

# Toll-like receptor 4 Asp299Gly/Thr399Ile polymorphisms are a risk factor for *Candida* bloodstream infection

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**ABSTRACT.** Toll-like receptor (TLR)-4 is an important pattern recognition receptor for *Candida albicans*, playing a role in innate host defense. We investigated whether there is an association between the *TLR4* Asp299Gly or *TLR4* Thr399Ile polymorphism, and the occurrence of *Candida* bloodstream infection. We performed a case-control study, involving 43 patients with a *Candida* bloodstream infection and 166 healthy individuals. *TLR4* Asp299Gly and Thr399Ile polymorphisms were assessed, as well as cytokine production after stimulation of peripheral blood mononuclear cells (PBMC) with *Candida albicans*. We observed that the prevalence of *TLR4* Asp299Gly polymorphism was found to be higher in patients with *Candida* bloodstream infection than in controls (26% versus 10%; OR 3.0; 95% CI 1.3-6.9). All patients bearing the Asp299Gly polymorphism were also positive for the Thr399Ile allele, a linkage well described in literature. IL-10 production was higher in *C. albicans*-stimulated PBMC from volunteers bearing the *TLR4* Asp299Gly polymorphism, and a similar tendency was observed in *TLR4* Asp299Gly heterozygous patients who had recovered from candidemia. These findings show that the *TLR4* Asp299Gly/Thr399Ile polymorphisms are associated with an increased susceptibility to *Candida* bloodstream infections, and an increased production of IL-10 is probably involved in this effect.

**Keywords:** TLR4 polymorphism, *Candida*, bloodstream infection, susceptibility, cytokine

In *Drosophila*, the Toll receptor is involved in ontogenesis and antimicrobial resistance [1]. In humans, 10 Toll-like receptors (TLR) have been identified [2], which play a crucial role in the host defense against infections. Activation of TLRs by microbial products results in release of proinflammatory cytokines, leading to activation of the innate immune system. TLR4 is crucial for host defense against disseminated candidiasis, as was demonstrated by a recent study showing that the TLR4-defective C3H/HeJ mouse strain is more susceptible to disseminated *Candida albicans* infection [3]. Two common mutations in the *TLR4* gene have been described, *TLR4* Asp299Gly and Thr399Ile, which are found to be in a strong linkage. Individuals heterozygous for these co-segregating mutations have a blunted airway responsiveness to inhaled LPS, while airway epithelial cells of these individuals were hyporesponsive to LPS *in vitro* [4]. Patients with these polymorphisms had an increased susceptibility to Gram-negative sepsis [5, 6].

Because TLR4 has been implicated in the host defense against *Candida* species [3], we have investigated the association of the *TLR4* polymorphism with candidemia. Here we show that the Asp299Gly and the co-segregating

Thr399Ile polymorphisms are associated with an increased risk of acquiring *Candida* bloodstream infection.

## METHODS

### Study subjects

The study population consisted of 43 non-neutropenic individuals, who had a *Candida* bloodstream infection (defined by at least one positive blood culture and clinical signs of infection) in the Radboud University Medical Center in Nijmegen, a tertiary care hospital, and 166 healthy volunteers. The healthy volunteers had no history of invasive *Candida* infections. This study was approved by the Regional Ethics Committee.

### *TLR4* polymorphisms

Genomic DNA was isolated from blood by using the Puregene DNA isolation kit (Gentra systems, BIOzym, the Netherlands). The DNA was stored at 4 °C until analysis.

### ***TLR4 896A > G (299Asp > Gly)***

To determine the *TLR4* Asp299Gly genotype, DNA was amplified with primers (forward primer: 5' ATACTTAGACTACTACCTCCATG 3', reverse primer 3' AAACCTCAAGGCTTGGTAGATC 5'; the bold C located in the forward primer is indicating a mutation, creating an *Nco*-I site). The polymerase chain reaction conditions were as follows: 5 minute initial denaturation at 94°C, followed by 37 cycles (94 °C for 30 seconds, 50 °C for 30 seconds and 72 °C for 30 seconds). The PCR products were digested with the restriction enzyme *Nco*-I (New England BioLabs, Beverly, MA, USA) and separated on a 2.5% agarose gel stained with ethidium bromide.

