

Toll-like Receptor 1 Polymorphisms Increase Susceptibility to Candidemia

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(See the editorial commentary by Bagni and Whitby, on pages 873–4.)

Background. Candidemia is a severe invasive fungal infection with high mortality. Recognition of *Candida* species is mediated through pattern recognition receptors such as Toll-like receptors (TLRs). This study assessed whether genetic variation in TLR signaling influences susceptibility to candidemia.

Methods. Thirteen mostly nonsynonymous single nucleotide polymorphisms (SNPs) in genes encoding TLRs and signaling adaptors MyD88 and Mal/TIRAP were genotyped in 338 patients (237 white, 93 African American, 8 other race) with candidemia and 351 noninfected controls (263 white, 88 African American). The SNPs significant in univariate analysis were further analyzed with multivariable logistic regression to determine association with clinical outcomes. Functional consequences of these polymorphisms were assessed via in vitro stimulation assays.

Results. Analyses of TLR SNPs revealed that 3 TLR1 SNPs (R80T, S248N, I602S) were significantly associated with candidemia susceptibility in whites. This association was not found in African Americans, likely due to lower power in this smaller study population. Furthermore, these TLR1 polymorphisms displayed impaired cytokine release by primary monocytes. No associations with susceptibility to candidemia were observed for SNPs in TLR2, TLR4, TLR6, TLR9, MyD88, or TIRAP.

Conclusions. Nonsynonymous SNPs in TLR1 are associated with impaired TLR1 function, decreased cytokine responses, and predisposition to candidemia in whites.

Candidemia (ie, bloodstream infection with *Candida* species) is the fourth most common nosocomial infection in immunocompromised patients and the systemic infections with the highest mortality rate, reaching 30%–40% in intensive care unit (ICU) patients [1, 2]. It is well established that the main risk factors for candidemia are

neutropenia, mucosal barrier injury, solid organ or stem cell transplantation, treatment with immunosuppressive drugs, use of broad-spectrum antibiotics, prolonged ICU stay, and central venous catheterization [3–5]. However, among patients with similar predisposing factors, the risk of systemic *Candida* infection and its clinical outcome vary significantly.

Several *Candida* species, of which *C. albicans* is the most prevalent, have been isolated from the bloodstream of patients with candidemia. However, in recent years there has been a trend toward increased prevalence of non-*albicans* species such as *C. glabrata* and *C. krusei*, with increasing reports of resistance to antifungal azoles [6, 7], which may be in part promoted by the frequent use of antifungal prophylaxis, preemptive therapeutic strategies in high-risk patients, or alteration of host status [8, 9].

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Antifungal prophylaxis remains the most effective way to prevent systemic *Candida* infection because of the lack of specific clinical and laboratory parameters to diagnose candidemia at an early stage [10, 11]. Due to all these facts, there is urgent need to identify host factors that predispose to candidemia in order to establish a well-defined predictive profile for patients at risk for developing infection.

Toll-like receptors (TLRs) are transmembrane proteins on immune cells that recognize conserved microbial molecular motifs designated as pathogen-associated molecular patterns. Toll-like receptors interact with several adaptor proteins, including myeloid differentiation primary response gene 88 (MyD88) and MyD88 adaptor-like/Toll-interleukin 1 receptor domain-containing adaptor protein (Mal/TIRAP), to activate transcription factors, leading to production of proinflammatory cytokines and subsequent induction of adaptive immunity. Recognition of *Candida* species by innate immune cells has been demonstrated to be mediated through several TLRs, which in turn leads to a potent host defense activation against the yeast [12]. Genetic variants of TLRs and their adaptors MyD88 and TIRAP have been associated with increased susceptibility to bacterial infections [13–17]. A number of studies have proposed associations of *TLR2* and *TLR4* polymorphisms with increased risk of aspergillosis and candidemia [18–21]. However, most of these studies have been performed in very small cohorts of patients, and a systematic genetic study investigating variation in pattern recognition receptors in a large cohort of patients at risk of candidemia, comprising infected patients and noninfected control patients, has not been performed to robustly interrogate the importance of this innate pathway for protection against *Candida* species.

In the present study, we hypothesized that certain polymorphisms in *TLR* genes or in genes encoding their signaling adaptors may influence host susceptibility to the most common invasive fungal disease, candidemia. To test this hypothesis, we analyzed the prevalence of mostly nonsynonymous single nucleotide polymorphisms (SNPs) in 5 candidate *TLR* genes and genes encoding their adaptors *MyD88* and *TIRAP* in a cohort of 338 candidemia patients and 351 noninfected controls that had similar underlying predisposing factors. The genetic study was further complemented and supported with functional assays that assessed the consequences of these specific polymorphisms on the cytokine production capacity of primary human blood mononuclear cells in order to validate the mechanistic association of the susceptibility trait.

