol.* 82: 1011–1018; 2007.

**Key Words:** innate immunity · cytokines · monocytes · human · Crohn's disease

## INTRODUCTION

Mycobacteria are an important group of pathological microorganisms causing disease in humans. Almost 2 billion people

are infected with *Mycobacterium tuberculosis* worldwide, and more than 2 million people die each year as a result of tuberculosis [1]. Other mycobacterial species, such as *Mycobacterium leprae*, are endemic in developing countries and are responsible for high morbidity and invalidate many people [2]. In developed countries, the prevalence of infections with mycobacteria is increasing, especially in patients with a compromised immune system. Under these circumstances, nonpathogenic mycobacteria may cause disease, such as *Mycobacterium avium* complex (MAC) in HIV-infected patients.

*Mycobacterium paratuberculosis* is a member of the MAC, which has been suggested to be associated with Crohn's disease (CD), a chronic granulomatous inflammation of the gut. This association is controversial, but some findings support a causative role of *M. paratuberculosis* in the pathogenesis of CD [3]. In cows, *M. paratuberculosis* causes Johne's disease, which is very similar to CD in humans [4]. Although *M. paratuberculosis* is not a known pathogen for humans, it can be cultured from intestinal biopsies of CD patients and even from the blood [5, 6]. Furthermore, there is some evidence that treatment of CD with antibiotics has a beneficial effect on the disease activity [7, 8].

It is thought that the innate immune response contributes to the difference in susceptibility to mycobacteria. The innate immune system constitutes the first line of defense against pathogens such as mycobacteria. The recognition of pathogen-associated molecular patterns by pathogen recognition receptors (PRR) is crucial for the initiation and coordination of this response. The importance of these receptors is emphasized further by the current concepts that the adaptive immune response is partly under control of the innate immune system [9, 10]. The extracellular TLR [11, 12] and C-type lectins [13, 14] are membrane-bound PRR, which are complemented by the intracellular nucleotide-binding oligomerization domain-like receptor (NLR) family [15, 16]. In animal models, it is well

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established that deficiencies of most of these receptors lead to an impaired immune response against pathogens and a higher susceptibility to infection [17].

Mutations in the LRR region of the nucleotide-binding oligomerization domain 2 (NOD2) gene, a member of the NLR family, are strongly associated with CD [18, 19]. NOD2 is expressed in leukocytes and epithelial cells of the gastrointestinal tract [20], and this intracellular receptor can recognize peptidoglycans (PGNs) of Gram-positive and Gram-negative bacteria [21]. Stimulation of NOD2 can induce production of cytokines [22], chemokines [23], and defensins [24, 25]. All these features suggest a key role for NOD2 in the innate immune response of the gastrointestinal tract. Not only does the intracellular PRR NOD2 seem to be important, but also, recognition of the commensal microflora and pathogens by extracellular TLRs in the gut is essential for the maintenance of the intestinal homeostasis and involved in the development of colitis [26]. Mutations in other genes of innate immunity have also been reported to be associated with susceptibility to CD [27, 28], which is considered to have a multifactorial pathogenesis, and there is consensus that the interaction of the innate immune system with the luminal bacterial flora is essential for developing the chronic inflammation of the intestinal mucosa [29]. However, it is still not clear whether pathogens, such as *M. paratuberculosis*, may cause or perpetuate inflammation in CD.

Despite the possible role of *M. paratuberculosis* in CD, there is not much known about the interaction of *M. paratuberculosis* and the innate immune system. A recent study has reported association of live *M. paratuberculosis* in blood cultures and biopsies with CD [6], and it has also been reported that CD patients with mutations in the NOD2 gene are infected more frequently with *M. paratuberculosis* than CD patients without these mutations [30]. Recently, we have shown that TLRs and NOD2 are nonredundant PRR of *M. tuberculosis* [31]. In the present study, we further explore the receptors involved in the recognition of the intracellular pathogen *M. paratuberculosis*. By using animal and human in vitro models, we were able to show that TLR2, TLR4, and NOD2 are involved in the recognition of *M. paratuberculosis*.

## MATERIALS AND METHODS

### Reagents and microorganisms

Synthetic tripalmitoyl-S-glycero-Cys-(Lys)4 (Pam3Cys) was purchased from EMC Microcollections (Tübingen, Germany). Muramyl dipeptide (MDP) and LPS (*Escherichia coli* serotype 055:B5) were purchased from Calbiochem (San Diego, CA, USA) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. The synthetic MDP was tested for contamination with lipoproteins or LPS in mice deficient in TLR2 or TLR4 (TLR2<sup>-/-</sup> or TLR4<sup>-/-</sup>, respectively), demonstrating the absence of contamination. Diaminopimelic acid-containing muramyl tripeptide (M-TriDAP) was obtained as described previously [32]. As a specific TLR4 antagonist, we used LPS derived from the cell membrane of the Gram-negative bacterium *Bartonella quintana*, which has been demonstrated to be a highly specific TLR4 antagonist [33].