### ***TLR4 1196 C > T (399Thr > Ile)***

For the determination of Thr399Ile genotype, DNA was amplified with primers (forward primer: 5' GCTGTTTTCAAAGTGATTTTGGGAGAA 3', reverse primer 5' CACTCATTTGTTCAAATTGGAATG 3'). The polymerase chain reaction conditions were as follows: 5 minute initial denaturation at 95°C, followed by 35 cycles (95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds). The PCR products were digested with the restriction enzyme *Hinf*-I (New England BioLabs, Beverly, MA, USA) and separated on a 4% agarose gel stained with ethidium bromide.

### ***Stimulation of peripheral blood mononuclear cells***

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque (Amersham Bioscience). The PBMC were stimulated with heat-killed *Candida albicans* blastoconidia (strain ATCC MYA-3573,  $2 \times 10^7$ /mL; heat-killed over 30 min. at 100 °C), heat-killed *C. albicans* hyphae ( $2 \times 10^5$ /mL, grown from *C. albicans* blastoconidia over 24 hours at 37°C in RPMI 1640 adjusted to pH 6.3, and heat-killed over 45 min. at 98°C; more than 95% of the blastoconidia were grown to hyphae which was checked by microscopy), or *E. coli* LPS (2 ng/mL, Sigma, St Louis, MO, USA).

The samples were incubated for 24h or 48h at 37 °C. Supernatants were collected and stored at -80 °C until tested. Cytokine production was tested in healthy volunteers or after recovery of patients from the *Candida* bloodstream infection.

The concentrations of TNF, IFN $\gamma$ , IL-8 and IL-10 were measured by ELISA (Pelikine, CLB). Detection limits of the assay were TNF, 0.015 ng/mL; IFN $\gamma$ , 2.5 pg/mL; IL-8, 3 ng/mL; IL-10, 1.25 pg/mL.

### ***Statistical analysis***

Genotype distributions were assessed, and Hardy-Weinberg equilibrium in the controls was checked for Mendelian inheritance. The strength of the association between *Candida* bloodstream infection and *TLR4* Asp299Gly and Thr399Ile polymorphism was expressed as an odds ratio (OR) together with a 95% confidence interval (CI). The differences in cytokine production between individuals bearing the wild-type allele and those with the mutated allele were analyzed using the Mann-Whitney U test. Values were considered significant at  $p < 0.05$ .

## **RESULTS**

### ***Toll-like receptor 4 polymorphisms***

Forty-three patients with a *Candida* bloodstream infection (defined by at least one positive blood culture and clinical signs of infection) and 166 control subjects from the general population, were assessed for the presence of *TLR4* Asp299Gly and Thr399Ile polymorphisms. The blood cultures of the 43 patients revealed *C. albicans*, 26 (60%), *C. tropicalis*, 2 (5%), *C. parapsilosis*, 8 (19%), *C. glabrata*, 5 (11%), and *C. lusitaniae*, 2 (5%). Eleven patients (26%) and 17 healthy control subjects (10%) were heterozygous for both the Asp299Gly and Thr399Ile mutation in the *TLR4* gene ( $p < 0.05$ , OR 3.0; 95%CI 1.3-6.9; table 1). No patients positive for only one of the polymorphisms were identified, whereas in the control group 2 out of 166 individuals were positive for either Asp299Gly or Thr399Ile polymorphism.

No differences in the presence of *TLR4* Asp299Gly and Thr399Ile polymorphisms were found between patients with *C. albicans* infection (7 out of 26 patients, 27%) and those with non-*albicans Candida* infections (4 out of 17 patients, 24%;  $p > 0.05$ ). In addition, no correlation between the presence of the *TLR4* Asp299Gly polymorphism and a particular *Candida* species was observed.