METHODS

Ethics Statement and Subjects

Adult subjects were enrolled after informed consent (or waiver as approved by the institutional review board) at Duke University Hospital (DUMC, Durham, North Carolina) and

Radboud University Nijmegen Medical Centre (RUNMC, Nijmegen, the Netherlands). Race of the patients was self-reported; all Dutch RUNMC patients were white. The study was approved by the institutional review boards at each study center, and enrollment occurred between January 2003 and January 2009. The study was performed in accordance with the Declaration of Helsinki.

Susceptibility to Infection

Infected subjects were enrolled consecutively and must have had ≥ 1 positive blood culture for a *Candida* species while hospitalized at the participating center. Noninfected controls must have been hospitalized with no history or evidence of candidemia or any invasive fungal infection. None of the control patients were on antifungal therapy/prophylaxis. Although it was impracticable to match controls on specific risk factors, noninfected controls were recruited consecutively from the same hospital wards/services as infected patients during the study period, with a similar balance of medical, surgical, and oncology patients in case and control groups. Immunocompromised status was a combined variable based on a broad definition including neutropenia, CD4⁺ lymphopenia (human immunodeficiency virus [HIV] associated or otherwise), chronic steroid use or receipt of immunosuppressive chemotherapy/immunosuppressant medications within 30 days prior to baseline, diabetes mellitus, and/or end-stage renal disease requiring hemodialysis. Acute renal failure was defined based on clinical diagnosis, including doubling of serum creatinine and/or requirement for acute dialysis (intermittent hemodialysis or continuous veno-venous hemofiltration dialysis). Intergroup comparisons between the 2 groups of noninfected subjects and between the 2 groups of infected subjects (at DUMC and RUNMC) were performed regarding similarity in genetic distribution of the studied SNPs prior to further statistical analysis of infected versus noninfected subjects.

Genetic Association With Clinical Outcomes

Due to the lack of detailed clinical data on the Dutch candidemia patients ($n = 40$) recruited at RUNMC, the correlations of the genetic data with clinical outcome was performed only for the patients ($n = 298$) from DUMC. Infected subjects at DUMC were followed prospectively for up to 12 weeks following diagnosis of candidemia to determine clinical outcome. Mortality data, including date of death, was ascertained from hospital records and/or the National Social Security Death Index. Disseminated infection was defined as the presence of *Candida* species at normally sterile body sites outside the bloodstream (excluding the urine). Persistent fungemia was defined as ≥ 5 days of persistently positive blood cultures.

Genetic Analysis

Genomic DNA was isolated from whole blood using standard procedures [22]. Coding nonsynonymous SNPs and a few SNPs

in untranslated regions of the analyzed genes were selected based on previously published associations with human diseases and/or known functional effects on protein function or gene expression. A total of 13 SNPs in *TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR9*, *TIRAP*, and *MyD88* were genotyped (Table 1) with the use of a mass spectrometry genotyping platform. Quality control was performed by duplicating samples within and across plates and by the incorporation of positive and negative control samples.

In Vitro Peripheral Blood Mononuclear Cell Stimulation Assays

Isolation and stimulation of peripheral blood mononuclear cells (PBMCs) was performed as described previously [23]. In brief, PBMCs obtained from 49 healthy white volunteers were incubated at 37°C for 24 hours with culture medium, the TLR1/2 agonist Pam3Cys (10 µg/mL; EMC Microcollections) or the TLR4 agonist lipopolysaccharide (*Escherichia coli* O55:B5 LPS, 10 ng/mL; Sigma-Aldrich). Cytokine production of interleukin 1 β (IL-1β), interleukin 6 (IL-6), and interleukin 8 (IL-8) was measured in the PBMC supernatants by enzyme-linked immunosorbent assay (R&D Systems).

Statistical Analysis

Chi-square tests of deviation from Hardy-Weinberg equilibrium for all 13 SNPs were calculated in cases and controls separately using PLINK statistical software [24]. Single locus tests of association were performed with logistic regression (or exact logistic regression, in the case of African Americans) using a dominant model (eg, minor allele homozygote combined with the heterozygote as risk genotypes) using SAS software version 9.2 (SAS Corporation). Model selection was based upon genotype frequencies. Standard summary statistics (odds ratio [OR] and 95% confidence interval [CI]) were reported for

these tests of association. Baseline demographic and clinical categorical variables in the case and control groups were compared by χ^2 analysis or Fisher exact test (as appropriate), and variables with $P < .05$ were analyzed as confounders of any SNP significantly associated with susceptibility to infection using logistic regression (or exact logistic regression as appropriate). Standard summary statistics for pairwise linkage disequilibrium (LD), D' , and r^2 were calculated using Haploview. Haplotype blocks were assigned using the D' confidence interval algorithm created by Gabriel et al [25].

