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Exploring the role of innate immune genetic variation in bacterial
vaginosis and vaginal bacterial colonization

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Abstract

Exploring the role of innate immune genetic variation in bacterial vaginosis and vaginal bacterial colonization

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Background. Bacterial vaginosis (BV) is a common, vaginal dysbiosis associated with adverse gynecological and reproductive health outcomes. BV-associated bacteria (BVAB) present microbe-associated molecular patterns to Toll-like receptors (TLRs), which modulate the innate immune response. However, the role of these TLRs in BV pathogenesis remains unclear. I hypothesized that individuals possessing single nucleotide polymorphism (SNP) genotypes associated with heightened innate immune responses (TLR1, TLR6, TLR4, and TLR5 “sufficiency”; TLR10, TOLLIP “deficiency”) are at decreased risk of BV and colonization with BV-associated bacteria (BVAB).

Methods. Women were enrolled in independent discovery and validation cohorts and genotyped for common, functionally-characterized SNPs: TLR1 rs5743618, TLR6 rs3821985, TLR4 rs4986790, TLR4 rs4986791, TLR5 rs5744168, TLR10 rs11096955, TLR10 rs4129009, and TOLLIP rs5743854. Genotype models (“sufficient” versus “deficient”) were based on previously published data. As my primary analysis, I compared TLR/TOLLIP sufficient and deficient study participants on the following outcomes: risk of clinically-defined BV, risk of microbiologically-defined BV, colonization with lactobacilli, and colonization with BVAB.

Results – Association with BV. In both the discovery and validation cohorts, TLR10 rs11096955 TG/GG (deficiency) associated with increased risk of clinically-defined BV. TOLLIP deficiency and TLR4 deficiency were both associated with increased risk of clinically-defined BV, but only in the discovery cohort.

Results – Association with bacterial colonization. TLR4, TLR10, and TLR6 deficiencies were associated with lower *Lactobacillus jensenii* concentrations. TOLLIP deficiency was associated with higher concentrations of *Megasphaera* types 1 and 2, *Gardnerella vaginalis*, and *Atopobium vaginae*. TLR4 deficiency was correlated with higher concentrations of *G. vaginalis*, *A. vaginae*, and total bacterial load. TLR5 deficiency was associated with lower concentration of flagellated species, BV-associated bacterium 1, and *Mobiluncus mulieris* (but not *Mobiluncus curtisii*).

Conclusions. Individuals possessing genotypes consistent with increased TLR-mediated responses—TLR10, TOLLIP deficiency—have increased risk of clinically-defined BV. The data presented here are consistent with TLR4 deficiency being realized as a deficiency of soluble TLR4 that affects concentrations of several Gram-positive bacteria. Finally, individuals with more robust TLR5 responses (TLR5 sufficiency) appear more susceptible to colonization with

specific, flagellated BVAB, although no differences in overall risk of clinically- or microbiologically-defined BV appear to exist. The data presented here are consistent with a model whereby some BVAB benefit from host innate immune response.

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DEDICATION

To my parents, for raising a daughter stubborn enough to finish a doctorate.

To my grandmothers, for being two of the first feminists I would ever know.

To my late sister, Aileen, a WSWOC who (I think) would have found this dissertation amusing.

To my spouse, Evan: let us never slide our backpacks off a snowfield above a cliff ever again.

To my daughter, Penelope Sylvia: for you, I hope anything is possible. I cannot wait to see who
you become.

Chapter 1. BACKGROUND

1.1 BACTERIAL VAGINOSIS

Bacterial vaginosis (BV) affects 29% of US women of reproductive age¹ and is a common reason to seek gynecologic care². BV is associated with increased risk of HIV acquisition³⁻⁷, sexually transmitted infections (STIs)⁸, cervicitis⁹, preterm labor¹⁰⁻¹², premature rupture of membranes¹³, and pelvic inflammatory disease^{14,15}.

Microbiologically, BV can be diagnosed using Gram stain of vaginal fluid with Nugent score¹⁶. More commonly, BV is diagnosed clinically based on the presence of 3 out of 4 clinical signs (Amsel's criteria): vaginal fluid pH above 4.5; thin, homogeneous vaginal discharge; amine odor on potassium hydroxide preparation (whiff test); and epithelial cells covered in adherent bacteria on wet mount of vaginal fluid ("clue cells")¹⁷. Although Nugent score is considered the "gold standard" of BV diagnosis, it is rarely used for point-of-care diagnostics¹⁸. While Amsel's criteria diagnosis of BV is usually concordant with Nugent score, discordant clinical and microbiological assessments are regularly observed. If some women have microbiological criteria for BV but lack clinical findings, host factors may play a role in modifying the clinical presentation of BV.

1.2 RISK FACTORS FOR BV

No animal or *in vitro* model captures the bacterial colonization patterns of the human vagina¹⁹. Thus, the models of BV pathogenesis developed thus far have relied mainly upon epidemiologic studies supplemented by some cell culture-based systems.

1.2.1 *Microbiological correlates of BV risk*

Using culture-dependent methods, detection of hydrogen peroxide-producing lactobacilli in vaginal fluid was found to be correlated with decreased risk of incident²⁰ and prevalent²¹ BV. More recently, use of molecular methods has demonstrated that *L. iners* is highly prevalent in women with and without BV²². Additional studies have identified virulence factors within the *L. iners* genome, indicating it may have a role as a vaginal pathobiont (reviewed in ²³). Other lactobacilli, including *L. crispatus* and *L. jensenii*, are highly sensitive and specific for the absence of BV^{22,24} and *L. crispatus* has been identified as a correlate of decreased risk of incident BV^{25,26}. Although earlier studies found protection associated with hydrogen-peroxide producing lactobacilli, hydrogen peroxide does not kill BVAB *in vitro*²⁷ and is not produced by lactobacilli in the low-oxygen environment of the vagina *in vivo*²⁸. *Lactobacillus* spp. are thought to provide protection via production of lactic acid²⁹⁻³¹, which has been shown to suppress growth of BVAB²⁷.

Women of African ancestry are at increased risk of BV¹ and often have diverse vaginal microbiota depleted of *L. crispatus*, *L. jensenii*, and *L. gasseri* despite a lack of clinical signs or symptoms of BV³². Even without signs or symptoms, these BV-like communities are associated with increased inflammation^{33,34} and risk of HIV acquisition³.

1.2.2 *Epidemiological risk factors suggest sexual transmission of BV-associated bacteria (BVAB)*

Behavioral risk factors for BV include increased number of sexual partners^{35,36} and sexual intercourse without condoms³⁶. Among women who have sex with women, partners tend to be concordant for BV status^{25,35,37,38}. BV can be treated with antibiotics³⁹, but at least 50% of women diagnosed with BV will experience recurrence within 6 months^{18,40}; sex with the same

partner increases risk of prevalent BV⁴¹ and recurrence of BV^{42,40,20}. In addition to these sexual risk factors, colonization of other body sites of patients and their partners with BVAB increases risk of BV²⁶. DNA from BVAB has been detected in gingival and rectal swabs collected from women¹⁸, and has also been detected in samples collected from men, including urethral swabs, urine, and penile swabs⁴³.

