Impaired dendritic cell function in Crohn's disease patients with NOD2 3020insC mutation

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Abstract: The nucleotide oligomerization domain 2 (NOD2) 3020insC (NOD2fs) mutation increases susceptibility to Crohn's disease (CD), but the mechanism remains controversial. Loss-of-function and gain-of-function phenotypes have been described as a result of NOD2fs. Here, we show that dendritic cells (DC) derived from CD patients homozygous for this mutation respond normally to purified Toll-like receptor (TLR) ligands but fail to up-regulate the costimulatory molecules CD80 and CD86 in response to the NOD2 ligand muramyl dipeptide (MDP). Moreover, they lack MDP-induced enhancement of TLR-mediated tumor necrosis factor α, interleukin (IL)-12, and IL-10 production, which is observed in control DC with intact NOD2. These data indicate that the NOD2fs mutation results in a loss-of-function phenotype in human myeloid DC and imply decreased immune regulation by IL-10 as a possible mechanism for this mutation in CD. *J. Leukoc. Biol.* 79: 860–866; 2006.

INTRODUCTION

Nucleotide oligomerization domain 2 (NOD2; CARD15) is the first gene identified within the IBD1 locus, and different NOD2 mutations are found to be associated with Crohn's disease (CD). Although mutations in NOD2 do not inevitably lead to the development of CD, individuals who are homozygous for the NOD2 3020insC (NOD2fs) mutation show a dramatically (up to 38-fold) increased susceptibility [1, 2]. Thus, homozygous NOD2fs individuals constitute a unique subset of patients to study the contribution of this mutation to the pathogenesis of CD.

NOD2 is a cytoplasmic protein that binds muramyl dipeptide (MDP), a motif common to peptidoglycan of Gram[+] and Gram[−] bacteria, resulting in nuclear factor (NF)-κB activation and cytokine induction [3]. Furthermore, NOD2 activation has been suggested to affect Toll-like receptor (TLR)-mediated responses [4, 5].

The mechanism by which NOD2fs contributes to CD remains largely unresolved. Recent data from several NOD2 mouse models have yielded surprisingly different conclusions. TLR2-induced interleukin (IL)-12 production by myeloid cells was found to be decreased after costimulation with MDP, an effect absent in NOD2^{−/−} mice, implying that NOD2 is a negative regulator of TLR2-mediated T helper cell type 1 (Th1) responses [6]. In line with this gain-of-function phenotype of knockout mice, NOD2fs knock-in mice showed enhanced NF-κB activity and IL-1β secretion in response to MDP as well as increased susceptibility to bacterial-induced intestinal inflammation [7]. However, another mouse study demonstrated that NOD2 activation in wild-type mice enhanced TLR-mediated cytokine production by bone marrow-derived macrophages, and NOD2-deficient animals had decreased cryptdin expression in terminal ileum cells and impaired antibacterial responses after challenge with *Listeria monocytogenes* [8], thus favoring a loss-of-function phenotype. Few studies have yet focused on the effect of NOD2fs in human dendritic cells (DC), which are the only antigen-presenting cells capable of activating naive T cells and the induction of immunity and tolerance [9]. Also, in the gut, DC are crucial for the induction of tolerance to the resident intestinal flora [10–12]. We therefore aimed to determine the consequence of NOD2fs for the responsiveness of DC to MDP, focusing on maturation and cytokine production. We specifically studied IL-12, tumor necrosis factor α (TNF-α), and IL-10, considering their importance in affecting inflammatory processes in general and the pathogenesis of CD in particular [13–16].

MATERIALS AND METHODS

Patients and genotyping of NOD2 variants

Blood was collected from 150 CD patients and 10 healthy volunteers. Polymerase chain reaction amplification of NOD2 gene fragments containing the polymorphic site 3020insC was performed in 50 µl reaction volumes containing 100–200 ng genomic DNA. The 3020insC polymorphism was analyzed by Genescan analysis on an ABI PRISM 3100 genetic analyzer, according to the protocol of the manufacturer (Applied Biosystems, Foster City, CA). Four CD

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patients were found to be homozygous for the NOD2fs mutation and were selected for further studies. As control groups, four patients with CD bearing the wild-type allele and five healthy volunteers homozygous for the wild-type NOD2 allele were included. Approval from the local regulatory committee was obtained.

