The Frameshift Mutation in Nod2 Results in Unresponsiveness Not Only to Nod2- but Also Nod1-activating Peptidoglycan Agonists*

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NOD2/CARD15 is the first characterized susceptibility gene in Crohn disease. The Nod2 1007fs (Nod2fs) frameshift mutation is the most prevalent in Crohn disease patients. Muramyl dipeptide from bacterial peptidoglycan is the minimal motif detected by Nod2 but not by Nod2fs. Here we investigated the response of human peripheral blood mononuclear cells (PBMCs) from Crohn disease patients not only to muramyl dipeptide but also to several other muramyl peptides. Most unexpectedly, we observed that patients homozygous for the Nod2fs mutation were totally unresponsive to MurNAc-L-Ala-D-Glu-muramyl peptides. We proposed that through a scavenger function, recognition protein S, a secreted protein known to interact with bacterial peptidoglycan is the minimal motif detected by Nod2 but not NOD2/CARD15. Nod2fs did not act as a dominant-negative molecule for Nod1, and to Gram-negative bacterial peptidoglycan. In contrast, PBMCs from a patient homozygous for the Nod2 R702W mutation, also associated with Crohn disease, displayed normal response to Gram-negative bacterial peptidoglycan. In addition, the blockage of the Nod1/M-TriDAP pathway could be partially overcome by co-stimulation with the Toll-like receptors agonists lipoteichoic acid or lipopolysaccharide. Investigation into the mechanism of this finding revealed that Nod2fs did not act as a dominant-negative molecule for the Nod1/M-TriDAP pathway, implying that the blockage is dependent upon the expression or activity of other factors. We demonstrated that PBMCs from Nod2fs patients express high levels of the peptidoglycan recognition protein S, a secreted protein known to interact with muramyl peptides. We proposed that through a scavenger function, peptidoglycan recognition protein S may dampen M-TriDAP-dependent responses in Nod2fs patients. Together, our results identified a cross-talk between the Nod1 and Nod2 pathways and suggested that down-regulation of Nod1/M-TriDAP pathway may be associated with Crohn disease.

Although Nod2 detects a muramyl dipeptide (MDP)3 motif found in peptidoglycans from all classes of bacteria (3–5), Nod1 detects a diaminopimelic acid (DAP)-containing muramyl tripeptide (M-TriDAP) found primarily in Gram-negative bacterial peptidoglycan (4, 6, 7). In addition to its role as an intracellular pattern recognition molecule, genetic evidence has identified NOD2 as the first susceptibility gene for Crohn disease (8, 9). Crohn disease is an inflammatory disorder affecting the digestive tract, the etiology of which remains largely unknown. However, the recent association between the disease and Nod2 on the one hand and between Nod2 and bacterial sensing on the other hand suggests that Crohn disease is likely a consequence of a breakdown in tolerance to the intestinal bacterial flora. Still, it remains unclear why Nod2 dysfunction is a risk factor favoring the onset of Crohn disease. Indeed, although Nod2fs is fully defective for peptidoglycan sensing, other Nod2 mutant proteins found in Crohn disease patients display only minor differences in peptidoglycan detection (10, 11).

Through the identification of new important functions of Nod2, substantial progress has been made over the past few years toward understanding the link between Nod2 mutations and Crohn disease (1, 2, 12). Indeed, Nod2 function has been shown to be related to intracellular bacterial killing (13), defensin activity due to its expression in Paneth cells (14–16), as well as the induction of the anti-inflammatory cytokine IL-10 (17). Also, Nod2−/− mice display an increased T17 profile of cytokine responses following stimulation with Toll-like receptor (TLRs) agonists (18), which is an observation compatible with some features of Crohn disease. Finally, a recent study has reported the characterization of a knock-in mouse homozygous for the Nod2fs mutation, thus mimicking the natural human mutation associated with Crohn disease (19). Most surprisingly, macrophages from these animals displayed increased response to MDP, which contradicts results obtained by groups studying human cells (5, 17, 20). It is still unclear why Nod2fs mutation seems to represent a loss-of-function in humans and a gain-of-function in the mouse model.