Cultures of *M. tuberculosis* H37Rv and *M. paratuberculosis* were grown to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco Laboratories, Detroit, MI, USA), washed three times in sterile saline, and resuspended in RPMI-1640 medium at the

various concentrations. Separate culture suspensions were washed three times in sterile saline; subsequently, the bacteria were heat-killed and disrupted in a bead-beater (Biospec Products, Bartlesville, OK, USA) using 0.1 mm glass beads.

### Genotyping of NOD2 variants

Blood was collected from 154 patients with CD and 10 healthy volunteers. PCR amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50  $\mu$ l reaction volumes containing 100–200 ng genomic DNA as described previously [34]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 genetic analyzer according to the protocol of the manufacturer (Applied Biosystems, Nieuwerkerk, The Netherlands).

Seven patients with CD were found homozygous for the 3020insC frameshift mutation, and four of them were investigated further in the cytokine studies. As control groups, five patients with CD bearing the wild-type alleles and five healthy volunteers homozygous for the wild-type *NOD2* allele were included.

### Isolation of PBMC and stimulation of cytokine production

After informed consent, venous blood was drawn from the cubital vein of patients and healthy volunteers into three 10 ml EDTA tubes (Monoject, s-Hertogenbosch, The Netherlands). Isolation of mononuclear cells (MNC) was performed as described elsewhere [30] 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 AB, Uppsala, Sweden). Cells were washed twice in saline and suspended in culture medium RPMI 1640, supplemented with gentamicin 10  $\mu$ g/ml, L-glutamine 10 mM, and pyruvate 10 mM. The cells were counted in a Coulter counter (Coulter Electronics, Mijdrecht, The Netherlands), and the number was adjusted to  $5 \times 10^6$  cells/ml.

MNC ( $5 \times 10^5$ ) in a 100- $\mu$ l vol were added to round-bottom, 96-well plates (Greiner, Alphen a/d Rijn, The Netherlands) and incubated with 100  $\mu$ l culture medium (negative control) or the various stimuli: *M. tuberculosis* sonicate (10  $\mu$ g/ml), *M. paratuberculosis* sonicate (10  $\mu$ g/ml), MDP (100 nM), LPS (10 ng/ml), or TLR4 antagonist (10  $\mu$ g/ml).

### Cytokine production by murine peritoneal macrophages

Resident peritoneal macrophages from TLR4<sup>-/-</sup>, TLR2<sup>-/-</sup>, MyD88<sup>-/-</sup>, or control C57Bl/6N mice (kindly provided by Dr. Shizuo Akira, Research Institute for Microbiological Diseases, Osaka University, Osaka, Japan) [35] and LPS2 [Toll-IL-1R domain-containing adaptor-inducing IFN- $\beta$  (TRIF)-defective] mice (kindly provided by Dr. Bruce Beutler, The Scripps Institute, La Jolla, CA, USA) [36] were harvested by injection of 4 ml sterile PBS containing 0.38% sodium citrate [37]. After centrifugation and washing, the cells were resuspended in RPMI 1640 containing 1 mM pyruvate, 2 mM L-glutamine, 100  $\mu$ g/ml gentamicin, and 2% fresh mouse plasma. Cells were cultured in 96-well microtiter plates (Greiner, Alphen a/d Rijn) at  $1 \times 10^5$  cells/well in a volume of 100  $\mu$ l. The cells were stimulated with purified LPS (1  $\mu$ g/ml), Pam3Cys (1  $\mu$ g/ml), sonicate of *M. tuberculosis* (10  $\mu$ g/ml), or sonicate of *M. paratuberculosis* (10  $\mu$ g/ml). After 24 h incubation at 37°C, the supernatants were collected and stored at -70°C until cytokine assays were performed.

### Cytokine measurements

Murine TNF- $\alpha$  and IL-1 $\beta$  concentrations were determined by specific radioimmunoassays as described [33, 34]. Murine IL-10 and human IL-10 and IL-1 $\beta$  were measured by commercial ELISA kits (Pelikine Compact, Sanquin, Amsterdam, The Netherlands), according to the instructions of the manufacturer. Human TNF- $\alpha$  concentrations were determined by specific ELISA [38].

### Signaling through human TLR2 and TLR4 in transfected cell lines

Chinese hamster ovary (CHO) fibroblasts stably transfected with human CD14 (3E10-CD14), a combination of CD14 and TLR2 (3E10-TLR2) or TLR4

(3E10-TLR4), were a kind gift from Dr. Robin Ingalls (Boston Medical Center, Boston University School of Medicine, Boston, MA, USA). Cell lines express inducible membrane CD25 under control of a region from the human E-selectin (endothelial leukocyte-adhesion molecule 1) promoter containing NF- $\kappa$ B-binding sites. Cells were maintained at 37°C and 5% CO<sub>2</sub> in HAM's F12 medium (Gibco, Invitrogen, Breda, the Netherlands), supplemented with 10% FCS, 0.01% L-glutamine, 50  $\mu$ g/mL gentamicin and 400 U/mL hygromycin, and 0.5 mg/mL G418 (for 3E10-TLR2) or 0.05 mg/mL puromycin (for 3E10-TLR4) as additional selection antibiotics. TLR2 and TLR4 expression was confirmed by flow cytometry (Coulter Epics XL-MCL, Beckman Coulter, Mijdrecht, the Netherlands) using PE-labeled anti-TLR2 (Clone TL2.1) or anti-TLR4 (Clone HTA125; Immunosource, Halle-Zoersel, Belgium).