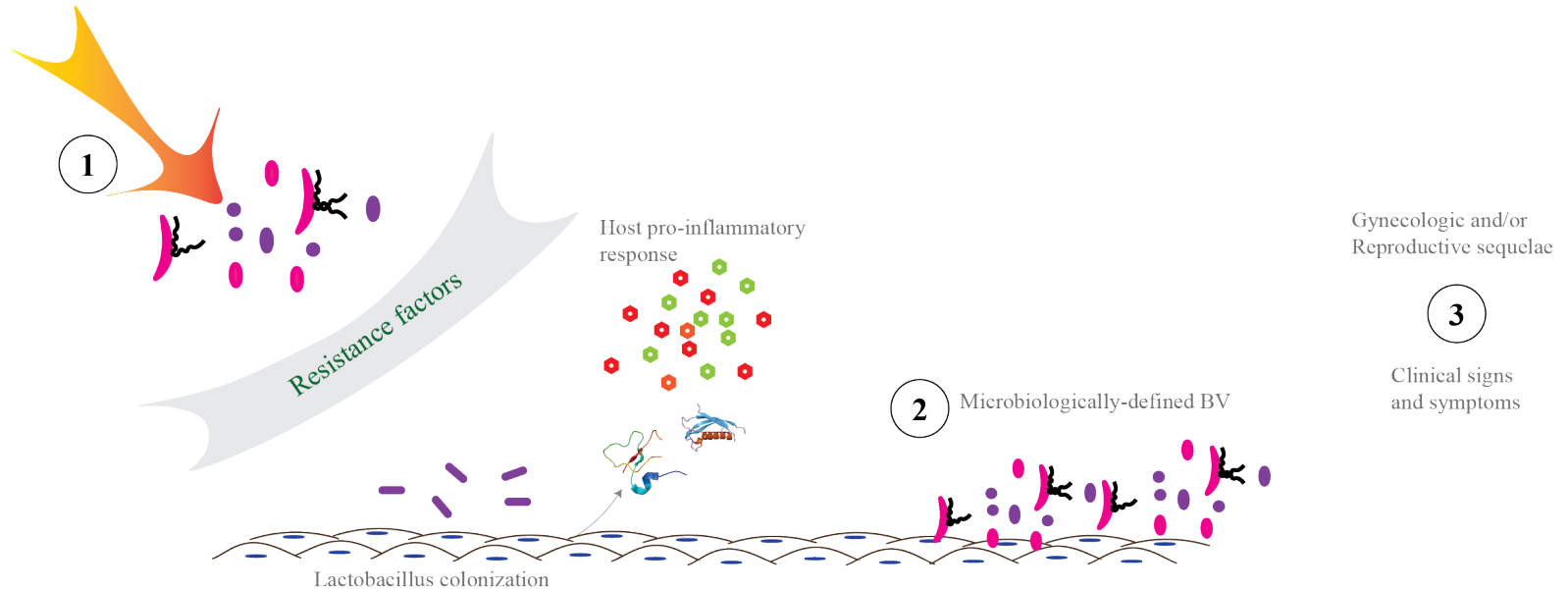
1.2.3 *Immune receptors and mediators correlated with BV*

While BV is not associated with an inflammatory response involving leukocytic infiltrate⁴⁴, prior studies describe pro-inflammatory cytokines increased in vaginal fluid of women with BV (reviewed in ⁴⁵), suggesting that immune response to certain BVAB may contribute to resistance or susceptibility to BV, or be a consequence of colonization. Genetic variation in IL-1 β , IL6, IL-8, IL-10, IL-1RN have been found to be associated with BV (reviewed in ⁴⁶). Single nucleotide polymorphisms (SNPs) in pattern recognition receptors (TLR1^{47,48}, TLR2⁴⁷, TLR4⁴⁸⁻⁵⁰, TLR7⁵¹, TLR9⁴⁸, CD14⁴⁷, and MD2⁴⁷) have been associated with risk of BV. However, many of the explored variants and SNPs have no functional characterization, making it difficult to formulate testable hypotheses about their role in BV pathogenesis^{48,49,51}. Other SNPs are in genes that have no clear mechanistic connection to BV pathogenesis, such as the SNPs identified in viral pattern recognition receptors, TLR7⁵¹ and TLR9⁴⁸. Additionally, many studies use a single method to assess BV, and thus cannot assess the impact of innate immune genetic variation on the appearance of clinical signs associated with BV versus asymptomatic microbiologically-diagnosed BV^{47,49-51}. Furthermore, these studies either focus on more commonly isolated BVAB with low specificity for BV^{47,49} or ignore the vaginal microbiota altogether^{48,50,51}.

1.3 CURRENT MODEL OF BV PATHOGENESIS

From these clinical studies and in vitro models of BV, I propose the following working model of BV pathogenesis (Figure 1.1). Under this model, BV develops due to a failure to clear BVAB from the vaginal environment. After an inoculation event (intercourse with a partner colonized with BVAB, inoculation from other body sites, etc.), BVAB encounter microbial and host resistance factors. Microbially, *Lactobacillus* spp. that colonize the vagina in “health” produce lactic acid and other factors that limit the growth of BVAB (e.g. antimicrobial proteins). The host immune response is thought to be important for control of BVAB growth: Toll-like receptor (TLR) recognition and downstream upregulation of inflammatory responses aid in the elimination of BVAB and prevention of BV. This inflammatory response may include the production of pro-inflammatory cytokines (symbolized as hexagons in Figure 1.1) or host antimicrobial proteins. This hypothesis places microbes as the center of BV pathogenesis, with BVAB colonization driving the phenotypes identified by Nugent scoring, clinical signs and symptoms, as well as gynecologic and obstetric sequelae.

Inoculation with BVAB



	0. Homeostasis	1. Inoculation with BVAB	2. Stable colonization with BVAB	3. Negative sequelae
Predisposing Factors	<p>Increased number of sexual partners^{35,36}</p> <p>Sex with same partner as previous episode of BV^{20,40-42}</p> <p>Extravaginal or partner colonized with BVAB^{25,26,35,37,38}</p>	<p>Presence of microbial factors blocking immune response to BVAB (hypothesized)⁵²</p> <p><i>L. iners</i> colonization²³</p>	<p>Colonization with BVAB1, <i>Mobiluncus</i> spp. (preterm birth)^{10,12}</p> <p><i>Parvimonas</i> spp., <i>Gemella asaccharolytica</i>, <i>Mycoplasma hominis</i>, <i>Sneathia</i> spp., <i>Eggerthella</i> spp., <i>Megasphaera</i> spp. (HIV)⁵³</p> <p><i>Megasphaera</i>, <i>Prevotella</i>, <i>Sneathia</i>, and other BVAB (HIV)³</p> <p>BVAB3 (cervicitis)⁹</p>	<p>Gynecologic and/or Reproductive sequelae</p>
Protective Factors	<p>Intercourse with condom³⁶</p> <p>Anal colonization with lactobacilli²⁶</p>	<p><i>L. crispatus</i> colonization^{25,26}</p> <p>Hormonal contraception⁵⁴</p>	<p>Colonization with lactobacilli (HIV)³</p> <p>Colonization with <i>L. jensenii</i> (cervicitis)⁹</p>	<p>Clinical signs and symptoms</p>

Figure 1.1. Current model of bacterial vaginosis pathogenesis

1.4 AIM OF DISSERTATION

In contrast to the model described in the previous section, it is possible that BV phenotypes are determined by a combination of microbial factors and host immunity. In this dissertation, I test whether the immune responses mediated by Toll-like receptors (TLRs) are beneficial to the prevention of BV and colonization with BVAB. I explore the role of genetic variation in TLRs and their negative regulators on: the risk of clinically- and microbiologically-defined BV; colonization with lactobacilli; and, colonization with BVAB. Each chapter examines SNPs associated with deficient responses in different TLRs or their regulators: in Chapter 2, I focus on SNPs in TLRs 1 and 6; Chapter 3 shifts to negative regulators of TLR signaling, TLR10 and TOLLIP; Chapter 4 explores the role of TLR4 deficiency—and TLR4 more broadly—in the lower female genital tract; Chapter 5 focuses on the relationship between TLR5, BV, and colonization with flagellated species of BVAB. Finally, in Chapter 6, I present a unified model of how TLR-mediated responses shape BV pathogenesis, and highlight remaining questions and future directions.

