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Clin. Vaccine Immunol. 2006, 13(3):423. DOI:
10.1128/CVI.13.3.423-425.2006.

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Nucleotide Oligomerization Domain 2 (Nod2) Is Not Involved in the Pattern Recognition of *Candida albicans*

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Received 10 September 2005/Returned for modification 18 October 2005/Accepted 20 December 2005

Nucleotide-binding oligomerization domain 2 (Nod2) pathways are known to interact with Toll-like receptor 2 (TLR2) and TLR4, which are pattern recognition receptors for *Candida albicans*. We observed that the prevalence of Nod2 polymorphisms was not increased in patients with *Candida* infections. *Candida*-induced cytokine production in individuals with Nod2 polymorphisms was unaffected. We conclude that Nod2 is unlikely to play an important role in the recognition of *Candida albicans*.

Candida albicans is a major fungal pathogen which causes invasive bloodstream infections in immunocompromised hosts (5), as well as mucosal infections, such as recurrent vulvovaginal candidiasis (RVVC) in women. Despite the importance of *C. albicans* in causing disease, little is known of how it is recognized by cells and how the innate immune system mounts an effective defense. Recently, a role for Toll-like receptor 2 (TLR2) and TLR4 in innate immunity against *C. albicans* has been established (9).

Analogous to the essential extracellular recognition functions mediated through TLRs (1), another important class of pattern recognition receptors comprises the nucleotide oligomerization domain (Nod) and caspase recruitment domain proteins, which are responsible for the intracellular recognition of microbial components like peptidoglycan (PGN) (4). It has been shown that homozygosity for the 3020insC frameshift variant of Nod2 gene is associated with decreased NF- κ B translocation after stimulation with peptidoglycan, leading to a decreased proinflammatory cytokine response (3). Patients homozygous for the 3020insC frameshift mutation are at high risk for the development of Crohn's disease (10). Recent data have shown that Nod2 modulates TLR2 signaling (13), and we have demonstrated synergistic effects of Nod2 with TLR2 and TLR4 (8). We therefore hypothesized that Nod2 contributes to the recognition of *C. albicans* by modulating TLR2- and TLR4-dependent *Candida* recognition.

In the present study, we have investigated whether Nod2 is involved in the recognition of *C. albicans* by using two experimental approaches. We assessed the prevalence of Nod2 polymorphisms in patients with *Candida* bloodstream infections and in patients with RVVC and investigated the role of Nod2 in cytokine stimulation by *C. albicans* blastoconidia.

Nod2 genotyping. Nod2 genotyping was performed for 40 non-neutropenic individuals who had *Candida* bloodstream infections (defined by at least one positive blood culture and clinical signs of infection) and 15 women with recurrent vulvovaginal candidiasis

who had had more than three culture-proven symptomatic episodes of a vulvovaginal *Candida* infection during a 12-month period. The 51 healthy volunteers were blood donors at Radboud University Nijmegen Medical Center.

DNA was extracted from whole blood by standard procedures. Genotyping was performed by multiplex pyrosequencing (11). Amplification of fragments containing the polymorphic sites 2104C \rightarrow T, 2722G \rightarrow C, and 3020insC was performed in multiplex with a three-primer system: a biotin-labeled universal primer (5'-biotin-GCTGCTCCGGTTCATAGATT-3') and two sequence-specific primers, one of which had a universal tail (Table 1). The 50- μ l PCR mixtures contained 100 to 200 ng genomic DNA, 10 pmol of each sequence-specific primer, 1 pmol of each sequence-specific primer with a universal tail and 9 pmol of the biotin-labeled universal primer, PCR Gold Buffer (Applied Biosystems) with 2.5 mM MgCl₂, 17 mM deoxynucleoside triphosphates, and 2 U *Taq* DNA polymerase (Applied Biosystems).

The samples were denatured at 94°C for 10 min and then subjected to 40 cycles of amplification (94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min) in a PTC-200 thermal cycler (MJ Research). The genotyping results were validated by conventional sequencing.

The frequency of carriage of the Nod2 variants in patients and controls is shown in Table 2. The frequencies of Nod2 variant carriage were 17.5% among patients with *Candida* bloodstream infections and 20% among patients with recurrent vulvovaginal candidiasis, whereas it was 16% in healthy volunteers. We also compared the frequencies of carriage of individual Nod2 variants to the frequency data from other studies reporting on healthy Dutch volunteers (Table 2). By using Fisher's exact test, no significant differences between *Candida*-infected patients and the healthy control groups were observed.