For stimulation experiments, 500  $\mu$ l cells in culture medium at a density of  $1 \times 10^5$ /mL were plated in 24-well culture plates. After an overnight incubation, cells were incubated with control medium, *M. tuberculosis* sonicate (10  $\mu$ g/ml), *M. paratuberculosis* sonicate (10  $\mu$ g/ml), Pam3Cys (10  $\mu$ g/ml), or LPS (1  $\mu$ g/ml) for 20 h, and thereafter, cells were harvested using trypsin/EDTA (Cambrex, East Rutherford, NY, USA) and prepared for flow cytometry (Coulter FACSscan). CD25 expression of the CHO cells was measured using FITC-labeled anti-CD25 (Dako, Glostrup, Denmark) and expressed as a percentage of CD25-positive cells.

## Stimulation of NOD cell lines and NF- $\kappa$ B translocation

Studies examining the activation of NF- $\kappa$ B by *M. paratuberculosis* sonicates in cells overexpressing NOD1 or NOD2 were carried out as described previously [19]. Briefly, human embryo kidney (HEK)293T cells were transfected overnight with 1 ng NOD1 or NOD2 plus 75 ng luciferase reporter plasmid. At the same time, sonicated, heat-killed *M. paratuberculosis* preparations were added to cell culture medium, and the NF- $\kappa$ B-dependent luciferase activation was then measured following 24 h of incubation. NF- $\kappa$ B-dependent luciferase assays were performed in duplicate, and data represent three independent experiments and expressed as a folds-over-mean increase.

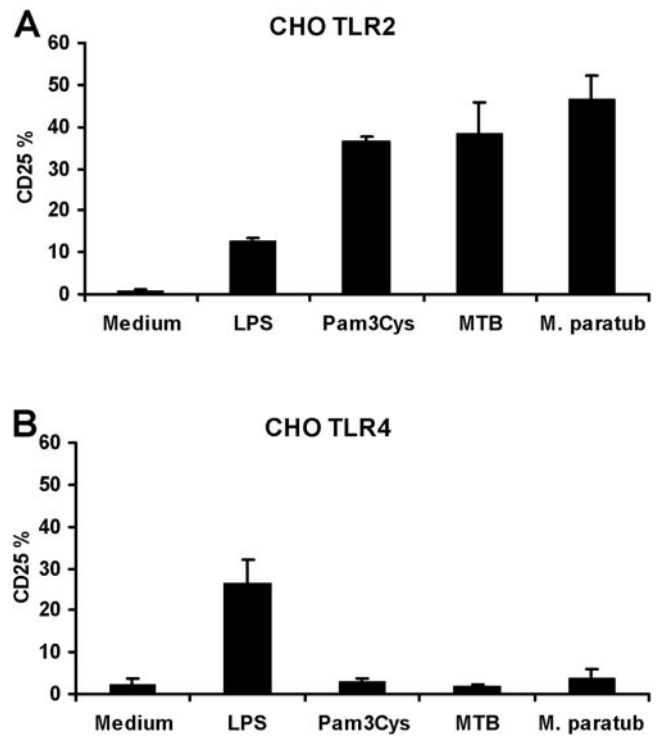
## Statistical analysis

The human experiments were performed in triplicate with blood obtained from patients and volunteers. The mouse experiments were performed twice in 10 mice per group, and the data are presented as cumulative results of all experiments performed. The differences between groups were analyzed by Mann-Whitney U test or Wilcoxon and where appropriate, by Kruskal-Wallis ANOVA test. The level of significance between groups was set at  $P < 0.05$ . The data are given as means  $\pm$  SEM.

## RESULTS

### *M. paratuberculosis* recognition by TLR2 and TLR4

To investigate the recognition of *M. paratuberculosis* by TLR2 and TLR4, both known to recognize *M. tuberculosis*, CHO cells transfected with human TLRs were stimulated with sonicated *M. paratuberculosis*. Whereas stimulation of CHO-TLR2 with *M. paratuberculosis* resulted in a robust expression of the reporter molecule CD25 ( $47 \pm 6\%$ ) as a measure of NF- $\kappa$ B activation (Fig. 1A), almost no expression of CD25 was seen after stimulation of CHO-TLR4 ( $8 \pm 3\%$ ; Fig. 1B). To verify these results in a more complex system, TLR knockout mice were used. Indeed, peritoneal macrophages of TLR2 $^{-/-}$  mice produced significantly less cytokines compared with control mice after stimulation with *M. paratuberculosis* (Fig. 2). After *M. paratuberculosis* stimulation, TLR4 $^{-/-}$  murine macrophages also produced significantly less cytokines than controls (TNF- $\alpha$ , Fig. 3A; IL-6, IL-10, and IL-1 $\beta$  also reduced, not

