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ORIGINAL ARTICLE

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Consequence of functional *Nod2* and *Tlr4* mutations on gene transcription in Crohn's disease patients

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Abstract The concept that mutations in germ-line encoded pattern recognition receptors with immune activating functions are associated with an increased incidence in Crohn's disease (CD) is gaining acceptance. Whether these mutations have similar or distinct effects on cellular physiology remains obscure. The incidence of three single nucleotide polymorphisms (SNPs) within the *Nod2* gene and one functional SNP within both the *Tlr4* and *Tlr5* gene in a Dutch cohort of 637 patients with inflammatory bowel disease and 127 controls was investigated. The functional consequence of mutant NOD2 and TLR4 was investigated by comparing gene expression profiles after stimulation of monocyte-derived dendritic cells (DCs) from homozygous TLR4- and NOD2-mutant patients with lipopolysaccharides and peptidoglycan, respectively. We observed that the R702W and 1007fs *Nod2* alleles and the A299G *Tlr4* alleles were significantly more prevalent in patients with CD as compared to healthy controls or patients with ulcerative colitis. The phenotype of TLR4- and NOD2-mutant DCs is distinct, but a large number of genes are up- or down-regulated concordantly. These data provide a concept for the genetic basis of CD; mutations in innate immunity cause similar effects on gene transcription and finally result in comparable clinical disease presentation.

Keywords Crohn's disease · Dendritic cells · NOD2 · TLR4

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Introduction

Clinical observations and experiments with genetically modified animals have demonstrated that inflammatory bowel disease (IBD) results from a deregulated mucosal immune response directed against the normal commensal flora in a

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genetic susceptible host [1]. Familial aggregation, concordance in monozygotic twins and ethnic differences strongly suggest the involvement of genetic factors in the development of IBD. Genetic predisposition was further substantiated with the discovery of susceptible loci on chromosome 5, 14 and 16 for Crohn's disease (CD), on chromosome 12 for ulcerative colitis (UC) and on chromosome 1, 6 and 19 for both CD and UC [2]. Three single nucleotide polymorphisms (SNPs), two missense variations and one frameshift variation in the *Nod2* gene are located within the IBD1 locus on chromosome 16 and independently associated with the development of CD [3, 4]. The sequence changes encoded by these three main variants are SNP 8 (R702W), SNP 12 (G908R) and SNP 13 (1007fs), especially the latter mutation is of interest because it leads to a truncated and non-functional protein [5]. Nod2 belongs to the class of pattern recognition receptors (PRRs) of the innate immune system that recognize evolutionary conserved pathogenic motifs or pathogen-associated molecular patterns (PAMPs). Although the amount of PAMPs recognized by PRRs is relatively limited, their expression by dentritic cells (DCs) is crucial for the generation of adaptive immune responses [6]. The subsequent recognition by specific antigen receptors on lymphocytes mediates tailored immune responses against pathogens, resulting either in immune activation or immune tolerance depending on the encountered pathogen and local environment. Different groups of PPRs exist, including the Nod family and the more extensively studied toll-like receptor (TLR) family [7, 8]. Both families of receptors share the ability to recognize PAMP and initiate signal transduction events that lead to transcription of specific genes and eventually to appropriate innate and adaptive immune responses [9]. Both TLR4 and TLR5 receptors recognize PAMPs of gram-negative bacteria, and for both genes, SNPs with functional consequences have been reported [10–12]. A possible role for TLR4 polymorphisms in IBD is further suggested by the observation that mice with a missense mutation of the *Tlr4* gene have a high incidence of spontaneous colitis [13, 14], and an association of the TLR4 Asp299Gly polymorphism with both CD and UC has been observed [15]. Hence, a picture is emerging that mutations in PAMP signalling in general associated with IBD and diminished PAMP signalling itself may be a contributing factor to the development of disease. If this notion is true, such mutations should be widespread in different IBD populations, and the functional consequences of these mutations should be similar. These considerations prompted us to investigate the prevalence of known SNPs in *Tlr4* and *Tlr5* in IBD and to address the functional consequences of these SNPs after PAMP-mediated signal transduction of DCs.

