NOD2 engagement induces proinflammatory cytokine production, but not apoptosis, in leukocytes isolated from patients with Crohn's disease

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ABSTRACT. ***Background.*** **NOD2/CARD15 is a member of the NACHT-LRR (NLR) family of proteins, which recognize the muramyl dipeptide motif from bacterial peptidoglycans. NOD2 has been shown to be involved in the pathogenesis of Crohn's disease. NLR proteins modulate inflammation and apoptosis, and several studies have implicated NOD2 in the induction of cytokines and inflammatory reactions. However, only scarce data are available regarding its role in apoptosis.** ***Donors and methods.*** **Neutrophils and lymphocytes isolated from the blood from four Crohn's disease patients homozygous for the loss-of-function 3020insC NOD2 mutation were examined for spontaneous and anisomycin-induced apoptosis. They were compared with cells from healthy controls and Crohn's disease patients bearing the wild-type NOD2 allele. Cytokine production after stimulation of mononuclear cells (MNCs) with muramyl dipeptide was assessed by specific immunoassays.** ***Results.*** **We observed that MNCs isolated from the blood of patients with Crohn's disease bearing the loss of function mutation in NOD2 displayed defective muramyl dipeptide-induced cytokine responses, but both granulocytes and lymphocytes from the same donors displayed normal apoptosis.** ***Conclusions.*** **NOD2 engagement by MDP mainly triggers cytokine activation and inflammatory reactions, but has negligible effects on cell apoptosis.**

Keywords: NOD2, apoptosis, Crohn's disease, cytokines

NOD-like receptors are intracellular receptors for bacterial peptidoglycans, which complement the recognition of pathogen-associated molecular patterns (PAMPs) by membrane-bound Toll-like receptors (TLR) [1]. 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 [2]. Mutations in the leucine-rich region (LRR) of the NOD2 gene are associated with Crohn's disease [3, 4], but how these NOD2 mutations act in the pathogenesis of the disease is only partially understood [5]. Several studies have shown the stimulatory effects of MDP/NOD2 interaction on cytokine production and inflammation [6, 7], and synergistic effects between NOD2 and TLR activation have also been reported [8, 9]. On the other hand, two recent studies have demonstrated that chronic NOD2 stimulation induces cross-tolerance to TLR stimuli, but this tolerance is absent in individuals bearing loss-of-function, NOD2 mutations [10, 11]. The role of NOD2 in the induction and modulation of inflammatory reactions is therefore well established.

However, in addition to their effects in inflammation, NLR proteins have also been implicated in apoptosis [12]. NOD2 is structurally similar to apoptotic protease-activating factor-1 that induces cell death [13]. Overexpression of both NOD1 and NOD2 was able to induce caspase-9-dependent apoptosis [14]. It has been hypothesized that modulation of apoptosis by NOD2 could play an important role in the pathogenesis of Crohn's disease [15]. These theoretical hypotheses, arguing for a role of NOD2 in apoptosis, have been boosted by a recent study that assessed the transcription profile induced by MDP in dendritic cells from patients with Crohn's disease, which showed that, in addition to inflammatory genes, MDP also modulated a gene cluster containing apoptotic factors [16].

In the present study, we addressed the question of whether recognition of MDP by NOD2 is important for both the induction of pro-inflammatory cytokines and for the modulation of apoptosis.

DONORS AND METHODS

Genotyping of NOD2 variants

Blood was collected from patients with Crohn's disease and healthy volunteers. PCR amplification of NOD2

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gene fragments containing the polymorphic site 3020insC was performed in 50µl reaction volumes containing 100-200 ng of genomic DNA as previously described [7]. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI-Prism 3100 Genetic Analyzer according to the manufacturer's protocol (Applied Biosystems). Four patients with Crohn's disease homozygous for the 3020insC mutation, were included in the study. As control groups, four patients with Crohn's disease and four healthy volunteers homozygous for the wild-type *NOD2* allele were included.