Data on susceptibility to infection were analyzed for whites separately from African Americans because allelic frequencies were expected to differ among these 2 populations and controls, and examination of race-stratified genotype frequencies for the tested polymorphisms confirms this expectation [26, 27]. Statistical analysis of the cytokine data was performed using the Mann-Whitney U test. A P value $< .05$ was considered statistically significant.

Within the infected DUMC cohort, allelic frequencies were further assessed in association with 3 prespecified clinical outcomes: (1) disseminated disease, (2) persistent fungemia, and (3) all-cause mortality at 30 days. This analysis was performed in the entire infected cohort of DUMC whites and African Americans because the progression of the disease once occurring is not expected to differ between white and African American patients and race was considered as covariate in the analysis. Variables with $P < .2$ were further assessed in a multivariable logistic regression model using backward elimination. Variables with $P < .05$ were retained in the final predictive model. Odds ratios and 95% CIs were reported for variables that remained significant in the final multivariable model.

RESULTS

Demographics

Three hundred thirty-eight adult patients (298 North American, 40 Dutch) and 351 adult controls (300 North American, 51 Dutch) had genetic and clinical data available for this analysis. Demographics for study subjects are presented in Table 2

Table 1. Genotyped Single Nucleotide Polymorphisms in Genes Encoding Toll-like Receptors and Associated Adaptor Molecules

Gene	SNP ID	Gene Region	Amino Acid Change
<i>MYD88</i>	rs4988453	Promoter	...
	rs6853	3' UTR	...
<i>TIRAP</i>	rs595022	Intron 1	...
	rs8177374	Exon 5	Nonsynonymous S180L
<i>TLR1</i>	rs4833095	Exon 4	Nonsynonymous S248N
	rs5743611	Exon 4	Nonsynonymous R80T
	rs5743618	Exon 4	Nonsynonymous I602S
<i>TLR2</i>	rs5743704	Exon 3	Nonsynonymous P631H
	rs5743708	Exon 3	Nonsynonymous R753Q
<i>TLR4</i>	rs4986790	Exon 3	Nonsynonymous D299G
	rs4986791	Exon 3	Nonsynonymous T399I
<i>TLR6</i>	rs5743810	Exon 1	Nonsynonymous S249P
<i>TLR9</i>	rs5743836	Promoter	...

Abbreviations: SNP, single nucleotide polymorphism; TLR, Toll-like receptor; UTR, untranslated region.

Table 2. Demographics for Duke University Hospital and Radboud University Nijmegen Medical Centre Adult Study Subjects

	Infected Cohort (n = 338), No. (%)	Noninfected Controls (n = 351), No. (%)
Race		
White	237 (70%)	263 (75%)
African American	93 (28%)	88 (25%)
Other	8 (2%)	0 (0%)
Sex		
Male	199 (59%)	184 (52%)
Female	139 (41%)	167 (48%)

Table 3. Baseline Patient Characteristics for Duke University Hospital Infected Cohort and Control Cohort, Including White and African American Adult Patients

Variable	Infected Cohort (n = 298)	Control Cohort (n = 300)	P Value
Median age, y (IQR)	58.7 (43.0–68.1)	58.7 (45.0–69.6)	.41
Primary admitting service/primary problem			
Medical	50%	54%	.31
Surgical	33%	31%	.64
Oncology	17%	15%	.36
Median length of hospitalization, d (IQR) ^a	9 (2–18)	6 (4–12)	.80
Immunocompromised state	59%	42%	<.0001
Hematopoietic stem cell transplantation	1%	0%	.12
Solid organ transplant	12%	2%	<.0001
Active malignancy ^b	32%	20%	.002
Solid tumor	23%	12%	.27
Leukemia	7%	5%	.29
Lymphoma	4%	4%	1
Chemotherapy within past 3 months	16%	11%	.03
Neutropenia (ANC <500 cells/mm ³)	10%	4%	.001
HIV infected	1%	0.1%	.06
Surgery within past 30 days	43%	48%	.25
Receipt of total parenteral nutrition	19%	4%	<.0001
Dialysis dependent	12%	7%	.02
Acute renal failure	34%	22%	.001
Liver disease	25%	4%	<.0001
Intensive care unit admission within past 14 days	49%	33%	<.0001
Central intravascular catheter	82%	41%	<.001
Peripheral intravenous catheter	100%	97%	.004
Baseline serum creatinine, mg/dL, median (IQR)	1.3 (0.8–2.3)	1.0 (0.8–1.5)	.001
Median baseline WBC count, cells/mm ³ (IQR)	10.5 (6.0–17.1)	8.6 (6.4–12.4)	.04
<i>Candida</i> species ^c			
<i>albicans</i>	42%	...	
<i>glabrata</i>	29%	...	
<i>parapsilosis</i>	16%	...	
<i>tropicalis</i>	13%	...	
<i>krusei</i>	4%	...	
Other <i>Candida</i> species	3%	...	