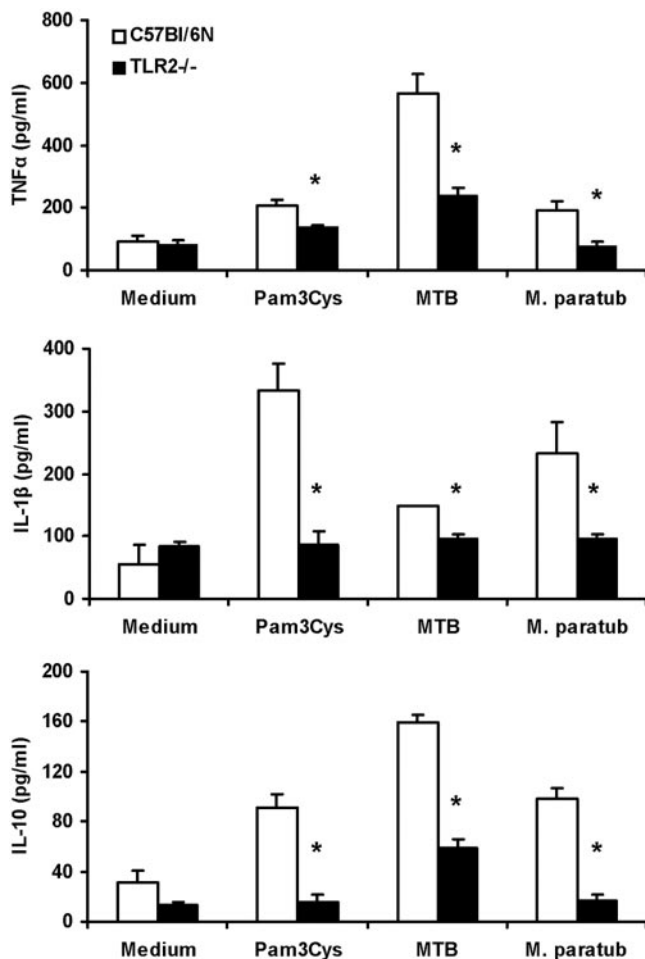


**Fig. 1.** *M. paratuberculosis* stimulates TLR2-transfected CHO cells predominantly and TLR4-transfected CHO cells modestly, and CHO cells cotransfected with TLR2 and CD14 induced potent expression of CD25 on the cell membrane as a measure for NF- $\kappa$ B activation, after stimulation with a sonicated *M. tuberculosis* (MTB) or *M. paratuberculosis* (10  $\mu$ g/ml) measured by FACS analysis (A). In contrast, cells transfected with CD14 and TLR4 were hardly activated by *M. tuberculosis* or *M. paratuberculosis* (B). LPS (1  $\mu$ g/ml) and Pam3Cys (5  $\mu$ g/ml) served as control stimuli for TLR4 and TLR2, respectively. Experiments were performed twice.

shown), although the difference was less pronounced, as seen in the macrophages of TLR2 $^{-/-}$  mice. To further investigate the role of TLR4 in the recognition of *M. paratuberculosis*, human PBMC were stimulated with sonicated *M. paratuberculosis* in the presence and absence of anti-TLR4. Under these conditions, the TNF- $\alpha$  production after stimulation with LPS was inhibited by over 50%, whereas no inhibitory effect on TNF was seen when cells were stimulated with *M. paratuberculosis* (Fig. 3B). In addition, IL-1 $\beta$  production induced by *M. paratuberculosis* was not influenced by TLR4 blockade (1255 pg/ml stimulation by *M. paratuberculosis* alone vs. 1860 pg/ml by *M. paratuberculosis* in the presence of the TLR4 antagonist). As heat-killing and sonication might influence the ability of *M. paratuberculosis* to stimulate TLR4, we next stimulated human PBMC with live *M. paratuberculosis*. Under these conditions, the cytokine production was decreased significantly in the presence of anti-TLR4 (Fig. 4). Thus, *M. paratuberculosis* is recognized by TLR2 and TLR4.

As TLR2 and TLR4 are involved in the recognition of *M. paratuberculosis*, MyD88 and TRIF knockout mice were used to decipher which adaptor molecule is important for the cytokine signal pathway. Stimulation of peritoneal macrophages of MyD88 $^{-/-}$  mice with *M. paratuberculosis* resulted in significant, reduced cytokine production compared with control





**Fig. 2.** TLR2 mediates recognition of *M. paratuberculosis*. Peritoneal macrophages of TLR2<sup>-/-</sup> mice were stimulated with Pam3Cys (10 μg/ml), sonicated *M. tuberculosis* (10 μg/ml), or sonicated *M. paratuberculosis* (10 μg/ml). Cytokines TNF-α, IL-1β, and IL-10 were measured in the supernatant by ELISA or radioimmunoassay (RIA) after 24 h incubation at 37°C. Groups of five mice/group were stimulated. Data presented as mean ± SEM and were compared by Mann-Whitney U test (\*, *P* < 0.05).

(Fig. 5A). Peritoneal macrophages of TRIF<sup>-/-</sup> mice stimulated with *M. paratuberculosis* produced significantly less IL-1β compared with control cells, whereas there was no difference in production of other cytokines (Fig. 5B). Thus, TLR signaling after stimulation with *M. paratuberculosis* is mediated through MyD88 and TRIF, although the MyD88-dependent pathway seems to be more important.