In the present study, we aimed to investigate the response of primary mononuclear cells isolated from Crohn disease patients not only to...
Nod2 Frameshift Mutation Blocks Nod1 Pathway

MDP but also to several muramyl peptides or peptidoglycan agonists. Most surprisingly, we observed that M-TriDAP, the specific agonist of Nod1, failed to induce cytokine response in PBMCs from Nod2fs patients, although it stimulated cells efficiently from either healthy donors or non-Nod2 Crohn disease patients. The importance of this result is further reinforced by the observation that cells from Nod2fs patients were totally unresponsive to peptidoglycan from Helicobacter pylori, which is an efficient activator of both Nod1 and Nod2 signaling pathways. Because Nod2fs was unable to act directly as a dominant-negative molecule for the Nod1 pathway, our results rather suggest that the blockage relies on additional factors expressed in Nod2fs cells. Accordingly, we provide evidence that PBMCs from Nod2fs patients express significantly higher levels of peptidoglycan recognition protein S (PGRP-S) than cells from healthy donors. We propose that PGRP-S expression contributes to the down-regulation of Nod1-dependent responses in Crohn disease patients with Nod2fs mutation. Therefore, these observations suggest the existence of an unexpected cross-talk between the Nod1 and Nod2 signaling pathways. Moreover, our results imply that defects in Nod1 function could participate in the development of Crohn disease. Together, this study provides a basis for the design of original therapeutic approaches for Crohn disease, aiming at establishing a functional Nod1 pathway in Nod2fs patients.

EXPERIMENTAL PROCEDURES

Preparation of Highly Purified Peptidoglycans from Gram-negative and Gram-positive Bacteria—Bacterial strains used to prepare peptidoglycans are the following: H. pylori 26695; Staphylococcus aureus COL (from Olivier Chesneau, Institut Pasteur); and Streptococcus pneumoniae R800. The peptidoglycans purification procedures were exactly as described previously (6, 21). Purity of samples was assessed by HPLC amino acid and saccharide analysis after HCl hydrolysis. Also, each peptidoglycan preparation was tested for the absence of LPS contamination using the Limulus amebocyte lysate assay as described previously (21). The absence of TLR2-detected contaminants (lipoproteins or lipoteichoic acids) was tested on thioglycollate-elicited mouse peritoneal macrophages from either C57Bl/6 or TLR2−/− mice as described previously (21).

Preparation of Muropeptides—DAP- and Lys-containing UDP-MurNAc-peptides were prepared as described previously (4, 22). M-TetraLYS, M-TriLYS, and M-TriDAP were generated by mild acid hydrolysis (0.1 M HCl, 10 min at 100 °C) of the corresponding UDP-MurNAc peptides. Replacement of meso-DAP by l-Lys in the peptidoglycan of Escherichia coli was obtained by overexpression in the latter species of the murE gene from S. aureus encoding UDP-MurNAc-l-Ala-d-Glu-l-Lys adding enzyme (23). Cells were harvested before cell lysis occurred, and their peptidoglycan was extracted and purified as described previously (24). In these conditions, about 50% of the DAP residues at the third position of the peptides was shown to be replaced by l-Lys. This peptidoglycan preparation was digested by StIy lytic transglycosylase in a reaction mixture (1 ml) consisting of 300 mM sodium acetate buffer, pH 4.5, 1 mg of PG (briefly sonicated for homogenization), and 100 μg of purified StIy enzyme (25). After overnight incubation at 37 °C, the reaction was stopped by adding 500 μl of 50 mM sodium phosphate buffer, pH 4.45 (HPLC eluent A), and 2 μl of phosphoric acid. The two main monomer products, Anh-GM-TetraLYS, and Anh-GM-TetraLYS were purified by HPLC on a column of nucleosyl SC-18 (4.6 × 250 mm, Alttech). Elution was performed at 0.6 ml/min with buffer A, using a gradient of methanol from 0 to 25% for 180 min. Detection was at 215 nm. The retention times of these two compounds were 67 and 80 min, respectively. They were further purified and desalted using a second HPLC step on the same column but this time using 0.1% trifluoroacetic acid and a gradient of methanol for elution. Their purity and composition were confirmed by amino acid and hexosamine analysis after acid hydrolysis of samples (6 M HCl, 16 h at 95 °C), using an Hitachi L8800 analyzer, as well as by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Anh-M-TetraLYS was obtained by treatment of Anh-GM-TetraLYS with E. coli NagZ β N-acetylgalactosaminidase. The reaction mixture (200 μl) contained 20 mM HEPES buffer, pH 7.4, 50 mM NaCl, 0.5 mM substrate, and 20 μg of purified NagZ enzyme (25). Anh-GM-TriLYS and Anh-M-TriLYS were generated by treatment of the corresponding tetrapeptide compounds with E. coli LdcA LD-carboxypeptidase. The reaction mixture (200 μl) contained 50 mM Tris-HCl buffer, pH 8.0, 0.5 mM substrate, and 20 μg of purified LdcA enzyme (25). In all cases, incubation was for overnight at 37 °C, and the products were purified by HPLC, and their identity was confirmed by the above described procedures.