1.5 METHODS

1.5.1 *Ethics statement*

All participants provided written informed consent to participate, and studies were approved by Institutional Review Boards at the Fred Hutchinson Cancer Research Center and the University of Washington.

1.5.2 *Discovery cohort enrollment*

Between October 2012 and June 2015, participants were enrolled from the Public Health, Seattle and King County Sexually Transmitted Diseases Clinic (PHSKC STD Clinic) and University of Washington Infectious Diseases/Virology Research Clinic. Women were eligible for enrollment if they were 18-50 years of age and either had prevalent BV (assessed clinically by Amsel's criteria) or had a remote history of BV (≥ 3 months). Women with BV were offered treatment with metronidazole or an alternative antibiotic treatment⁵⁵. Pregnant women, women who were menstruating at time of initial sample collection, or women who had visible cervicovaginal epithelial disruption were excluded from study participation. For the purposes of the analyses presented here, participants were included if they successfully collected at least one home-collected vaginal swab and had a complete Nugent score at enrollment.

During study visits, participants completed a computer-assisted survey to self-report demographic and behavioral information, including sexual, medical, and reproductive history. Experienced clinicians assessed women for BV using Amsel's criteria¹⁷. Pelvic examinations were performed using non-lubricated speculum, and study clinicians obtained vaginal fluid samples using cotton-tipped swabs. These samples were used for pH measurement, saline and potassium hydroxide microscopy, and were also rolled onto a glass slide for Gram staining and subsequent microbiological BV diagnosis by Nugent score¹⁶. In addition to vaginal samples, clinicians collected gingival swabs at enrollment. Women diagnosed with vaginal yeast or *Trichomonas vaginalis* were offered treatment as appropriate⁵⁶. Study clinicians also collected vaginal fluid samples for DNA extraction and quantitative PCR using Epicentre© foam swabs (Madison, WI). Participants returned to the clinic for exams occurring 30, 60, 90, and 180 days, and then approximately one-year post-enrollment.

Study participants self-collected vaginal swabs as previously described²⁴: two vaginal swabs daily for up to 90 days, followed by weekly swabs for 90 days. Self-reported vaginal symptoms and sexual activities were recorded via paper and web-based home diaries.

A subset of participants were selected for analysis based on completeness of daily sample collection and of BV at a clinic visit (defined by Amsel's criteria or Nugent score), or if found to have Nugent score of (7-10) in at least 2 out of 3 consecutive days via home collection. For women with incident or prevalent BV, samples were selected based on windows into (14- 30 days) and out of BV (minimum 3 days of Nugent 0-3 or minimum 7 days 4-6 for negative window). For women who did not develop BV during the timeframe observed, samples were selected over the course of consecutive menstrual cycles.

1.5.3 *Validation cohort enrollment*

Between September 2012 and July 2013, women were enrolled from PHSKC STD Clinic in a 2:1 ratio of BV-positive to BV-negative (Amsel's). Women with BV were offered antibiotic treatment per CDC guidelines⁵⁶. Women were eligible to participate if they were greater than 18 years of age, had not yet undergone menopause, and reported having current or previous male partners. Women were ineligible if they reported no sexual intercourse in the past month or were pregnant. Samples were collected by study clinicians as described above. Data from the entire cohort was used in the analysis.

1.6 DNA EXTRACTION AND QUANTITATIVE PCR

Bacterial DNA was extracted using the MoBio/Qiagen BiOstic Bacteremia DNA Isolation kit (MoBio, Carlsbad, CA; Qiagen, Germantown, MD), as previously described⁵⁷.

Concentrations of vaginal bacterial DNA from 11 species associated with BV or vaginal health were determined via quantitative PCR.

The following genus/species-specific quantitative PCR assays were performed on DNA extracted from discovery cohort participants' vaginal swabs: 16S rRNA gene broad-range (to assess total bacterial load), *Lactobacillus jensenii*, *Lactobacillus crispatus*, *Lactobacillus iners*, *Atopobium vaginae*, BVAB1, BVAB2, BVAB3 (*Mageibacillus indolicus*), *Eggerthella* spp., *Gardnerella vaginalis*, *Megasphaera* Type 1 and 2, and *Sneathia* spp. (as described previously^{24,53,58}). In addition to these assays, *Lactobacillus gasseri* colonization was assessed in the validation cohort, which was developed using a probe based TaqMan format. Reactions contained 1X TaqMan FAST Advanced Master Mix (Thermo Fisher, Waltham MA), 0.8 μ M forward primer (5'- ATTTGGTGCTTGCACCAG-3') modified from Kassinen et al⁵⁹, 0.8 μ M of our lab developed reverse primer (5'- CAGAACCATCTTTTAAACTCTAGA-3') and 150 nM probe (5'-6FAM-AGGTGTTATCCCAGTCTCTTMGBNFQ-3'). The assay was run on a QuantStudio 6 instrument (Thermo Fisher, Waltham MA) using the standard cycling setting and the following conditions, 50°C 2 min, 95°C pre-melt 10 min, and 45 cycles of 95°C melt 30 sec, 60°C annealing 30 sec, 72°C extension 30 sec. Standards were run in duplicate with an assay sensitivity of 2.5 gene copies.

1.7 SINGLE NUCLEOTIDE POLYMORPHISMS (SNPs) IN INNATE IMMUNE GENES

Host genomic DNA was extracted from self-collected vaginal swabs (discovery cohort) , or from clinician-collected saliva or gingival swabs (validation cohort) using MoBio Bacteremia single tube DNA extraction kits, as previously described⁵³. For the discovery cohort, DNA was extracted from a home-collected vaginal swab collected during a BV-negative timeframe. For study participants who were BV-negative at enrollment, the home collected vaginal swab closest

in time to their enrollment date was used. For study participants with BV at time of enrollment, we used a home collected vaginal swab closest in time to a follow up clinic visit at which the participant was BV-negative. For study participants with BV at enrollment and all follow up clinic visits, we used a vaginal swab collected on a date during which the study participant had vaginal fluid pH less than 4.5.

SNPs were genotyped using commercially available TaqMan assays (Thermo Fisher) and were used as single-SNP assays run on a QuantStudio6 or in multiplex using the Fluidigm Biomark platform.

