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

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Candida albicans is a major fungal pathogen which causes invasive bloodstream infections in immunocompromised hosts, 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 maintains an effective defense. Recently, a role for Toll-like receptor 2 (TLR2) and TLR4 in innate immunity against C. albicans has been established.

Analogous to the essential extracellular recognition functions mediated through TLRs, 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). It has been shown that homozygosity for the 3020insC frameshift variant of Nod2 gene is associated with decreased NF-κB translocation after stimulation with peptidoglycan, leading to a decreased proinflammatory cytokine response. Patients homozygous for the 3020insC frameshift mutation are at high risk in cytokine stimulation by Candida albicans.

The frequency of carriage of the Nod2 variants in patients and controls is shown in Table 1. The 50-μl 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 deoxyribonucleoside 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.

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...
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 \( \mu \text{g} / \text{ml} \), and 10 mM pyruvate. The cells were counted in a hemacytometer, and the number of cells was adjusted to 5 \( \times 10^6/\text{ml} \). Heat-killed \( C. \) albicans blastoconidia (10^7 CFU/ml, strain ATCC MYA 3573), zymosan (100 \( \mu \text{g} / \text{ml} \); Sigma, St. Louis, MO), \( E. \) coli lipopolysaccharide (LPS; 1 ng/ml; Sigma), and \( S. \) aureus peptidoglycan (1 ng/ml; Sigma) were added to 5 \( \times 10^5 \) mononuclear cells in 100 \( \mu \text{l} \) 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

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

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primer</th>
<th>Primer sequence (5′–3′)</th>
<th>Modification</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2104C→T</td>
<td>Forward primer</td>
<td>TTCTGGCAGGGCTTGT</td>
<td>Biotin</td>
<td>129</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>AGCCGCCTCCTGCCGTATAGATTTCCAGACACCAGGGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequence primer</td>
<td>ACATTGGAGAAGGCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2722G→C</td>
<td>Forward primer</td>
<td>TAGAGGAGGAGGACTGTAGTT</td>
<td></td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>AGCCGCCTCCTGCCGTATAGATTTCCCCCTCGTCACCCAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequence primer</td>
<td>CTTTTAACCTTTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3020insC</td>
<td>Forward primer</td>
<td>AGCCGCTCCTGCCGTATAGATTTCTTTTCTTCAGGTTGTCCA</td>
<td></td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>TCCTTACACACTTCCAGGATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sequence primer</td>
<td>TGTCATTCCTTTCAAGGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SNPs, single-nucleotide polymorphisms.

### Table 2. Frequency of carriage of Nod2 polymorphisms in patients with \( C. \) candida bloodstream infection, patients with recurrent vulvovaginal candidiasis, and healthy volunteers, as well as data from Dutch healthy controls retrieved from the literature

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients with the following Nod2 variants/total no. of patients tested (%)</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C. ) candida bloodstream infection (( n = 40 ))</td>
<td>5/40 (12.5) 1/40 (2.5) 1/40 (2.5)</td>
<td>17.5</td>
</tr>
<tr>
<td>RVVC (( n = 15 ))</td>
<td>3/15 (20) 0/15</td>
<td>20</td>
</tr>
<tr>
<td>Control (( n = 51 ))</td>
<td>2/49 (4) 2/51 (4) 4/51 (8)</td>
<td>16</td>
</tr>
<tr>
<td>Controls from literature</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laine et al. (6)</td>
<td>10/97 (10.3) ND</td>
<td>ND</td>
</tr>
<tr>
<td>Murillo et al. (7)</td>
<td>9/152 (5.9) 3/152 (2)</td>
<td>NA</td>
</tr>
</tbody>
</table>

*One of the patients is homozygous.

b In two cases, genotyping failed.

\( \text{ND} \), not determined.

\( \text{NA} \), not applicable.

![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 \( \mu \text{g} / \text{ml} \)). Data are represented as means ± standard errors of the means. *P < 0.05, Mann-Whitney U test.](http://cvi.asm.org)
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-κB in cells overexpressing Nod1 and Nod2 were carried out as described previously (2). In brief, 1 × 10⁶/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-κB-dependent luciferase activation was measured following 24 h of incubation. NF-κB-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 diaminopimelate-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 (10), among patients with *Candida* bloodstream infections and women with RVVC is similar to that among healthy volunteers. In addition, the nod2 302fsnC 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.

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**REFERENCES**