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Basic Study

***NOD2*- and disease-specific gene expression profiles of peripheral blood mononuclear cells from Crohn's disease patients**

Holger Schäffler, Maria Rohde, Sarah Rohde, Astrid Huth, Nicole Gittel, Hannes Hollborn, Dirk Koczan, Anne Glass, Georg Lamprecht, Robert Jaster

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Author contributions: Schäffler H, Rohde S and Jaster R designed the study; Huth A, Schäffler H and Lamprecht G took responsibility for patient care and follow-up; Rohde M, Rohde S, Hollborn H, Jaster R and Koczan D (microarray studies) performed the experiments; Gittel N, Huth A and Schäffler H collected the samples and performed the clinical characterization of the patients; Glass Ä performed the biostatistics; all authors analyzed the data; and Schäffler H and Jaster R wrote the manuscript.

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Abstract

AIM

To investigate disease-specific gene expression profiles of peripheral blood mononuclear cells (PBMCs) from Crohn's disease (CD) patients in clinical remission.

METHODS

Patients with CD in clinical remission or with very low disease activity according to the Crohn's disease activity index were genotyped regarding nucleotide-binding oligomerization domain 2 (*NOD2*), and PBMCs from wild-type (WT)-*NOD2* patients, patients with homozygous or heterozygous *NOD2* mutations and healthy donors were isolated for further analysis. The cells were cultured with vitamin D, peptidoglycan (PGN) and lipopolysaccharide (LPS) for defined periods of time before RNA was isolated and subjected to microarray analysis using Clariom S assays and quantitative real-time PCR. *NOD2*- and disease-specific gene expression profiles were evaluated with repeated measure ANOVA by a general linear model.

RESULTS

Employing microarray assays, a total of 267 genes were identified that were significantly up- or downregulated in PBMCs of WT-*NOD2* patients, compared to healthy donors after challenge with vitamin D and/or a combination of LPS and PGN ($P < 0.05$; threshold: ≥ 2 -fold change). For further analysis by real-time PCR, genes with known impact on inflammation and immunity were selected that fulfilled predefined expression criteria. In a larger cohort of patients and controls, a disease-associated expression pattern, with higher transcript levels in vitamin D-treated PBMCs from patients, was observed for three of these genes, *CLEC5A* ($P < 0.030$), *lysozyme* (*LYZ*; $P < 0.047$) and *TREM1* ($P < 0.023$). Six genes were found to be expressed in a *NOD2*-dependent manner (*CD101*, $P < 0.002$; *CLEC5A*, $P < 0.020$; *CXCL5*, $P < 0.009$; *IL-24*, $P < 0.044$; *ITGB2*, $P < 0.041$; *LYZ*, $P < 0.042$). Interestingly, the highest transcript levels were observed in patients with heterozygous *NOD2* mutations.

CONCLUSION

Our data identify *CLEC5A* and *LYZ* as CD- and *NOD2*-associated genes of PBMCs and encourage further studies on their pathomechanistic roles.

Key words: Peripheral blood mononuclear cells; Gene expression; *NOD2*; *Lysozyme*; Crohn's disease; *CLEC5A*

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Core tip: Peripheral blood mononuclear cells (PBMCs) are a useful tool to study peculiarities of the immune response in the context of Crohn's disease (CD). Here, we investigated whether PBMCs from patients with CD, even at the stage of clinical remission, exhibit altered gene expression profiles after challenge with pathogen-associated molecular patterns and vitamin D. For *TREM1*, *lysozyme* and *CLEC5A*, disease-associated expression patterns, with higher transcript levels in patient-derived PBMCs, were observed. The two latter genes, along with four other transcripts, also showed *NOD2*-dependent expression profiles. *TREM1* and

CLEC5A may act with *NOD2* in a regulatory network with a pathophysiological role in CD.