1.8 STATISTICAL ANALYSIS

Data cleaning and statistical analyses were performed using R version 3.4.1⁶⁰. Our main outcome of interest was incident (discovery cohort) or prevalent (validation cohort) BV, using microbiological diagnosis (Nugent) as our primary measure and clinically-defined BV (Amsel's) as a secondary measure. We used Cox proportional hazards regression to estimate the effect of TLR/TOLLIP deficiency on time to first diagnosis with BV after enrollment (R package “survival” version 2.41.3⁶¹). In the validation cohort, we used logistic regression to estimate the relationship between TLR/TOLLIP deficiency and odds of BV. Because we used a hypothesis driven approach and focused on only 9 SNPs with functional characterization, no correction for multiple comparisons was made when examining correlations between SNPs and risk (or prevalence) of BV. Additionally, because a targeted approach was used to examine the relationship between genetic variation in TLR/TOLLIP SNPs and colonization with specific vaginal bacteria in two, independent cohorts, no correction for multiple comparisons was made when analyzing the correlations between SNPs and colonization with bacteria.

To explore mechanisms underlying relationships between TLR/TOLLIP deficiency and BV, we conducted a secondary analysis comparing vaginal bacterial colonization in deficient and sufficient study participants. Because vaginal bacterial colonization is highly correlated with BV status, any differences between susceptibility to BV between TLR/TOLLIP deficient/sufficient participants could drive differences in colonization. Thus, we controlled for differences in BV susceptibility by matching women with genotypes associated with “deficiency” to women with genotypes associated with “sufficiency” using R package MatchIt (3.0.1)⁶². MatchIt creates a distance matrix for a set of matching variables provided by the user and minimizes the distance between cases and controls. Because recent history of BV is a strong predictor of whether a participant will develop BV in the near future, discovery cohort participants were matched on BV by Nugent score at enrollment. Additionally, participants were matched on age, race, sex with men, women, or both, and hormonal contraception use. Hormonal contraceptives reported by study participants included: oral contraceptive pills, patch, depot-medroxyprogesterone injection, Mirena intrauterine device (IUD), and Implanon.

Because the distance-based algorithm implemented by MatchIt can still result in some imbalance between cases and controls, we assessed quality of matching “deficient” to “sufficient” study participants in a variety of ways. For the discovery cohort, these data are presented as a set of stereotyped figures (Appendix B). We examined survival curves comparing time to next diagnosis with BV and used Cox proportional hazards regression to estimate the effect of TLR/TOLLIP deficiency (panel A). Examining the time course of all included observations, we compared the percentage of deficient and sufficient study participants positive for BV by Nugent score (panel B). Finally, we compared each group in aggregate, examining the percentage of days with Nugent diagnosis of BV, average age in each group, race, sexual

preference, and hormonal contraception use (panel C). In contrast, the data from the validation cohort were cross-sectional. To assess adequacy of matching in the validation cohort, we compared deficient cases and sufficient controls by Nugent diagnosis of BV, age, race, hormonal contraception usage, and vaginal douching (Appendix C).

Bacterial colonization analyses included all daily, home-collected samples after a study participant's first Nugent score over 7 (with end of daily collection determined by a gap in collection longer than 14 days). Differences between matched "deficient" cases and "sufficient" controls in bacterial colonization were determined by mixed effects linear modeling (lme4 version 1.1.14), with statistical significance determined using a likelihood ratio test and ANOVA to compare a mixed effects model with "deficiency"/ "sufficiency" as a covariate to a mixed effects model with only subject-level random effects. In the validation cohort, study participants were matched on BV by Nugent score at enrollment, age, race, douching (ever), and hormonal contraception use. Within these cross-sectional data, we used two-sample t-tests and report p-values and mean difference in colonization (16S rRNA gene copies per swab) between matched "deficient" and "sufficient" populations.

In all analyses, p-values were considered as a measure of the probability of obtaining a more extreme statistical estimate under the null hypothesis. No specific p-value cut off was used.

Chapter 2. SINGLE-NUCLEOTIDE POLYMORPHISMS

ASSOCIATED WITH DEFICIENCIES OF TOLL-LIKE RECEPTOR-1 AND -6 ARE NOT ASSOCIATED WITH RISK OF BV

Adapted from: ⁶³

2.1 INTRODUCTION

Prevalence of BV is higher among individuals of African descent and those who report Hispanic or other ethnicity^{64,40,1,65}. Furthermore, asymptomatic BV—the presence of microbiologically-defined BV without clinical signs or symptoms—is more common in women of African ancestry³². Some have posited that differences in sexual networks between African/African-Americans versus Europeans/European-Americans may drive differences in vaginal bacterial colonization⁶⁶. Others have suggested that innate immune genetic variation may be a contributor, as innate immune SNPs that are more prevalent in African ancestry genetic backgrounds have been linked to increased risk of BV⁴⁶.

Although SNPs in TLR1 have been linked to risk of BV^{48,50}, these studies do not examine characterized SNPs, making it difficult to design meaningful mechanistic studies. Furthermore, no SNPs in TLR6 have been linked to BV. TLR1 and TLR6 form heterodimers with TLR2 and mediate immune responses to extracellular lipopeptides, which are present on most bacteria, including those involved in BV. Because TLRs 1 and 6 genetic variation is tightly linked to ancestry, genetic variation in TLR1 and TLR6 may be a source of some of the variability of BV prevalence by race and ethnicity. In this chapter, we test the hypothesis that genetic variants in TLR1 and TLR6 are correlated with risk of BV and vaginal bacterial colonization.

2.2 MATERIALS AND METHODS: SELECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS

We considered TLR1 rs5743618, TLR2 rs3804099, and TLR6 rs3821985 as common SNPs that have been associated with cytokine response in peripheral blood mononuclear cells (PBMCs). TLR2 rs3804099 has been associated with changes in cytokine response in a single study, in which PBMCs stimulated with *Legionella pneumophila*; synthetic ligands were not tested⁶⁷. Thus, we focused our analyses on TLR1 rs5743618, which has been robustly characterized⁶⁸, and TLR6 rs3821985, which has been shown to associate with PBMC cytokine responses to a variety of synthetic ligands⁶⁹. Genotype models were based on previous studies (Appendix A).

2.3 RESULTS

2.3.1 *Participant characteristics*

To determine whether TLR pathway polymorphisms are associated with susceptibility to BV, we examined independent discovery and validation cohorts. The discovery cohort was drawn from a longitudinal study of the vaginal microbiota, designed to follow the kinetics of vaginal bacterial colonization. The longitudinal discovery cohort was composed of 214 women (Table 2.1), of whom 125 were BV-negative by clinical criteria at enrollment and 123 were BV-negative by Nugent score. Approximately half of enrollees in this cohort were Caucasian and one-third were African-American. Concordant with previous studies^{1,40}, African-Americans were more likely to be BV-positive. African-Americans comprised 26% of the BV-negative population, but comprised 46% of the BV-positive population (by clinical criteria). Caucasians

comprised 56% of the BV-negative population, and 46% of the BV-positive population. History of BV was more common among participants with BV at enrollment.