### ***Stimulation of peripheral blood mononuclear cells***

To test the possible mechanisms of increased susceptibility to *C. albicans* infection, we investigated cytokine stimulation by *Candida blastoconidia* or *hyphae* in healthy volunteers, and in patients with and without the *TLR4* Asp299Gly polymorphism. PBMC from one healthy volunteer homozygous for the *TLR4* Asp299Gly polymorphism, 3 healthy volunteers heterozygous for the *TLR4*

**Table 1**  
*TLR4* Asp299Gly and Thr399Ile polymorphisms in patients with *Candida* bloodstream infection and healthy control subjects

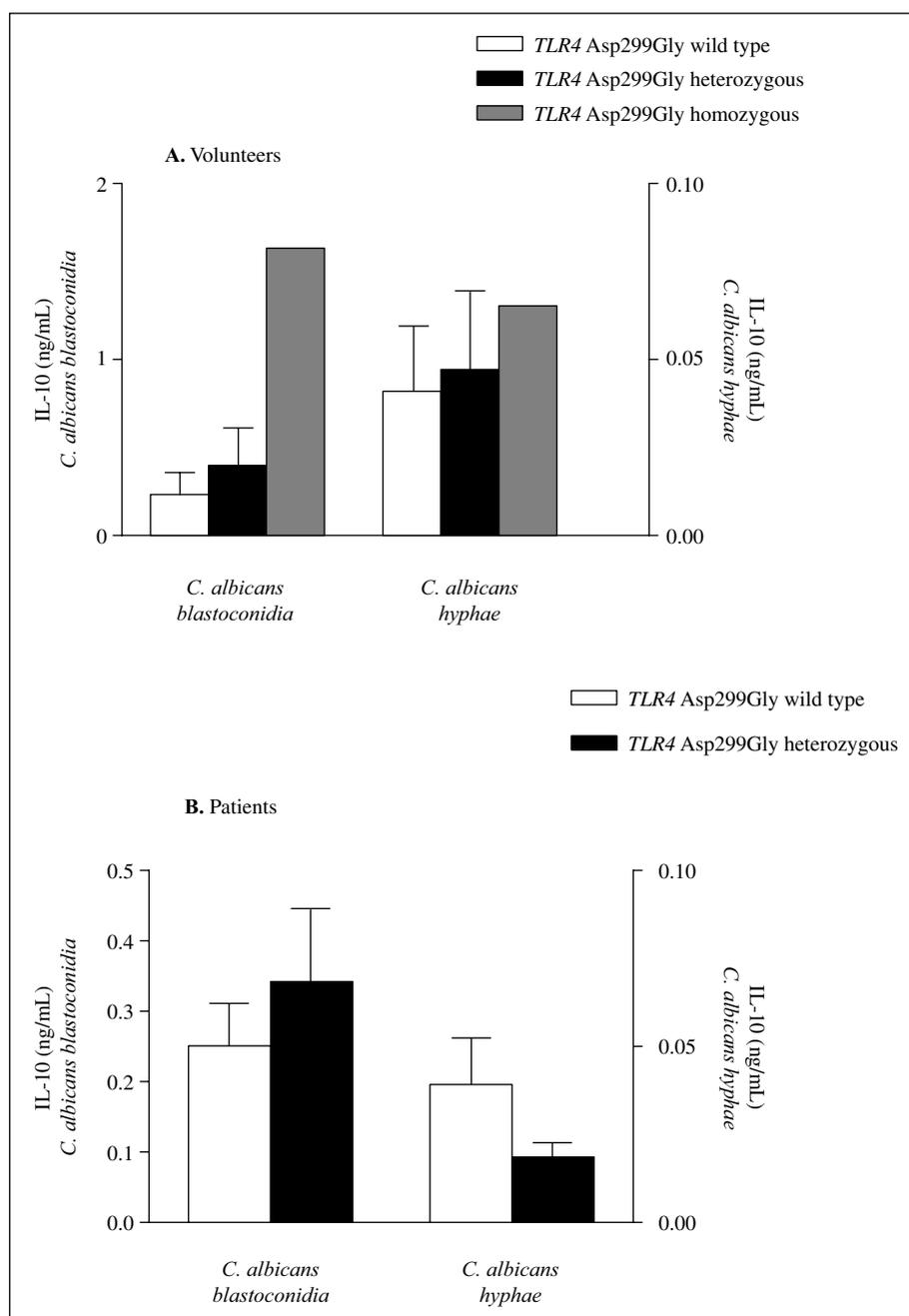
|                      | <b>Candidemia</b> | <b>Controls</b>           | <b>Odds ratio (95% CI)</b> |
|----------------------|-------------------|---------------------------|----------------------------|
| <b>TLR4 genotype</b> |                   |                           |                            |
| 299+ / 399+          | 32/43 (74%)       | 147/166 (89%)             |                            |
| 299- / 399-          | 11/43 (26%)       | 17/166 (10%) <sup>a</sup> | 3.0 (1.3-7.0)              |
| 299- / 399+          | 0/43 (0%)         | 1/166 (0.6%)              | 1.5 (0.06-38)              |
| 299+ / 399-          | 0/43 (0%)         | 1/166 (0.6%)              | 1.5 (0.06-38)              |
| <b>Total</b>         | <b>43</b>         | <b>166</b>                |                            |