Isolation of mononuclear cells and generation of monocyte-derived DC (moDC)

After informed consent, 80 ml venous blood was drawn from the cubital vein of patients and healthy volunteers into 10 ml EDTA tubes (Monoject, s-Hertogenbosch, The Netherlands). Total blood was diluted 1/1 using phosphate-buffered saline (PBS) containing 0.45% sodium citrate (dilution solution), and mononuclear cells were isolated by density gradient centrifugation using Lymphoprep (1.077 g/ml, Axis Shield PoC AS, Oslo, Norway), according to the manufacturer's instructions. Cells were washed once in dilution solution at 1800 rotations per minute (rpm) for 8 min to remove Lymphoprep remains and further washed (1500 rpm/5 min) using ice-cold dilution solution containing 1% fetal calf serum (FCS) until clear supernatant was obtained. After counting, cells were resuspended in RPMI containing 2% human serum (HS) at a density of $10–12.5 \times 10^6$ cells/ml and plated in T25 or T75 culture flasks (Costar, Corning, NY) at 37°C for a period of 1 h. Nonadherent cells were removed by washing 3× with PBS, and adherent cells were cultured in RPMI 1640 supplemented with 100 U/ml antibiotic-antimycotic (Invitrogen, Carlsbad, CA), 10 mM L-glutamine, and 10% FCS. To generate moDC, 400 U/ml IL-4 and 800 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF; Schering-Plough, Kenilworth, NJ) were added to the culture medium, which was refreshed, and complete cytokines were added at Day 3. On Day 6, immature DC were harvested using cold PBS, and cells were stimulated as indicated below. An average yield of 2.3×10^6 (SD 0.7×10^6) immature DC was obtained per donor.

Stimulation of DC

For cytokine analysis, immature moDC (50×10^3) in 100 μl medium were added to round-bottom, 96-well plates (Costar) with 50 μl culture medium (negative control) or 50 μl medium containing the various stimuli, purified lipopolysaccharide (LPS; *Escherichia coli*, 100 ng/ml), synthetic Pam3 Cys-Ser-(Lys)4 .3HCl (Pam$_3$Cys; 10 μg/ml, EMC Microcollections, Germany), synthetic polyionisicpolycytidylic acid [poly(I:C); 20 μg/ml, Sigma-Aldrich, St. Louis, MO] in the presence or absence of MDP (5 μg/ml, Sigma-Aldrich), interferon-γ (IFN-γ; 400 U/ml), or a combination of both MDP and INF-γ for a period of 24 h. For flow cytometry, immature moDC (0.6×10^6) in a 1.5-ml vol were added to 12-well plates (Costar) and incubated with 20 μg/ml poly(I:C) or 10 μg/ml Pam$_3$Cys for a period of 24 h, after which, the expression of costimulatory molecules was analyzed using flow cytometry.

Flow cytometric analysis of costimulatory molecule expression on DC

After 24 h incubation with indicated TLR ligands, cells were harvested and washed using ice-cold PBA (PBS containing bovine serum albumin and azide).

Cells (50×10^3 cells/well) were added to a v-bottom, 96-well plate (Costar) and stained using CD80 or CD86-specific mouse anti-human immunoglobulin G1 (IgG1) antibodies or the appropriate isotype control (BD PharMingen, San Diego, CA) on ice for a period of 30 min. Cells were washed twice in ice-cold PBA and incubated with phycoerythrin-labeled goat anti-mouse Ig (BD Phar-Mingen) on ice for a period of 30 min. After double washings, cells were incubated in 200 μl PBA, and the expression of CD80 and CD86 was analyzed via flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). Analysis was done using WinMDI 2.8 software.

Cytokine measurements

After 24 h stimulation, supernatants were harvested and stored at −80°C until cytokine analysis was performed. IL-12 concentrations in supernatants were measured using an IL-12p70-specific sandwich enzyme-linked immunosorbent assay (Pierce-Endogen, Rockford, IL). TNF-α and IL-10 levels were determined using commercially available kits (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's instructions. Data analysis was done with Bio-Plex Manager software (Bio-Rad Laboratories).

Statistical analysis

Differences in the increase in mean fluorescence intensity (MFI) of CD80 and CD86 after 24 h stimulation with TLR ligands or MDP between individuals homozygous for NOD2fs (n=4) and those with intact NOD2 (four CD patients and five healthy controls) were analyzed using the Student's *t*-test. Stimulation experiments for cytokine analysis were performed in duplo. Differences in cytokine production between cells from patients homozygous for the NOD2fs polymorphism (n=4) and cells from individuals bearing the wild-type allele (four CD patients and five healthy controls) were analyzed using the Student's *t*-test.