Schäffler H, Rohde M, Rohde S, Huth A, Gittel N, Hollborn H, Koczan D, Glass A, Lamprecht G, Jaster R. *NOD2*- and disease-specific gene expression profiles of peripheral blood mononuclear cells from Crohn's disease patients. *World J Gastroenterol* 2018; 24(11): 1196-1205 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v24/i11/1196.htm> DOI: <http://dx.doi.org/10.3748/wjg.v24.i11.1196>

INTRODUCTION

Inflammatory bowel diseases (IBD) are chronic intestinal disorders and mainly consist of the two entities Crohn's disease (CD) and ulcerative colitis (UC)^[1,2]. The clinical course of IBD is characterized by intermittent periods of relapses and remission, which are unpredictable in clinical practice. The pathogenesis of IBD is multifactorial, including genetic and environmental factors, and involves an inappropriate activation of the mucosal immune system, which is triggered by the intestinal microbiota in genetically predisposed individuals^[1-5]. In Caucasian populations, nucleotide-binding oligomerization domain 2 (*NOD2*) has emerged as one of the main susceptibility genes for CD^[6-8]. *NOD2* is an intracellular pattern recognition receptor sensing muramyl dipeptide (MDP)^[9,10], a fragment of peptidoglycan (PGN), but also PGN by itself^[11,12] and, upon ligand binding, induces activation of the transcription factor NF- κ B^[13]. However, *NOD2* activation via PGN is dependent on a TLR2 co-stimulatory signal^[14].

In addition, the environment, *e.g.*, vitamin D deficiency, also affects the development and clinical course of IBD^[15-17]. Vitamin D deficiency has a high prevalence in IBD patients^[17,18]. We have recently shown that clinical factors, *e.g.*, the use of tumor necrosis factor (TNF)- α inhibitor, are associated with significant changes in vitamin D levels^[19]. Vitamin D was originally mainly implicated in bone health, regulating calcium and phosphate metabolism^[20,21], but recent evidence has shown that vitamin D also profoundly impacts the innate and adaptive immune system^[22-24]. Underscoring its role in the pathogenesis of CD, vitamin D was shown to be an inducer of *NOD2* gene expression^[25]. Using peripheral blood mononuclear cells (PBMCs) and dendritic cells, Dionne *et al.*^[26] showed that 1, 25-vitamin D acts as a modulator of the innate immune system. However, little is known about the effects of vitamin D and the presence of *NOD2* mutations on different gene expression levels in CD. The aim of our study was therefore to further characterize different gene expression profiles in CD patients and healthy controls correlating to *NOD2* mutation status and vitamin D pretreatment. We identified different genes associated with the presence of CD and mutations in the *NOD2*

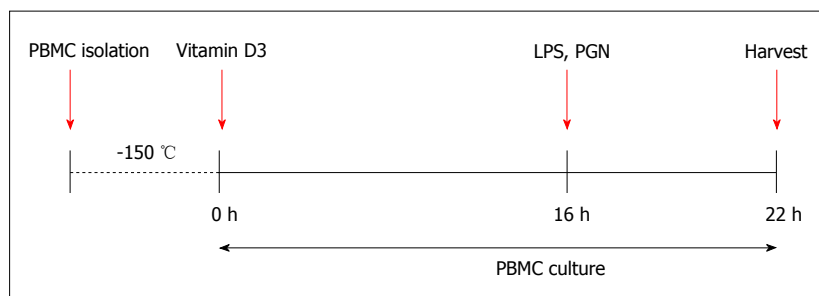


Figure 1 Protocol of peripheral blood mononuclear cells treatment.

gene. Follow-up studies on these genes may provide novel insights into the pathogenesis of CD and could contribute to the establishment of biomarkers to better predict the clinical course of the disease.

MATERIALS AND METHODS

Patients and controls

Sixteen patients with CD were recruited from the Rostock University Medical Center. The disease activity was determined via the Crohn's disease activity index (CDAI)^[27]. Furthermore, all patients were classified according to the Montreal classification^[28], and age, gender and disease-specific medication were recorded. Six healthy volunteers without immune-mediated gastrointestinal or other autoimmune disorders served as controls. EDTA blood samples were drawn from all participants for genotyping studies and isolation of PBMCs. Plasma levels of vitamin D and C-reactive protein (CRP) were determined using routine laboratory methods.

The study was approved by the ethics board of the University of Rostock (A-2015-0042). Written informed consent was obtained from each participant prior to enrollment.