Methods

Study population

The Medical Ethical Committee of the Academic Medical Center in Amsterdam approved the collection and analysis of DNA from patients and controls. After informed consent, peripheral blood samples were obtained at the outpatient IBD clinic of the Academic Medical Center; DNA was isolated from peripheral blood according to routine procedures and stored in the IBD gene bank of the Academic Medical Center [16]. Control genomic DNA was isolated from peripheral blood of healthy personnel of the Academic Medical Center. The clinical diagnosis was confirmed using conventional clinical, radiologic, endoscopic and histologic criteria. For this study, 411 consecutive DNA samples from CD patients, 226 consecutive DNA samples from UC patients and 137 consecutive DNA samples from controls were screened for the presence of the SNPs described below. Samples from patients with indeterminate colitis were excluded from the study population. The mean age of all CD patients is 40.7 years and 65% is female; the mean age of all UC patients is 44.4 years and 47% is female.

Genotyping

Polymerase chain reaction (PCR) restriction fragment-length polymorphism assay was used for genotyping three SNPs within the *Nod2* gene (R702W, G908R and 1007fs), one SNP within the *Tlr4* gene (A299G) and one SNP within the *Tlr5* gene (N392ST, followed by a stop codon). Genomic DNA was amplified using primers that were chosen to create a different restriction site in the mutant allele (G908R, 1007fs and A299G) and wild-type allele (R702W) (Table 1). The amplified product was digested overnight with the indicated restriction enzymes and fragments separated on a 3% agarose gel.

Dendritic cell culture, final maturation and RNA isolation

All cultures were performed in Iscove's modified Dulbecco's medium with 1% fetal calf serum (FCS) (HyClone,

Table 1 Primers and restriction enzymes used for rflp-PCR of indicated SNPs

SNP	Primers	Restriction enzyme
R702W	GCACAACCTTCAGATCACAGCA GCTGGCGGGATGGAGTGGAAG	Msp1
G908R	CAGTGAGGCCACTCTGGGATTG AAAACTGCAGGATAGACTCT	Cfo I
3020Cins	ATGTGTCTAAGGGACAGGTGAA CTGAGGTTCGGAGAGCTA	Nla IV
A299G	GATTAGCATACTTAGACTACTAC CTCCATGGATCAACTTCTGAAA AAGCATTCCCAC	Cfo I
N392ST	CGAAATTTCTATGGACTATTCTC CAAGGAAAAGCTGTTCTA	Dde I

Lagan, UT, USA) and gentamycin (86 mg/l; Sigma, St. Louis, MO, USA). Peripheral blood of patients with homozygous inactivating mutations of NOD2 and TLR4 was used to generate immature DCs; all patients were in remission at the time of inclusion. Monocytes were isolated after Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and Percoll (Amersham Biosciences AB, Uppsala, Sweden) gradient centrifugation, and 2×10^6 monocytes were cultured in the presence of recombinant human (rhu) granulocyte–macrophage colony-stimulating factor (GM-CSF) (500 U/ml; Schering-Plough, Uden, The Netherlands) and rhu IL-4 (250 U/ml; Pharma Biotechnology). After 6 days, DCs were stimulated for 4 h with peptidoglycan (100 ng/ml; Fluka, Buchs, Switzerland) or lipopolysaccharide (100 ng/ml; Sigma), respectively. Subsequently, DCs were dissolved in 2 ml of Trizol (Life Technology, Gaithersburg, MD, USA). One milliliter of chloroform was added to the RNA–Trizol solution, vortexed for 15 s and incubated for 3 min at room temperature. After centrifugation [15 min at 40,000×*g* (4°C)], the upper layer was mixed with 2.5 ml of isopropyl alcohol and incubated for 10 min at room temperature. Subsequently, the samples were centrifuged at 25.000×*g* for 10 min (4°C), and the pellet was washed with 5 ml of 75% alcohol and dried. Finally, the pellet was dissolved in 20 μl of water and incubated for 10 min at 60°C.