RESULTS

TLR activation of NOD2fs DC leads to normal maturation and cytokine production

To determine whether the NOD2fs mutation affects TLR-mediated DC activation, we analyzed DC maturation (a hallmark of activation) via the expression of CD80 and CD86 on moDC from healthy controls, CD patients with intact NOD2, and CD patients homozygous for NOD2fs. For patient characteristics, see **Table 1**. DC were stimulated with synthetic or highly purified TLR ligands to avoid possible effects of contamination with enterotoxins. As shown in **Figure 1A**, irrespective of their NOD2 genotype, DC stimulated with the synthetic TLR1/2 ligand Pam$_3$Cys or TLR3 ligand poly(I:C) responded with similar up-regulation of the DC

TABLE 1.   Overview of General Patient Characteristics, Relevant Clinical Information, and Prescribed Medication at the Time of Study

Donor	Age	Sex	Disease duration (years)	Fistulae	Resections	Medication
Healthy control	26	M	*	no	no	
Healthy control	36	F	*	no	no	
Healthy control	35	F	*	no	no	
Healthy control	36	M	*	no	no	
Crohn (NOD2 +/+)	56	M	28	no	no	#
Crohn (NOD2 +/+)	53	M	34	yes	no	†,§
Crohn (NOD2 +/+)	48	M	29	yes	no	†,§
Crohn (NOD2 +/+)	60	M	11	yes	yes	‡
Crohn (3020insC)	61	F	27	yes	yes	†,#,§
Crohn (3020insC)	57	M	38	no	yes	#
Crohn (3020insC)	41	M	19	yes	yes	†
Crohn (3020insC)	54	F	36	yes	yes	§

None of the patients included received prednisolone during the study. Three patients (two NOD2fs CD patients and one NOD2+/+ CD patient) had been treated with Infliximab, the last infusion being more then 6 months prior to study. Taking into account the half-life of 9 days, no relevant residual Infliximab activity could be expected. †, azathioprine; ‡, 6-mercaptopurine; #, budesonide; §, mesaline.
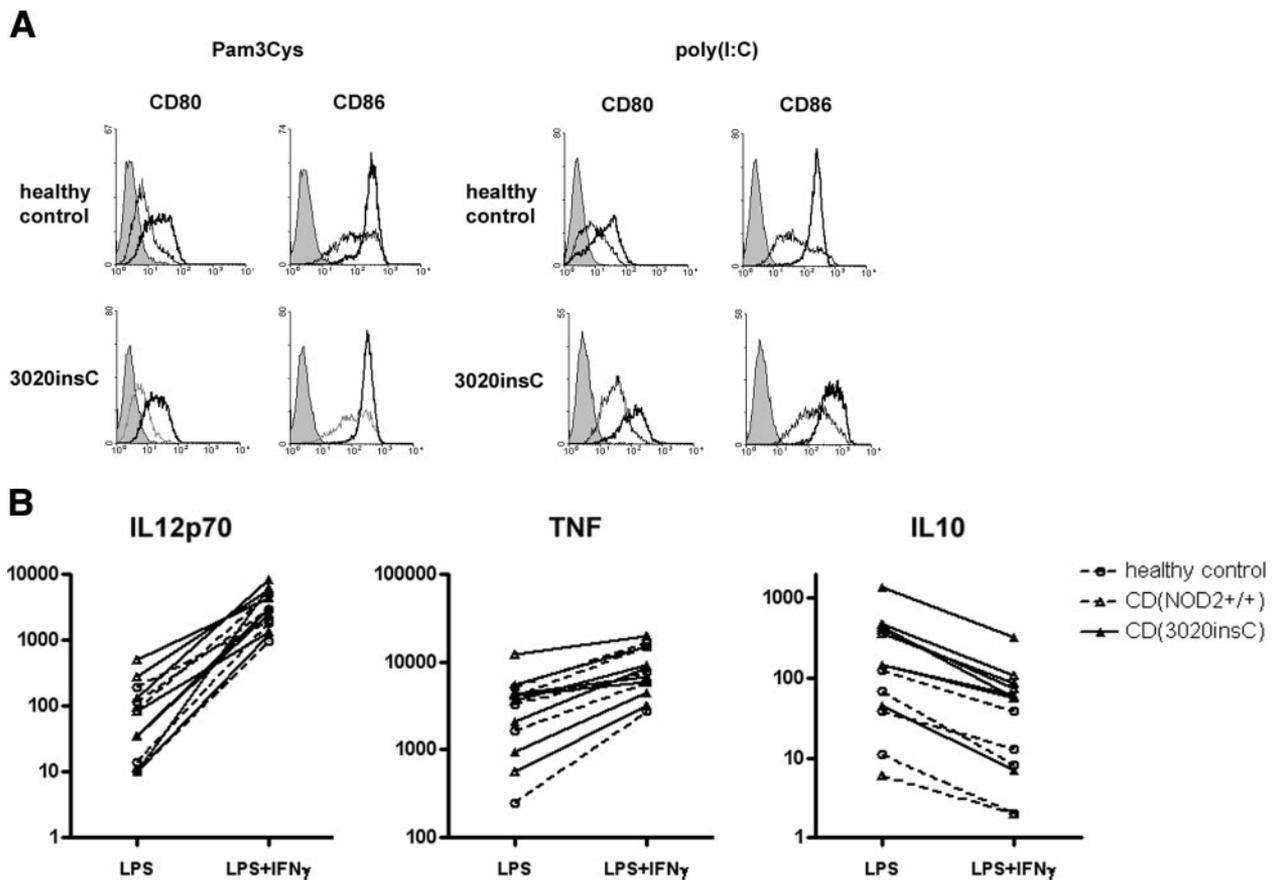
A