Isolation, culture and treatment of PBMCs

PBMCs were isolated from EDTA venous blood using density-gradient centrifugation over Pancoll (PAN-Biotech, Aidenbach, Germany). Immediately after isolation, PBMCs were resuspended in cryopreservation medium [fetal calf serum (FCS) supplemented with 10% dimethyl sulfoxide (DMSO)] and stored at -150 °C until required. After thawing, the cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin (all reagents from Biochrom/Merck, Berlin, Germany), and exposed to 1 α ,25-dihydroxyvitamin D3 (Santa Cruz Biotechnology, Dallas, TX, United States) at 40 nmol/L as indicated. After an incubation period of 20 h at 37 °C in a 5% CO₂ humidified atmosphere, lipopolysaccharide (LPS; 1 μ g/mL; Sigma-Aldrich, Deisenhofen, Germany) and peptidoglycan (PGN; 10 μ g/mL; Sigma-Aldrich) were added to the cells as indicated, and incubation continued for another 6 h (Figure 1). Subsequently, the

cells were lysed in RTL Plus buffer, which was included in the RNeasy Plus Kit (Qiagen, Hilden, Germany), and subjected to RNA isolation (see below).

NOD2 genotyping

DNA was isolated from whole blood using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol. All patients and controls were genotyped with respect to the three major mutations in the *NOD2* gene (SNP 8; R702W, NCBI reference SNP ID: rs2066844, SNP 12; G908R, NCBI reference SNP ID: rs2066845 and SNP 13; 1007fs, NCBI reference SNP ID: rs2066847). The corresponding regions of the *NOD2* gene were amplified by PCR using a Taq PCR Master Mix Kit (Qiagen) and primers as specified in Table 1. The following PCR conditions were used: 5 min, 94 °C; 1 min, 94/60/72 °C (45 cycles); 7 min, 72 °C; 4 °C. After Sanger sequencing (Seqlab, Göttingen, Germany), the data were analyzed using the software Chromas, version 2.6. Individuals with no SNP mutations were considered wild-type (WT) for *NOD2*.

Microarray analysis of RNA expression profiles

RNA was extracted employing an RNeasy Plus Kit according to the manufacturer's protocol. Total RNA samples were quantified with a spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, United States), and their integrity was confirmed using the Agilent Bioanalyzer 2100 with an RNA Nano chip kit (both from Agilent Technologies, Waldbronn, Germany).

Expression profiling was performed using 200 ng RNA and the Affymetrix Human Clariom S Assay (Affymetrix/Thermo Fisher Scientific), which interrogates over 20000 well-annotated genes. Therefore, the so-called Whole Transcriptome protocol was employed. T7 promoter tags were introduced into all RNA molecules by using N6 3'-ends for DNA strand synthesis, before RNA strand replacement according to Eberwine^[29] was conducted. Non-labeled aRNA was produced by *in vitro* transcription. All RNA molecules were amplified in a linear manner, avoiding a 3' bias. Using purified aRNA as a template, a new strand-identical single-strand DNA was produced by adding random primers and dNTPs (including dUTP, which replaced a limited

Table 1 Primer for *NOD2*-genotyping

| SNP | Primer |
|-----|--|
| 8 | Forward: 5'-CCTCTCAATGTGGCAGGC-3' Reverse: 5'-CTCTGCATCTCGTACAGGC-3' |
| 12 | Forward: 5'-ATGGAGGCAGGTCCACTTTG-3' Reverse: 5'-TTACCTGAGCCACCTCAAGC-3' |
| 13 | Forward: 5'-GATGGTACTGAGCCTTTGTGA-3' Reverse: 5'-CAGACTCCAGGATGGTGTGTCAT-3' |

amount of dTTP). After digestion with RNase H, endpoint fragmentation was performed with uracil-DNA-glycosylase in combination with apurinic/apyrimidinic endonuclease 1, and biotinylated dNTPs were added to the 3'-ends of the single-stranded DNA fragments with deoxynucleotidyl transferase. Subsequently, hybridization of the microarrays was performed at 45 °C in a GeneChip® Hybridization Oven 645 (Affymetrix/Thermo Fisher Scientific). After overnight incubation, the microarrays were scanned using the GeneChip Scanner 3000 (Affymetrix/Thermo Fisher Scientific) at 0.7 µm resolution.