DNA micro-arrays

For investigating the DC function, a purpose-designed DNA micro-array was employed, for which 347 inflammatory-relevant human cDNA clones were obtained from Research Genetics (Inchinnan, Scotland). Each cDNA clone was individually sequence-verified in our laboratory and spotted in triplicate on hybond N+ filter (Amersham, Buckinghamshire, UK); in addition, a glyceraldehyde phosphate dehydrogenase (GAPDH) clone was spotted as an invariant control. The quality of isolated RNA was assessed by visual inspection of the ribosomal RNA bands after gel electrophoresis. Five microgram of total RNA was used as template to produce radioactive-labelled probes, and water was added to the sample to get a start volume of 13 μl. Then, 2 μl of Oligo dT-primer (Amersham) was added to the solution before incubating the mixture at 70°C for 10 min. After 2 min on ice followed by an addition of 6 μl ×5 first-strand buffer, 1 μl 0.1 M DTT, 1.5 μl dNTPs (16 mM dCTP, dGTP, dTTP, 100 um dATP) and 8 μl of ^{33}P α dATP 2500 ci/mmol (Amersham), the mixture was incubated for 5 min at 42°C. Next, 2 μl of Superscript RT (Gibco BRL, Cheshire, England, UK) was added to the mixture and incubated at 42°C for 1 h. Subsequently, 1 μl of Superscript RT was added, and the mixture was incubated for another hour to complete the reaction. Non-incorporated nucleotides were removed before the filter was prehybed for at least 2 h in 20 ml of hybmix (5× SSC, 5× Denhardts, 0.5% SDS). Then, the array was exposed to a radioactive-labelled probe for 72 h at 65°C, the hybridisation mix was discarded and the array was washed once with buffer 1 (2× SSC, 0.1% SDS) and twice with buffer 2 (0.2 SSC, 0.1% SDS) at 65°C for an hour. Subsequently, the array was placed between two plastic sheets and exposed to a photosensitive plate for 48 h before it was analysed by a Phosphor Imager 2.0 (PE Applied Biosystems, Foster City, CA, USA). The photos of the radioactive spots were imported into AIDA Array Metrix (Raytest, Straubenhardt, Germany) to measure the signal of the spots; the average and standard deviation of every triplet were calculated. Subsequently, the micro-arrays were normalized to account for overall differences in spot intensities due to variability in experimental conditions. The arrays were normalized by adjusting every intensity measure according to a scaling factor that was derived from the intensities of GAPDH on different arrays. GAPDH is assumed to be unaffected by stimulation, and therefore, the small differences observed across arrays reflect variability in experimental conditions. After normalization, the difference in gene expression between unstimulated and stimulated DCs was determined.

Multiplex ligation-dependent probe amplification

To validate the DNA micro-array, mRNA expression of MIP-1β and IL-1β was analysed using a multiplex ligation-dependent probe amplification (MLPA) as described by Spek et al. [17]. In short, total RNA was used to prepare cDNA with gene-specific reverse transcriptase (RT) primers; total RNA (10–50 ng) was mixed with RT buffer (final concentration 50 mM Tris–HCL pH 8.5, 75 mM KCl, 3 mM MgCl2, 10 mM DTT), dNTPs (2.5 nmol) and 500 fmol of each gene-specific RT-primer (MRC-Holland, Amsterdam, The Netherlands, http://www.mrc-holland.com). Subsequently, samples were heated for 1 min at 80°C, incubated for 5 min at 45°C and for 15 min at 37°C with 6 U MMLV RT (Promega, Leiden, The Netherlands). Then, relative amounts of each cDNA were determined by MLPA using cDNA-specific probes that overlap an exon boundary; after heat inactivation of the RT by incubation at 98°C for 2 min, 1.5 μl of probe mix containing 0.5–4 fmol of each individual synthetic MLPA probe oligonucleotide (MRC-Holland) and 1.5 μl of MLPA hybridization buffer [1500 mM KCl, 300 mM Tris–HCl pH 8.5, 1 mM ethylenediaminetetraacetic acid (EDTA)] was added. Next, the hybridization mixture was heated for 1 min at 95°C min and incubated for 16 h at 60°C; subsequently, ligation of hybridized probes was performed for 15 min at 54°C after the addition of 31 μl ligation mixture [2.6 mM MgCl2, 5 mM Tris–HCl pH 8.5, 0.013% v/v non-ionic detergents, 0.2 mM nicotinamide adenine dinucleotide (NAD), 1 U Ligase-65 (MRC-Holland] [18]. Finally, after the inactivation of Ligase-65 by incubating the samples for 5 min at 98°C, the ligation products were amplified by PCR (Applied Biosystems, Warrington, UK).