Table 2.1. Discovery Cohort Demographics

	BV ^{-a}		BV ^{+a}		Total		p _{χ²}
	N = 125		N = 89		N = 214		
	N	%	N	%	N	%	
<i>Race</i>							
AIAN/NHPI ^b	6	5%	1	1%	7	3%	0.04
Asian	2	2%	2	2%	4	2%	
African-American	33	26%	41	46%	74	35%	
Caucasian	70	56%	41	46%	111	52%	
Other	12	10%	4	4%	16	7%	
Two or more races	2	2%	0	0%	2	1%	
<i>Ethnicity</i>							
Hispanic	9	7%	6	7%	15	7%	0.90
Non-Hispanic	100	80%	72	81%	172	80%	
Refuse to report	16	13%	11	12%	27	13%	
<i>Age</i>							
18-30	58	46%	44	49%	102	48%	0.31
31-40	31	25%	26	29%	57	27%	
41-50	34	27%	16	18%	50	23%	
Refuse to report	2	2%	3	3%	5	2%	
<i>Hormonal Contraception</i>							
Yes	42	34%	30	34%	72	34%	1.00
No	82	66%	59	66%	141	66%	
Refuse to report	1	1%	0	0%	1	0%	
<i>History of BV</i>							
Yes	75	60%	73	82%	148	69%	<0.01
No	46	37%	13	15%	59	28%	
Refuse to report	4	3%	3	3%	7	3%	

^aBacterial vaginosis diagnosed by Amsel's criteria.

^bAmerican Indian, Alaska Native, Native Hawaiian, Pacific Islander.

The validation cohort was composed of 45 BV-negative and 66 BV-positive women (Table 2.2). Similar demographic patterns were seen in this cohort with regard to race, ethnicity, and history of BV, with a wider age range of pre-menopausal women (ages 18-59), and fewer women reporting use of hormonal contraceptives (21%).

Table 2.2. Validation Cohort Demographics

	BV ^{-a}		BV ^{+a}		Total		p _{χ²}
	N = 45		N = 66		N = 111		
	N	%	N	%	N	%	
<i>Race</i>							
AIAN/NHPI ^b	0	0%	2	3%	2	2%	0.01
Asian	1	2%	5	8%	6	5%	
African-American	9	20%	27	41%	36	32%	
Caucasian	29	64%	21	32%	50	45%	
Other	6	13%	8	12%	14	13%	
Two or more races	0	0%	3	5%	3	3%	
<i>Ethnicity</i>							
Hispanic	5	11%	5	8%	10	9%	0.75
Non-Hispanic	36	80%	45	68%	81	73%	
Refuse to report	4	9%	16	24%	20	18%	
<i>Age</i>							
18-30	24	53%	40	61%	64	58%	0.25
31-40	9	20%	12	18%	21	19%	
41-50	7	16%	9	14%	16	14%	
51-60	5	11%	2	3%	7	6%	
Refuse to report	0	0%	3	5%	3	3%	
<i>Hormonal Contraception</i>							
Yes	12	27%	11	17%	23	21%	0.35
No	32	71%	51	77%	83	75%	
Refuse to report	1	2%	4	6%	5	5%	
<i>History of BV</i>							
Yes	24	53%	50	76%	74	67%	0.01
No	21	47%	14	21%	35	32%	
Refuse to report	0	0%	2	3%	2	2%	

^a Bacterial vaginosis diagnosed by Amsel's criteria.

^b American Indian, Alaska Native, Native Hawaiian, Pacific Islander.

2.3.2 *TLR1 deficiency is not associated with risk of BV*

In the discovery cohort, women with TLR1 deficiency trended towards decreased risk of clinically-defined BV (Figure 2.1B, $p = 0.11$), but not microbiologically-defined BV (Figure 2.1B, $p = 0.30$). TLR1 rs5743618 segregates with race⁴⁰ and TLR1 deficiency occurs more frequently among individuals of European descent. When we control for Caucasian race using Cox proportional hazards regression, the trend towards decreased risk of clinically-defined BV associated with TLR1 deficiency is abolished (adjusted hazard ratio = 0.717, 95% CI: [0.30, 1.69], $p = 0.44$). Thus, our data suggest that TLR1 deficiency is not associated with risk of BV.

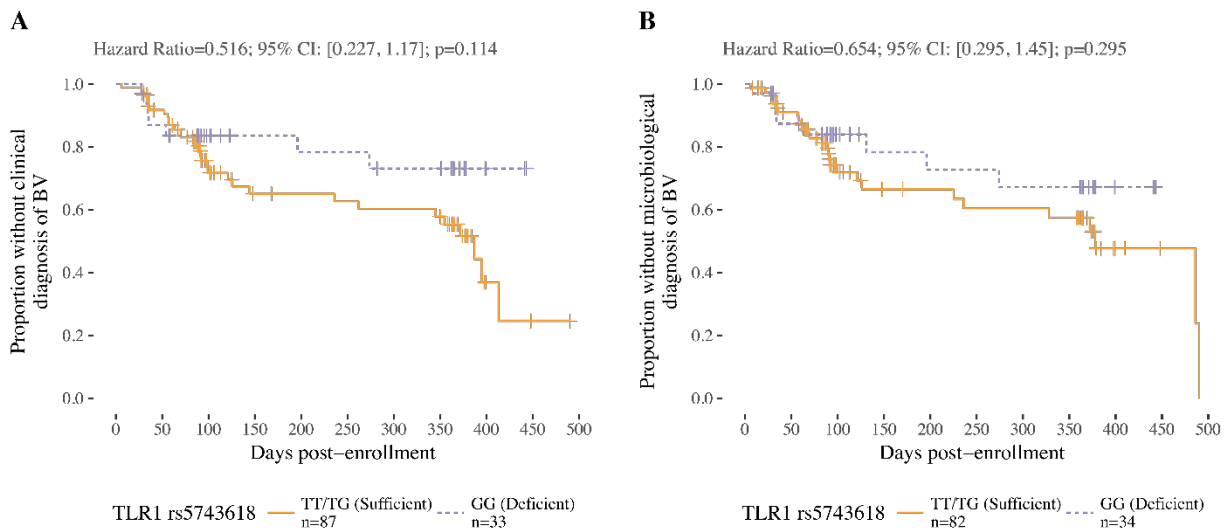


Figure 2.1. TLR1 deficiency and risk of BV in the discovery cohort

(A) Women with TLR1 deficiency trend towards decreased risk of clinically-defined BV (HR=0.516, $p = 0.11$). (B) Women with TLR1 deficiency have similar risk of microbiologically-defined BV to TLR1 sufficient women (HR=0.654, $p = 0.29$).

2.3.3 *TLR1 deficiency is not associated with colonization with BVAB*

TLR1 deficient cases were matched to TLR1 sufficient controls in the discovery cohort (Appendix B). After matching, we examined the average difference in bacterial colonization associated with TLR1 deficiency. We found that TLR1 deficiency was not associated with statistically significant differences in bacterial colonization (Figure 2.2).

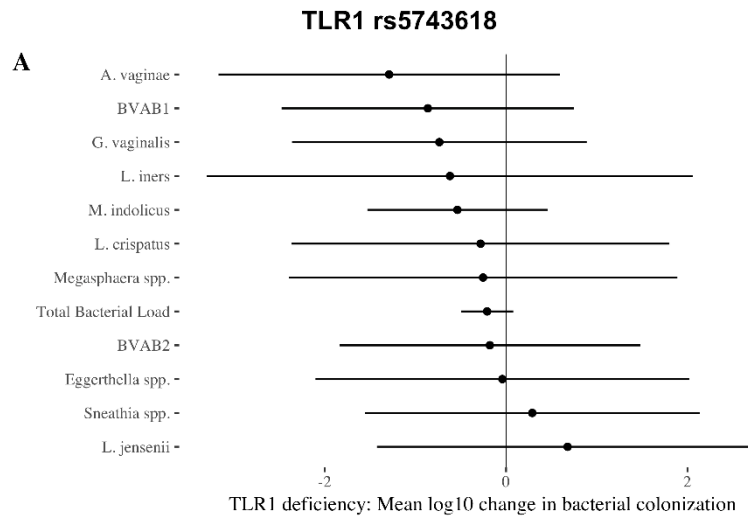


Figure 2.2. Concentration of vaginal bacteria in TLR1 sufficient and deficient women
Dot represents estimated log₁₀ difference in 16S rRNA gene copies per swab associated with each assay; line represents 95% confidence interval of the estimate. Vertical line denotes zero, or no difference in bacterial colonization in TLR1 deficient women.