Primary data analysis was performed with the Affymetrix Transcriptome Analysis Console software version 3.1.0.5 including the Robust Multiarray Average module for normalization. Gene expression data were log-transformed. A change was considered significant when the ANOVA *P*-value met the criterion *P* < 0.05 at fold changes >|2|, *i.e.*, expression increments or declines larger than two. Along with the publication of the manuscript, our complete microarray data will be available in the Gene Expression Omnibus database (GEO accession number: GSE110186).

Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Unless indicated otherwise, reagents from Thermo Fisher Scientific were used in all subsequent steps. Cellular RNA prepared as described above was treated with a DNA-free kit to remove traces of genomic DNA, and 250 ng of RNA per sample was reverse transcribed into cDNA using TaqMan™ Reverse Transcription Reagents and random priming. Using a ViiA 7 sequence detection system (Thermo Fisher Scientific), target cDNA levels were quantified by real-time PCR. Therefore, qPCR MasterMix (Eurogentec, Seraing, Liège, Belgium) and the following human-specific TaqMan™ gene expression assays with fluorescently labeled MGB probes were used: Hs00355476_m1 (*CCL20*), Hs00188627_m1 (*CD101*), Hs00370621_m1 (*CLEC12A*), Hs04398399_m1 (*CLEC5A*), Hs01902549_s1 (*CLEC7A*), Hs01099660_g1 (*CXCL5*), Hs01114274_m1 (*IL24*), Hs00167304_m1 (*ITGAM*), Hs00164957_m1 (*ITGB2*), Hs00426232_m1 (*LYZ*), Hs00234007_m1 (*MSR1*), Hs01065279_m1 (*PECAM1*), Hs00218624_m1 (*TREM1*), and Hs99999905_m1 (*GAPDH*). PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C/ 1 min at 60 °C. Relative amounts of target mRNA

in PBMCs were expressed as $2^{-(\Delta Ct)}$ values.

Statistical analysis

Real-time PCR data were analyzed with repeated-measures ANOVA. Mean group differences were compared for "disease" (patients with CD vs controls) and "NOD2-status" (WT, heterozygote, homozygote), as well as for (within-subject factors) "vitamin D application" (yes vs no) and "stimulation" (LPS, PGN, LPS + PGN, controls), employing a *general linear model* for repeated measurements. Age was considered a covariate in the disease model because disease groups were not balanced by age, and *NOD2* groups were tested post hoc by LSD. Normal distribution of measurements was assessed using the Kolmogorov-Smirnov test. *P* < 0.05 was considered statistically significant. All data were processed using IBM® SPSS® Advanced Statistics 22.0.

RESULTS

PBMCs provide an easily accessible tool to investigate disease-associated peculiarities of the antipathogenic immune response of patients with CD. To study transcripts in an unbiased manner, we initially chose a microarray approach. Therefore, PBMCs from healthy individuals and patients with CD in remission (*n* = 3 each; all of them *NOD2*-WT) were pretreated with vitamin D3 for 20 h before they were challenged simultaneously with LPS and PGN for 6 h. Subsequently, global gene expression was analyzed employing Clariom S assays, and data were compared with those of untreated controls. Table 2 gives an overview of the significant differences between patients with CD and controls under identical conditions of PBMC treatment.

Under basal conditions and any treatment regimen, genes upregulated in patients with CD exceeded downregulated genes both in number and maximum change. Complete lists of the 267 genes are presented as Supplementary Table 1.

Many of the differentially expressed genes are well-known modulators of immune cell functions in the context of innate and adaptive immunity, and unsurprisingly, some of them have previously been implicated in the pathogenesis of CD, including several immune cell receptors, cytokines/chemokines and their cognate receptors and the antimicrobial peptide lysozyme^[30-36]. The latter transcript was found to be upregulated in PBMCs of patients with CD in response to LPS/PGN treatment, independent of the presence or absence of vitamin D3. Intriguingly, expression of various genes was synchronously up- or downregulated under different conditions, suggesting a robustness of the expression profile against external perturbations.