Table 2 Genotype and allele frequencies of NOD2 SNPs in Dutch cohorts of CD patients, UC and HCs

Group	Compound mutant (%)	Compound/ homozygous mutant (%)		1 (wild type) 2 (mutant)	R702W (%)	G908R (%)	1007fs (%)
HC *n*=137	1.5 (*n*=2)	2.2 (*n*=3)	Genotype	1 1	93.4	90.5	94.2
				1 2	5.8	9.5	5.8
				2 2	0.7	–	–
			Allele	1	96.4	95.3	97.1
				2	3.6	4.7	2.9
CD *n*=411	3.9 (*n*=17)	7.4 (*n*=30)[?]	Genotype	1 1	82.3[†]	90.7	87.2
				1 2	15.7[†]	8.4	12.3
				2 2	2.0[†]	1.0	0.5
			Allele	1	90.2[§]	94.8	93.4*
				2	9.8[§]	5.2	6.6*
UC *n*=226	–(*n*=0)	2.7 (*n*=6)	Genotype	1 1	88.5	96.0[‡]	93.8
				1 2	8.8	4.0[‡]	6.2
				2 2	2.7	–	–
			Allele	1	92.9	98.0[#]	96.9
				2	7.1	2.0[#]	3.1

[?] χ^2=4.8, p=0.028 compared to HC, RR 3.5, 95 %Cl 1.07–11.84
[†] χ^2=10.0, p=0.007 compared to HC
[§] χ^2=10.3, p=0.001 compared to HC
* χ^2=5.3, p=0.022 compared to HC
[‡] χ^2=4.5, p=0.033 compared to HC
[#] χ^2=4.4, p=0.036 compared to HC

Statistics

Case–control association analyses were performed using the χ^2 statistics (SPSS 11.5.2, SPSS Inc., Chicago, IL, USA) and odds ratios (ORs) were calculated. Genotype ORs were estimated based on control genotype frequencies calculated assuming Hardy–Weinberg equilibrium, and a 95% confidence interval (CI) was constructed according to Woolf: $\sqrt{(1/\mathrm{AA}+1/\mathrm{GG}+1/g+1/a)}$ and 95% lower interval=$2^{(\ln -3.09\times\mathrm{var})}$ [18], in which AA and GG are total homozygous and wild-type individuals and a and g are total mutant and wild-type alleles. To determine whether expression of genes measured in the mutant DCs correlated, we plotted every gene in a two-dimensional coordinate system reflecting the expression in the NOD2 and TLR4 mutants. Subsequently, the Spearmann rank correlation was calculated on the intensities (SPSS) for genes that showed a positive correlation and genes that showed a negative correlation.

Results

Incidence of the R702W, G908R and 1007fs mutations of the *Nod2* gene

The *Nod2* gene variants R702W, G908R and 1007fs were determined in 137 healthy controls (HCs), 411 CD patients and 226 UC patients; patients with an indeterminate phenotypic colitis were not screened for these mutations. The NOD2 allele and genotype frequencies are summarized (Table 2).

The allele frequency of the R702W mutation was higher in CD patients (9.8%) compared to normal controls (3.6%) (χ^2=10.3, p=0.001); eight CD patients and one HC were identified as homozygous individuals for the R702W mutation. The allele frequency of the R702W mutation in UC patients (7.1%) was not significantly changed compared to HCs or CD patients (Table 2). In CD patients, the allele frequency for the G908R mutation (5.2%) was not significantly different from the allele frequency of HCs (4.7%); however, four homozygous individuals were present in the CD group, with none in the HCs. In the cohort of UC patients, the allele frequency of the G908R mutation was significantly lower compared to HCs (2.0% for UC and 4.7% for HC; χ^2=4.4, p=0.036), and no homozygous UC patients were present (Table 2). Finally, the allele frequency of the 1007fs mutation in CD patients appeared to be significantly increased compared to HCs (6.6% for CD and 2.9% for HC; χ^2=5.3, p=0.022), and no homozygous HCs were identified while two CD patients had the 1007fs mu-

Table 3 Genotype and allele frequencies of TLR4 (A299) and TLR5 (N392ST) SNPs in Dutch cohort of CD patients, UC patients and HCs