Reagents

Synthetic MDP and anisomycin was purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of polymorphonuclear neutrophils and mononuclear cells

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 polymorphonuclear neutrophils and mononuclear cells (MNC) was performed by differential centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Pharmacia Biotech). For the isolation of a purified lymphocyte population, magnetic beads coated with anti-CD3 antibodies were used (Miltenyi Biotec).
Because apoptosis of granulocytes and lymphocytes plays an important role in Crohn's disease, apoptosis was assessed in these two cell populations. Proinflammatory cytokine production is due to the close interaction of monocytes and lymphocytes that are both present in the mononuclear cell population isolated from the patients (containing 20% monocytes and 80% lymphocytes). In order to assess cytokine production, the mixed MNC population was stimulated with the various stimuli as described below.

Assessment of apoptosis

Cells were resuspended at a concentration of 5.0 x 10^6/mL in RPMI 1640 medium (Dutch modification; Invitrogen, Paisley, United Kingdom) and incubated at 37°C. For spontaneous apoptosis, cells were incubated for 24 h in the absence of serum, and apoptosis was assessed as described below. In lymphocytes, apoptosis was induced by the protease inhibitor anisomycin (Sigma-Aldrich, St Louis, MO, USA), which was added at a concentration of 20 µg/mL. At different time points (0, 5, and 24 hours) the reaction was stopped. At 0, 5, and 24 hours, cells were stained with annexin-V–FITC and PI (Apoptest-FITC; VPS Diagnostics, Hoeven, The Netherlands) and apoptosis was analyzed by flow cytometry (Coulter XL; Beckman Coulter, Fullerton, CA, USA).

Cytokine stimulation

5 x 10^5 MNC 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), MDP (10 nM). After 24 h, the supernatants were collected and stored at -70°C until assayed. Human TNF-α concentrations were determined by specific ELISA [41]. IL-1β was measured using a commercial ELISA kit (R&D Systems, MN, USA), according to the manufacturer's instructions.

Statistical analysis

The differences between groups were analyzed by the Mann-Whitney U test or Wilcoxon's test. The level of significance between groups was set at $p < 0.05$. The data are given as means ± SD.

Ethical considerations

The study was approved by the Ethical Committee of the Radboud University Nijmegen, and the patients gave written consent.

RESULTS

MDP effects on cytokine production and apoptosis

When freshly-isolated mononuclear cells (MNC) from healthy volunteers were stimulated with 10 µg/mL of MDP, they released significant amounts of the pro-inflammatory cytokines tumor necrosis factor (TNF, 454 ± 112 pg/mL) and interleukin-1β (IL-1β; 298 ± 45 pg/mL). In contrast, MDP did not induce apoptosis in either lymphocytes *(figure 1)* or neutrophils (not shown), independently of the presence or absence of NOD2 mutations in volunteers or patients.

Spontaneous and MDP-induced apoptotsis of blood PMN and lymphocytes from donors with wild-type NOD2 or homozygous for the 3020insC NOD2 mutation

Under incubation conditions in the absence of serum, neutrophils undergo significant spontaneous apoptosis. No differences between spontaneous neutrophil apoptosis in control patients bearing the wild-type NOD2 genotype and Crohn's disease patients homozygous for the 3020insC NOD2 mutation were apparent *(table 1)*. In addition, incubation of neutrophils with MDP did not significantly increased apoptosis in either patients with or without NOD2 mutations *(table 1)*.
In contrast to neutrophils, lymphocytes do not undergo spontaneous apoptosis. In order to investigate apoptosis of lymphocytes, cells were incubated with anisomycin and apoptosis assessed after 24 h. As shown for neutrophils, there were no defects in lymphocyte apoptosis in Crohn's disease patients homozygous for the 3020insC NOD2 mutation compared to cells from patients bearing the wild-type NOD2 *(figure 2A, B)*. Similarly, neutrophil apoptosis did not differ between bearers of wild-type or mutated NOD2 *(figure 2A)*. In contrast, the proinflammatory cytokine response (TNF and IL-1β) to MDP stimulation was significantly lower in cells from patients with the mutated NOD2 *(figure 2C)*.

