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Journal

Genes & Immunity, 16(5)

ISSN

1466-4879

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Publication Date

2015-07-01

DOI

10.1038/gene.2015.13

Peer reviewed

HHS Public Access

Author manuscript

Genes Immun. Author manuscript; available in PMC 2016 January 01.

Published in final edited form as:

Genes Immun. 2015 ; 16(5): 362–365. doi:10.1038/gene.2015.13.

Toll-like receptor gene variants and bacterial vaginosis among HIV-1 infected and uninfected African women

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Conflict of Interest

The authors declare no conflict of interest.

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Abstract

Bacterial vaginosis (BV) is a common vaginal syndrome associated with altered microflora that increases the risk of preterm delivery and acquisition of sexually transmitted diseases. The cause of BV is unknown although toll-like receptors (TLRs), that are central to innate immune responses, may be important. We evaluated associations between TLR SNPs and BV among HIV-1 infected and uninfected African women. Logistic regression was used to assess associations between SNPs (N=99) in *TLRs 2-4*, *7-9* and BV (as classified by Nugent's criteria). Among HIV-1 uninfected women, *TLR7* rs5743737 and *TLR7* rs1634323 were associated with a decreased risk of BV while *TLR7* rs179012 was associated with an increased risk. TLR2 SNP rs3804099 was associated with a decreased risk of BV among HIV-1 infected women. Our findings indicate that there may be differences in TLR association with BV among HIV-1 infected and HIV-1 uninfected women.

Introduction

Bacterial vaginosis (BV) is a clinical disorder associated with changes in the vaginal microflora and studies have shown it affects nearly 50% of African women[1, 2]. It is associated with significant health consequences, and increases risk of a number of sexually transmitted infections (STIs), including acquiring [3, 4] and transmitting [2] HIV-1. Despite its high prevalence and significant sequelae, the pathogenesis of BV has not been entirely elucidated, thus impeding development of effective treatment and prevention interventions.

The shift from healthy vaginal flora to flora characteristic of BV is not well understood. To date no individual pathogen has been definitively linked to BV causation; however, in the context of BV, the vaginal ecosystem transitions from one dominated by Lactobacillus spp., to a more diverse microflora. This transition raises the possibility that host innate pathogen sensors, such as the TLRs, may play a role in these changes. TLRs are expressed in epithelial cells, leukocytes and dendritic cells of the female genital tract and both *in-vitro* and *in-vivo* studies have suggested that they may be important in the immune response to BV[5]. However, which TLRs and the mechanism by which they contribute to BV is unclear. Furthermore, no studies have evaluated TLR associations with BV susceptibility among HIV-1 infected women. We sought to explore the role of TLRs in BV by evaluating the association of *TLR* polymorphisms with this dysbiosis. In this study we aimed to test the hypothesis that single nucleotide polymorphisms (SNPs) in *TLR2*, *TLR3*, *TLR4*, *TLR7*, *TLR8*, *TLR9*, or associated TLR signaling genes *MYD88, TIRAP and ACAA1*, are associated with BV incidence in HIV-1 infected and uninfected African women.

Results & Discussion

Population characteristics

Of the 372 women included in this analysis, 216 (58%) were HIV-1 infected, including 195 (90%) who had HIV-1 at enrollment and 21 (10%) who acquired HIV-1 during follow-up (Table 1). Among HIV-1 infected women, 165 (76%) had BV at >1 visit and 51 (24%) had no BV during follow-up. HIV-1 infected women with BV were less likely than women without BV to be East African (69% versus 84%; p=0.05), had a lower CD4+ T cell count at study enrollment (434 versus 520 cells/mm^3; p=0.02), higher plasma HIV-1 RNA levels (4.7 versus 4.4 log_{10} copies/mL at enrollment; p=0.03) and also higher genital herpes (HSV-2) prevalence (99% versus 92%; p=0.01). HIV-1 infected women with and without BV had the same median age (29 years). Among 156 (42%) women who were HIV-1 uninfected: 105 (67%) had BV and 51 (33%) had no BV. HIV-1 uninfected women with and without BV had similar distributions of East Africans (86% versus 78%; p=0.36), were of similar age (27 versus 28; p=0.11) and had similar prevalence of HSV-2 (94% versus 90%; p=0.34).