### *M. paratuberculosis* recognition of NOD1 and NOD2

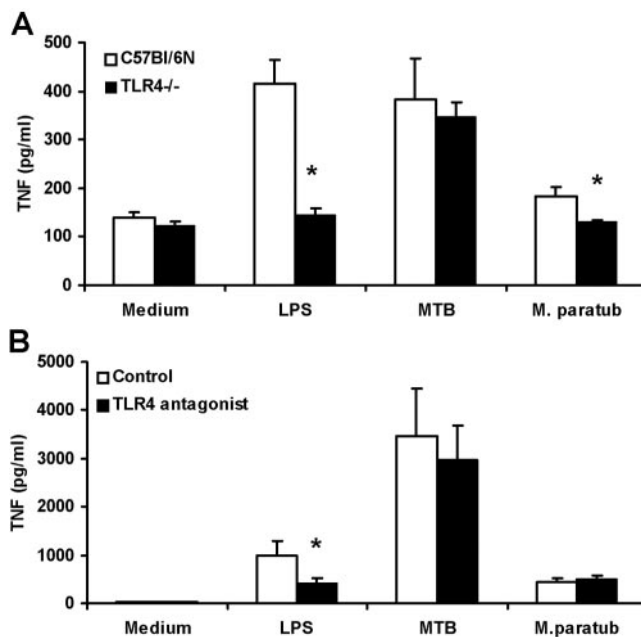
To investigate the role of NOD1 and NOD2 in the recognition of *M. paratuberculosis*, HEK293 cells transfected with human NOD1 or NOD2 and a NF-κB-driven, luciferase-reporter system were stimulated with sonicated *M. paratuberculosis*. NOD2-transfected cells responded in a dose-dependent NF-κB activation, expressed as fold-increase luciferase activity, whereas NOD1-transfected cells responded marginally to high concentrations of *M. paratuberculosis* (Fig. 6). To explore the

role of NOD2 recognition of *M. paratuberculosis* in human cells, MNC of CD patients homozygous for the 3020insC frame-shift mutation (NOD2fs), known to be nonfunctional, were stimulated with sonicated *M. paratuberculosis* or a control TLR2 ligand (the lipopeptide Pam3Cys). When stimulated with Pam3Cys, TNF production was similar in cells isolated from healthy volunteers (202 ± 55 pg/ml), CD patients with wild-type NOD2 (213 ± 35 pg/ml), and CD patients homozygous for the 3020insC mutation (175 ± 53 pg/ml). After stimulation with *M. paratuberculosis*, cytokine production of NOD2fs cells was less than 30% of that by wild-type NOD2 allele (NOD2wt) and controls (Fig. 7). Thus, NOD2 is involved in the *M. paratuberculosis*-induced cytokine production by human primary PBMC.

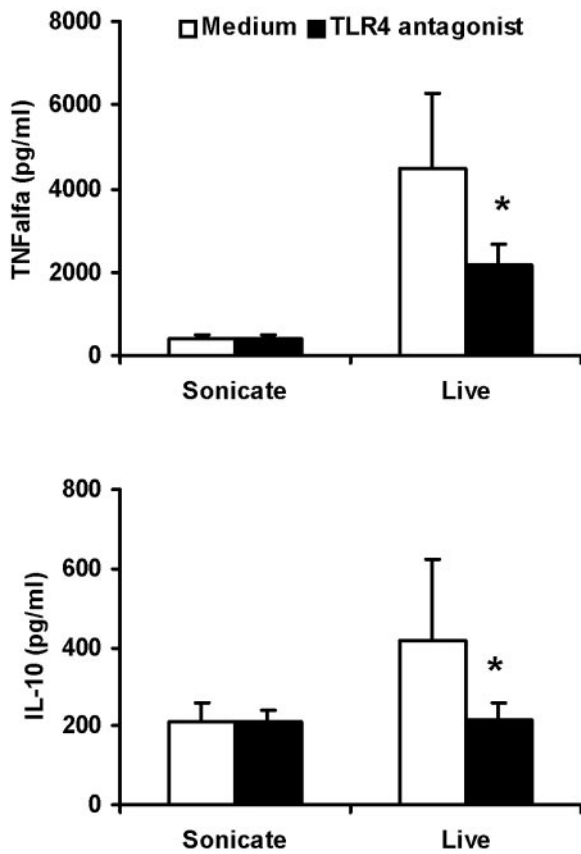
## DISCUSSION

In this study, we demonstrate that TLR2, TLR4, and NOD2 are PRR for *M. paratuberculosis*, which are responsible for mediating cytokine production and stimulation of host defense.

In the first part of the study, we show that murine and human TLR2 recognize sonicated *M. paratuberculosis*. The role of TLR2 for *M. tuberculosis* recognition was already established in different experimental models [31, 39], and it is known that



**Fig. 3.** TLR4 involvement in *M. paratuberculosis* induced cytokine production. Peritoneal macrophages of TLR4<sup>-/-</sup> mice were stimulated with LPS (1 μg/ml), sonicated *M. tuberculosis* (10 μg/ml), or sonicated *M. paratuberculosis* (10 μg/ml). TNF-α was measured in the supernatant by RIA after 24 h incubation at 37°C. Groups of five mice/group were stimulated (A). Human MNC of five healthy volunteers were stimulated with LPS (10 ng/ml), MTB (10 μg/ml), or sonicated *M. paratuberculosis* (10 μg/ml) in the presence or absence of TLR4 antagonist. TNF-α was measured in the supernatant by ELISA after 24 h incubation at 37°C (B). Data presented as mean ± SEM and were compared by Mann-Whitney U test (mice) and Wilcoxon paired test (human; \*, *P* < 0.05).