Group (*n*)		1 (wild type) 2 (mutant)	A299G (%)	N392ST (%)
HC (*n*=137)	Genotype	1 1	90.5	83.2
		1 2	9.5	15.3
		2 2	–	1.5
	Allele	1	95.3	90.9
		2	4.7	9.1
CD (*n*=411)	Genotype	1 1	84.9	88.6
		1 2	13.6	11.2
		2 2	1.5[§]	0.2
	Allele	1	91.7	94.2
		2	8.3	5.8
UC (*n*=226)	Genotype	1 1	88.9	87.2
		1 2	10.6	12.4
		2 2	0.4	0.4
	Allele	1	94.6	93.4
		2	5.4	6.6

[§] OR 6.26 95%Cl=1.50-26.0

Table 4 Disease phenotype and genotype of NOD2 (R702W, G908R and 1007fs) and TLR4 (A299G) SNPs form CD patients

Group (*n*)	Genotype 1 (wild type) 2 (mutant)	Upper GI (%)	Ileum (%)	Ileocolonic (%)	Colon (%)	Fistula (%)
wild type (171)	1 1	52.0	17.5	46.8	25.1	25.1
R702W (61)	2 2	55.6	16.7	66.7	16.7	11.1
	1 2	48.0	22.4	53.1	24.5	18.0
G908R (31)	2 2	0	0	100.0	0	0
	1 2	50.0	26.1	47.8	26.1	15.0
1007fs (47)	2 2	33.3	0	100.0	0	0
	1 2	45.5	19.5	41.5	39.0	24.2
A299G (53)	2 2	33.3	40.0	40.0	20.0	50.0
	1 2	35.7	9.3	48.8	41.9	21.4

tation in both alleles. UC patients did not have more mutant alleles than HCs (3.1% for UC), and no homozygous UC patients were identified (Table 2). In total, 17 CD patients were compound heterozygous for the *Nod2* gene, compared to zero UC patients and two HCs. Thirty CD patients (7.4%), six UC patients (2.7%) and three HCs (2.2%) had a compound or homozygous risk allele. Mutations in the *Nod2* gene were associated with an increased risk for CD (χ^2=4.8, p=0.028; OR 3.5, 95% CI 1.07–11.84) and not for suffering from UC.

Incidence of the A299G substitution of the *Tlr4* gene and the N392ST substitution of the *Tlr5* gene

We screened our IBD gene bank for one functional SNP of the *Tlr4* gene (A299G) and one functional SNP of the *Tlr5* gene (N392ST) using rflp-PCR (Table 3); again, patients with indeterminate colitis were not screened.

The allele frequency of the A299G mutation was higher in CD patients (8.3%) compared to HCs (4.7%) and UC patients (5.4%). Six CD patients were homozygous for the A299G mutation compared to zero in the HCs (OR 6.26, 95%CI 1.50–26) and one in the UC population (OR 1.81, 95%CI 0.10–32) (Table 3). It appeared that heterozygous mutations of the A299G mutation did not increase the risk for developing CD (13.6% for CD and 9.5% for HC). The allele frequency of the N392ST mutation of the *Tlr5* gene was higher in HCs than in CD or UC patients (5.8% for CD, 6.6% for UC and 9.1% for HC); therefore, the presence of this mutation does not increase the likelihood of suffering from IBD. Phenotypic characteristics from CD patients, including disease localization and the presence of fistulizing complications, were extracted from the IBD gene bank and linked to the R702W, G908R and 1007fs SNPs of the *Nod2* gene and to the A299G SNP of the *Tlr4* gene (Table 4).

Patients with NOD2 mutations were prone to have ileocolonic disease (50.4% for wild-type patients and 66.7, 100 and 100% for the R702W, G908R and 1007fs homozygous patients, respectively). Finally, patients with the A299G SNP more often had isolated ileal involvement (19.9% for wild-type patients and 40.0% homozygous patients) or fistulizing disease (23.0% for wild-type patients and 50.0% for homozygous patients).

Responses of dentritic cells from patients with a NOD2 and TLR4 mutation to PGN and LPS

The observation that both *Nod2* and *Tlr4* gene mutations are independently associated with CD raised the question whether such mutations have similar effects on immune activation after appropriate stimulation. Hence, we investigated the effect of such mutations on inflammatory gene expression in NOD2 and TLR4 mutant DCs, respectively. These cells express a variety of germ-line PRRs, including NOD2 and TLR4, and are crucial in generating adaptive T cell responses. To this end, DCs were generated from three CD patients who were homozygous for the 1007fs mutation in *NOD2* and two CD patients who were homozygous for the A299G mutation in *TLR4* (Table 5).