Pam3Cys

CD80   CD86

poly(I:C)

CD80   CD86

B

Fig. 1. TLR activation is not affected in NOD2fs DC. (A) DC were generated by culturing monocytes in the presence of IL-4 and GM-CSF for a period of 6 days. DC from healthy controls or NOD2fs patients were stimulated with synthetic TLR ligands Pam$_3$Cys (10 μg/ml) or poly(I:C; 20 μg/ml) for 24 h. CD80 and CD86 expression on the cell surface was analyzed by flow cytometry. Maturation responses shown are representative of six healthy controls and four NOD2fs patients. Medium control and indicated TLR stimulation are represented by thin and thick lines, respectively. Filled histogram indicates isotype-matched, control monoclonal antibodies (mAb). (B) Production of IL-12, TNF-α, and IL-10 by DC from healthy controls (n=5), NOD2$^{+/+}$ CD patients (n=4), or homozygous NOD2fs CD patients (n=4). Cells were stimulated with LPS (*E. coli*, 100 ng/ml) alone or LPS in combination with 400 U/ml IFN-γ for a period of 24 h, after which, cytokine concentrations in culture supernatants were analyzed.

maturation markers CD80 and CD86. The minor differences observed in absolute expression levels between different individuals fall within the normal donor variation. We note that a similar up-regulation of those molecules was observed when using (healthy control) DC generated with HS instead of FCS (data not shown). To assess cytokine responses of DC from NOD2fs patients in response to TLR activation, we measured IL-12, TNF-α, and IL-10 after stimulation with TLR ligands in the presence or absence of IFN-γ, which is known to be present in increased amounts in the gut of CD patients and can enhance TLR and NOD2 responses. No significant difference in cytokine induction by LPS alone (IL12p70, $P=0.31$; TNF-α, $P=0.32$; IL-10, $P=0.18$) or in combination with IFN-γ (IL12p70, $P=0.23$; TNF-α, $P=0.44$; IL-10, $P=0.16$) between NOD2fs DC and control DC was observed (Fig. 1B). These findings indicate that DC maturation and cytokine production following TLR activation are not altered in DC from CD patients homozygous for NOD2fs.

MDP stimulation results in modest maturation and cytokine responses in control but not NOD2fs DC

Next, the response of DC to the NOD2 ligand MDP was investigated. Based on initial titration experiments, a concen-

tration of 5 μg/ml MDP was used. As shown in **Figure 2A**, MDP treatment resulted in a weak but consistent up-regulation of CD80 and CD86 on DC from healthy donors and CD patients expressing wild-type NOD2. In contrast, NOD2fs DC invariably failed to increase the expression of these costimulatory molecules. Cytokine analysis further revealed that in contrast to what is observed after TLR4 activation with LPS, no IL-12 was detected, and only weak TNF-α and IL-10 responses [mean 1.7% (0–11%) and 0.7% (0–3%) of LPS response, respectively] were observed following MDP stimulation in a number of control DC but not NOD2fs DC (Fig. 2B). These results indicate that NOD2 triggering alone does not induce significant cytokine production in DC but does result in modest DC maturation, which is abrogated completely in DC homozygous for the NOD2fs mutation.