Cell Lines and Reagents—HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. Prior to transfection, HEK293T cells were seeded into 24-well plates at a density of 1 × 105 cells/ml as described previously (26). MDP LD (l-alanine d-glutamine) was from Calbiochem and reported to be 98% pure by TLC. Highly purified E. coli LPS (strain O55:B5) was from Invitrogen. Artificially synthesized LTA was kindly provided by Dr. Corinna Hermann (Konstanz University, Germany). Anti-Nod2 rabbit polyclonal antibody was from Cayman Chemical (Ann Harbor, MI), and anti-β-tubulin monoclonal antibody was from Sigma.

Expression Plasminids and Transient Transfections—The expression plasmids for Nod1 and Nod2 were the kind gift from Gabriel Nuñez (University of Michigan Medical School, Ann Arbor, MI) and have been described previously (27). The expression vector for Nod2fs was from M. Giovannini (INSERM U674, CEPH, Paris, France). Constructs for Rip2 and MyD88 were kind gifts from M. Thome (ISREC, Lausanne, Switzerland) and M. Muzio (Mario Negri Institute, Milan, Italy), respectively. The NF-κB luciferase reporter plasmid was from Stratagene. Transfections and Western blotting experiments were carried out in HEK293T cells as described previously (26).

NF-κB Activation Assays—Studies examining the synergistic activation of NF-κB by muramyl peptides in cells overexpressing Nod2 were carried out as originally described by Inohara et al. (27). Briefly, HEK293T cells were transfected overnight with the expression vectors of interest plus 75 ng of NF-κB luciferase reporter plasmid. At the same time, muramyl peptides were added to the cell culture medium, and the synergistic NF-κB-dependent luciferase activation was then measured following 24 h of co-incubation. NF-κB-dependent luciferase assays were performed in duplicate, and the data represent at least three independent experiments. Data show means ± S.E.

Genotyping of NOD2 Variants—Blood was collected from 74 patients with Crohn disease and 10 healthy volunteers. PCR amplification of NOD2 gene fragments containing the polymorphic sites 3020insC (for Nod2fs) and C2104T (for R702W) was performed in 50-μl reaction volumes containing 100–200 ng of genomic DNA, as described previously (17). 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 aan den IJssel, The Netherlands).

Four patients with Crohn disease were found homozygous for the 3020insC mutation; one patient with Crohn disease was found homozygous for the C2104T mutation, and they were further investigated in the cytokine studies. As control groups, five patients with Crohn disease heterozygous for the 3020 insC NOD2 mutation, five patients with Crohn disease bearing the wild type allele, and five healthy volunteers...
homozygous for the wild type NOD2 allele were included. The cells isolated from the four groups of patients were isolated and tested at two separate occasions. The study was approved by the Ethical Committee of the Radboud University, Nijmegen, The Netherlands.

Isolation of Mononuclear Cells 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). The PBMC fraction was obtained by density centrifugation of blood diluted 1:1 in pyrogen-free saline over Ficoll-Paque (Amersham Biosciences). Cells were washed twice in saline and suspended in culture medium (RPMI 1640 DM) supplemented with 10 \( \mu \)g/ml gentamicin, 10 mM L-glutamine, and 10 mM pyruvate. 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. 5 \( \times \) 10^6 PBMCs in a 100-\( \mu \)l volume were added to round-bottom 96-well plates (Greiner, Alphen aan den Rijn, The Netherlands) and were incubated with either 100 \( \mu \)l of culture medium (negative control) or the following various stimuli: 50 nM of the various muropeptide preparations, 10 \( \mu \)g/ml of the purified peptidoglycans, 100 ng/ml highly purified E. coli LPS (strain O55:B5), 5 \( \mu \)g/ml of artificially synthesized LTA (kindly provided by Dr. Corinna Hermann, Konstanz University, Germany), or a combination of stimuli as described under “Results and Discussion.”