Peripheral blood mononuclear cell (PBMC) stimulation. Five healthy controls who were wild type for the Nod2 3020insC polymorphism, six patients with Crohn's disease who were wild type for the Nod2 polymorphism and four patients with Crohn's disease who were homozygous for the 3020insC polymorphism were investigated. The patients with Crohn's disease have been described earlier (8) and were not treated with medication prone

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TABLE 1. Primer sequences for detection of 2104C→T, 2722G→C, and 3020insC frameshift mutations

SNPs ^a used for PCR	Primer	Primer sequence (5'-3') ^b	Modification	Fragment length (bp)
	Universal primer	GCTGCTCCGGTTCATAGATT	Biotin	
2104C→T	Forward primer	TTCCTGGCAGGGCTGTT		129
	Reverse primer	AGCGCTGCTCCGGTTCATAGATTCCAGACACCAGCGGGC		
	Sequence primer	ACATTGGAGAAGGCC		
2722G→C	Forward primer	TAGAGGGAGGAGGACTGTTAGTT		140
	Reverse primer	AGCGCTGCTCCGGTTCATAGATTCCCCCTCGTACCCAC		
	Sequence primer	CTTTAACCTTTTCA		
3020insC	Forward primer	AGCGCTGCTCCGGTTCATAGATTCTTCTTTCCAGGTTGTCCA		127
	Reverse primer	TCCTTACCAGACTCCAGGATG		
	Sequence primer	TGTCATTCTTTCAAGGG		

^a SNPs, single-nucleotide polymorphisms.

^b Italics indicate the positions of the universal parts of the primers.

to modulate cytokines (steroids, anti-tumor necrosis factor [anti-TNF]) either before or during the experiments. Mononuclear cells were isolated from peripheral blood by density gradient centrifugation over Ficoll-Hypaque (Amersham Biosciences, Sweden); washed twice in sterile phosphate-buffered saline; and resuspended in RPMI 1640 supplemented with 10 mM L-glutamine, gentamicin at 10 µg/ml, and 10 mM pyruvate. The cells were counted in a hemacytometer, and the number of cells was adjusted to 5×10^6 /ml. Heat-killed *C. albicans* blastoconidia (10^7 CFU/ml, strain ATCC MYA 3573), zymosan (100 µg/ml; Sigma, St. Louis, MO), *Escherichia coli* lipopolysaccharide (LPS; 1 ng/ml; Sigma), and *Staphylococcus aureus* peptidoglycan (1 ng/ml; Sigma) were added to 5×10^5 mononuclear cells in 100 µl in a 96-well microtiter plate (Greiner, Alphen a/d Rijn, The Netherlands) and were incubated for 24 h at 37°C in a 5% CO₂ atmosphere.

When the PBMCs were stimulated with purified *E. coli* LPS (1 ng/ml), a TLR4 ligand, the levels of production of TNF and interleukin-10 (IL-10) in the patients homozygous for the 3020insC variant did not differ from those in patients with

Crohn's disease or healthy volunteers with wild-type Nod2 alleles (Fig. 1A and B). The cytokine responses by cells carrying the Nod2 frameshift mutation variant were not affected after stimulation with *C. albicans* blastoconidia or zymosan, a yeast cell wall particle, or TLR2 ligand. After stimulation with PGN, cells homozygous for the frameshift mutation variant