2.3.4 *TLR6 deficiency is not associated with risk of bacterial vaginosis*

In an unadjusted analysis, TLR6 deficient women trend towards increased risk of both clinically- and microbiologically-defined BV (Figure 2.3). However, the minor allele frequency

of TLR6 rs3821985 is 0.694 in an African genetic background, and ranges from 0.311 (Admixed American) to 0.396 (East Asian) in all other groups studied in the 1000 Genomes Project⁷⁰.

Similar to TLR1, we hypothesized that race may be confounding the relationship between TLR6 deficiency and risk of BV. Indeed, after adjusting for African-American race, the trend towards increased risk of clinically-defined BV associated with TLR6 deficiency vanished (adjusted hazard ratio = 1.04, 95% CI: [0.42, 2.60], $p = 0.93$). The trend towards increased risk of microbiologically-defined BV in TLR6 deficient women was also abolished after adjusting for race (adjusted hazard ratio = 0.442, 95% CI: [0.55, 4.37], $p = 0.40$). Thus, our data suggest that TLR6 deficiency is not associated with risk of BV.

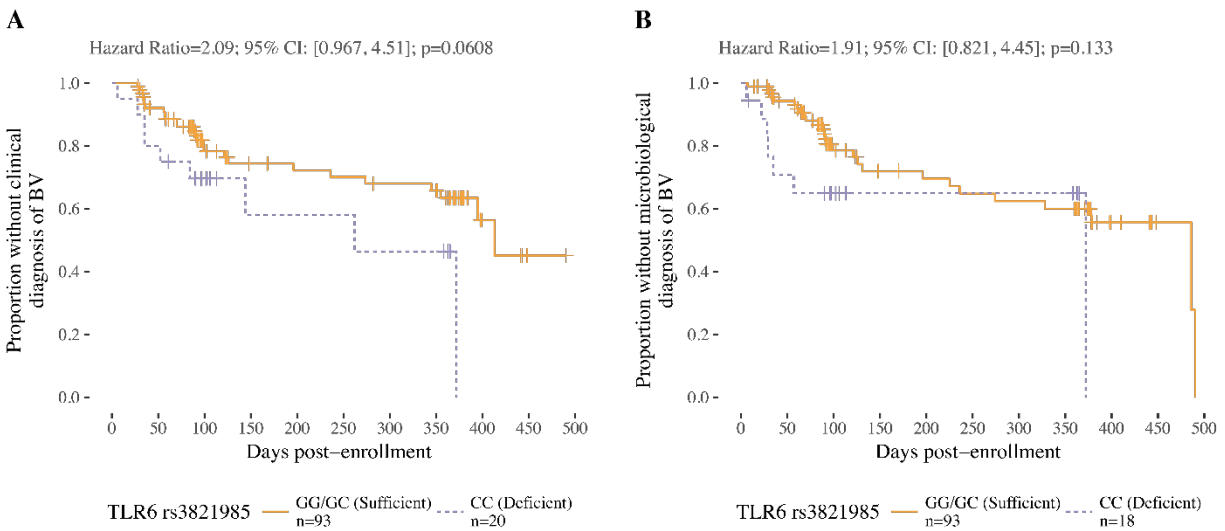


Figure 2.3. TLR6 deficiency and risk of BV in the discovery cohort

(A) In an unadjusted analysis, women with TLR6 deficiency trend towards increased risk of clinically-defined BV (HR=2.09 $p = 0.06$). (B) Women with TLR6 deficiency have similar risk of microbiologically-defined BV to TLR6 sufficient women (HR=1.91, $p = 0.13$).

2.3.5 *TLR6 deficiency is associated with shifts in Lactobacillus colonization*

Comparing vaginal colonization of TLR6 deficient women with TLR6 sufficient matched controls (Appendix B), we found that TLR6 deficient women had significantly less *L. jensenii* colonization (Figure 2.3B, mean \log_{10} = -2.07 16S copies per swab; $p = 0.01$) and a correspondingly higher *L. iners* colonization (Figure 2.3C, mean \log_{10} difference = 2.54 16S copies per swab; $p = 0.01$). No other statistically significant differences in vaginal bacterial colonization were found.

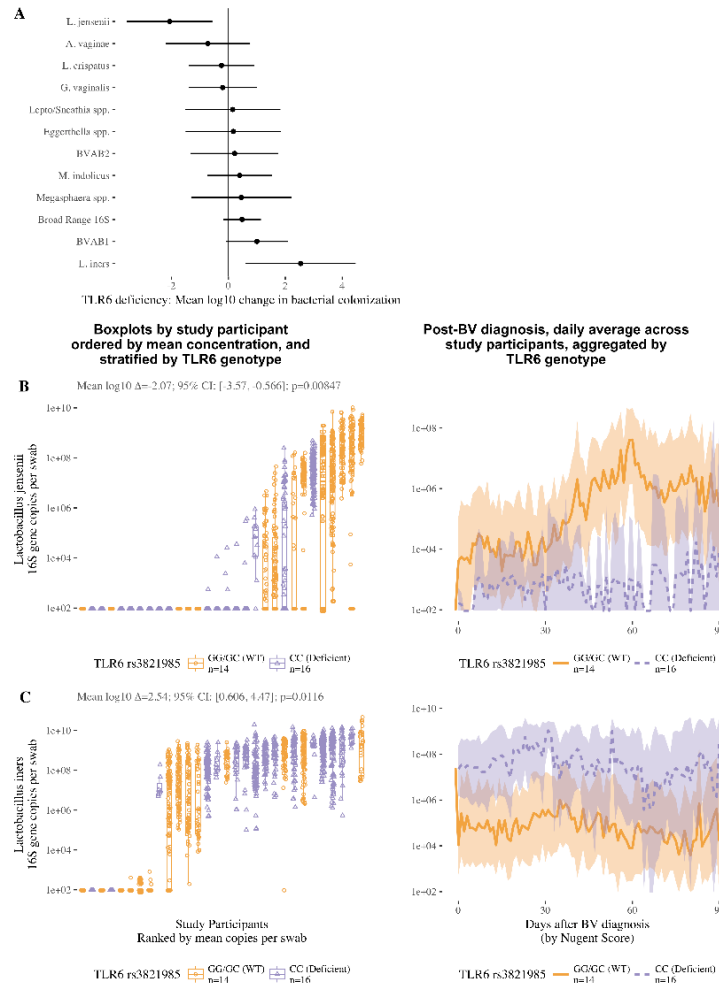


Figure 2.4. Shifts in *Lactobacillus* colonization associated with TLR6 deficiency in the discovery cohort

(A) Difference in concentration of vaginal bacteria between TLR6 sufficient and deficient women. Dot represents estimated log₁₀ difference in 16S rRNA gene copies per swab associated with each assay; line represents 95% confidence interval of the estimate. Vertical line denotes zero, or no difference in bacterial colonization in TLR6 deficient women. (B)-(C) Colonization across time in TLR6 deficient cases and matched TLR6 sufficient controls, divided into separate boxplots with overlaid scatterplots for each study participant to show within-subject variability (left), and depicted as a daily, moving average aggregated by TLR6 deficiency versus sufficiency (right, shading depicts 95% bootstrapped confidence interval). (B) TLR6 deficiency is associated with a -2.07-log₁₀ 16S rRNA gene copies per swab lower *L. jensenii* concentration (p = 0.008). (C) TLR6 deficiency is associated with a 2.54-log₁₀ 16S rRNA gene copies per swab higher *L. iners* colonization (p = 0.01).