Lack of cross-talk between NOD2 and TLR signals in NOD2fs DC

We and others have reported recently that MDP can increase cytokine production when combined with TLR-activating stimuli [5, 17]. Considering the important role of IL-12 in CD, we first studied the release of this major Th1-driving cytokine in DC from healthy controls after activation with purified LPS. As
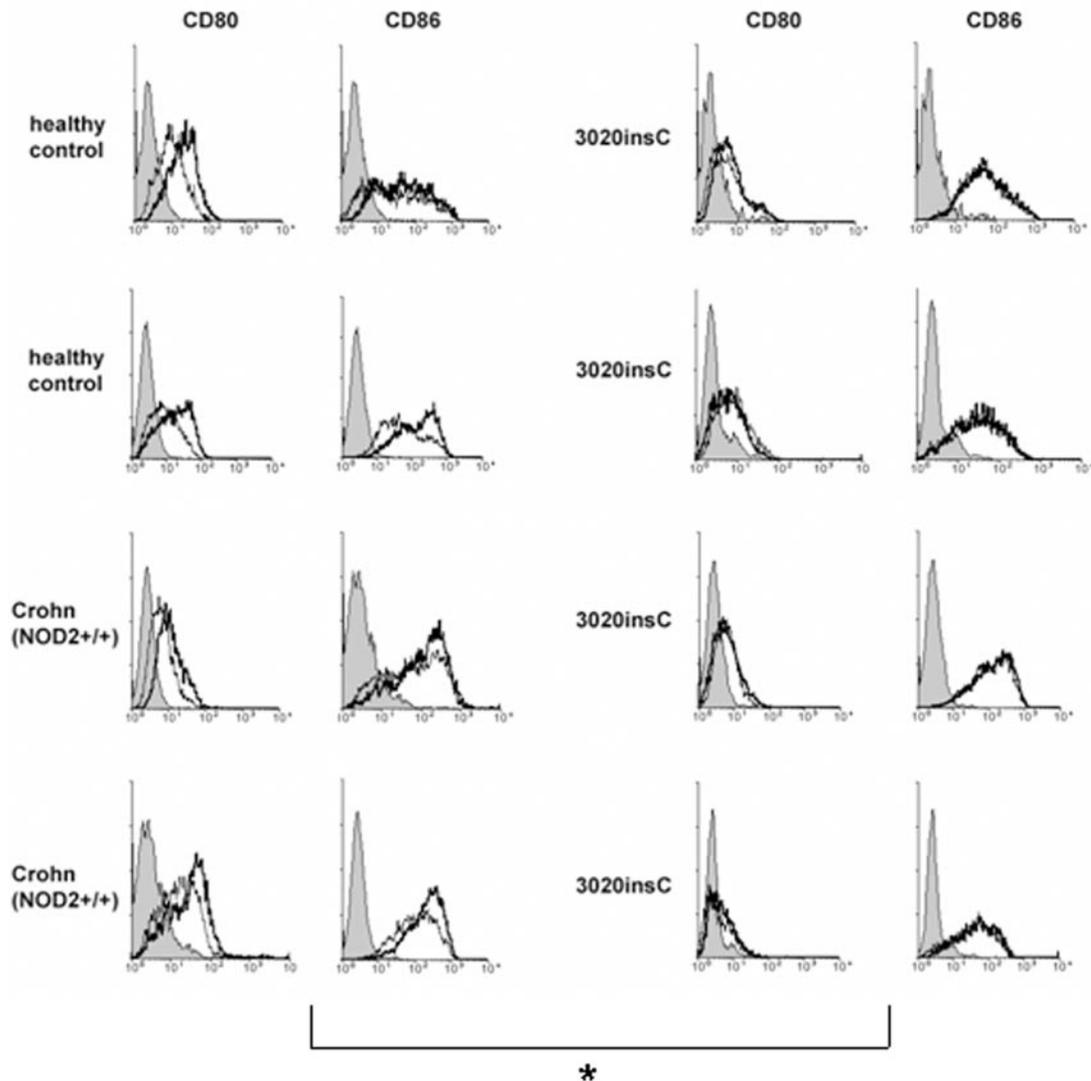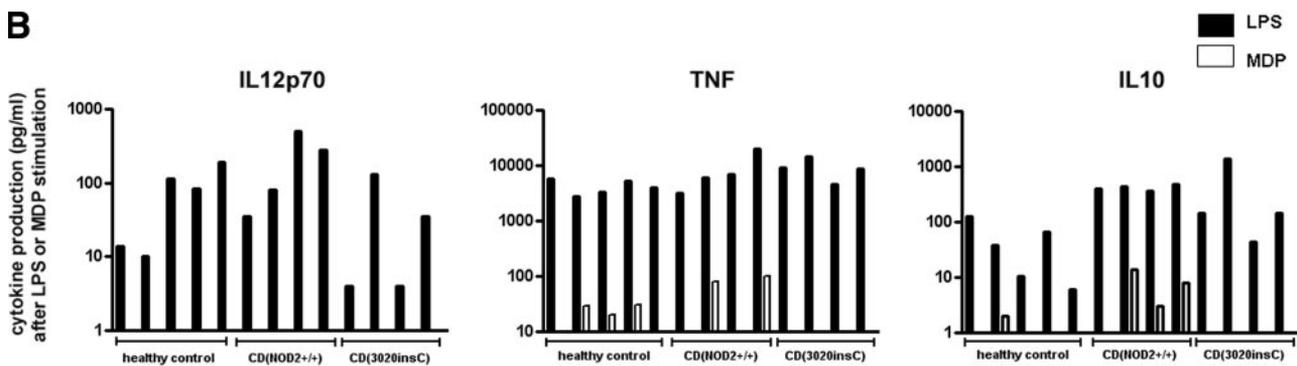
Fig. 2. NOD2fs DC show no maturation or cytokine production after stimulation with MDP. (A) Representative examples of the expression of CD80 and CD86 on DC from healthy controls (n=5), NOD2$^{+/+}$ CD patients (n=4), or NOD2fs CD patients (n=4) after stimulation with 5 μg/ml N-acetylmuramyl-L-alanyl-D-isoglutamine hydrate (MDP) for a period of 24 h. Medium control and MDP stimulation are indicated by thin and thick lines, respectively. Filled histogram represents isotype-matched, control mAb. Increase in MFI of CD80 and CD86 after MDP stimulation was calculated. Differences between DCs from donors with intact NOD2 and NOD2fs DC were compared using Student's t-test (*, P<0.05). (B) IL-12, TNF-α, and IL-10 levels (pg/ml) in culture supernatant, comparing stimulation of cells with 100 ng/ml-purified LPS or 5 μg/ml MDP for a period of 24 h. No IL-12 could be detected after stimulation with MDP, whereas some, but not all, control DC responded with the production of low levels of TNF-α or IL-10.