BV-TLR associations in HIV-1 uninfected women—Among HIV-1 uninfected women, the intronic tagSNP *TLR7* rs5743737 was associated with a decrease in BV risk even after correcting for multiple comparisons (odds ratio [OR] = 0.14, 95% confidence interval [CI]: 0.04, 0.37; $p=5\times10^{-5}$, $p_{corrected}=0.005$), with 30% of women with one or two copies of the *TLR7* rs5743737 minor allele (AG or GG) developing BV compared to 74% of women with the AA genotype. Similarly, the intronic tagSNP *TLR7* rs1634323 was associated with decreased risk of BV (OR= 0.20, 95% CI: 0.09, 0.46; $p=1\times10^{-4}$, $p_{corrected}=0.01$). Specifically, 42% of women with the *TLR7* rs1634323 minor allele (AG or GG) developed BV compared to 75% with two copies of the A allele. The intronic candidate SNP *TLR7* rs179012 (which had previously been found associated with lower plasma HIV-1 set-point in HIV-1 infected individuals [6]) was associated with increased risk of BV (OR=2.39, 95% CI: 1.06, 5.79; p = 0.04). Among women with the TLR7 rs179012 minor allele (AG or GG), 78% developed BV during follow-up compared to 62% of women who carried two copies of the A allele.

BV-TLR associations in HIV-1 infected women—Among HIV-1 infected women, SNPs in TLR7 did not have a statistically significant association with BV. However, the synonymous *TLR2* 816 C/T candidate SNP (rs3804099) was associated with reduced risk of BV (OR = 0.43; 95% CI: 0.21, 0.84; p = 0.01). This SNP has previously been associated with a lower plasma HIV-1 set-point in HIV-1 infected Africans (Table 1)[6]. Additional analyses comparing women with normal flora (Nugent's score = 0-3) to women with BV (Nugent's score = 7-10) resulted in point estimates with the same direction of risk as the analyses that included women with intermediate flora (Nugent's score =4-6).

Discussion—Our study is the first to evaluate associations between TLR SNPs and BV in African HIV-1 infected and uninfected women. We found that SNPs in TLR2 and TLR7 may contribute to BV incidence in African women and that these genetic associations may be modified by HIV-1 status. Interestingly, two SNPs previously associated with HIV-1 set-point in the same cohort were associated with BV among women in our analysis, which may

underscore a complex relationships between BV, HIV-1 and innate immune responses. A recent study among HIV-1 infected African-American adolescents found SNPs in TLR1, TLR2, TLR4, and TLR9 to be associated with an increased risk of BV [7]. Our findings in an African cohort suggest that TLR7 gene variants may be differentially associated with BV occurrence in HIV-1 infected and uninfected women. Notably, no previous published genetic epidemiology studies have evaluated the association of TLR7 with BV development. Of interest in this regard is recent data that suggests HIV-1 may effectively disrupt TLR7 function[8]. This could explain why we do not observe the strong protective effect from TLR7 rs5743737 and rs1634323 variants in HIV-1 infected women, and may also explain the generally high rates of BV in HIV-1 infected women [1]. The mechanism by which TLR7, which is typically understood to mediate protection against viral infections, may protect against BV, a disease commonly perceived as of bacterial etiology, is not clear.

Among the SNPs we found to be associated with BV outcomes, two are intronic and one is synonymous and, similarly to most SNP-association studies, will require future studies to elucidate how these SNPs are linked to functional characteristics influencing BV susceptibility. One possibility is that intronic SNPs influence gene splicing or regulation[9]. For instance, the intronic TLR7 rs179012 SNP is predicted to impact potential transcriptional binding sites[10]. Alternatively, associations of nonfunctional SNPs with BV could be due to these tagSNPs being in linkage disequilibrium (LD) with a causal SNP. The TLR7 rs5743737 and TLR7 rs1634323 SNPs from our study are located in areas of high LD on chromosome X that include splicing and functional variants [10].

