NOD2 and Toll-Like Receptors Are Nonredundant Recognition Systems of Mycobacterium tuberculosis

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Infection with Mycobacterium tuberculosis is one of the leading causes of death worldwide. Recognition of M. tuberculosis by pattern recognition receptors is crucial for activation of both innate and adaptive immune responses. In the present study, we demonstrate that nucleotide-binding oligomerization domain 2 (NOD2) and Toll-like receptors (TLRs) are two nonredundant recognition mechanisms of M. tuberculosis. CHO cell lines transfected with human TLR2 or TLR4 were responsive to M. tuberculosis. TLR2 knock-out mice displayed more than 50% defective cytokine production after stimulation with mycobacteria, whereas TLR4-defective mice also released 30% less cytokines compared to controls. Similarly, HEK293T cells transfected with NOD2 responded to stimulation with M. tuberculosis. The important role of NOD2 for the recognition of M. tuberculosis was demonstrated in mononuclear cells of individuals homozygous for the 3020insC NOD2 mutation, who showed an 80% defective cytokine response after stimulation with M. tuberculosis. Finally, the mycobacterial TLR2 ligand 19-kDa lipoprotein and the NOD2 ligand muramyl dipeptide synergized for the induction of cytokines, and this synergism was lost in cells defective in either TLR2 or NOD2. Together, these results demonstrate that NOD2 and TLR pathways are nonredundant recognition mechanisms of M. tuberculosis that synergize for the induction of proinflammatory cytokines.


Introduction

Worldwide, 2 billion people are currently believed to be infected with Mycobacterium tuberculosis, with an estimated death toll of 2 million patients each year [1]. M. tuberculosis is an intracellular pathogen capable of infecting and surviving within the host’s mononuclear cells (MNCs), and a coordinated response of the innate and adaptive immune systems is required for an effective host defense. This involves sequestration of the microorganism in macrophages within organized granulomas, and elimination of the pathogen through a combination of killing mechanisms and apoptosis of host macrophages [2]. These responses are coordinated by T-helper 1-type proinflammatory cytokines, which are synthesized by phagocytes upon recognition of pathogen-associated molecular patterns of mycobacteria by pattern recognition receptors (PRRs).

Toll-like receptors (TLRs) are believed to be an important pattern recognition system of M. tuberculosis. A soluble, heat-stable mycobacterial fraction was initially reported to signal through TLR2, whereas heat-labile components associated with the cell wall were reported to signal through TLR4 [3]. Later, several components of mycobacteria were identified as being responsible for TLR2-dependent activation: the 19-kDa lipoprotein [4], lipomannan [5], phosphatidyl-myo-inositol mannoside [6], but not mannosyl-caped lipoarabinomannan from virulent M. tuberculosis, which mainly has anti-inflammatory effects through its interaction with the mannose receptor and dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin, also termed DC-SIGN [7,8]. Despite an abundance of in vitro data regarding the recognition of mycobacterial structures by TLR2 and TLR4, knock-out mice deficient for these receptors display remarkably little enhanced susceptibility to infection with M. tuberculosis. TLR4−/− mice showed variable responses to the challenge with M. tuberculosis, with either normal resistance to infection [9,10] or chronic pneumonia and increased mortality [11,12]. In one study, TLR2−/− mice had a decreased clearance of the bacteria and developed chronic pneumonia when infected with a low dose of microorganisms [13], whereas in other studies only minor effects have been found.

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Abbreviations: CHO, Chinese hamster ovary fibroblasts; HEK293T, human embryonic kidney 293T cell; LPS, lipopolysaccharide; LRR, leucine-rich repeat; MDP, muramyl dipeptide; MNC, mononuclear cell; NOD, nucleotide-binding oligomerization domain; PRR, pattern recognition receptor; SD, standard deviation; TLR, Toll-like receptor; TNF, tumor necrosis factor

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The Role of TLR2 and TLR4 in the Recognition of M. tuberculosis

In addition to representing a major healthcare problem in developing countries, concern is also growing about the increased incidence of tuberculosis in developed countries, especially in immunocompromised patients such as those with AIDS, transplantation, and immunosuppressive therapy. The present study describes the pathways that enable leukocytes to recognize M. tuberculosis, and demonstrates for the first time that NOD2, member of a new class of intracellular receptors, is an independent recognition mechanism for mycobacteria. NOD2 acts together with the earlier-described Toll-like receptors for the activation of host defenses during the encounter of leukocytes with M. tuberculosis. Understanding the mechanisms through which the cells of the immune system recognize M. tuberculosis can be an important step in designing new therapeutic approaches, as well as improving the limited success of current vaccination strategies.