After stimulation with ligands for NOD2 and TLR4 (PGN and LPS, respectively), the changes in gene tran-

Table 5 Disease phenotype and genotype of patients involved in gene array experiment

Patient ID	Phenotype						Genotype			
							1 (wild type)		2 (mutant)	
	Sex	Age	Disease duration	Disease localization	Medication	Surgery	R702W	G908R	1007fs	A299G
1	Male	24	7	Perianal	Azathioprine	No	11	11	11	22
2	Male	45	13	Ileocolonic	Azathioprine	No	11	11	11	22
3	Female	39	18	Ileocolonic	None	Yes	11	11	22	11
4	Female	36	8	Ileocolonic	Enterocort	Yes	11	11	22	11
5	Female	36	13	Ileum	Azathioprine	No	11	11	22	11

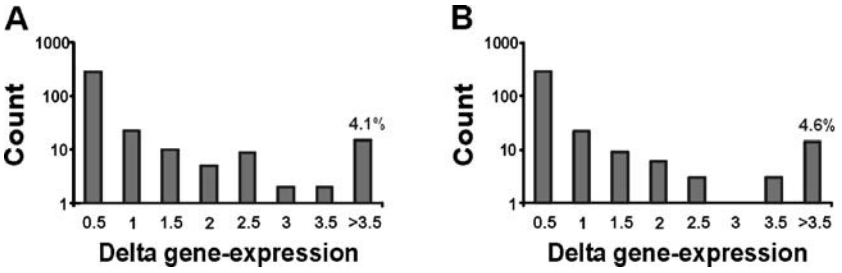

Fig. 1 Delta gene expression of NOD2 and TLR4 mutant DCs. After excluding unreliable spots and normalization for the expression of GAPDH, the difference in gene-expression between unstimulated and stimulated NOD2 (**a**) and TLR4 (**b**) mutant DCs was calculated and plotted in a histogram. It appeared that 4.1% of all genes from PGN-stimulated Nod2-mutated DCs were more than three times up-regulated, whereas the corresponding number for LPS-stimulated Tlr4-mutated DCs was 4.6%, respectively

scription were assessed using a purpose-designed DNA micro-array. After 4 h of stimulation, RNA was isolated as described in the experimental procedures, and the gene expression profile was compared to non-stimulated DCs of the same donor. An appreciable subset of genes appeared to be more than 3.5 times up-regulated compared to the unstimulated condition in both NOD2- and TLR4-mutated DCs (4.1% and 4.6% for NOD2 and TLR4 mutant DCs, respectively; Fig. 1), and thus, the mutations do not render the cells unresponsive for these conserved microbial motifs.

The effects on gene transcription were confirmed employing an RT-MLPA procedure, which was shown earlier to be a successful tool for quantifying the effects of PRR activation on inflammatory gene expression [19]. It appeared that the effects on MIP-1β and IL-1β gene expression as detected by the DNA array corresponded closely with the effects determined by MLPA (Fig. 2).

NOD2 mutations and TLR4 mutations have common and distinct effects on gene expression

We next investigated differences in ligand-induced gene expression between NOD2- and TLR4-mutated DCs. To this end, aggregate normalized expression was calculated, and we ranked the genes according to their difference in gene expression, comparing unstimulated and stimulated DCs. Genes that were up-regulated by stimulation were assigned a positive hierarchic rank, and genes that were down-regulated were assigned a negative hierarchic rank. This approach allowed us to determine whether regulation of gene expression in NOD2- and TLR4-mutated DCs was concordant. The results demonstrated that the genes expressed in NOD2- and TLR4-mutated DCs can be roughly divided in two groups of positively and negatively correlated genes. Nevertheless, overall, a positive correlation is observed (Fig. 3; r=0.350, p<0.01). A large group of genes was co-regulated, the rank number either going up (Table 6) or going down in both NOD2- and TLR4-mutated cells after stimulation (Fig. 3; r=0.889, p<0.01). In addition, a second substantial set of genes displayed negative correlation when rankings of NOD2 and TLR4 mutant cells were compared (Fig. 3; r=−0.712, p<0.01). Hence, mutations in distinct PRR cause similar but not identical changes in stimulated DCs.