Abbreviations: ANC, absolute neutrophil count; HIV, human immunodeficiency virus; IQR, interquartile range; WBC, white blood cell.

^a Until index blood culture for cases.

^b Subjects could have >1.

^c 16 subjects had >1 species isolated.

(including Dutch and North American subjects), and major clinical features among the infected and noninfected cohort are presented in Table 3 (including the North American subjects only). As expected, the groups were similar in regard to primary admitting service/primary presenting problem.

Genetic Analysis

Data from adult patients (n = 338) and controls (n = 351) were included in the analysis for genetic association with susceptibility to infection. The intergroup comparison between the Dutch RUNMC and white DUMC noninfected controls and between the infected subjects recruited at DUMC and RUNMC

revealed a similar genetic distribution of the genotyped SNPs, which allowed the groups to be merged into 1 group of noninfected controls and 1 group of infected subjects (data not shown). A post hoc analysis of the interaction between SNPs and race supported our stratification of this analysis by race. Genotyping of candidemia patients and noninfected controls for polymorphisms in genes encoding TLRs and their adaptor molecules MyD88 and Mal/TIRAP revealed a significantly different distribution of 3 *TLR1* polymorphisms when comparing infected patients and noninfected controls in white adults (Table 4). All genotyped SNPs were in Hardy-Weinberg equilibrium (data not shown). Linkage disequilibrium analysis

Table 4. Distribution of *TLR1* Genotypes in the White (245 Adult Candidemia Patients, 263 Noninfected Adult Controls) and African American (93 Adult Candidemia Patients, 88 Noninfected Adult Controls) Cohorts

Whites (245 Infected, 263 Noninfected) MAF				P Value	OR (95% CI) ^b
<i>TLR1</i> R ^a 80T	GG ^a	GC	CC	.02	1.82 (1.12–2.98)
Noninfected	88.2%	9.9%	1.91%	0.068	
Infected	80.2%	18.9%	0.86%	0.103	
<i>TLR1</i> S248N ^a	GG	GA	AA ^a	.04	0.68 (.47–.96)
Noninfected	5.3%	41.8%	52.9%	0.262	
Infected	7.2%	30.1%	62.7%	0.223	
<i>TLR1</i> I602S ^a	TT	TG	GG ^a	.02	0.69 (.49–.80)
Noninfected	7.6%	44.7%	47.7%	0.300	
Infected	10.2%	33.1%	56.8%	0.268	
African Americans (93 infected, 88 noninfected) MAF				P value	OR (95% CI) ^c
<i>TLR1</i> R ^a 80T	GG ^a	GC	CC	.62	2.92 (.23–155.70)
Noninfected	98.9%	1.1%	0%	0.006	
Infected	96.8%	3.2%	0%	0.016	
<i>TLR1</i> S ^a 248N	GG	GA	AA ^a	.09	3.86 (.71–39.15)
Noninfected	63.6%	28.4%	7.95%	0.222	
Infected	69.9%	27.9%	2.15%	0.161	
<i>TLR1</i> I ^a 602S	TT	TG	GG ^a	.60	3.12 (.25–166.70)
Noninfected	76.1%	20.5%	3.4%	0.137	
Infected	80.2%	18.7%	1.1%	0.105	

Abbreviations: CI, confidence interval; MAF, minor allele frequency; OR, odds ratio; TLR, Toll-like receptor.

^a Reference (equals major) allele in entire study population.

^b Logistic regression, dominant model.

^c Exact logistic regression.

revealed that alleles at rs4833095 (S248N) and rs5743618 (I602S) were strongly in LD ($r^2 = 0.8$) with each other but not with alleles at rs5743611 (R80T; $r^2 < 0.03$) (Figure 1). No associations were observed with polymorphisms in *TLR2*, *TLR4*, *TLR6*, *TLR9*, *MyD88*, and *TIRAP* and susceptibility to candidemia in whites (data not shown).