Synopsis

Tuberculosis is one of the most prevalent infections worldwide, with 2 billion people believed to be infected, and 2 million deaths each year. In addition to representing a major healthcare problem in developing countries, concern is also growing about the increased incidence of tuberculosis in developed countries, especially in immunocompromised patients such as those with AIDS, transplantation, and immunosuppressive therapy. The present study describes the pathways that enable leukocytes to recognize M. tuberculosis, and demonstrates for the first time that NOD2, member of a new class of intracellular receptors, is an independent recognition mechanism for mycobacteria. NOD2 acts together with the earlier-described Toll-like receptors for the activation of host defenses during the encounter of leukocytes with M. tuberculosis. Understanding the mechanisms through which the cells of the immune system recognize M. tuberculosis can be an important step in designing new therapeutic approaches, as well as improving the limited success of current vaccination strategies.

Results

The Role of TLR2 and TLR4 in the Recognition of M. tuberculosis

TLR2 and TLR4 have been suggested to recognize bacterial structures of M. tuberculosis [20]. Indeed, a sonicate of M. tuberculosis strongly activated a Chinese hamster ovary fibroblast (CHO) cell line cotransfected with human TLR2 and CD14, whereas cells transfected with CD14 alone or a combination of CD14 and TLR4 displayed no signaling upon activation with the sonicated mycobacterial preparation (Figure 1A). However, when cells were stimulated with a preparation of whole mycobacteria, both TLR2 and TLR4-transfected cells were activated, although TLR2 activation was stronger (Figure 1A).

In line with these data, macrophages isolated from TLR2−/− mice displayed a 50%-75% reduction in tumor necrosis factor (TNF) production after stimulation with both M. tuberculosis preparations (Figure 1B), whereas TLR4-deficient macrophages showed a 30%-40% reduction of TNF release only when stimulated with the whole mycobacteria (Figure 1C). To confirm the role of TLR4 in the stimulation of cytokines by M. tuberculosis, we stimulated human MNCs with the whole mycobacterial preparation in the absence or presence of 10 μg/ml of a blocking anti-TLR4 antibody. TLR4 blockade completely inhibited lipopolysaccharide (LPS)-induced TNF secretion, and reduced M. tuberculosis-induced TNF secretion from 0.9 ± 0.2 to 0.5 ± 0.2 ng/ml (p < 0.05). These data confirm the role played by TLR2 and TLR4 in the recognition of M. tuberculosis; however, the significant remaining production of cytokines induced by M. tuberculosis...
in TLR2<sup>−/−</sup> or TLR4-defective mice points to the presence of additional signaling pathway(s) for cytokine induction.

The Role of Intracellular Recognition Systems in the Recognition of *M. tuberculosis*

The role of internalization in cytokine induction by *M. tuberculosis* was assessed by blocking it with cytochalasin B, an inhibitor of actin polymerization. Blocking internalization of *M. tuberculosis* partially inhibited *M. tuberculosis*-induced cytokine release in freshly isolated human MNCs stimulated with sonicated *M. tuberculosis* (1 × 10<sup>9</sup> microorganisms/ml), but not zymosan (1 µg/ml). Data are presented as mean ± SD (n = 5; *p < 0.05).