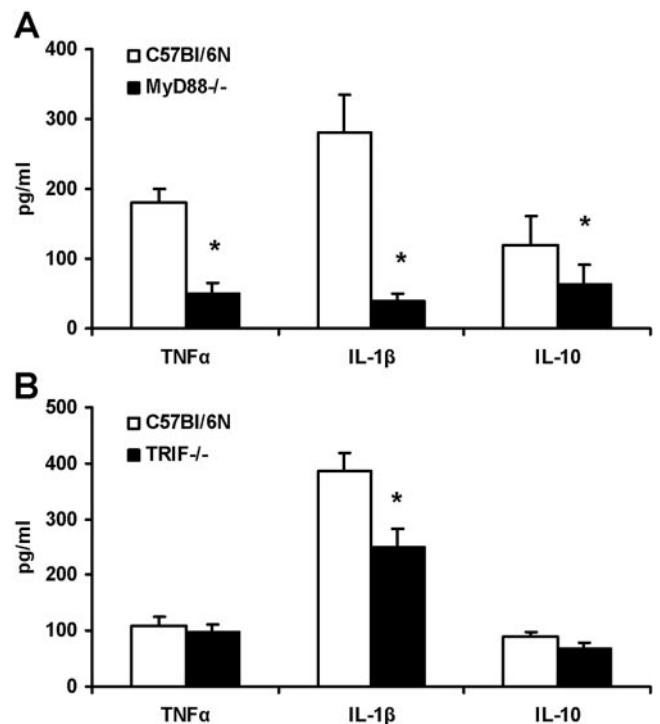
**Fig. 4.** Live *M. paratuberculosis* stimulates TLR4 in human. Human MNC of five healthy volunteers were stimulated with sonicated *M. paratuberculosis* ( $10^7$   $\mu$ g/ml) or live *M. paratuberculosis* ( $10^7$ /ml) in the presence or absence of TLR4 antagonist. TNF- $\alpha$  and IL-10 were measured in the supernatant by ELISA after 24 h incubation at 37°C. Data presented as mean  $\pm$  SEM and compared by Wilcoxon paired test (\*,  $P < 0.05$ ).

19-kD lipoprotein of *M. tuberculosis* is recognized by TLR2 [40, 41]. Which structure of *M. paratuberculosis* engages TLR2 is yet unknown, but various immune-active lipoproteins, such as 19-kD lipoprotein [42], 22-kD lipoprotein [43], and 34-kD lipoprotein [44, 45], have been identified in *M. paratuberculosis*. It is therefore tempting to speculate that *M. paratuberculosis* lipoproteins may be recognized by TLR2.

The role of TLR4 in the recognition of mycobacteria is more controversial. Sonicated *M. tuberculosis* has failed to stimulate human TLR4 in various experimental models. Conversely, it seems that TLR4 plays a beneficial role in tuberculosis infection models in mice [46, 47], although not all groups could confirm these findings [48]. One remarkable aspect is that TLR4 responds differently to intact compared with sonicated mycobacteria [31], indicating that the sonication procedure changes the structure of certain cell wall components, which are important for TLR4 ligation. Our data show that TLR4 does not play a role in the recognition of sonicated *M. paratuberculosis* in human PBMC and transfected CHO cells. However, the role of TLR4 becomes apparent if human MNC are infected with live *M. paratuberculosis*, as under these conditions, cytokine responses were reduced by inhibition of TLR4. By contrast, macrophages from TLR4 $^{-/-}$  mice did produce less