No significant associations with susceptibility to candidemia were observed in the African American cohort, neither with SNPs in *TLR1* (Table 4) nor in the other analyzed genes (data not shown).

Association of Polymorphisms With Clinical Outcome

Among the 298 DUMC adult infected subjects, 18% (54) developed disseminated disease and 15% (45) experienced persistent fungemia; the mortality rate was 28% (83) and 39% (116) at 30 and 90 days after initial blood culture, respectively. The most common type of infection complicating the bloodstream was abdominal abscess/peritonitis (55%) followed by hepatosplenic candidiasis (13%) and endocarditis/vascular sites (11%). Persistence occurred at similar rate among those with and without central catheters (13% vs 15%; $P = .67$) and with and without central catheter removal by day 5 (19% vs 25%; $P = .33$). Median (interquartile range) time to antifungal therapy in those with and without persistence (2 [1.75–3] days

vs 2 [1–3] days; $P = .36$) was also similar in those with and without persistence. Univariate and subsequent multivariate analysis assessing baseline patient characteristics and polymorphisms in *TLR1*, *TLR2*, *TLR4*, *TLR6*, *TLR9*, *MyD88*, and *TIRAP* (data not shown) indicated that there was no association between presence of polymorphisms at these loci and outcomes such as dissemination, persistence, and mortality.

In Vitro Stimulation Assays

Functional consequences of the 3 *TLR1* polymorphisms were studied by stimulating PBMCs obtained from 49 white, healthy volunteers bearing different *TLR1* haplotypes with Pam3Cys, a specific agonist of the TLR1/TLR2 heterodimer. Cytokine production capacity of IL-1 β , IL-6, and IL-8 was measured after 24 hours of stimulation (Figure 2). Unstimulated cells produced no detectable cytokine concentrations (data not shown). Dramatically decreased cytokine production was observed in cells bearing 1, 2, or 3 multilocus genotypes in *TLR1* that are associated with higher susceptibility to candidemia compared with haplotypes of wild-type alleles. Cytokine responses in relation to previously reported associations of *TLR2* (R753Q; Pam3Cys stimulation) and *TLR4* (D299G/T399I; LPS stimulation) polymorphisms with candidemia were also evaluated but revealed no significant differences (Figure 3).

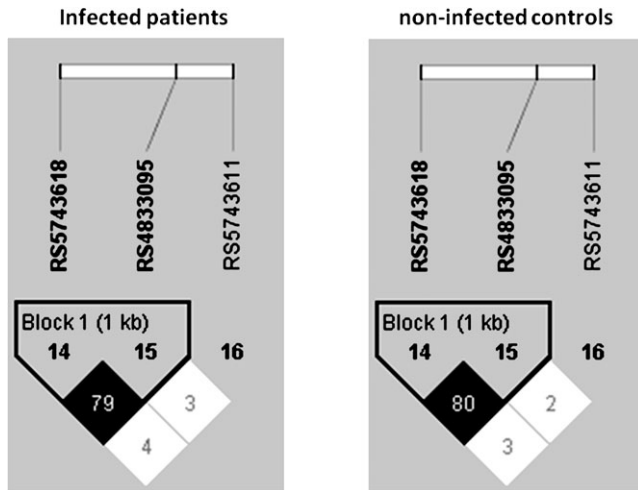


Figure 1. Linkage disequilibrium (LD) analysis of *TLR1* single nucleotide polymorphisms R80T (rs5743611), S248N (rs4833095), and I602S (rs5743618) in white individuals. Pairwise LD was assessed using the correlation coefficient (r^2). Percentage correlation between the 2 markers is noted in each box.

DISCUSSION AND CONCLUSIONS

The present study demonstrates the association of 3 *TLR1* polymorphisms with an increased susceptibility for candidemia in white patients. In addition to the genetic association between *TLR1* polymorphisms and candidemia, cells isolated from individuals bearing the *TLR1* variants that predispose to candidemia have shown functional consequences with decreased cytokine responses upon in vitro stimulation with specific TLR1/TLR2 ligands. In contrast to *TLR1*, no influence of *TLR2*, *TLR4*, *TLR6*, *TLR9*, *MyD88*, or *TIRAP* SNPs on the susceptibility to candidemia was observed.

Several TLRs have been reported to be involved in recognition of *C. albicans*, among which TLR2 mediates recognition of phospholipomannan [28], TLR4 of O-bound mannan [29], and TLR9 of fungal DNA [30]. Toll-like receptor 6 has also been demonstrated to contribute to *Candida*-mediated cytokine responses [31], whereas TLR1 forms functional heterodimers with TLR2 [32]. Because identified genetic variants of TLRs have recently been reported to influence function of these proteins and susceptibility to infections [33], we assessed the role of SNPs in these receptors and their adaptor molecules MyD88 and Mal/TIRAP for potential insights into the susceptibility to *Candida* bloodstream infections.