For in-depth analysis, we selected a panel of 11 genes from the list of candidates shown in Table 2 that fulfilled the following criteria: (1) differential expression in PBMCs from patients with CD and controls under basal conditions and/or under at least two

Table 2 Numbers and maximum changes of up- and downregulated genes in peripheral blood mononuclear cells from Crohn's disease patient *vs* identically treated controls

| Treatment of PBMCs | Upregulated genes | Downregulated genes |
|----------------------|-------------------|---------------------|
| Untreated | 85 (59-fold) | 39 (39-fold) |
| Vitamin D3 | 25 (21-fold) | 12 (9-fold) |
| LPS/PGN | 54 (6-fold) | 15 (5-fold) |
| Vitamin D3 + LPS/PGN | 29 (15-fold) | 8 (5-fold) |

$P < 0.05$; Threshold: ≥ 2 -fold change. PBMCs: Peripheral blood mononuclear cells; LPS: Lipopolysaccharide; PGN: Peptidoglycan.

Table 3 Genes selected for real-time PCR studies

| Transcript | Fold changes: patients <i>vs</i> controls | | | | Details on function/ reasons to study |
|------------|---|-----------|----------|-----------|--|
| | Basal | + D, -L/P | -D, +L/P | + D, +L/P | |
| MSR1 | 9.56 | 13.19 | | 14.72 | macrophage scavenger receptor ^[49] , differentially expressed in 3 of 4 groups expressed on various immune cells; inhibits expansion of colitogenic T cells ^[30] |
| CD101 | 2.66 | | | | |
| CLEC5A | | 2.82 | | 3.11 | C-type lectin member 5A, pattern recognition receptor; involved in antibacterial/ antiviral defense ^[43] |
| CLEC7A | 4.53 | | | 3.94 | C-type lectin member 7A, pattern recognition receptor; control of fungal infections ^[50] |
| CLEC12A | 6.04 | 4.52 | | | C-type lectin member 12A, pattern recognition receptor, inhibits cell death-induced inflammation ^[51] |
| ITGAM | 2.18 | | | | CD11b; integrin αM ; expressed by many immune cells; polymorphisms linked to autoimmunity ^[52] |
| LYZ | | | 3.51 | 2.03 | antimicrobial enzyme; essential role in innate immunity; increased production linked to CD ^[31] |
| PECAM1 | 3.15 | | | | CD31; implicated in transendothelial leukocyte migration in experimental colitis ^[32] |
| CCL20 | -2.33 | | -5.08 | | chemokine expressed by neutrophils, enterocytes, B-cells and dendritic cell; IBD predilection gene ^[33] |
| CXCL5 | -38.89 | | -5.32 | | regulates neutrophil homeostasis and chemotaxis; increased serum levels in IBD patients reported ^[34] |
| IL-24 | | | -3.49 | -4.82 | Involved in host defence against bacteria and fungi; increased expression in patients with active IBD ^[35] |
| TREM1 | | | | | amplifier of antimicrobial immune responses and inflammation in experimental colitis and IBD ^[36] |

$P < 0.05$; Threshold: ≥ 2 -fold change. Positive values refer to genes upregulated and negative values to genes downregulated in CD patients. LPS: Lipopolysaccharide; PGN: Peptidoglycan; PBMCs: Peripheral blood mononuclear cells. CD: Crohn's disease; IBD: Inflammatory bowel diseases.

treatment regimens (vitamin D3, LPS+PGN and their combination, respectively) and (2) an established or potential role in inflammation and/or regulation of the immune response. Table 3 shows details regarding all selected genes as well as a twelfth gene, *TREM1*, that was included as a control as an established vitamin D-responsive gene with immunomodulatory function^[37]. Interestingly, three pro-inflammatory mediators, *CCL20*, *CXCL5* and *IL-24*, displayed lower expression levels in patients with CD, which might be a consequence of their disease-specific medication (see below).

The expression profiles of the selected genes were subsequently studied by real-time PCR. In addition to WT-*NOD2* patients and healthy controls ($n = 6$ each, including the samples previously analyzed by microarray technology), we also included patients with heterozygous and homozygous mutations of *NOD2* ($n = 5$ each). Furthermore, we refined the protocol of PBMC treatment using LPS and PGN both in combination and as individual factors (Supplementary Table 2).