Discussion

We investigated the incidence of the three known SNPs of the *Nod2* gene in our IBD DNA cohort consisting of 137 HCs, 411 CD patients and 226 UC patients. The three mutations for NOD2 have been previously reported in

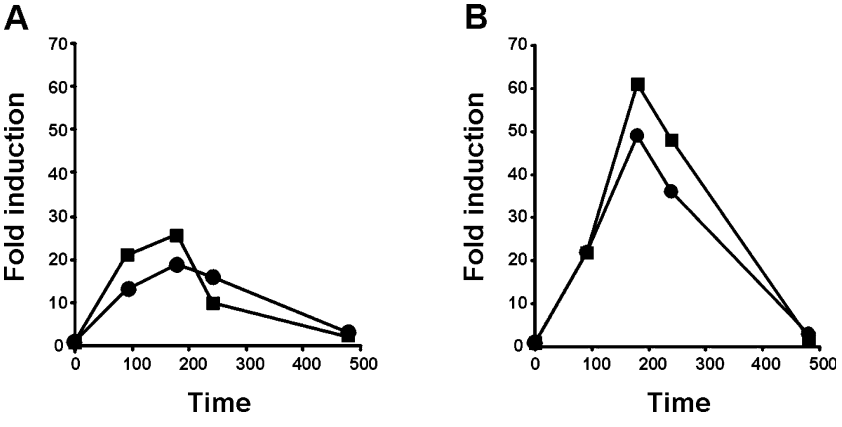

Fig. 2 Example of MLPA analysis. Peripheral blood from healthy volunteers was used to isolated peripheral blood mononuclear cells (PBMCs), which were subsequently stimulated with LPS (100 ng/ml) for 0, 90, 180, 240 and 480 min, respectively. Fold induction of IL-1β (**a**) and MIP-1β (**b**) mRNA is presented for DNA micro-array (*squares*) and MLPA (*rounds*). No significant difference exists for the results obtained between DNA micro-array and MLPA overtime for both IL-1β (p<0.57) and MIP-1β (p<0.20), using a single factor ANOVA with a two-factor ANOVA without replication 95% CI

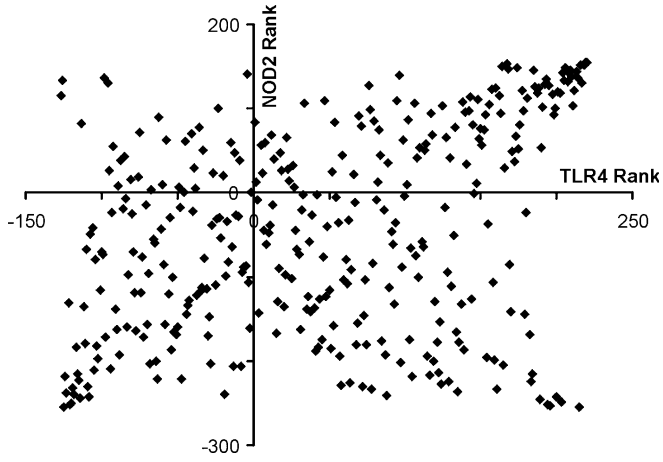

Fig. 3 Comparison of gene expression between NOD2 and TLR4 mutant DCs. The genes were given a rank according to the amount of difference in gene expression between the stimulated and unstimulated DCs. The highest rank was assigned to genes that showed the highest increase and vice versa for both the TLR4 mutant and NOD2 mutant DCs. Both the NOD2 and TLR4 ranking are normally distributed and a Spearman correlation of 0.350 exists between the NOD2 and TLR4 rank ($p<0.01$). Furthermore, correlation for genes in the first and third quadrant is 0.889 (Spearman, $p<0.01$) and for genes in the second and fourth quadrant is −0.712 (Spearman, $p<0.01$)

0% of Japanese and Chinese CD patients and up to 25% of CD patients in North America and Europe [19–21]. The observed incidence of NOD2 in our cohort of CD patients is lower than originally published but in agreement with more recent data from European countries [22, 23]. Hence, it is likely that other genes with similar function as NOD2 are involved in the pathogenesis of CD.