Cytokine Measurements—For detection of cytokine concentrations in the supernatants, BioPlex 100 system (Bio-Rad) was used. The kits were used as indicated by the manufacturer, and the sensitivity for all cytokines was \( \leq 20 \) pg/ml.

Real Time PCR Studies—Total RNA was isolated from cells using RNeasy kits (Macherey Nagel, Hoerd, France) according to the manufacturer’s instructions. RNA quantification was performed using spectrophotometry. After treatment at 37 °C for 30 min with 20–50 units of RNase-free DNase I (Roche Diagnostics), oligo(dT) primers (Roche Diagnostics) were used to synthesize single-stranded cDNA. miRNAs were quantified using SYBR green master mix (Applera, Courtaboef, France) with specific human oligonucleotides in a GeneAmp ABIprism 7000 (Applera, Courtaboef, France). The following primers were used:
Nod1, sense GTAAGGTGCTAAGCGAAGA and antisense TCTGATTCTGGATAAGCCAT; hPepT1, sense CCGCCTCCCAGGTCTCAA and antisense GGTGCATGCCGCTAATCC; PGRP-S, sense GCAGCACTACCACATGAAGACACT and antisense GAGCCGTCTTCTCCAATCA; PGRP-L, sense ACTGAGGGCTGCTGGGACCA and antisense GGCCTCAGTGAATTCCTTGG; Rip2, sense AAATGGATCATTAAATGAACTCCTACATAG and antisense TTCATGCAGGATGCGAAATC. In each assay, calibrated and no-template controls were included. Each sample was run in duplicate. SYBR green dye intensity was analyzed using the Abiprism 7000 SDS software (Applied, Courtaboeuf, France). All results were normalized to the /H/H9252-actin, an unaffected housekeeping gene.

**Statistical Analysis**—The human experiments were performed in triplicate with blood obtained from patients and volunteers. The differences between groups were analyzed by the Mann-Whitney U test and where appropriate by the Kruskal-Wallis analysis of variance test. The level of significance between groups was set at p < 0.05. The data are given as means ± S.E.