shown in **Figure 3A**, IL-12 was undetectable after MDP stimulation, alone or in combination with IFN-γ. In contrast, TLR4 activation using LPS resulted in an appreciable amount of IL-12 (120 pg/ml), which was further enhanced by IFN-γ. In the presence and absence of IFN-γ, additional stimulation with MDP resulted in a significant increase in IL-12 as compared with LPS or LPS/IFN-γ alone. Thus, the reported synergy between TLRs and NOD2 for the production of cytokines also applies for IL-12 release by human DC.

Next, we studied the consequences of the NOD2fs mutation on cytokine production following combined NOD2/TLR stimulation in DC. Cells were treated with LPS, Pam$_3$Cys, or poly
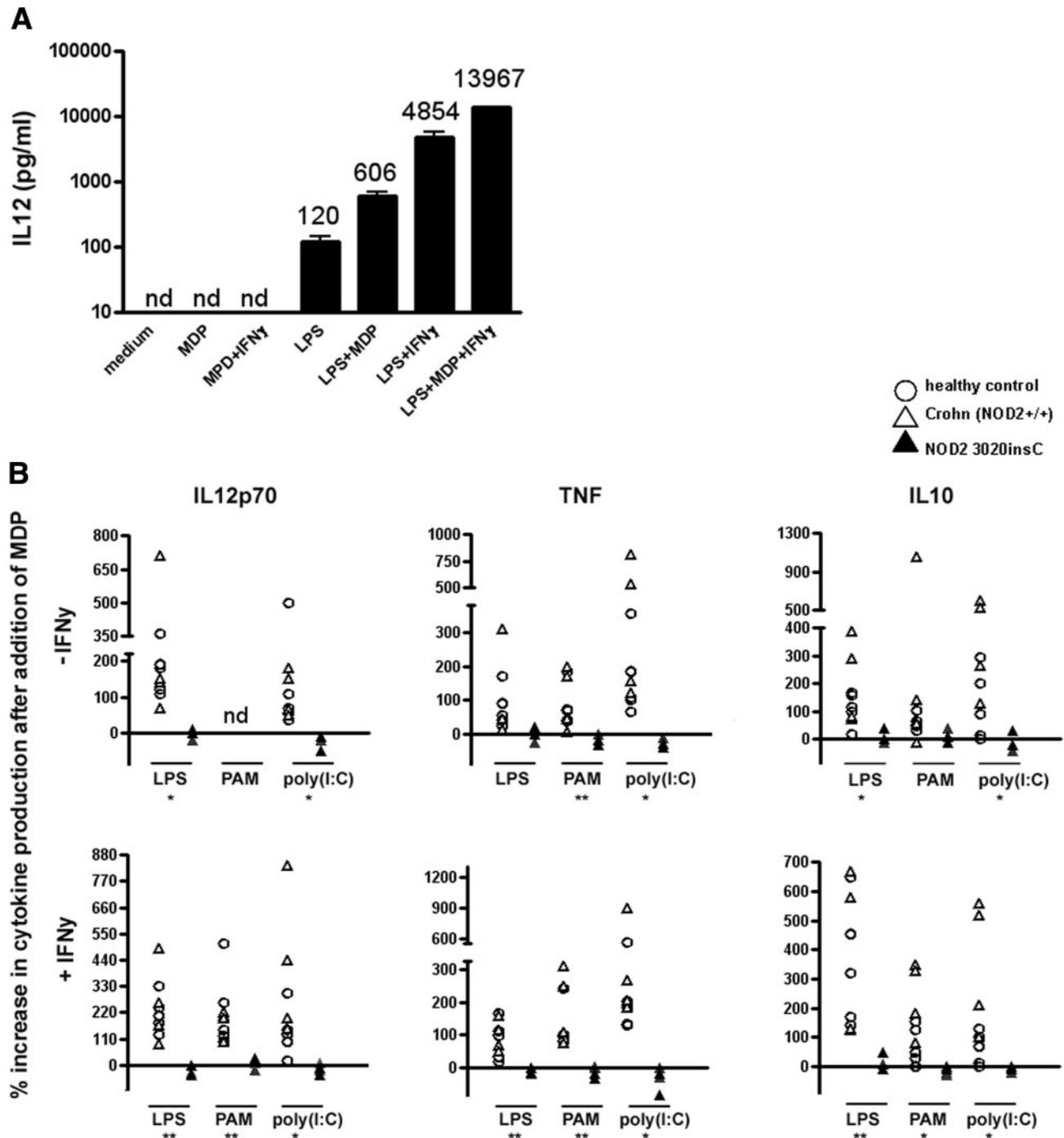

Fig. 3. The NOD2fs mutation abrogates the synergy between TLR and NOD2 signals for release of cytokines. (A) IL-12 production by DC from healthy controls was measured after stimulation with 5 μg/ml MDP, 100 ng/ml purified LPS, or a combination of both in the presence or absence of 400 U/ml IFN-γ. Data shown are the mean + SD of triplicate measurements of one of four independent experiments. nd, Not detectable. (B) IL-12, TNF-α, and IL-10 levels were measured following stimulation of DC from healthy controls (n=5), NOD2$^{+/+}$ CD patients (n=4), or NOD2fs CD patients (n=4) with 100 ng purified LPS, 10 μg/ml Pam$_3$Cys (PAM), or 20 μg/ml poly(I:C), alone or in combination with 5 μg/ml MDP for 24 h. Experiments were performed without (upper panel) or with (lower panel) additional IFN-γ stimulation. Displayed is the percentage increase in cytokine production as a result of the addition of MDP compared with TLR stimulation alone. Differences in the observed increases in cytokine levels between control DC and NOD2fs DC were analyzed using Student's *t*-test (*, $P<0.05$, and **, $P<0.01$).

(I:C) in the presence or absence of IFN-γ, with or without additional stimulation with MDP. IL-12, TNF-α, as well as IL-10 production was measured. Results are depicted as the percent increase in cytokine levels, comparing TLR activation alone with TLR activation combined with MDP (Fig. 3B). In control DC, stimulation with TLR ligands in the presence of MDP resulted in a significant increase in IL-12, IL-10, and TNF-α levels compared with TLR activation alone, although this effect was not significant for all cytokines measured or all TLR ligands used. The synergistic effect of MDP stimulation on TLR-induced cytokine release was completely absent in DC from NODfs patients. In the presence of IFN-γ, there was a statistically significant difference for all cytokines measured after stimulation with the selected TLR ligands and MDP, indicating that in a proinflammatory Th1 environment, the effect of NOD2fs could become even more apparent.

Together, these results demonstrate that the 3020insC mutation renders DC unable to mature in response to MDP and abrogates the synergy between NOD2 and TLRs for the production of pro- and anti-inflammatory cytokines.