The clinical characteristics, laboratory findings and

the medication of all 16 patients are shown in Table 4. Except for one person with a slightly increased CDAI of 166, all patients presented with a CDAI of < 150 , indicating disease remission^[27]. The CRP-values of 13 patients were in the normal range (below 5 mg/L). In the remaining three patients, modestly elevated CRP-values (all below 14 mg/L) were detected. Disease activity in all patients could still be considered low. All but two patients presented with vitamin D levels below 75 nmol/L, suggesting an insufficiency or even deficiency (levels below 50 nmol/L). This finding was not unexpected, as all of the samples were collected during the European winter season. As a consequence, a vitamin D substitution therapy was initiated, if appropriate. Three of the patients were on steroids (> 10 mg prednisolone/d) at the time of the study, 7 received azathioprine, and 12 were treated with anti-TFN- α antibodies. Healthy controls consisted of 3 males and 3 females with an age range from 25 to 53 years.

For statistical data analysis, a general linear model repeated measure was chosen to assess mean differ-

Table 4 Characteristics of the patients -nucleotide-binding oligomerization domain 2 status, classification and activity of the disease, C-reactive protein and vitamin D levels, and medication at the time of the study

| No. | Sex | Age | NOD2 | Montreal classification | CDAI | CRP (mg/L) | Vitamin D (nmol/L) | Prednisolon (> 10 mg/d) | Azathio-prine | Anti-TNF- α |
|-----|-----|-----|-----------------|-------------------------|------|------------|--------------------|-------------------------|---------------|--------------------|
| 1 | M | 24 | WT ¹ | A2 L3 L4 B1 | 121 | 2.58 | 104.0 | Yes | No | Yes |
| 2 | M | 28 | WT ¹ | A2 L3 B3p | 46 | < 1.0 | 62.9 | No | No | Yes |
| 3 | M | 64 | WT ¹ | A3 L3 B2p | 111 | 4.58 | 27.0 | Yes | No | Yes |
| 4 | F | 60 | WT | A2 L3 B2 | 100 | 2.27 | 72.5 | No | No | Yes |
| 5 | F | 62 | WT | A3 L3 B3p | 82 | < 1.0 | 58.2 | Yes | Yes | Yes |
| 6 | M | 46 | WT | A2 L3 L4 B2p | 110 | 4.97 | 32.9 | No | No | Yes |
| 7 | M | 38 | HO | A2 L3 B3p | 92 | 13.30 | 40.2 | No | Yes | No |
| 8 | M | 64 | HO | A3 L3 B1 | 34 | < 1.0 | 53.0 | No | No | Yes |
| 9 | F | 48 | HO ² | A2 L3 B2 | 54 | < 1.0 | 67.6 | No | No | Yes |
| 10 | M | 54 | HO | A2 L3 B2p | 103 | 1.63 | 51.9 | No | Yes | No |
| 11 | M | 26 | HO | A1 L3 B2p | 120 | 7.86 | 31.0 | No | Yes | Yes |
| 12 | M | 27 | HT | A2 L3 B1 | 106 | 1.41 | 47.2 | No | No | Yes |
| 13 | M | 37 | HT | A2 L3 B2p | 166 | 4.56 | 37.9 | No | Yes | No |
| 14 | F | 48 | HT | A2 L1 B3p | 115 | 1.01 | 55.2 | No | Yes | Yes |
| 15 | M | 57 | HT | A1 L3 B2p | 132 | 12.30 | 52.3 | No | No | Yes |
| 16 | M | 47 | HT | A2 L3 B3 | 60 | 2.62 | 92.0 | No | Yes | No |

¹Patients who were also included into microarray analysis; ²SNP8 mutation. All other HO/HT mutations refer to SNP13. NOD2: Nucleotide-binding oligomerization domain 2; CDAI: Crohn's disease activity index; CRP: C-reactive protein; TNF : Tumor necrosis factor; WT: Wild-type; HO: Homozygous mutation; HT: Heterozygous mutation.