**RESULTS AND DISCUSSION**

In the search for MDP-derived muramyl peptides that could stimulate the Nod2 signaling pathway, we generated several molecules differing in the length of their peptidic moiety, including M-TriLys and M-TetraLys (Fig. 1A). These molecules were then tested for their ability to activate Nod2 by using co-transfection assays in HEK293T epithelial cells and measuring NF-κB activity as a read-out (Fig. 1B). By using such tests, we reported previously that the activation of Nod2 was maximal with the addition of 10 pmol of MDP/ml of culture medium (leading to a concentration of 10 nM) (4). Here a larger range of muramyl peptide concentration was used (10–250 nM) to allow for the identification of even weak inducers of the Nod2 pathway. Through this approach, we observed that MDP and M-TriLys activated Nod2 with similar efficiency, whereas M-TetraLys represented a poor agonist (Fig. 1B). These results are consistent with our previous observations showing that the length of the muramyl peptide stem peptide is a key requirement for induction of Nod2 (4). Because our goal was to use MDP-derived muramyl peptides to stimulate primary human PBMCs (see below), we searched for other MDP-derived molecules that could represent the optimal negative controls for M-TriLys and M-TetraLys agonists. We took advantage of our previous observation that the sugar moiety of muramyl peptides also plays a key role for optimal activation of Nod2 (4). We generated modified forms of M-TriLys and M-TetraLys in which the MurNAc moiety is dehydrated to form anhydro-muramyl peptides (see Fig. 1A), and we observed that this subtle modification was sufficient to abolish stimulation of Nod2 (Fig. 1B). Therefore, anhydro-M-TriLys and anhydro-M-TetraLys were subsequently used as control inac-
Quality control tests were performed along the purification steps to ensure the purity of the peptidoglycan polymer is a crucial feature. Several addition, our observation that M-Tetra Lys was a poor inducer of Nod2 in vitro was reinforced by our findings that this agonist only marginally induced cytokine secretion from human PBMCs, and that this effect was further blunted in cells from Nod2fs patients (Fig. 2A). More importantly, our results identified MDP and M-Tri Lys as potent activators of human PBMC responses, and we confirmed that the detection of these muramyl peptides depends upon Nod2 because Nod2fs cells were not stimulated by MDP and M-Tri Lys, (Fig. 2A). In addition, our observation that M-Tetra Lys was a poor inducer of Nod2 in vitro was reinforced by our findings that this agonist only marginally induced cytokine secretion from human PBMCs, and that this effect was further blunted in cells from Nod2fs patients (Fig. 2A). More importantly, our results showed that Nod2-independent Crohn disease patients still reacted to MDP and M-Tri Lys, thus demonstrating that the inability of cells from Nod2fs patients to detect these agonists resulted from their Nod2 mutation and was not an indirect consequence of the disease. Consequently, this observation also suggested that defects in muramyl peptide sensing is not the sole cause of Crohn disease development. Second, the conclusion that sensing of M-Tri Lys in PBMCs from healthy donors and non-Nod2 Crohn disease patients depends on Nod2 is reinforced by the observation that anhydro-M-Tri Lys failed to stimulate these cells, which is in agreement with the results obtained in HEK293T cells (see Fig. 1B). Finally, we aimed to use M-Tri DAP in order to stimulate PBMCs in a Nod1-dependent but Nod2-independent manner. M-Tri DAP stimulation induced cytokine secretion in human PBMCs from healthy donors or non-Nod2 Crohn disease patients (Fig. 2A). However, M-Tri DAP was strikingly unable to stimulate PBMCs from Nod2fs patients. This result was unexpected because M-Tri DAP is a specific activator of Nod1 but not of Nod2. Therefore, these results identified an unexpected link between Nod2 mutations and the Nod1 signaling pathway.

Muramyl peptides are naturally occurring degradation products of peptidoglycan, which are useful tools to study precisely the involvement of signaling pathways dependent upon the specific activation of Nod1 or Nod2. However, in physiological situations, macrophages would likely encounter the presence of both intact peptidoglycan polymers together with muramyl peptides. Therefore, we aimed to investigate the response of PBMCs from the same individuals to peptidoglycans from H. pylori, S. pneumoniae, and S. aureus. We decided to use peptidoglycan from H. pylori because it is the prototype of Gram-negative bacterial peptidoglycan (DAP-type peptidoglycan) and is relatively easy to purify. Similarly, peptidoglycan from S. pneumoniae was chosen because it represents a classical peptidoglycan (Lys-type peptidoglycan) from Gram-positive bacteria. Finally, peptidoglycan from S. aureus was also included in this study because it is widely studied; however, because of the extremely high degree of peptidic cross-linking found in this peptidoglycan, its structure is less representative of Gram-positive bacterial peptidoglycan than that of S. pneumoniae. For such studies, the level of purification of the peptidoglycan polymer is a crucial feature. Several quality control tests were performed along the purification steps to ensure that other cell wall contaminants are excluded, such as LPS, lipoproteins, or LTA. To this end, the absence of LPS contamination was assayed by the Limulus amebocyte lysate test, showing that purified peptidoglycans contained less than 4 pg of LPS/ml of sample (data not shown). To address the difficult question of contamination by lipoproteins or LTA, we took advantage of our recent observation that contaminant-free peptidoglycans failed to stimulate thioglycollate-induced peritoneal macrophages from mice (21). Our purified peptidoglycans failed to induce the secretion of TNFα or IL-6 from peritoneal mouse macrophages (data not shown), showing that only traces amounts, if any, of lipoproteins or LTA contaminants were present in our peptidoglycan preparations. These peptidoglycan preparations were then added (each at 10 μg/ml) to the human PBMCs from the same individuals as described above, and the cytokines were measured after overnight stimulation (Fig. 2B). As a control, cells were also stimulated with LPS (100 ng/ml). First, by analyzing the cytokine response of cells from the healthy donors, we noticed that purified peptidoglycans activated human PBMCs, which contrasts with the lack of response in mouse peritoneal macrophages (see above). The reason for this discrepancy remains unknown, but it strongly correlates with the blunted response of mouse macrophages to muramyl peptides.4 Second, we observed that cells from non-Nod2 Crohn disease patients also responded to peptidoglycans as well (or even slightly more, depending on the cytokines) as the healthy donors (Fig. 2B). Finally, we found that PBMCs from the Nod2fs group of patients were totally unresponsive to the three peptidoglycans used in this study, regardless of the cytokine analyzed. More importantly, these cells were still fully responsive to LPS stimulation, thus demonstrating that Nod2fs PBMCs did not display a global unresponsiveness to any stimulation. Again, these results are in agreement with the data from cells stimulated with muramyl peptides (see Fig. 2A).