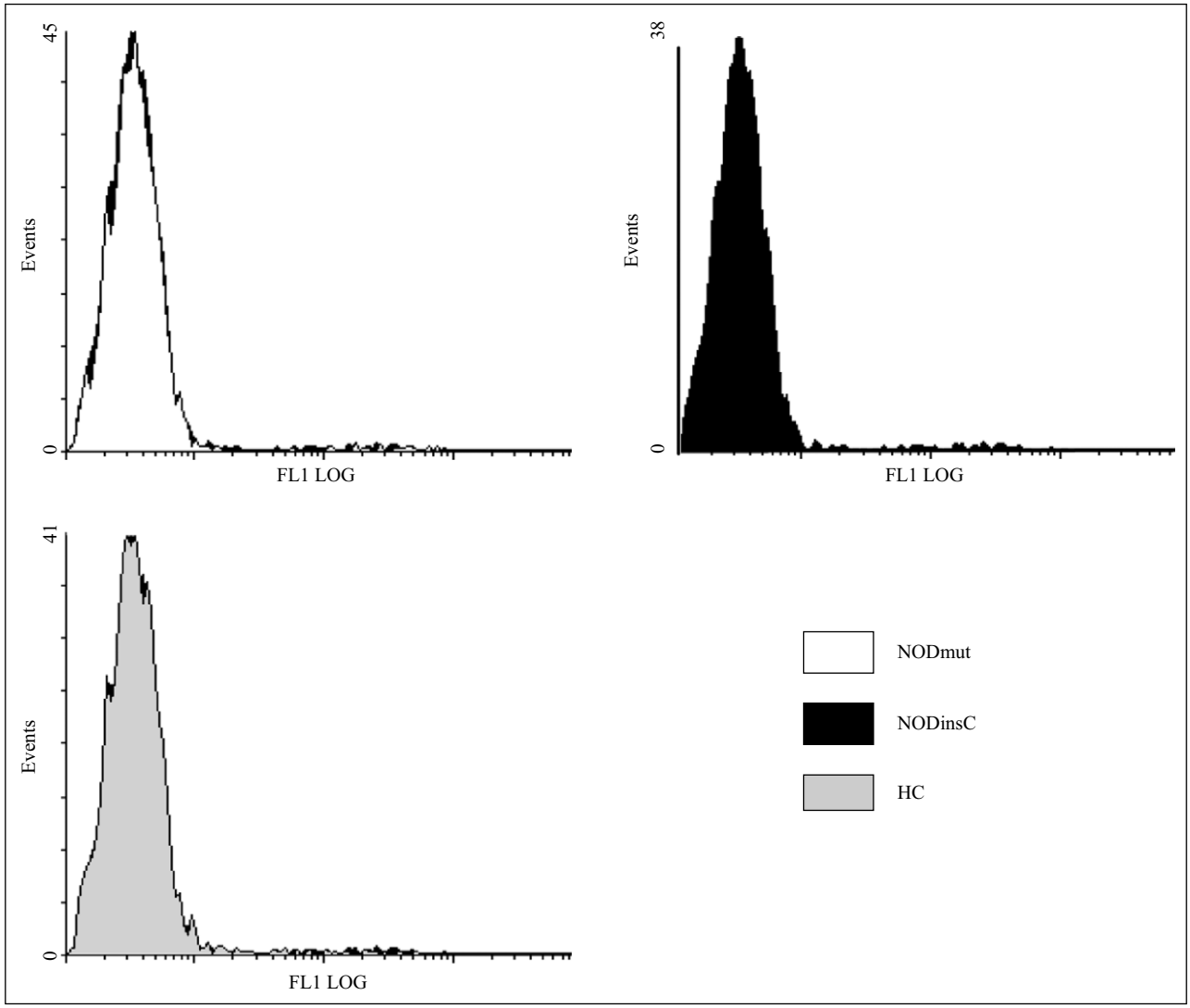

Figure 1
MDP effects on neutrophil and lymphocyte apoptosis. The figure presents annexine positivity of lymphocytes after 24 h stimulation with MDP (10 μg/mL), indicating the absence of apoptotic cells, independent of the presence of NOD2. The results presented are from one out of three representative experiments.