Figure 2. Blockade of internalization of *M. tuberculosis* impairs recognition and Cytokine Production

Blockade of *M. tuberculosis* internalization by cytochalasin B (20 µg/ml) impairs TNF (A) and IL-10 (B) stimulation in human MNCs stimulated with sonicated *M. tuberculosis* (1 × 10<sup>9</sup> microorganisms/ml), but not zymosan (1 µg/ml). Data are presented as mean ± SD (n = 5; *p < 0.05). DOI: 10.1371/journal.ppat.0010034.g002


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Discussion

In the present study, we investigated the role of TLRs and NODs, the two most important classes of PRRs in the recognition by macrophages of *M. tuberculosis*. Although we confirmed the role of TLR2 and TLR4 for in mycobacterial recognition, strong residual activity was detectable in cells lacking TLR2, which suggests the existence of TLR-independent recognition mechanisms; this idea is supported by a study demonstrating MyD88-independent pathways of macrophage stimulation by *M. tuberculosis* [22]. Using cell lines transfected with NOD1 and NOD2, as well as primary MNCs defective in these receptors, we demonstrated that, in addition to TLRs, NOD2 represents a nonredundant recognition system of *M. tuberculosis*. We also demonstrated that mycobacterial TLR2 and NOD2 ligands synergize for the production of proin
flam m atory cytokines, and that this synergism is lost in cells lacking either of these receptors.

Several studies have demonstrated the role of TLR2 and TLR4 in the recognition of \textit{M. tuberculosis}. The 19-kDa lipoprotein [4], lipom annan [5], and phosphatidyl-myositol mannoside [6], all components of mycobacteria, have been identified as being responsible for TLR2-dependent activation, whereas heat-labile components associated with the cell wall were found to signal via TLR4 [3]. A role for TLRs in antimycobacterial defense was also suggested by the enhanced susceptibility to \textit{M. tuberculosis} infection in mice deficient for MyD88, an adapter molecule shared by almost all TLR family members [15]. Similarly, TLR2\textsuperscript{-/-} mice had a decreased clearance of the bacteria and developed chronic pneumonia when infected with low doses of microorganisms [9,13,14], whereas TLR4\textsuperscript{-/-} mice showed variable responses to infection [10].

Our data confirm the important role played by TLRs, and especially TLR2, in the recognition of \textit{M. tuberculosis}, but at the same time demonstrate strong TLR-independent induction of cytokines by this microorganism. The contribution of TLR4 was found to be less crucial in our study, and could be observed only when cells were stimulated with intact microorganisms.

Because \textit{M. tuberculosis} is an intracellular pathogen, we hypothesized that intracellular recognition systems could contribute to the sensing of mycobacteria and stimulation of innate immunity. To test this hypothesis, we studied the effect of blocking the internalization of \textit{M. tuberculosis} with cytochalasin B. Blocking internalization of \textit{M. tuberculosis} partially inhibited \textit{M. tuberculous}-induced cytokine release in MNCs, whereas cytochalasin B potentiated the cytokine induction by zymosan, likely due to prolonged stimulation of receptors at the cell surface by zymosan. The differential effects of cytochalasin B on \textit{M. tuberculosis}- or zymosan-induced cytokine secretion demonstrate that, in addition to the interaction with cell-membrane bound TLRs, \textit{M. tuberculosis} is recognized by and induces cytokine production through intracellular receptors.

NOD2 and NOD1 are members of the expanding CATERPILLER family of proteins, which share an LRR domain similar to that found in TLRs [23]. NOD2 has been linked genetically to increased risk for Crohn’s disease [16,17], and is a sensor of bacterial peptidoglycans [18]. When HEKs transfected with either NOD1 or NOD2 were stimulated with \textit{M. tuberculosis} cell wall preparations, both of them—but most markedly those transfected with NOD2—showed a dose-dependent response. These data are consistent with the conclusion that NOD2 is a general sensor of bacteria through the detection of MDP, a peptidoglycan substructure present in bacterial cell walls [18]. However, the fact that NOD1 was found to be a poor sensor of \textit{M. tuberculosis} not only in NOD1-transfected HEK cells, but also through the lack of a defective response in NOD1\textsuperscript{-/-} macrophages, is somewhat puzzling. Indeed, NOD1 detects diaminopimelic acid (DAP)-type peptidoglycans, and earlier reports had suggested that \textit{M. tuberculosis} peptidoglycan is of this category. Further investigation is therefore required to discover why NOD1 detects \textit{M. tuberculosis} poorly.