<sup>a</sup> One out of 17 healthy controls is homozygous, all others are heterozygous.

Asp299Gly polymorphism, and 8 healthy volunteers bearing the wild type alleles, were stimulated with *C. albicans*. After stimulation with *C. albicans* blastoconidia or hyphae, PBMC of the individuals bearing the Asp299Gly polymorphism, especially those from the homozygous healthy volunteer, showed a clear trend towards increased IL-10 production compared to PBMC of the 8 wild-type controls, although no statistical significance was reached due to the low number of volunteers with the *TLR4* polymorphism (figure 1A). No significant differences were found in the production of TNF between the homozygous, heterozygous and wild-type control subjects (figure 2A). Similarly, the release of IL-8 and IFN $\gamma$  was identical after

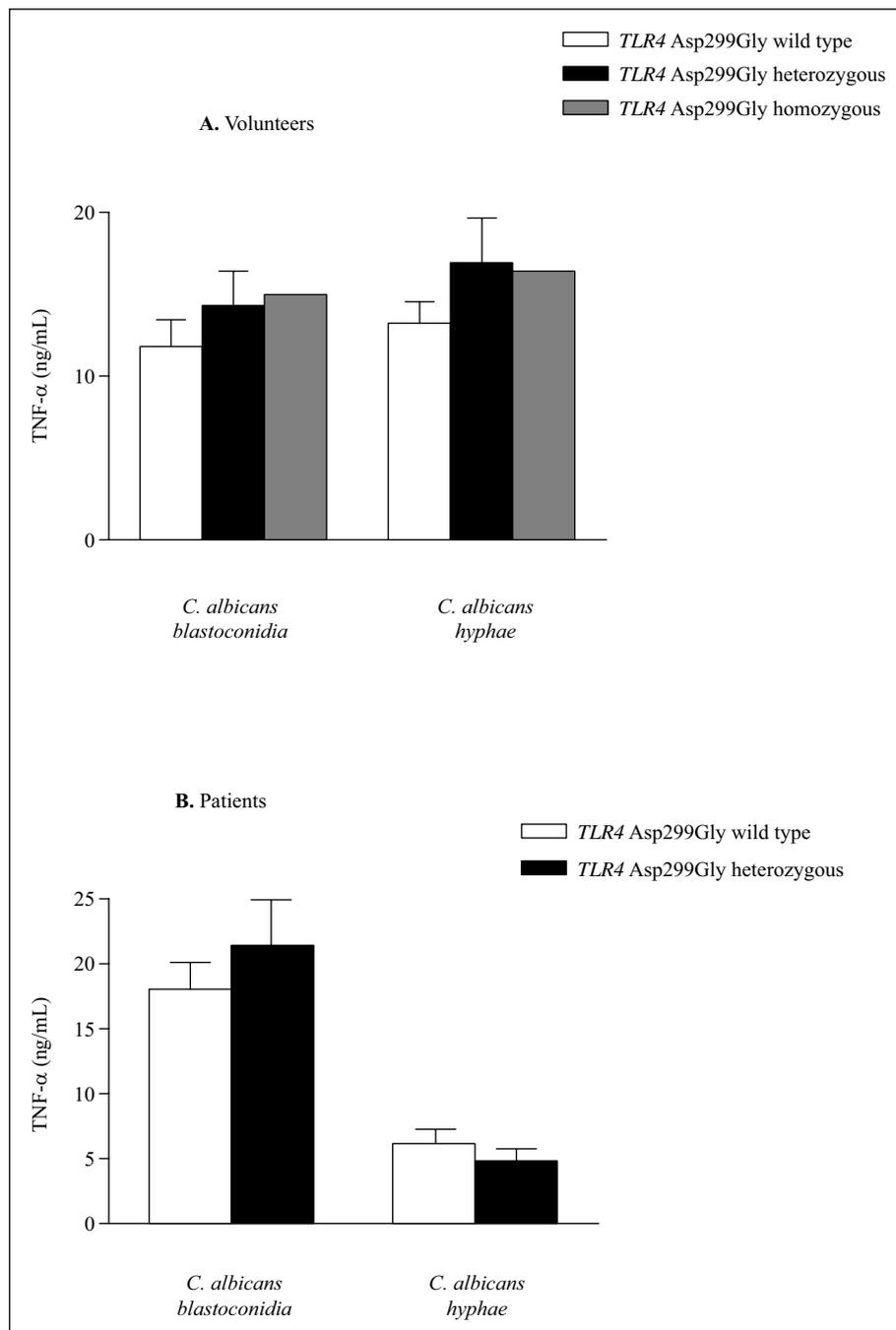
stimulation of PBMC from individuals with the wild-type *TLR4* allele or the *TLR4* Asp299Gly polymorphism (data not shown).