Our observation that PBMCs from Nod2fs patients are unresponsive to Gram-positive bacterial peptidoglycans allows us to draw some important conclusions. First, this result shows that Nod2 is a key sensor

### TABLE ONE

|                  | CTR | RPMI | LPS | PG H. pylori |
|------------------|-----|------|-----|-------------|
| **Nod2fs**       |     |      |     |             |
| TNF              | 0   | 198 ± 47 | 58 ± 25 |
| IL-1β            | 0   | 987 ± 169 | 382 ± 152 |
| IL-10            | 0   | 470 ± 87 | 78 ± 44 |
| IL-1α            | 103 ± 51 | 3368 ± 319 | 1580 ± 287 |
| **Nod2fs RPMI**  |     |      |     |             |
| TNF              | 0   | 267 ± 54 | 0   |
| IL-1β            | 0   | 1815 ± 447 | 14 ± 7 |
| IL-10            | 0   | 573 ± 66 | 0   |
| IL-1α            | 127 ± 42 | 4860 ± 823 | 75 ± 50 |
| **Nod2fs LPS**   |     |      |     |             |
| TNF              | 0   | 222   | 60   |
| IL-1β            | 0   | 1085  | 665  |
| IL-10            | 3   | 656   | 72   |
| IL-1α            | 265 | 3560  | 2700 |

4 S. E. Girardin, unpublished results.
of the Gram-positive bacterial peptidoglycan. Second, this observation suggests that, within the peptidoglycan polymer, the MDP and M-TriLys motifs are the key structures that drive the response of the host through their detection by Nod2. Third, our assumption that these peptidoglycans are free of bacterial contaminants is reinforced by this result, because unpurified peptidoglycans would have induced a TLR-driven response. In the case of the Gram-negative bacterial peptidoglycan, it was anticipated that, in the cells from Nod2fs patients, the defective Nod2 sensing could be compensated by the activation of the Nod1 signaling pathway. Indeed, unlike Gram-positive bacterial peptidoglycan, Gram-negative bacterial peptidoglycan is able to stimulate both Nod1 and Nod2. The lack of Nod1-dependent signaling in Nod2fs cells (Fig. 2B) again suggests that a functional Nod2 signaling pathway is required for Nod1-driven signaling to take place. This result confirms and extends the conclusions from the study of muramylpeptide stimulation of PBMCs (see Fig. 2A). Taken together, it can be concluded that any peptidoglycan sensing (dependent upon Nod2, Nod1, or any uncharacterized peptidoglycan sensor) is abrogated in PBMCs from Nod2fs patients. This defect is not found in non-Nod2 Crohn disease patients, therefore suggesting that lack of peptidoglycan sensing contributes to the onset of Crohn disease, the pathology can also arise from other causes.

FIGURE 3. Expression of Nod1 in PBMCs from 13 individuals by real time PCR. Expression of Nod1 was analyzed by real time PCR in cells from healthy donors (CTR), Crohn disease patients without defects in Nod2 (Crohn), or Crohn disease patients homozygous for the frameshift mutation (Nod2fs). Expression of Nod1 is reported to the internal control β-actin.