Table 5 differentially expressed transcripts in vitamin D-pretreated peripheral blood mononuclear cells from Crohn's disease patients and controls (with and without additional stimulation with lipopolysaccharide and peptidoglycan, respectively, age adjusted)

| Gene | P value | Upregulated in |
|---------------|---------|----------------|
| <i>CLEC5A</i> | 0.030 | CD patients |
| <i>LYZ</i> | 0.047 | CD patients |
| <i>TREM1</i> | 0.023 | CD patients |

CD: Crohn's disease.

ences between groups. Comparing controls to CD patients, no significant differences were detected when samples with and without vitamin D incubation were considered conjointly, due to the strong overlaying effect of vitamin D. However, focusing on the subgroup of vitamin D treated samples, disease-related differences were observed (no. 5-8 in Supplementary Table 2). Here, with age adjustment, three genes displayed a disease-dependent expression pattern (Table 5): consistent with the microarray data, significantly higher *CLEC5A* and *LYZ* transcript levels were observed in PBMCs of patients with CD. The same finding was observed for *TREM1*, which was included due to its vitamin D-dependent expression pattern but not because of the microarray results. Together, these findings suggest that CD-associated changes in gene expression could be attributed to treatment protocols that include vitamin D, as analyses in the remaining subgroup (w/o vitamin D) did not show any differences (all $P > 0.20$).

We also analyzed the influence of *NOD2* mutations on the expression of the gene panel described above. Considering all treated and untreated samples (Supplementary Table 2), a significant effect of the *NOD2* status was observed for 5 of these genes, including *CLEC5A* and *LYZ*, and one additional gene from Table 2, integrin

subunit beta 2 (*ITGB2*) (Table 6). With a P -value of 0.053, *TREM1* just missed statistical significance.

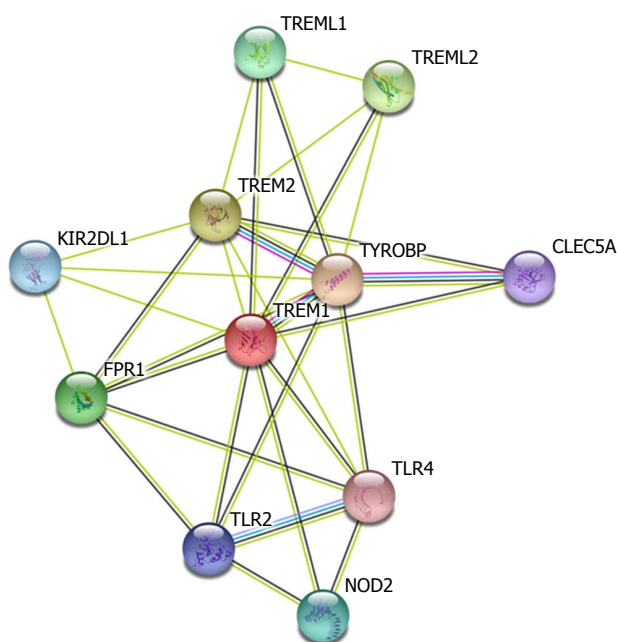
Unexpectedly, heterozygous CD patients displayed the highest expression levels for all of the genes, whereas no statistically significant differences between persons with WT-*NOD2* (patients and controls) and homozygous *NOD2* mutations were detected. The phenomenon is apparently unrelated to medication, which was very similar in the groups of patients with heterozygous and homozygous *NOD2* mutations (Table 4). This conclusion is also supported by statistical evaluations, which did not show any significant association between treatment with prednisolone, azathioprine or anti-TNF- α and expression of *CLEC5A*, *LYZ* and *TREM1* in PBMCs of patients with CD.

DISCUSSION

Many studies have shown that numerous risk genes of CD code for molecules involved in host defense against pathogens, such as nucleotide-binding oligomerization domain 2 (*NOD2*), *ATG16L1*, and those implicated in the T helper type 17 (Th17) pathway^[38-43]. Here, we tested the hypothesis that PBMCs of patients with CD, even at the stage of clinical remission, exhibit an

Table 6 Transcripts with a *NOD2*-dependent expression pattern

| Gene | P value | Highest levels |
|---------------|---------|----------------|
| <i>CD101</i> | 0.002 | Heterozygotes |
| <i>CLEC5A</i> | 0.020 | Heterozygotes |
| <i>CXCL5</i> | 0.009 | Heterozygotes |
| <i>IL-24</i> | 0.044 | Heterozygotes |
| <i>ITGB2</i> | 0.041 | Heterozygotes |
| <i>LYZ</i> | 0.042 | Heterozygotes |

**Figure 2** Network analysis using the STRING database^[48]. The network was derived employing human TREM1 as the search term (<https://string-db.org/cgi/network.pl?taskId=PmXpOD7RMwaM>).

altered gene expression profile upon challenge with pathogen-associated molecular patterns (PAMPs) and/or the immunomodulatory hormone vitamin D, which has previously been shown to exert differential effects on the expression of *NOD2*- and TLR-induced cytokines in the context of CD^[26].