Table 1
MDP effects on neutrophil apoptosis in individuals with wild-type NOD2 or homozygous for the 3020insC NOD2 mutation. Polymorphonuclear neutrophils from patients with Crohn's disease bearing the wild-type NOD2 allele (NOD2wt) and patients with Crohn's disease homozygous for the 3020insC NOD2 mutation (NOD2insC) were incubated for 24 h with either control RPMI1640 medium (without serum) to assess spontaneous apoptosis, or in medium with 10 μg/ml of MDP. Each experiment assessed in triplicate, apoptosis in neutrophils from one patient with and one patient without NOD2 mutations. Data are presented as percentage apoptosis

	Control medium		MDP	
	NOD2wt	NOD2insC	NOD2wt	NOD2insC
Expt. 1	44	42	61	66
Expt. 2	48	57	44	50
Expt. 3	50	54	58	ND
Expt. 4	56	52	62	54

ND: not determined.

DISCUSSION

The role of NOD2 in the susceptibility to Crohn's disease is firmly established. However, the pathophysiological mechanisms responsible for this association are poorly understood. While NOD2 has been shown to be a receptor for the peptidoglycan components of microorganisms and its minimally active motif MDP [2], inducing production of cytokines and defensins [8, 17], these findings do not explain the exacerbated inflammatory reactions in the intestinal mucosa of Crohn's disease patients. It has been therefore hypothesized that mechanisms in addition to inflammation may also contribute to the effects of NOD2. Considering the role of a dysregulated apoptotic process in the pathophysiology of autoimmune diseases in general [18], and for Crohn's disease in particular [19], it has been hypothesized that defective apoptotic processes in individuals bearing NOD2 mutations may contribute to the disease mechanisms [20]. It was therefore