Inactivating mutations in the TLR4 gene have been associated with inflammatory conditions, and mice with an inactivating mutation of TLR4 suffer from spontaneous colitis [24, 25]. Therefore, we screened our IBD cohort for the frequency of the A299G mutation of the *Tlr4* gene,

Table 6 Mean intensity of three spots from non-stimulated TLR4 or NOD2 DCs was subtracted from the mean intensity of three spots from LPS-stimulated (TLR4 mutant) or PGN-stimulated (NOD2 mutant) DCs, respectively

TLR4	NOD2	Gene
56.13	23.57	MIP1β macrophage inflammatory protein 1 beta precursor
40.06	11.10	ICAM3 intercellular adhesion molecule 3 precursor (ICAM3)
19.70	7.77	IL1β interleukin-1 beta precursor 1 (IL-1beta); catabolin
14.02	7.92	CYP26A1 cytochrome P450, subfamily XXVIA, polypeptide I
18.65	1.61	RPML3 ribosomal protein, mitochondrial, L3

The difference in absolute intensityof TLR4 and NOD2 mutant DCs is depicted, together with the abbreviation and full description of the gene in question. The table shows the five genes being most upregulated in both TLR4 and NOD2 mutant DCs of Crohn's disease patients

showing that patients with a homozygous mutant allele for this SNP had an increase for suffering from CD, while an increased risk for UC is absent. CD is a complex hereditable disease of multiple genes, in which the intestinal microbial environment might be responsible for triggering disease. Therefore, it would be interesting to determine the co-segregation of the A299G mutation with other mutations of the Tlr4 gene (i.e. T399I or T46C).

Thus, CD but not UC seems to be associated with the inability to recognize conserved microbial motifs [26] and decreased activation of pro-inflammatory signal transduction events [5, 27, 28]. It is not clear how a decreased ability to recognize microbial motifs finally results in an uncontrolled mucosal inflammation, but it is possible that PRRs are important for both the clearance of pathogens and the induction of tolerogenic immune responses [29, 30]. Our data indicate that it is an oversimplified view to suggest that mutations in PRR always result in a predisposition to develop CD, as we demonstrate that mutations in the *Tlr5* gene are not more abundant in our CD cohort than in HCs. The mutation studied causes a truncation of TLR5 that is a receptor for flagellin and is associated with decreased cell signalling and increased susceptibility for *Legionella pneumophila* infections. Investigators have shown that serum immunoglobulin G levels against flagellin are increased in colitic mice [31]. However, this increase might be a normal immunological response during colonic inflammation resulting from increased permeability and bacterial translocation.

The A299G substitution of the *Tlr4* gene and the 1007 frameshift mutation of the *Nod2* gene locate to the leucine-rich repeats of both proteins. The leucine-rich repeats constitute the actual binding site for conserved microbial motifs, and mutations within these regions leave the protein non-functional and unable to induce signal transduction upon appropriate stimulation [32, 33]. Normally, both NOD2 and TLR4 activation lead to translocation of nuclear factor-kappa B (NF-κB) to the nucleus and specific gene transcription. However, the signal transduction events that lead to NF-κB translocation differ; TLR4-mediated NF-κB activation is mediated via TRAF-6 and IRAK-1, while NOD2 mediated NF-κB activation is mediated via RICK [9, 34]. Signal transduction events are often not limited to the activation of one transcription factor, and it might therefore be expected that also NF-κB independent genes will be influenced.

We decided to investigate the functional consequences of TLR4 and NOD2 mutations in DCs because there is increasing evidence that mucosal DCs are critically involved in the initiation of mucosal inflammation in CD [35]. DCs have been shown to have a mature phenotype in the lamina propria of IBD patients and are known to express a wide variety of PRRs including NOD2 and TLR4 [36]. Therefore, NOD2- and TLR4-mutated DCs from CD patients were stimulated with PGN and LPS, respectively. Differences in gene transcription between unstimulated and stimulated cells were analysed by micro-array, and subsequently, gene expression profiles of NOD2- and TLR4-mutated DCs were compared. Although different stimulations

were used, concordant effects on gene expression were observed and this observation might reflect comparable clinical presentation.

In conclusion, mutations in PRR are associated with comparable effects on gene expression and a higher risk for developing CD. Although a direct link between NOD2 and TLR4 mutations and the development of CD has not been shown, it is remarkable that these mutations do not occur in UC. A picture emerges in which mutations in a specific set of genes induce the development of comparable features of disease presentation via activation of a common signal transduction pathways. It is tempting to speculate that mutations in the innate immunity, especially in DCs, are a defining characteristic for the development of CD and not for the development of UC.

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