DISCUSSION

CD is associated with high levels of the NF-κB-induced cytokines IL-12 and TNF-α, and mAb against these cytokines are effective therapies for CD [18–20]. It is striking that decreased NF-κB activation and defective cytokine responses have been reported in human and mouse studies as a consequence of the CD-associated NOD2fs mutation, providing evidence for a loss-of-function phenotype [1, 8, 21, 22]. However, two recent studies in mice challenged this view [6, 7]. Watanabe and colleagues [6] suggested that NOD2 is a negative regulator of TLR2-induced Th1 responses, as measured by IL-12 production. Wild-type cells stimulated with the TLR2 ligand Pam$_3$Cys plus MDP displayed lower IL-12 production than cells from NOD2$^{-/-}$ mice, which were stimulated similarly [6]. In addition, Maeda et al. [7] developed a mouse model in which the NOD2fs variant was introduced into the mouse NOD2 locus and showed that mutant mice exhibited elevated NF-κB activation and IL-1β secretion following MDP stimulation, suggesting that the NOD2fs mutation would lead to a gain-of-function phenotype. This discrepancy between recent mouse and human data published necessitates further studies in, preferentially, human subjects. A lot of studies have used transfected human cell lines, but in these systems, NOD2 expression is well above physiological levels with unknown outcome. Also, the use of (patient-derived) peripheral blood mononuclear cells (PBMC) complicates identification of the effect of NOD2fs in specific cell types within this mixed population.

DC are present in the intestinal mucosa, where they sense the content of the intraluminal bacterial milieu, and they are crucial in the decision process between tolerance and immunity [12, 23]. We therefore decided to determine the consequence of the NOD2fs mutation in myeloid DC obtained from patients homozygous for this NOD2 polymorphism. Our results indicate that intact NOD2 is essential for the recognition of MDP by human DC and support the view that the NOD2fs

mutation in CD patients results in a loss-of-function phenotype in these cells. To avoid the unwanted effect of possible contamination with, for instance, endotoxins, we used only synthetic or highly purified TLR and NOD2 ligands during our study. DC from CD patients bearing the NOD2fs mutation responded with a normal up-regulation of the costimulatory molecules CD80 and CD86 after TLR activation but failed to do so in response to MDP. The production of IL-12p70, TNF-α, and IL-10 was not significantly different between DC from control and NOD2fs DC after TLR activation. MDP induced low cytokine responses in some but not all DC carrying intact NOD2, and NOD2fs DC did not produce any cytokines following MDP stimulation alone. Finally, in control DC, stimulation with TLR ligands plus MDP enhanced the production of IL-12, TNF-α, and IL-10 compared with those levels obtained following TLR activation alone. This synergy between NOD2 and TLRs was completely absent in DC obtained from patients with the NOD2fs polymorphism. These results are in concordance with the recent findings by van Heel et al. [22], who showed that PBMC from homozygous NOD2fs donors display significantly decreased TNF-β and IL-1β production following combined activation of TLRs and NOD2.

In the presence of IFN-γ, which is known to increase NOD2 and TLR expression and function [24–26], the observed differences were even more significant. The absence of an increase in IL-12 production by simultaneous TLR2 and NOD2 activation in NOD2fs DC is at variance with findings reported in NOD2-deficient mice [6]. The reason for this discrepancy is currently unknown but might be related to differences between the NOD2fs mutation and complete absence of NOD2 or (species-related) differences between human DC and mouse splenocytes. In addition, other mutations in NOD2 might have different consequences, for instance, in the case of Blau syndrome [27].

The inflammatory phenotype found in CD patients is hard to explain by a decreased TNF-α and IL-12 production by NOD2fs DC after combined TLR/NOD2 activation. However, we now demonstrate that NODfs DC also produce decreased amounts of the anti-inflammatory cytokine IL-10 under these conditions. IL-10 is crucially involved in preventing excessive immune responses, including down-regulation of IL-12 and TNF-α production [28]. We therefore postulate that deficient IL-10-mediated immune suppression is dominant over the reduction in TNF-α and IL-12 levels, which is also observed after TLR/NOD2 stimulation in NOD2fs DC. As DC instruct the adaptive immune system via the release of specific cytokine combinations, a disturbed organization of this cytokine production could induce unwanted T cell responses. Indeed, studies using therapeutic IL-10 administration or IL-10-deficient mice have demonstrated the importance of this cytokine in preventing mucosal inflammation [29, 30]. Furthermore, IL-10 is a multifunctional cytokine that is also crucial for the development of suppressor T cells, which are involved intricately in controlling intestinal immune responses [31–33]. NOD2fs-related reduction in IL-10 levels could thus result in a defective counter-regulation of the effect of proinflammatory cytokines with concomitant Th1 responses and thereby contribute to the perpetuation of chronic inflammation characteristic for CD.

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