Among the genes and SNPs tested, we found that 3 polymorphisms in *TLR1* were associated with increased susceptibility to development of candidemia in hospitalized patients at risk for this fungal disease. Although 2 of these polymorphisms (S248N and I602S) were strongly (80%) linked, the third (R80T) was not linked to the first 2 polymorphisms. When the 2 groups

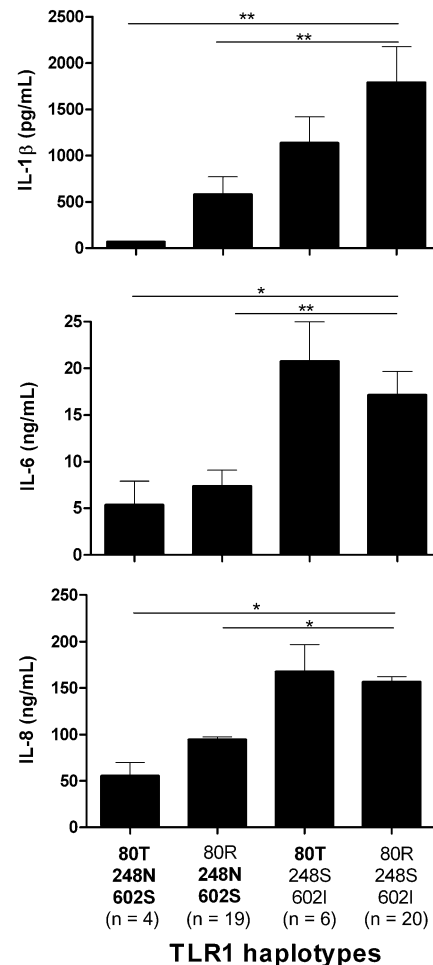


Figure 2. Correlation of *TLR1* haplotypes with cytokine measurements of interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and interleukin 8 (IL-8) 24 h after in vitro stimulation of peripheral blood mononuclear cells from healthy volunteers with the Toll-like receptor 1 (TLR1)/TLR2 agonist Pam3Cys (10 μ g/mL). On the x-axis the different *TLR1* haplotypes are depicted with protective genotypes (not bold) and risk genotypes (bold). Data are presented as means \pm SEM (Mann-Whitney *U* test, * $P \leq .05$, ** $P \leq .005$).

of subjects recruited at DUMC and RUNMC were considered separately, this trend of association could be observed also in the smaller RUNMC group. Due to the common Western European ancestry of the 2 groups, they were analyzed together. Importantly, frequencies of the *TLR1* polymorphisms in the white noninfected controls were fairly equal compared with the general population, as assessed by genotyping 152 white healthy controls for these SNPs (data not shown), indicating that *TLR1* polymorphisms confer a higher risk for developing candidaemia, but not for underlying predisposing factors. The observation that all 3 SNPs influence susceptibility to candidemia strengthens the conclusion that there is an important role for TLR1 in influencing the