FIGURE 4. Nod2fs does not act as a dominant-negative molecule on the Nod1/M-TriDAP pathway. A, human HEK293 epithelial cells were transfected with several constructs that trigger the NF-κB pathway: Nod1 (1 ng of DNA added; simultaneously, addition of 50 nM M-TriDAP); Nod2 (0.5 ng of DNA added; simultaneously, addition of 50 nM MDP); Rip2 (50 ng); MyD88 (1 ng). Co-transfection of the above constructs was then performed together with increasing amounts (1, 5, and 20 ng) of expression vector for Nod2fs, and the activity of a NF-κB-driven luciferase reporter gene was measured. NF-κB activations are presented as a percentage of the value obtained in the absence of Nod2fs expression. Data show the mean ± S.E. of duplicate experiments. Experiments were performed three times with similar results. B, the response of PBMCs from healthy donors (CTR; n = 4) and Crohn disease patients heterozygous for the Nod2fs mutation (heterozygote; n = 4) to H. pylori peptidoglycan (PG) was analyzed. Intracellular IL-1α was measured from cell lysates. H. pylori peptidoglycan (10 μg/μl) and LPS (100 ng/ml) were added directly to the cell culture medium for 18 h.
In an attempt to better understand the origin of the defective Nod1-dependent signaling in cells from Nod2fs patients, we aimed to define whether such a defect was specific to the Nod2fs mutation. We took advantage that one individual in our cohort of Crohn disease patients had been genotyped as homozygous for the Nod2 R702W mutation, which is the second most frequent Nod2 mutation associated with increased risk for Crohn disease. Most strikingly, PBMCs from this patient were responsive to \textit{H. pylori} peptidoglycan to a similar extent as the control group (TABLE ONE), which contrasts with the results obtained from Nod2fs patients. Obviously, these results will need further confirmation by analyzing larger populations of patients carrying mutations.

Next, we investigated whether Nod1 expression was decreased in Nod2fs PBMCs. Nod1 expression was analyzed by real time PCR on 13 individuals (6 "CTR", 3 "Crohn," and 4 "Nod2fs"). Even though expression of Nod1 was found quite variable among individuals, no correlation could be observed between expression levels of Nod1 and the three groups analyzed. Therefore, the lack of the Nod1-dependent response in Nod2fs PBMCs cannot be accounted for by a defect in Nod1 expression in these cells. One likely explanation for the defective Nod1/M-TriDAP pathway in cells from Nod2fs patients is that the Nod2fs protein would simply behave as a dominant-negative molecule for Nod1 signaling. To test this hypothesis, HEK293 cells were transfected with Nod2 plus M-TriDAP (1 ng of Nod1; 50 nM M-TriDAP, according to standard procedures) in the presence of increasing amounts of Nod2fs. Clearly, Nod2fs was unable to block the Nod1/M-TriDAP pathway (Fig. 4A), even at the highest dose tested (50 ng of Nod2fs DNA transfected).

Also, we found no effect of Nod2fs overexpression on NF-κB activation triggered by either Rip2 or MyD88 (Fig. 4A), whereas the molecule blocked only partially the NF-κB activation induced by Nod2/MDP (~45%). Therefore, in light of these results, one can rule out a direct effect of Nod2fs on Nod1 signaling. Accordingly, if Nod2fs protein had behaved as a dominant-negative molecule on the Nod1 pathway, it is likely that patients heterozygous for the Nod2fs mutation would have displayed altered sensing of \textit{H. pylori} peptidoglycan. This was indeed not the case (Fig. 4B; similar results were obtained with the other cytokines measured (data not shown)).

Next, we analyzed whether the lack of response of Nod2fs cells to M-TriDAP was still observed in the case of co-stimulation with other agonists. Indeed, it is well characterized that muramyl peptides act in synergy with TLR agonists to induce cytokine secretion from PBMCs (28–32). Therefore, we stimulated PBMCs from individuals in our three groups (CTR, Crohn, and Nod2fs) with M-TriDAP, LPS (TLR4 agonist), or LTA (TLR2 agonist) either alone or in combination (M-TriDAP + LPS or M-TriDAP + LTA). For the three groups of individuals, we observed that M-TriDAP could function in synergy with LPS or LTA to potentiate cytokine secretion (Fig. 5; similar results were obtained with the other cytokines measured (data not shown)). These results are in agreement with the recent evidence presented in two independent studies, in which PBMCs from healthy donors were used (23, 24). As a control, PBMCs were also stimulated with MDP in combination with LPS or LTA. As reported previously (17), synergistic induction of cytokine secretion was observed in the case of healthy individuals or non-Nod2 Crohn disease patients but not in the Nod2fs group (Fig. 5). Together, these results suggest that even though M-TriDAP does not directly induce cytokine secretion in Nod2fs PBMCs, the blockage can be partially overcome in the case of co-stimulation with TLR ligands.