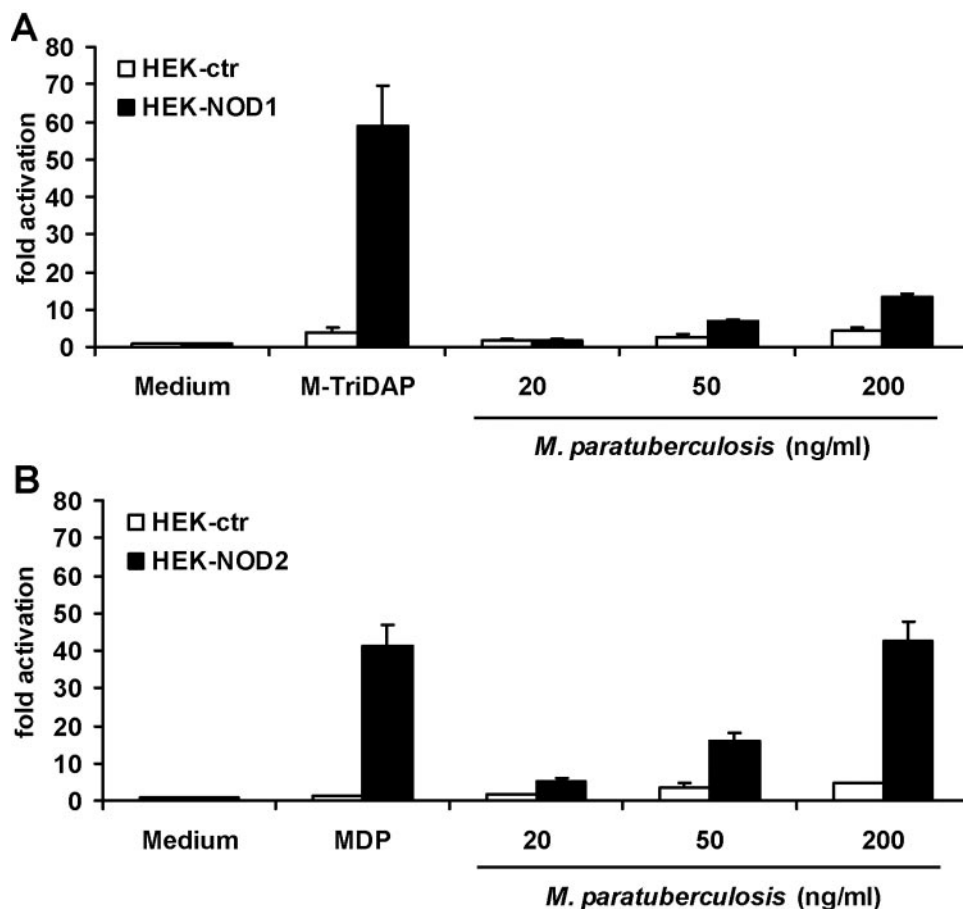
TNF- $\alpha$  after stimulation with sonicated *M. paratuberculosis*. This finding indicates that there are differences between the interaction of murine and human TLR4 with *M. paratuberculosis*. In addition, the interaction of leukocytes with components of live *M. paratuberculosis* at the level of the cell membrane is responsible for the TLR4-induced signaling. In contrast, NOD2 is an intracellular receptor, and recognition of *M. paratuberculosis* by NOD2 most likely requires prior internalization and digestion of the microorganism, leading to presentation of NOD2-stimulating motifs, as is probably the case in sonicated *M. paratuberculosis*. The structures of *M. paratuberculosis* responsible for interaction with human TLR4 are not known, but data obtained with *M. tuberculosis* indicate that structures sensitive for heat inactivation are responsible [39]. This might explain the different response after recognition of sonicated and living *M. paratuberculosis* by human TLR4.

The intracellular signals induced by TLRs leading to cytokine production are mediated by a limited number of adaptor molecules [49]. For TLR4, there are two intracellular pathways mediating its effects, represented by the adaptor molecules MyD88 and TRIF, whereas TLR2 depends entirely on the MyD88 pathway. We investigated which of the adaptor molecules are involved in the TLR signaling induced by *M. paratuberculosis*. The data from MyD88 $^{-/-}$  and TRIF $^{-/-}$  mice showed that MyD88 is important for the induction of TNF- $\alpha$ , IL-10, and IL-1 $\beta$ , whereas TRIF only seems to be important for



**Fig. 5.** *M. paratuberculosis* induced cytokine production is TLR-adaptor molecules MyD88- and TRIF-dependent. Peritoneal macrophages of MyD88 $^{-/-}$  mice (A) or TRIF $^{-/-}$  (B) were stimulated with sonicated *M. paratuberculosis* ( $10^7$   $\mu$ g/ml). Cytokines TNF- $\alpha$ , IL-1 $\beta$ , and IL-10 were measured in the supernatant by ELISA or RIA after 24 h incubation at 37°C. Groups of six mice/group were stimulated. Data presented as mean  $\pm$  SEM and compared by Mann-Whitney U test (\*,  $P < 0.05$ ).

**Fig. 6.** NOD1 and NOD2 recognize *M. paratuberculosis* in transfected HEK cells. HEK293 cells with a luciferase reporter construct (HEK-ctr) were transfected with NOD1 (A) or NOD2 (B) and stimulated with *M. paratuberculosis*. MDP (1  $\mu\text{g/ml}$ ) and TriDAP (1  $\mu\text{g/ml}$ ) served as control stimuli for NOD2 and NOD1, respectively. The NF- $\kappa\text{B}$ -dependent luciferase activation was measured following 24 h of incubation. NF- $\kappa\text{B}$ -dependent luciferase assays were performed in duplicate, and data represent three independent experiments.