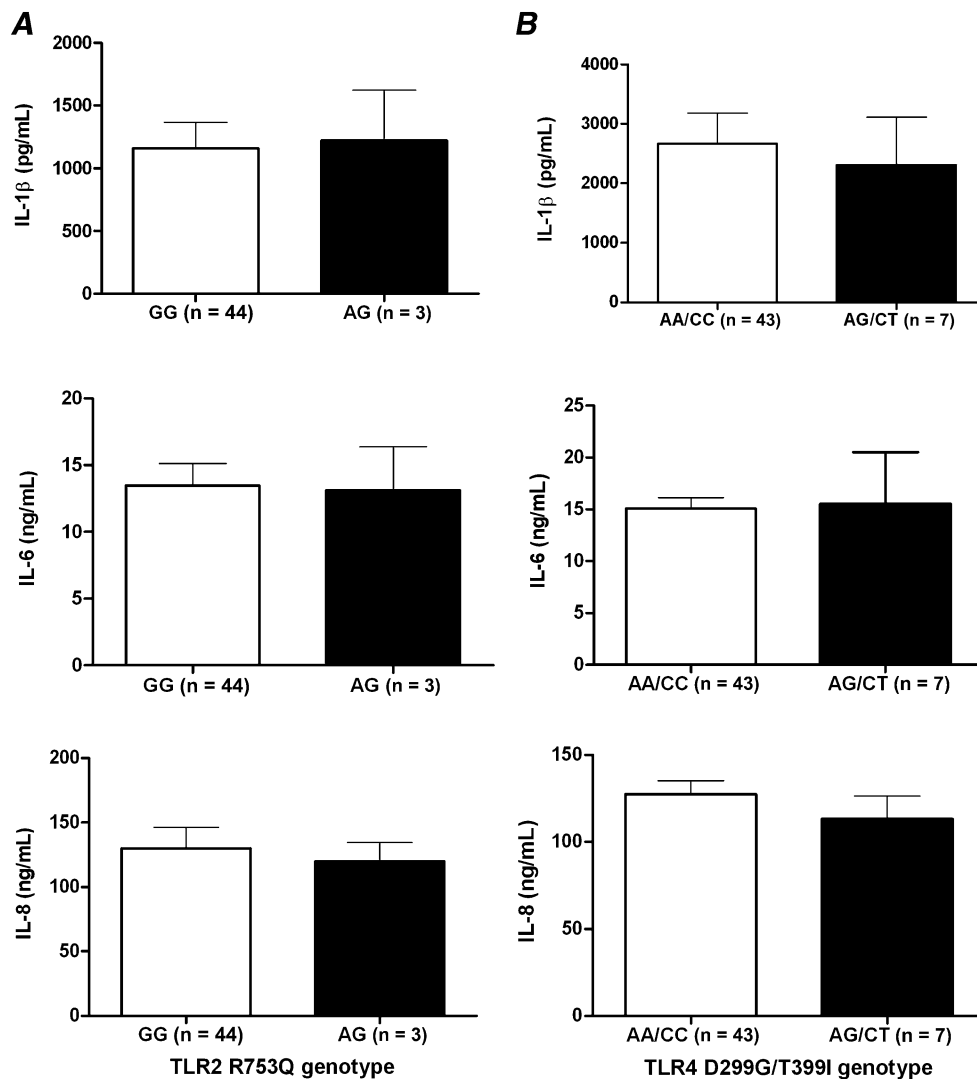


Figure 3. Correlation of *TLR2* R753Q (A) and *TLR4* D299G/T399I (B) genotypes with cytokine measurements of interleukin 1 β (IL-1 β), interleukin 6 (IL-6), and interleukin 8 (IL-8) 24 h after in vitro stimulation of peripheral blood mononuclear cells from healthy volunteers with either Pam3Cys (A, 10 μ g/mL) or lipopolysaccharide (B, 10 ng/mL). Data are presented as means \pm SEM. Abbreviation: TLR, Toll-like receptor.

susceptibility to the infection. Moreover, the genetic data were complemented by functional studies demonstrating that *TLR1* genotypes predisposing to candidemia are associated with decreased proinflammatory cytokine release upon stimulation.

One study in mice did not identify a nonredundant role of TLR1 in the host defense against disseminated candidiasis [31]. Hence, our data suggest that important differences exist between the role of TLR1 in mice and humans. Furthermore, the high inocula mouse model of disseminated candidiasis may very well differ from the clinical course of infection in humans with their heterogeneous and dynamic risk factors.

Toll-like receptor 1 is known to form heterodimers with TLR2, which has been shown to be involved in *Candida* recognition. For one of the *TLR1* polymorphisms, I602S, it was

demonstrated that the TLR1 protein bearing 602S is associated with impaired cell surface trafficking, resulting in a decreased availability of TLR1 on the cell membrane [34]. Consequently, lower availability of TLR1 could skew the configuration of TLR2 from heterodimers attached to TLR1 toward predominantly TLR2 homodimers or TLR2/TLR6 heterodimers. This, in turn, could alter intracellular signaling and cytokine production induced after *Candida* species encounter innate immune cells, which might result in deleterious effects during systemic fungal infections.

Toll-like receptor 1 could also exert its effect on antifungal host defense through beta-defensin-3, which activates immune cells through TLR1/TLR2 [35]. Beta-defensin-3 has an important lytic activity against *C. albicans*, and its expression

is induced in mucosal layers after fungal recognition [36]. As a consequence, diminished TLR1 function could impair mucosal antifungal defense, enabling *Candida* to invade the tissue defenses and reach the bloodstream. Of note, the main route of *Candida* species to invade the bloodstream and cause invasive infection is indeed through crossing the epithelial layers of the skin and the intestine [37]. Furthermore, it cannot be excluded that other still unidentified endogenous danger-associated molecular patterns, which are possibly elicited in the context of candidemia, are able to induce TLR1 signaling and contribute to activation of antifungal mechanisms.