In the validation cohort, no differences in *Lactobacillus* colonization were observed (Figure 2.5).

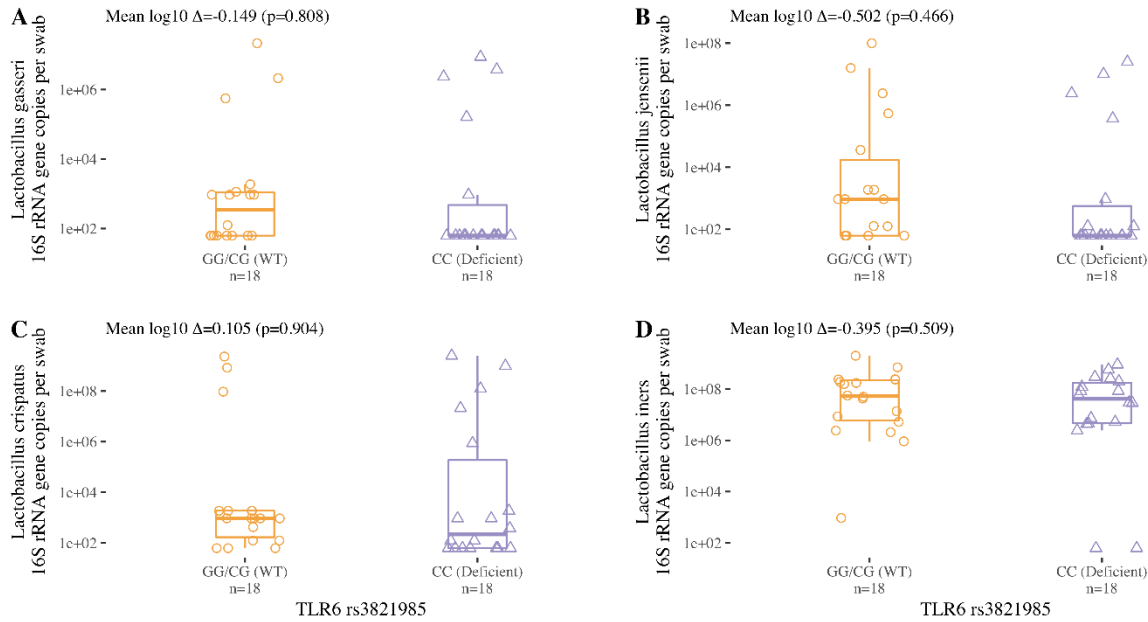


Figure 2.5. TLR6 deficiency is not associated with differences in *Lactobacillus* colonization in the validation cohort

Boxplots with overlaid scatterplots depicting distribution of cross-sectionally measured *Lactobacillus* concentration (each point represents a different study participant). In the validation cohort, no significant differences in concentrations of (A) *L. gasseri*, (B) *L. jensenii*, (C) *L. crispatus*, or (D) *L. iners*.

2.4 DISCUSSION

In this chapter, we fail to find a significant association between TLR1 and TLR6 deficiency and BV after adjusting for race. However, other innate immune genes in the same locus may have a relationship with BV. In particular, TLRs 1 and 6 share a high degree of sequence identity with a neighboring gene, TLR10⁷¹.

While TLR1 deficiency was not associated with any differences in vaginal bacterial colonization by qPCR, TLR6 deficiency was correlated with higher *L. iners* colonization and lower *L. jensenii* colonization. These results suggest that *L. iners* may produce a lipopeptide capable of agonizing TLR6, which results in clearance of *L. iners* from the vagina. Alternatively, TLR6 may be agonized by a product of *L. jensenii* which leads to the maintenance of its colonization.

Chapter 3. DEFICIENCIES IN NEGATIVE REGULATORS OF TOLL-LIKE RECEPTOR SIGNALING ARE ASSOCIATED WITH INCREASED RISK OF CLINICALLY-DEFINED BACTERIAL VAGINOSIS AND ALTERED BACTERIAL COLONIZATION

Adapted from: ⁶³

3.1 INTRODUCTION

TLRs 1 and 6 are highly linked with TLR10; these three TLRs share a high degree of homology and are the result of a gene duplication event. TLR10 is expressed on the cell surface, but has no known microbial ligand. TLR10 has been characterized as a negative regulator of both MyD88- and TRIF-dependent signaling^{72,73}. While mice do not possess a functional TLR10 gene, transgenic mice expressing human TLR10 exhibit hypo-inflammatory phenotype⁷⁴. Furthermore, genotypes of several SNPs, including rs11096955 TG/GG and rs4129009 TC/CC, are correlated with *increased* cytokine production in PBMCs, consistent with TLR10 “deficiency”, or impairment of TLR10’s negative regulation of innate immunity^{71,72,74,75}. Although deficiencies in TLR1 and TLR6 were not associated with risk of BV, we hypothesized that negative regulation of TLR response may increase susceptibility to BV.

In addition to TLR10, we hypothesized negative regulation of innate immune response modulated by TOLLIP would also be associated with decreased innate immune recognition of BVAB thereby leading to increased risk of BV. Unlike TLR10, TOLLIP has narrower function,

and mainly regulates MyD88-dependent TLR signaling⁷⁶⁻⁷⁹. Furthermore, genetic variation in TOLLIP has been shown to affect susceptibility to *Mycobacterium* infection^{78,79}.

3.2 MATERIALS AND METHODS: SELECTION OF SINGLE NUCLEOTIDE POLYMORPHISMS

TLR10 polymorphisms were initially selected based on existing studies indicating correlations between the minor alleles of rs11096955, rs4129009, rs1109657, and rs11466653 and increased cytokine response of PBMCs stimulated with TLR ligands and heat inactivated bacteria¹⁷. rs11466653 was not examined further due to its low minor allele frequency^{70,80}. We confirmed that rs11096955 and rs1109657 were in complete linkage disequilibrium and examined rs11096955 and rs4129009 in the discovery cohort. TOLLIP SNP rs5743854 is an expression quantitative trait locus, with individuals with GG genotype (“deficiency”) expressing significantly less TOLLIP which results in increased expression of pro-inflammatory cytokines⁷⁹.

3.3 TOLL-LIKE RECEPTOR-10 (TLR10)

3.3.1 *TLR10* rs11096955 TG/GG is associated with increased risk of clinically-defined BV

TLR10 deficiency resulting from SNP rs11096955 TG/GG was associated with increased risk of clinical (Amsel’s) BV in both the discovery and validation cohorts. In the discovery cohort, TLR10 SNP rs11096955 TG/GG (deficiency) was associated with a 2.23-fold increased risk of clinically-defined BV (Figure 3.1A, 95% CI: [1.02, 4.88], $p = 0.04$). In the validation cohort, TLR10 SNP rs11096955 TG/GG (deficiency) was associated with 2.86-times increased odds of clinically-defined BV (Figure 3.1C, 95% CI: [1.28, 6.38], $p = 0.01$). In both discovery and validation cohorts, however, TLR10 SNP rs11096955 TG/GG (deficiency) was not

associated with any significant difference in risk of microbiologically-defined (Nugent) BV (Figure 3.1B-C).