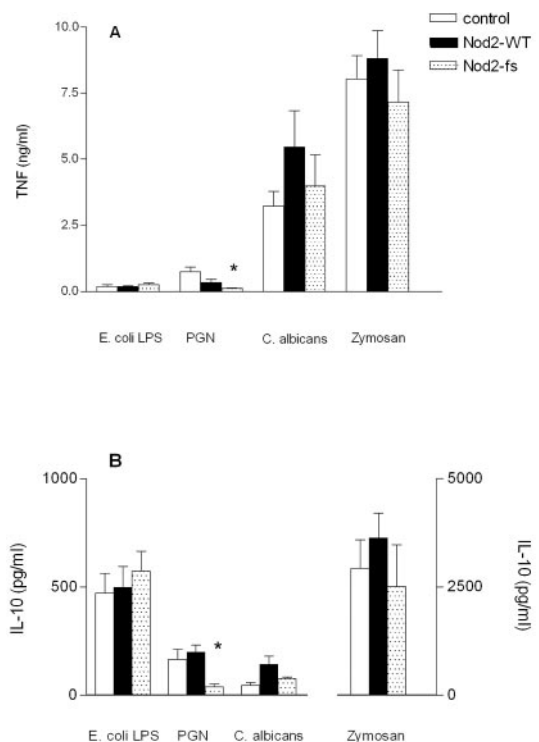


FIG. 1. Production of TNF (A) and IL-10 (B) by the PBMCs of five healthy volunteers bearing the wild-type allele for Nod2 3020insC variant (control), six patients with Crohn's disease bearing the wild-type alleles for Nod2 3020insC variant (Nod2-WT), and four patients with Crohn's disease homozygous for this variant (Nod2-fs). The PBMCs were stimulated with *E. coli* LPS (1 ng/ml), *S. aureus* peptidoglycan (1 ng/ml), heat-killed *C. albicans* 10^7 CFU/ml, and zymosan (100 µg/ml). Data are represented as means \pm standard errors of the means. *, $P < 0.05$, Mann-Whitney U test.

TABLE 2. Frequency of carriage of Nod2 polymorphisms in patients with *Candida* bloodstream infection, patients with recurrent vulvovaginal candidiasis, and healthy volunteers, as well as data from Dutch healthy controls retrieved from the literature

Group	No. of patients with the following Nod2 variants/total no. of patients tested (%)			Total (%)	
	2104C→T	2722G→C	3020insC		
<i>Candida</i> bloodstream infection ($n = 40$)	5/40 (12.5) ^a	1/40 (2.5)	1/40 (2.5)	17.5	
RVVC ($n = 15$)	3/15 (20)	0/15	0/15	20	
Control ($n = 51$)	2/49 ^b (4)	2/51 (4)	4/51 (8)	16	
Controls from literature	Laine et al. (6)	10/97 (10.3)	ND ^c	5/97 (5.2)	NA ^d
	Murillo et al. (7)	ND	9/152 (5.9)	3/152 (2)	NA

^a One of the patients is homozygous.

^b In two cases, genotyping failed.

^c ND, not determined.

^d NA, not applicable.

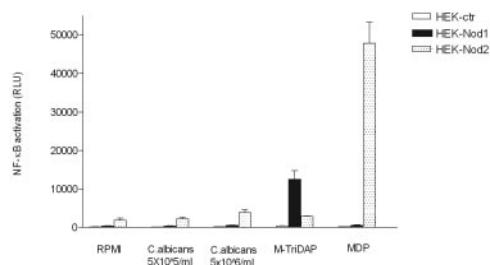


FIG. 2. Stimulation of HEK cells transfected with Nod1 or Nod2. Cells were stimulated with heat-killed *Candida albicans* blastoconidia (5×10^5 CFU/ml and 5×10^6 CFU/ml), MDP (100 nM), and TriDAP (100 nM).

showed significantly lower levels of TNF and IL-10 production compared to that by Nod2 wild-type cells (Fig. 1A, B).

Stimulation of Nod1- and Nod2-transfected HEK cells. Studies examining the activation of NF- κ B in cells overexpressing Nod1 and Nod2 were carried out as described previously (2). In brief, 1×10^6 /ml HEK293T cells were transfected overnight with 1 ng of either Nod1 or Nod2 plus 75 ng luciferase reporter plasmid. At the same time, heat-killed *Candida albicans* was added to cell culture medium, and the NF- κ B-dependent luciferase activation was measured following 24 h of incubation. NF- κ B-dependent luciferase assays were performed in duplicate. As shown in Fig. 2, the *Candida*-induced stimulation of HEK cells transfected with Nod1 or Nod2 is similar to that of unstimulated cells. Muramyl dipeptide (MDP; 100 nM) and diamino pimelate-containing tripeptide muropeptide (TriDAP; 100 nM) served as control stimuli for Nod2 and Nod1, respectively.

The innate detection of fungal products has been shown to involve both of the extracellular receptors TLR2 and TLR4. We have previously shown a role of TLR4 in disseminated *Candida* infections, due to decreased chemokine production and recruitment in TLR4-defective mice challenged with an intravenous *Candida* infection (9). In patients with a *Candida* bloodstream infection, we have also recently demonstrated an increased prevalence of the TLR4 Asp299Gly polymorphism, indicating that the variant of TLR4 leads to increased susceptibility to this infection (12). However, in the present study, we demonstrate that the prevalence of Nod2 polymorphisms among patients with *Candida* bloodstream infections and women with RVVC is similar to that among healthy volun-

teers. In addition, the Nod2 3020insC frameshift polymorphism does not impair cytokine release in response to *C. albicans* or fungal components. These data argue that Nod2 is unlikely to play a major role in susceptibility to *Candida* infections.

Mihai G. Netea was supported by a VIDI grant of The Netherlands Organization for Scientific Research.

We thank Marc Bijen for genotyping.

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