To test whether the data in transfected cell lines could be reproduced in primary cells, we stimulated MNCs isolated from Crohn’s disease patients homozygous for the 3020\textsuperscript{insC} null-allele allele with \textit{M. tuberculosis}. This mutation leads to the deletion of the last 32 amino acids of the LRR region responsible for the detection of peptidoglycan, and we have recently shown that cells isolated from these patients are completely unable to recognize MDP or gram-positive peptidoglycan [21]. In line with the hypothesis that NOD2 is involved in the recognition of mycobacteria, both peritoneal macrophages from NOD2-deficient mice and MNCs isolated from patients homozygous for the 3020\textsuperscript{insC} mutation of NOD2 were found to synthesize significantly less cytokines after stimulation with \textit{M. tuberculosis}. Interestingly, the very strong defect in the response to \textit{M. tuberculosis} of the cells of patients with the NOD2 3020\textsuperscript{insC} mutation suggests that...
NOD1 is not able to compensate for the defective NOD2 recognition. This is in line with the weak stimulation of NOD2-transfected HEKs by M. tuberculosis and our recent finding that NOD2 is needed for normal signaling by NOD1 ligands such as Mur-Tri-DAP [24]. The reverse is not true in the case of NOD1: Macrophages harvested from NOD1−/− mice responded normally to M. tuberculosis, demonstrating that the absence of NOD1 can be compensated by other recognition systems, most likely NOD2.

The finding of strongly reduced cytokine production after stimulation with M. tuberculosis in cells of patients with a defective NOD2 and of NOD2 knock-out mice demonstrates that NOD2 is a key sensor of M. tuberculosis in mammalian cells. Interestingly, inhibition of both NOD2 and TLR2 systems blocked stimulation of cytokines by M. tuberculosis by substantially more than 50%. This suggests that the signaling pathways induced by these receptors interact and potentiate each other, and, conversely, that defects in one pathway lead to a loss of synergy. We have recently shown that NOD2 signals strongly synergize with specific TLR pathways such as TLR2, TLR4, and TLR3 [25]. We therefore investigated whether NOD2 activation leads to similar synergistic cytokine stimulation by TLR2 ligands specifically derived from M. tuberculosis. Indeed, MDP had strong synergistic effect on the cytokine production induced by the 19-kDa lipoprotein of M. tuberculosis, and this synergy was lost both in individuals homozygous for the NOD2 3020insC mutation and in macrophages harvested from TLR2−/− mice.

An issue yet to be resolved is represented by the mechanism through which mycobacterial peptidoglycans come in contact with NOD2. NOD2 is an intracytoplasmic molecule, while M. tuberculosis remains located mainly in phagosomes. Although shedding of cell wall components from the microorganism is likely responsible for the release of peptidoglycans that are ultimately recognized by NOD2, the precise mechanism through which peptidoglycans translocate from the phagosome into the cytoplasm remains to be identified.

The data presented in this study demonstrate that NOD2 and TLRs are two nonredundant recognition mechanisms of M. tuberculosis. Both are essential for effective activation of the proinflammatory cytokine production by M. tuberculosis, and they strengthen each other’s activity through synergistic effects. This demonstrates that host cells sense the presence of M. tuberculosis using multiple recognition systems in which different classes of receptors, in this case NOD2 and TLRs, interact with each other.

The involvement of NOD2 in the recognition of M. tuberculosis has several implications. First, it is possible that NOD2 is involved in recognition of other gram-positive bacteria with cell walls rich in peptidoglycans. The recent report of NOD2 serving as a receptor for Streptococcus pneumoniae supports this idea [26]. Second, our data suggest that NOD2 is involved in the recognition of other Mycobacteria species. From this point of view, M. paratuberculosis is of particular interest, due to its possible involvement in the
The MNC fraction was obtained by density centrifugation of blood was performed as described elsewhere [29], with minor modifications. For the below), or combinations of MDP and 19-kDa lipoprotein. The intracellular pathways induced by NOD2 and TLR2 during recognition of mycobacterial components synergize, and the stimulation of cytokine production by M. tuberculosis is greatly impaired in individuals with NOD2 mutations.

Materials and Methods

Reagents and microorganisms. Synthetic Pam3Cys and 19-kDa lipoprotein were purchased from EMC Microcollections (Tubingen, Germany). MDP and LPS (E. coli serotype O55:B5) were purchased from Calbiochem (San Diego, California, United States) and Sigma (St. Louis, Missouri, United States), respectively. The synthetic MDP was beneficial for stimulation with lipoproteins or LPS in mice deficient in TLR2 or TLR4, respectively. No defect in cytokine production was apparent in these mice after stimulation with MDP, demonstrating the absence of contamination.