Since TLR7 is thought to be activated by single-stranded viral RNA, and the strongest associations of TLR7 variants with BV outcomes were in HIV-uninfected women, our findings raise a speculative hypothesis that innate responses through TLR7 may provide homeostatic support to the vaginal microflora through an inflammatory responses against bacteriophages targeting Lactobacilli. Bacteriophages with RNA genomes are well described [11], and recent studies have suggested that Lactobacillus-associated bacteriophage may be present in the context of BV [12-14]. Furthermore, host inflammatory responses to endosymbiotic Trichomona virus through TLR3 have been reported in the context of genitourinary infection with *Trichomonas vaginalis* [15]. If our findings of TLR7 variants and BV are corroborated, modifiers of TLR7 function could be evaluated as possible interventions to treat or prevent BV.

Our findings require replication, particularly given our convenience sampling based on prior BV and TLR genotyping data. Furthermore, this African cohort had high (95%) HSV-2 seroprevalence while the prior study of African-American adolescents had low HSV-2 seroprevalence (11%)[7]. Given the reported association between HSV-2 shedding and TLR2 variation, further study is warranted to understand how HSV-2 seroprevalence may modify the relationship of TLR variation with BV [16]. *In vitro* studies of monocytes have shown that BV-related ligands may stimulate TLR2 mediated release of pro-inflammatory cytokines [17]. While it is uncertain how these *in vitro* findings relate to our genetic association study, these data underscore the importance of further study to better evaluate the role of TLRs in relation to BV and other genital tract infections.

Methods

Participants were from a prospective cohort of HIV-1 serodiscordant heterosexual couples (one partner HIV-1 infected and the other HIV-1 uninfected) described in detail elsewhere [18]. Couples were recruited based on the HIV-1 infected partner being dually infected with HSV-2 and with $CD4^+ > 250$ cells/mm^3 at enrollment. Thus, women in this analysis could either be HIV-1 infected (at enrollment or during follow-up) or HIV-1 uninfected. BV outcome classification used Nugent's criteria applied to vaginal secretion swabs prospectively collected from all women at enrollment and quarterly follow-up visits [19]. Women with BV had a Nugent's score = 7-10 at any visit; women without BV had normal or intermediate flora (Nugent's score = 0-6) at all visits with BV testing. Additionally, we performed a separate analysis that only included women with normal flora (Nugent's score 0-3) among women without BV (excluding women with intermediate flora, Nugent's score = 4-6).

DNA was isolated from archived whole blood using Puregene DNA purification (Qiagen, Valencia, CA). Genotyping was performed using an Illumina Custom Oligo Pooled Assay for124 SNPs in *TLR2* (n=9), *TLR3* (n=13), *TLR4* (n=22), *TLR7* (n=40), *TLR8* (n=25), *TLR9* (n=3), *MYD88* (n=4) and *TIRAP* (n=8); 117 of these are haplotype-tagging SNPs (tagSNPs) chosen to represent common variation across the genes as previously described [6]. The remaining 7 SNPs are candidate SNPs that have previously been associated with BV or HIV-1 outcomes [6, 7, 20]. SNPs previously implicated in HIV-1 outcomes were considered candidate SNPs since we have previously shown in this cohort that HIV is associated with BV[21] and TLRs are associated with HIV-1 outcomes [6].

In total, TLR genotypes and longitudinal BV data were available from 392 women. HIV-1 infected women and initially HIV-1 uninfected women had a similar average number of visits at which BV was assessed. Women were excluded from downstream analyses if their reported sex did not match genotypic sex (n=9), if they exhibited genotypic missingness >10% (n=4), or exhibited relatedness to participants as determined by Identity by State>95% (n=7). Thus, 372 women were included in the analyses. Of the 124 genotyped SNPs, 25 were excluded for call rate <95% (n=7) or minor allele frequency (MAF) <5% (n=18), leaving 99 SNPs for the final analyses. No SNPs violated Hardy-Weinberg Equilibrium.