Initial microarray experiments identified more than 200 genes with different expression patterns among patients with CD and controls. Based on predefined expression criteria, genes with roles in inflammation and immunity were selected for in-depth analysis by real-time PCR. A disease-associated expression pattern was identified for *CLEC5A*, *lysozyme* and *TREM1*. Six genes, including *CLEC5A* and *lysozyme*, displayed a *NOD2*-dependent expression pattern. With respect to *lysozyme* and *TREM1*, our findings are consistent with previous reports, which found that increased levels of both proteins in serum were implicated in the pathophysiology of IBD^[44,45]. To the best of our knowledge, however, this is the first report of an association between *CLEC5A* expression and CD. *CLEC5A* has most recently been identified as an important receptor in innate immunity

by neutrophil trap formation and secretion of different proinflammatory cytokines after stimulation with *Listeria monocytogenes*^[43]. This finding is especially interesting, as defective bacterial clearance was shown to play a crucial role in the pathogenesis of CD^[46,47]. Of note, both *CLEC5A* and *TREM1* proteins can be linked to the product of the best-established CD risk gene, *NOD2*, by the STRING database^[48] (Figure 2).

In conclusion, we found that PBMCs of patients with CD display alterations in their response to vitamin D and PAMPs. Disease-associated and *NOD2*-dependent gene expression profiles are preserved even at the stage of clinical remission. Our data identify *CLEC5A*, *LYZ* and *TREM1* as genes of particular interest for follow-up studies. We hypothesize that these genes may act in a common network relevant to CD pathogenesis. Establishment of biomarkers to better predict the clinical course of the disease remains a long-term goal of our studies.

ARTICLE HIGHLIGHTS

Research background

In Crohn's disease (CD), the interplay of genetic and environmental factors converges at the level of an altered antipathogenic immune response, which is incompletely understood. Peripheral blood mononuclear cells (PBMCs) provide a useful tool to study elements of the immunopathogenesis of the disease *in vitro*.

Research motivation

Currently, there is a lack of biomarkers to predict the clinical course of CD. Furthermore, the development of specific therapies would benefit from an improved mechanistic understanding of the pathogenesis of the disease.

Research objectives

The aim of this study was to identify disease-specific gene expression profiles of PBMCs from patients with CD in clinical remission. Specifically, we were interested in alterations of the gene expression profile after challenging PBMCs with pathogen-associated molecular patterns (PAMPs) and the immunomodulatory hormone vitamin D.

Research methods

PBMCs from patients with CD and healthy donors were cultured with vitamin D, peptidoglycan (PGN) and lipopolysaccharide (LPS), before RNA was isolated and subjected to microarray analysis and quantitative real-time PCR. Disease-specific gene expression profiles were evaluated by *general linear model repeated measure* analysis, paying particular attention to the well-established CD risk gene *NOD2*.

Research results

Microarray experiments yielded a total of 267 genes that were significantly up- or downregulated in PBMCs of patients with CD, compared to healthy donors, after challenge with vitamin D and/or a combination of LPS and PGN. For further analysis by real-time PCR, genes with roles in inflammation and immunity were selected. For three of these genes, *CLEC5A*, *lysozyme* and *TREM1*, a disease-associated expression pattern was validated. Six genes, including *CLEC5A* and *lysozyme*, were found to be expressed in a *NOD2*-dependent manner.

Research conclusions

PBMCs of patients with CD display alterations of their response to vitamin D and PAMPs that are preserved even at the stage of clinical remission. *CLEC5A*, *TREM1* and *NOD2* may act in a common network relevant to CD pathogenesis.

Research perspectives

Follow-up studies on alterations of the antipathogenic immune response may provide novel insights into the pathogenesis of CD and may also help to establish biomarkers to better predict the clinical course of the disease.

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