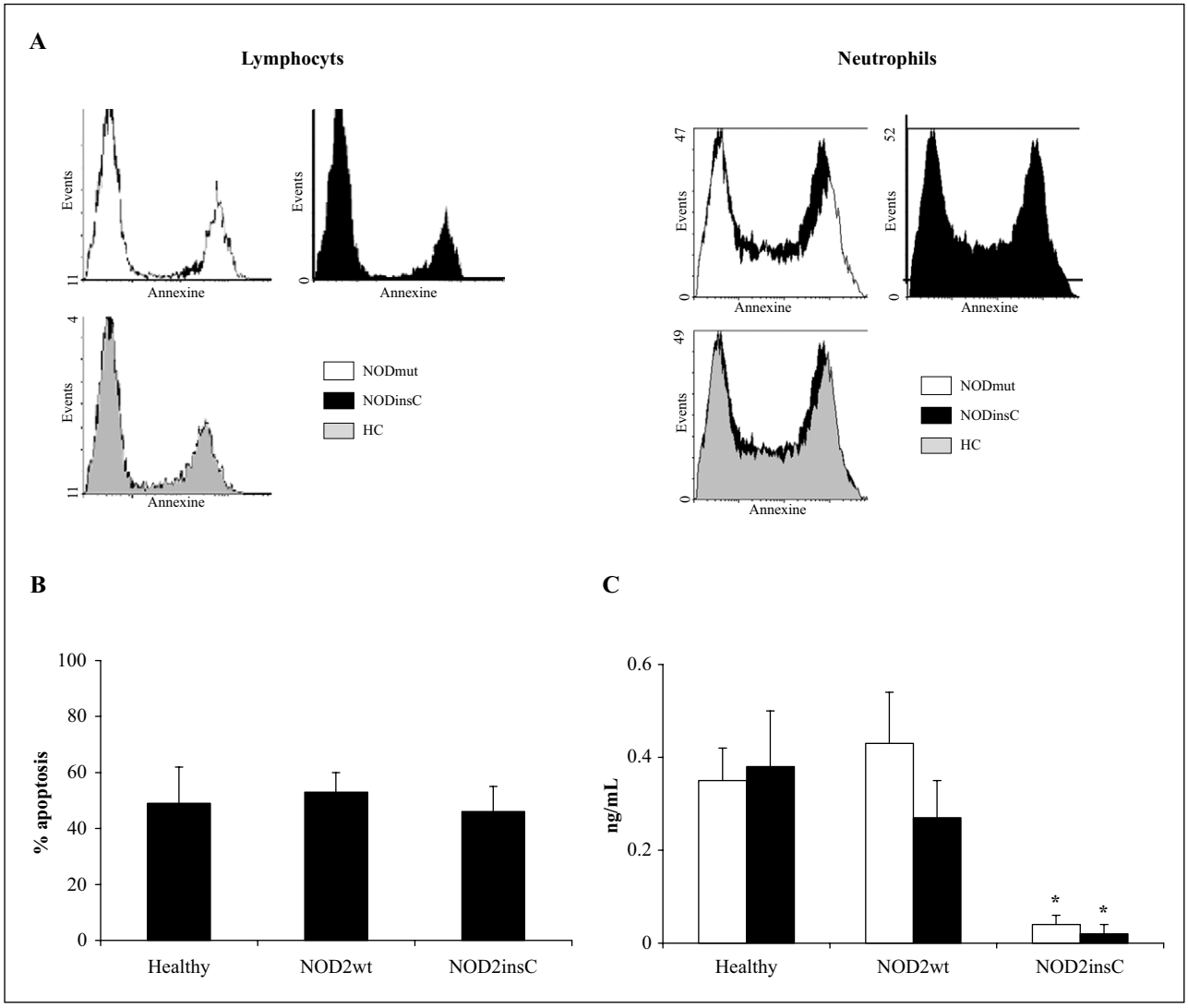

Figure 2

Apoptosis in lymphocytes and neutrophils of Crohn's disease patients homozygous for the 3020insC mutation. In order to investigate apoptosis, cells were incubated with anisomycin, and apoptosis assessed after 24 h. There were no defects in anisomycin-induced lymphocyte apoptosis in Crohn's disease patients homozygous for the 3020insC NOD2 mutation compared to cells from healthy volunteers or patients bearing the wild-type NOD2 (**Panel A**). Neutrophil apoptosis was also identical between the three groups. **Panel B** presents means and SD of all four experiments performed for lymphocyte apoptosis. In contrast, the TNF (open bars) and IL-1β (closed bars) response to MDP stimulation was significantly lower in those cells with mutated NOD2, compared to cells isolated from healthy donors and Crohn's disease patients bearing the wild-type NOD2 allele (**Panel C**). Data are presented as means ± SD (* $p < 0.05$).

surprising that no study to date has investigated the role of NOD2 in apoptosis in patients with Crohn's disease bearing NOD2 mutations.

The present study has used complementary approaches: on the one hand we have investigated whether NOD2 engagement by MDP in cells isolated from healthy individuals can modulate apoptosis, and on the other hand we assessed apoptosis in various cell types from Crohn's disease patients homozygous for the 3020insC loss-of-function NOD2 mutation. MDP strongly stimulated production and release of proinflammatory cytokines in cells from healthy volunteers, but did not influence programmed cell death in neutrophils, lymphocytes or monocytes. In line with this, apoptotic processes were normal in individuals bearing NOD2 mutations, despite the complete loss of cytokine production upon stimulation with MDP. These data indicate that while MDP/NOD2 interactions strongly stimulate cytokine production, NOD2-mediated signals do not significantly influence programmed cell death. In a recent gene profiling study of dendritic cells from patients with Crohn's disease, it has been reported that MDP induces transcription of a cluster of anti-apoptotic genes [16]. However, these effects were present both in patients bearing wild-type NOD2 alleles and patients with loss-of-function NOD2 alleles, demonstrating that these effects were not NOD2-dependent [16]. This suggests that any putative effect of MDP on apoptosis may be mediated by a different cellular sensor; other NLR receptors such as NALP3 and NALP1 have been shown to recognize MDP [21, 22].

The main role of NOD2 in mediating inflammation, but not apoptosis, has consequences not only for pathophysiology of Crohn's disease, but also for host defenses against infections. NOD2 has been implicated in the recognition of important bacterial pathogens such as mycobacteria, pneumococcus and *Chlamydia spp.* [23-25], and

apoptotic processes play a role in host defense against some of these microorganisms, and especially in tuberculosis [26]. The present study suggests however, that the role of NOD2 in these infections is most likely mediated by the induction of proinflammatory cytokines, rather than mediation of apoptosis.

In conclusion, in this study we have investigated for the first time apoptosis in patients with Crohn's disease homozygous for loss-of-function mutations in NOD2. Our data do not support a role for NOD2 in the mediation of apoptosis, and suggest that the influence of NOD2 in Crohn's disease is mediated through alternative mechanisms, most likely involving cytokine induction and inflammatory reactions.

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