In order to gain more insights into the mechanism responsible for the blockage of the Nod1 pathway in Nod2fs cells, real time PCR analysis of selected candidate genes was performed in PBMCs isolated from our three groups of individuals (6 healthy donors (CTR), 3 Crohn disease patients without mutations in the NOD2 gene (Crohn), and 4 Nod2fs patients). The selected genes were the following and were chosen based on their potential roles in muramyl peptide signaling: PGRP-L, PGRP-S, RIP2, and hPEPT1 (NOD1 expression was also presented as a compar-
(ison). Indeed, PGRP-L and PGRP-S both interact with muramyl peptides and peptidoglycan (33–35). Rip2 is a crucial adaptor protein downstream of both Nod1 and Nod2 pathways (26, 36–40). Finally, hPepT1 has been demonstrated to allow for the internalization of muramyl dipeptide, at least in epithelial cells (41). Even though differences in expression were noted for these genes among the groups tested (Fig. 6, A–E), all except one case, these variations were not judged statistically significant (95% confidence) using the Mann-Whitney test for nonparametric samples. The only strong correlation was found in the Nod2fs group, in which a significant increase of PGRP-S expression was observed as compared with the control group of healthy donors (Fig. 6C; \( p < 0.05 \)). Because PGRP-S is a secreted protein known to bind muramyl peptides, one can speculate that excessive expression of PGRP-S by Nod2fs PBMCs might contribute to dampening the response of these cells to muramyl peptides, thereby acting as a scavenger molecule (Fig. 6F). In this context, it is interesting to note that PGRP-SC1B, a *Drosophila* homolog of mammalian PGRP-S, has been proposed to modulate the innate immunity in the fruit fly also via a scavenger function (42). Further investigation will be required to define precisely the role of PGRP-S in muramyl peptide-dependent signaling in both normal and inflammatory conditions.

The defective Nod1 function in PBMCs from Nod2fs patients was, at least in part, overcome when cells were co-stimulated with TLR ligands, such as LPS or LTA (see Fig. 5). This observation strongly suggests that in cells expressing functional TLRs, the defective Nod1 pathway may not have a crucial impact on the etiology of Crohn disease. However, this defect could prove of critical importance in epithelial cells lining the mucosal surfaces. Indeed, these cells are permanently in contact with microbes and microbial products, and therefore down-regulation of TLR function represents a common mechanism to avoid constitutive inflammation due to the microbial flora (43). Accordingly, by using *ex vivo* experiments, we have been able to show that intestinal epithelial cells detect nonflagellated bacteria exclusively through Nod1 (6). As a consequence, it can be envisioned that defective function of Nod1 in intestinal epithelial cells from Nod2fs patients may participate in the establishment of Crohn disease. This hypothesis has recently found strong support through the identification of genetic variation in Nod1 associated with predisposition to inflammatory bowel disease (44). The results presented here may have an impact on the design of new therapeutic treatments for Crohn disease. Nod2 1007fs mutation represents one-third to one-half of the Nod2 mutations found in Crohn disease patients. In this group of patients, a therapeutic approach aimed...
at restoring functional Nod1 signaling can be envisioned. Indeed, up until now, the idea of targeting Nod1 pathway in Crohn disease patients was not envisioned in this way, because it was assumed that Nod1 remained fully functional. Restoring a functional Nod1 pathway in Nod2Δc cells would have the important advantage of restoring partial homeostasis of the intestinal mucosa vis à vis the microbial environment. Therefore, if Crohn disease is a consequence of a breakdown in the tolerance to the intestinal bacterial flora, such tolerance could be restored through Nod1-dependent sensing of Gram-negative bacterial components of the microbial environment. Because such therapy would rely on a fine balance defined by the host itself, it would be likely less aggressive than other treatments, such as those acting to reduce the inflammation induced by the disease. However, one can question whether restoring the sensing of Gram-negative bacterial species only is sufficient to promote intestinal homeostasis to the microbial environment. This issue is of crucial importance for both our fundamental understanding of the etiology of Crohn disease and the rational design of new therapeutics aimed at treating the pathology.

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