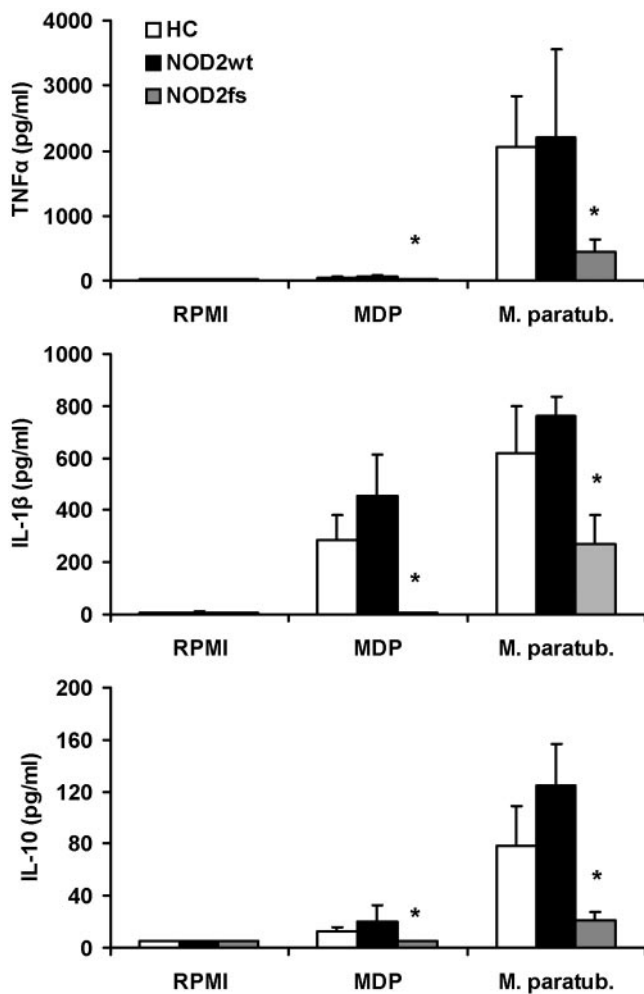


the induction of IL-1 $\beta$ . This is in line with early studies showing that TRIF is involved in the IL-1 $\beta$  production [50]. In addition, TRIF-mediated signaling is associated mainly with IFN- $\beta$  production.

NLRs are a class of intracellular receptors, which complement the extracellular TLRs. Among the NLRs, NOD1 and NOD2 are the main receptors for PGN, and NOD1 recognizes the PGN of Gram-negative bacteria, whereas NOD2 recognizes the PGN of Gram-negative and Gram-positive bacteria. We have shown recently that NOD2 is involved in the recognition of *M. tuberculosis* and acts synergistically with TLR2 and TLR4 for the induction of cytokines [31]. When HEK cells transfected with NOD2 were stimulated with sonicated *M. paratuberculosis*, a dose-dependent effect was observed, suggesting that NOD2 recognizes *M. paratuberculosis*. In contrast, NOD1 only showed a marginal stimulation. We were able to confirm this finding in human primary cells. The MNC of CD patients homozygous for the 3020insC mutation, which are nonresponsive for MDP [51], had a significant, reduced cytokine response after stimulation with sonicated *M. paratuberculosis*. By sonicating *M. paratuberculosis* or during killing by macrophages, fragments of the complete bacteria will be exposed and be able to stimulate the different PRR. How small these components are and how far they are degraded still remain unclear. For stimulation of the intracellular receptors, these fragments have to be taken up by leukocytes and digested further by enzymes in the leukocytes before they can stimulate receptors such as

NOD2. The PGN of mycobacteria contains muramyl peptides with the N-acetylglucosamine-N-glycolylmuramic acid backbone and the L-Ala-D-Glu-*meso*-DAP-D-Ala peptide side-chain [52]. This muramyl peptide contains motifs, such as L-Ala-D-Glu-*meso*-DAP (TriDAP), which can stimulate NOD1 [32]. Although it is known that the N-acetylmuramyl-L-Ala-D-Glu (MDP) structure is the minimal motif to stimulate NOD2 [32], our data also suggest that a N-glycolylmuramic acid-containing dipeptide can stimulate NOD2. As shown previously, the NOD2 3020insC mutation also leads to an impaired cytokine production after stimulation of NOD1 in human primary cells, suggesting that NOD1 and NOD2 are needed for an efficient response of the cells to NOD1 ligands [53]. Therefore, the lack of functional NOD2 in Crohn's patients with 3020insC mutation may lead to defective responses to mycobacterial PGNs, which contain strong NOD2-binding motifs but also weaker NOD1 ligands. In conclusion, as *M. paratuberculosis* is an intracellular pathogen, NOD2 might play an important role in the defense against this mycobacterium.

In the present study, we describe for the first time the receptors and mechanisms involved in the recognition of *M. paratuberculosis*. TLRs, such as TLR2 and TLR4, as well as NOD2 are independent recognition systems of *M. paratuberculosis*. It is tempting to speculate about the consequences of the finding that *M. paratuberculosis* is recognized by NOD2, as NOD2 polymorphism and *M. paratuberculosis* are associated with CD. One might hypothesize that a defective recognition of



**Fig. 7.** Human NOD2 is a receptor for *M. paratuberculosis*. MNC isolated from four patients with CD homozygous for the 3020insC *NOD2* mutation (NOD2fs), five patients with the NOD2wt, and five healthy volunteers with wild-type *NOD2* (HC) were stimulated with 10  $\mu\text{g/ml}$  *M. paratuberculosis*. TNF- $\alpha$ , IL-1 $\beta$ , and IL-10 were measured after 24 h stimulation at 37°C by ELISA. MDP (100 nM), LPS (data not shown), and Pam3Cys (data not shown) served as control stimuli. Data are presented as means  $\pm$  SEM and compared by Mann-Whitney U test (\*,  $P < 0.05$ ).

*M. paratuberculosis* by cells of Crohn's patients with NOD2 mutations is involved in the pathogenesis of CD.

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