In addition, cytokine production by PBMC from 5 candidemia patients bearing the *TLR4* Asp299Gly polymorphism was assessed. For each heterozygous patient, an age- and sex- matched patient with candidemia bearing the wild-type *TLR4* gene was selected as a control. Similar to the healthy volunteers bearing the polymorphism, there was a tendency towards increased IL-10 after *C. albicans* blastoconidia stimulation, although this did not reach statistical significance (figure 1B). No significant difference



**Figure 1**

**A)** Interleukin (IL)-10 production after stimulation of PBMC from healthy volunteers, with *C. albicans* blastoconidia or hyphae *in vitro*. Bars represent mean  $\pm$  SD for 8 subjects with the wild-type *TLR4*, 3 subjects heterozygous for the *TLR4* Asp299Gly polymorphism, and 1 subject homozygous for the *TLR4* Asp299Gly polymorphism. **B)** IL-10 production by PBMC from 10 patients who had recovered from *Candida* bloodstream infection with or without the *TLR4* Asp299Gly polymorphism (mean  $\pm$  SD; 5 subjects in each group).

**Figure 2**

**A)** Tumor necrosis factor (TNF)- $\alpha$  production after stimulation of PBMC from healthy volunteers with *C. albicans* blastoconidia or hyphae *in vitro*. Bars represent mean  $\pm$  SD for 8 subjects with the wild-type *TLR4*, 3 subjects heterozygous for the *TLR4* Asp299Gly polymorphism, and 1 subject homozygous for the *TLR4* Asp299Gly polymorphism. **B)** TNF- $\alpha$  production by PBMC from 10 patients who had recovered from *Candida* bloodstream infection with or without the *TLR4* Asp299Gly polymorphism (mean  $\pm$  SD; 5 subjects in each group).

in TNF production was found between patients with and without the *TLR4* Asp299Gly polymorphism (figure 2B).

## DISCUSSION

In this study, we have demonstrated that the *TLR4* Asp299Gly and co-segregating Thr399Ile polymorphisms are associated with an increased risk for *Candida* bloodstream infection, and that this effect is possibly mediated through increased IL-10 production upon *Candida* stimulation. To our knowledge, this is the first genetic factor shown to affect the host susceptibility to invasive mycoses.