Analysis of TLR SNP associations with BV was performed using logistic regression. We adjusted for population stratification using three principal components (PC) that were derived by applying a modified EIGENSTRAT method [22] to the ~10^6 SNPs included in our previous genome-wide association study [23]. We evaluated statistical significance of tagSNPs associations by applying a Bonferroni correction cutoff of Pcorrected < 0.0005 reflecting N=99 tagSNPs. P-values for candidate variant associations were not corrected since they represented confirmation of variants previously reported to be associated with HIV-1 or BV outcomes. We did not include HSV-2 as a covariate since it may be in the causal pathway (see Discussion) for TLR2 SNPs in HIV-1 infected women. P-values defining significant candidate variant associations were not corrected since they represented confirmation of variants previously reported with significant associations with HIV-1 outcomes. We also evaluated false-discovery rate adjustment [24] and found that yielded

similar results. All analyses were performed in R and assumed a dominant model of inheritance.

Acknowledgments

The specimens and data for this analysis were collected in the context of the Partners in Prevention HSV/HIV Transmission Study (Bill and Melinda Gates Foundation Grant #26469) with BV analysis augmented through support from NIH NAID R01 AI-083034. Genotyping was supported through NIH R21 AI073115. The authors are grateful for the women who participated in this study and the Partners in Prevention Study team in Africa and Seattle who provided clinical and laboratory data.

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Table 1

Description of the cohort by HIV-1 and BV status

	HIV-1 Infected[b] *(n =216)*			*HIV-1 Uninfected (n =156)*			***Total* (n =372)**
	BV (n=165)	***No BV n=(51)***	***p-value***	***BV (n=105)***	***No BV (n=51)***	***p-value***	
Age, years	29 (24, 34)	29 (25, 33)	0.81	27 (22, 31)	28 (24, 33)	0.11	28 (24, 33)
East African	114 (69%)	43 (84%)	0.05	90 (86%)	40 (78%)	0.36	287 (77%)
CD4+ Count (enrollment; cells/mm^3)	434 (330, 575)	520 (408, 755)	0.02	-	-		445 (334, 604)
Plasma HIV-1 RNA (enrollment; log_{10})	4.7 (4.0, 5.1)	4.4 (3.9, 4.9)	0.03	-	-		4.7 (4.0, 5.0)
HSV-2 Infected	164 (99%)	47 (92%)	0.01	99 (94%)	46 (90%)	0.34	353 (95%)

a. Numbers (%) are provided for categorical variables and medians (inter-quartile ranges) are provided for continuous variables.

[b] HIV-1 infected prior to first BV diagnosis

Table 2

TLR genotypes significantly associated with BV risk in HIV-1 infected and HIV-1 uninfected women.

Gene Variants					
HIV-1 INFECTED		**BV - positive**	**BV - negative**	**OR (95%CI)**	**p-value**
***TLR2* - rs3804099 Exon 3 (C/T) Synonymous [Candidate SNP]**	***CC***	94 (84%)	18 (16%)	**0.43 (0.21, 0.84)**	0.01*
	CT/TT	71 (68%)	33 (32%)		
HIV-1 UNINFECTED					
***TLR7* - rs179012 Intron (A/G) [Candidate SNP]**	**AA**	63 (62%)	39 (38%)	**2.39 (1.06, 5.79)**	0.04*
	AG/GG	42 (78%)	12 (22%)		
***TLR7* - rs1634323 Intron (A/G) [TagSNP]**	**AA**	89 (75%)	29 (25%)	**0.20 (0.09, 0.46)**	1×10^{-4}**
	AG/GG	16 (42%)	22 (58%)		
***TLR7* - rs5743737 Intron (A/G) [TagSNP]**	**AA**	98 (74%)	35 (26%)	**0.14 (0.04, 0.37)**	5×10^{-5}**
	AG/GG	7 (30%)	16 (70%)		

*Significant at p-value threshold of $p<0.05$

**Significant at p-value threshold of $p<5\times10^{-4}$ (with Bonferroni correction for N=99 comparisons)