Cultures of M. tuberculosis H37Rv were grown to mid-log phase in Middlebrook 7H9 liquid medium supplemented with oleic acid/albumin/dextrose/catalase (Difco, Becton-Dickinson, Palo Alto, California, United States), washed three times in sterile saline, and resuspended in RPMI 1640 medium at the various concentrations. Separate culture suspensions were sonicated for 10 min on ice, in order to obtain cell lysates.

Genotyping of NOD2 variants. Blood was collected from 74 patients with Crohn's disease and ten healthy volunteers. PCR amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50-µl reaction volumes containing 100–200 ng of genomic DNA as previously described [21]. 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 a/d IJssel, The Netherlands).

Four patients with Crohn's disease were found to be homozygous for the 3020insC mutation, and they were further investigated in the cytokine studies. As control groups, five patients with Crohn's disease who were heterozygous for the 3020insC NOD2 mutation, five patients with Crohn's disease bearing the wild-type allele, and five healthy volunteers homozygous for the wild-type allele were included. None of the patients with Crohn's disease used immunosuppressive medication at the time of the study.

Isolation of MNCs 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, Hettich-Bengusche, The Netherlands). Isolation of MNCs was performed as described elsewhere [29], with minor modifications. The MNC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia, Cambridge, UK) and centrifuged at 3000 rpm for 30min. The isolated MNCs were washed three times in saline supplemented with 5% FCS, and then used to prepare whole blood culture. The MNCs were incubated with either 100 µl of culture medium (negative control), or combinations of MDP and LPS (10 ng/ml), or Pam3Cys (10 µg/ml), or LPS (10 µg/ml). The influence of internalization of M. tuberculosis on cytokine production was investigated by adding 20 µg/ml cytochalasin B during stimulation with the microorganism. Positive control stimulation for the effects of cytochalasin B was provided by stimulation of cells with zymosan (1 µg/ml; Sigma). All stimuli were checked for the contamination with LPS in the Limulus amoebocyte lysate assay and found to be negative. Cytochalasin B did not influence cell viability (unpublished data).

To evaluate the role of TLR4 in the induction of cytokines, cells were preincubated with 10 µg/ml of a blocking monoclonal anti-TLR4 antibody (eBioscience, San Diego, California, USA). TLR4-defective macrophages (Resident peritoneal macrophages. Resident peritoneal macrophages from either ScCr (TLR4-defective) or C57Black/J (TLR4 control) mice, TLR2−/− or control TLR2+/+ mice (kindly provided by S. Akira, Osaka, Japan), NOD−/− and NOD mice, were purchased from E. Abraham, Denver, Colorado, United States, or NOD−/− and NOD−/− (from M. Giovannini, CEPH, Paris), backcrossed to the seventh generation into the C57Black/6J background, were harvested by injection of 4 ml of sterile PBS containing 0.5% sodium citrate [31]. After centrifugation and washing, the cells were resuspended in RPMI 1640 medium supplemented with 10% FCS, 50 µM 2-ME, 10 mM L-glutamine, 10 mM pyruvate, 2 mM L-glutamine, 100 µg/ml gentamicin, and 2% fresh mouse plasma. Cells were cultured in 96-well microtiter plates (Greiner) at 1 X 105 cells/well, in a volume of 100 µl. The cells were stimulated with purified LPS (1 µg/ml), Pam3Cys (1 µg/ml), FK-156 ligand of murine NOD1 (1 µg/ml). After 24 h incubation at 37 °C, cell supernatants were collected and stored at −70 °C until cytokine assays were performed.

Cytokine measurements. Human and murine TNFα concentrations were determined by specific ELISAs as described [32,33]. IL-10 and IL-6 were measured by a commercial ELISA kits (Pelikine Complex, CLB, Amsterdam, The Netherlands), according to the instructions of the manufacturer.

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 unpaired Student t-test, and where appropriate by paired t-test. The level of significance between groups was set at P < 0.05. The data are given as means ± standard deviation (SD).

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**Competing Interests.** The authors have declared that no competing interests exist.

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