Previously we have shown that *TLR4* is required for the host defense against disseminated candidiasis in an experimental mouse model [3]. *TLR4* defective C3H/HeJ mice were more susceptible to *C. albicans* infection through impaired chemokine expression and neutrophil recruitment [3]. These findings are in line with those in the present study, showing an association of the *TLR4* Asp299Gly polymorphism with *Candida* bloodstream infections in humans. Previously, a relationship between *TLR4* polymorphisms and a higher incidence of Gram-negative bacterial infections was found in a set of ICU patients [5, 6], but no relationship was found between the

*TLR4* Asp299Gly polymorphism and severity of meningococcal disease [7]. However, incidence of other, rare *TLR4* polymorphisms has been associated with an increased risk of meningococcal sepsis [8]. In women with vaginal colonization by *C. albicans*, Morr  et al. did not find an association between the *TLR4* polymorphism and *Candida* colonization [9]. This is not in disagreement with our findings since firstly, these patients were colonized rather than having signs of active infection, and secondly, specific T-cell-mediated immunity is the main line of defense against mucosal candidiasis, whereas innate immune responses, in which TLR4 recognition is crucial, are responsible for defense against disseminated candidiasis.

The original observation by Arbour et al. [4] that common mutations in *TLR4* are associated with LPS hyporesponsiveness in humans has been disputed. In recent studies, healthy individuals heterozygous for the *TLR4* Asp299Gly polymorphism did not show defects in proinflammatory cytokine production after stimulation with LPS [10-12]. These later findings are in line with our data that showed no difference in proinflammatory cytokine production between individuals heterozygous for *TLR4* Asp299Gly polymorphism and individuals bearing the wild type allele, after stimulation with *C. albicans*. It is however interesting to observe that patients who recovered from candidemia displayed a lower production of TNF after stimulation with *Candida hyphae* (figure 2A and 2B). It is tempting to speculate that this difference might have contributed to their susceptibility to candidiasis. However, this was independent of the presence of the Asp299Gly polymorphism. It is suggestive that other, as yet unknown factors, may also influence the variation of TNF production. It is of great interest however that a clear trend towards increased IL-10 release was observed after stimulation with *Candida* in healthy individuals heterozygous for the *TLR4* Asp299Gly polymorphism. Although a similar trend was observed in patients with candidemia, it did not reach statistical significance. This is probably due to the small size of the groups and a bias inherent to our study, since only the surviving patients were tested, and not those who died early during their candidemia, who may have had higher IL-10 concentrations. The small size of both the cohort of patients with candidemia as well as the groups of individuals with *TLR4* polymorphisms tested for cytokine production, is a limitation of the present study. However, the correlation between the presence of the *TLR4* Asp299Gly polymorphism and the increased IL-10 production suggest a biological phenomenon of true significance. IL-10 has been shown to inhibit the action of human monocytes against *C. albicans* [13]. In mice with disseminated candidiasis, the absence of IL-10 augmented innate and acquired antifungal immunity by increasing the production of proinflammatory cytokines, and this leads to reduced fungal growth [14]. These findings are corroborated by a study by Roilides et al. demonstrating strongly elevated serum concentrations of IL-10 in non-surviving patients with invasive aspergillosis [15].

We have recently demonstrated that during *Candida* infection, TLR4 induces a Th1 cytokine profile [16]. In contrast, the role of TLR2 is more controversial. We have shown that TLR2-mediated signals lead to IL-10 release and generation of CD4+CD25+ T-regulatory cells, suppressing the innate immunity against candidiasis [16], and these data are supported by other studies showing improved host

defense mechanisms against *Candida* in TLR2-/- mice [17]. In contrast, others have reported an increased susceptibility to candidiasis in TLR2-/- mice [18]. Differences in the experimental design and/or *Candida* strains may be responsible for these differences.

The notion that TLR4 induces a more proinflammatory cytokine profile, while TLR2 has a more pronounced anti-inflammatory bias [16, 19] may suggest that, in patients with a *TLR4* polymorphism, a defective TLR4-mediated signaling indirectly leads to a suppressive Th2 cytokine response, through unaffected TLR2-induced IL-10 production.

In conclusion, our results show an increased prevalence of the *TLR4* Asp299Gly and co-segregating Thr399Ile polymorphisms in patients with a candidemia, demonstrating that *TLR4* polymorphisms are associated with an increased susceptibility for *Candida* bloodstream infection. Although no definite conclusions about the pathophysiological mechanism can be drawn from our data due to the small group size, the mechanism is of great interest. The mechanism through which these polymorphisms influence the innate immune response probably involves a shift towards anti-inflammatory IL-10 production, suppressing the innate host defense against *C. albicans*.

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