In contrast to the effects of *TLR1* SNPs in white patients, no significant association of *TLR1* polymorphisms with susceptibility to candidemia was detected in patients of African American origin. This is likely due to the smaller sample size of African Americans in our study population and consequently lower statistical power as well as a different distribution of these polymorphisms compared with those observed among whites. It has been previously noted that there are large differences in the genetic distribution of these *TLR1* polymorphisms between African American and white populations [38]. In particular, the alleles encoding S248N and I602S polymorphisms are distributed very differently throughout the populations, and this may provide a partial explanation for the absence of association of *TLR1* polymorphisms with candidemia in our smaller African American cohort. On the other hand, no substantial differences were observed between white and African American patients with regard to basic clinical characteristics.

Clinical parameters of infection comprising dissemination, persistent fungemia, and 30-day mortality were not associated with any of the polymorphisms analyzed in this study, including polymorphisms in *TLR1*. This may imply that TLR1 is mainly involved in the early host response, thereby preventing bloodstream infection with *Candida* rather than determining the outcome of established candidemia. Of note, previous studies with mouse models of disseminated candidiasis in *TLR1* knockout mice have only addressed the latter condition [31], which may provide an additional explanation for the differences observed in the role of TLR1 in candidemia between mice and humans.

Our results demonstrating important effects of *TLR1* polymorphisms on *Candida* bloodstream infections are strengthened by studies reporting similar effects on genetic susceptibility to aspergillosis [19], bacterial sepsis [39], or mycobacterial infections [40]. Interestingly, a recent study on the evolutionary genetics of TLRs reported a high variation in *TLR1*, with important evolutionary pressures exerted by infections on this receptor [38]. We must, however, concede that these specific pressures were most likely exerted by bacterial infections and not by fungal infections that are primarily caused by modern

medical circumstances (eg, chemotherapy, stem cell transplantation, and invasive procedures).

Among DUMC whites and African Americans, a number of baseline risk factors were significantly more common among infected subjects on univariate analysis (data not shown). Although these differences were unavoidable due to the nature of the pathologies associated with candidemia, none of these variables were significantly associated with presence of the *TLR1* polymorphisms in either infected patients or noninfected controls. Therefore it is highly unlikely that they are confounders of the association between *TLR1* polymorphisms and candidemia (data not shown).

Recent studies have demonstrated that TLR2, TLR4, TLR6, and TLR9 are also implicated in recognition of components of *Candida* species. Polymorphisms in *TLR2* have been reported as a risk factor for candidemia in a small number of patients [21], and we have reported the association of *TLR4* D299G/T399I polymorphisms in a small cohort of patients [20]. These data could not be confirmed in the present study that investigated by far the largest cohort of patients with candidemia, further emphasizing the importance of recruiting cohorts of sufficient size and comparing them to controls at similar risk of infection. In contrast to *TLR1*, no effects of *TLR6*, *TLR9*, *TIRAP*, or *MyD88* polymorphisms on susceptibility to *Candida* bloodstream infections were apparent.

Our study is subject to several limitations. Although we had a relatively large sample for a study of genetic association of an infectious disease, we had a small sample of African American patients, which limited our ability to detect differences in this segment of the population, except large differences in allelic frequency. In addition, the associations we report for TLR1 polymorphisms among whites were not significant enough to withstand adjustment for multiple comparisons. However, we demonstrated that genetic variants of *TLR1* associated with higher susceptibility result in a suppressed function of TLR1, as revealed by impaired induction of cytokine responses upon activation of innate immune cells through TLR1. Together, these complementary genetic and immunologic data support a role of this receptor in antifungal host defense against candidemia.

In conclusion, the present study demonstrates the association between *TLR1* polymorphisms and susceptibility to candidemia. We also found that these same TLR1 polymorphisms are associated with impaired function of TLR1 and decreased cytokine responses. Although this correlation between genetic and functional data strengthens the conclusion of the study, further research is warranted to elucidate the exact role of TLR1 signaling in host defense against *Candida* species in humans and how knowledge on polymorphisms in *TLR1* could be used in risk assessment for strategies of prophylactic, empiric, or preemptive therapies.

Notes

Acknowledgments. T. S. P., E. v. d. V., D. K., and M. O. performed the experiments; E. v. d. V. and D. K. selected SNPs and designed the genotyping assays; M. D. J., P. B. S., B. D. A., J. C. Y., G. M. L., J. R. P., B. J. K., and M. G. N. managed the collection of the cohort; M. D. J., D. R. V. E., and W. K. S. performed the clinical statistical analysis; T. S. P., M. D. J., J. R. P., T. W., and M. G. N. designed the study and wrote the manuscript. All authors read and approved the final manuscript. All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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