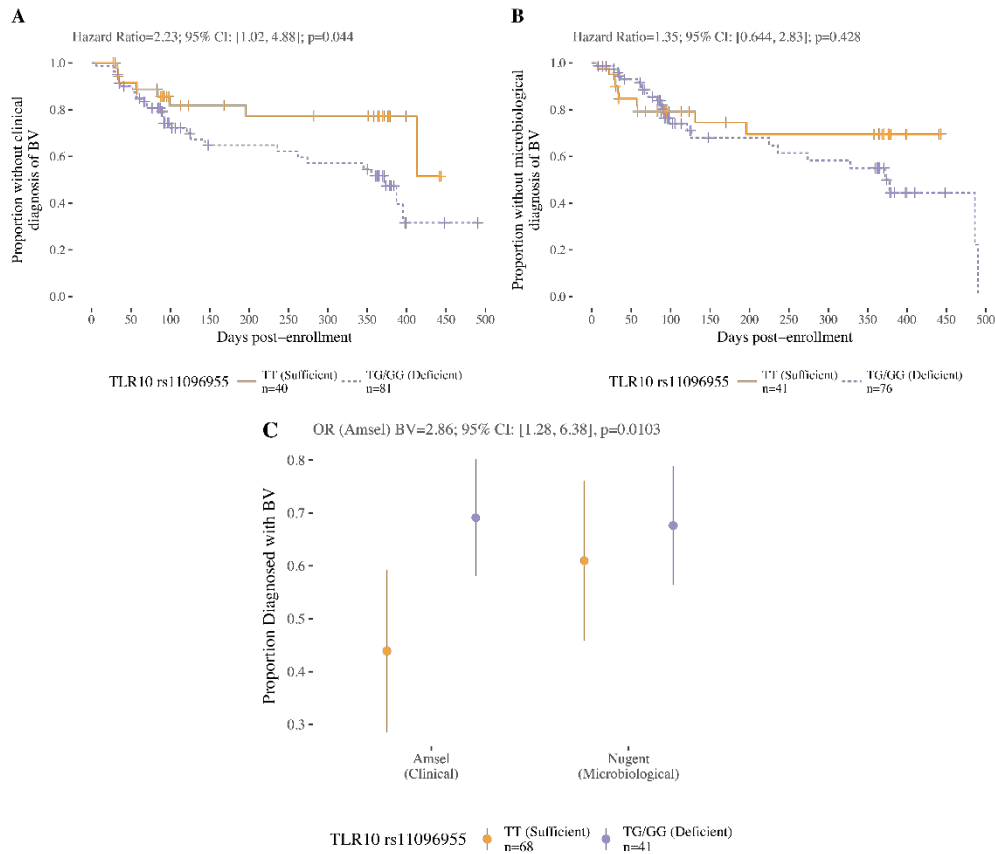


Figure 3.1. TLR10 deficiency is associated with increased risk of clinically-defined BV (A) Women in the discovery cohort with rs11096955 TG/GG genotype are at 1.99-fold increased risk of clinically-defined BV compared to women with TLR10 rs11096955 TT genotype. (B) In the discovery cohort, women with rs11096955 TG/GG have similar risk of microbiologically-defined BV to women with rs11096955 TT genotype. (C) Estimate of proportions of women with BV diagnosis (point) with 95% confidence interval (line). Women with rs11096955 TG/GG genotype are at increased risk of clinically-defined BV (adjusted odds ratio, aOR = 2.63, 95% confidence interval = [1.16, 5.97], p = 0.02), while there are no significant differences in microbiologically-defined BV.

TLR10 deficiency resulting from SNP rs4129009 TC/CC genotype was not associated with any significant differences in clinical or microbiological BV risk in the discovery cohort (Figure 3.2A-B). However, in the validation cohort, TLR10 rs4129009 TC/CC (deficiency) was associated with 3.11-times increased odds of clinical—but not microbiological—BV (Figure 3.2C).

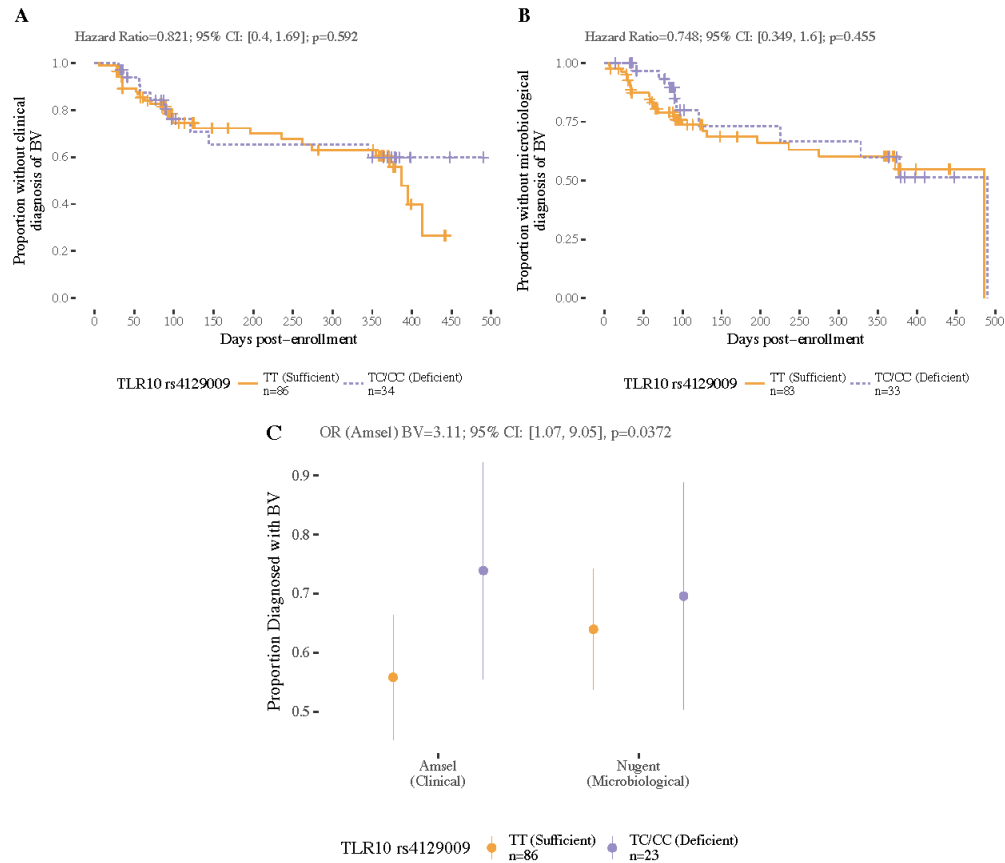


Figure 3.2. Relationship between TLR10 rs4129009 TC/CC and BV

(A) Women in the discovery cohort with rs4129009 TC/CC genotype are at similar risk of clinically-defined BV compared to women with rs4129009 TT genotype. (B) In the discovery cohort, women with TLR10 rs4129009 TC/CC genotype (“deficient”) are at similar risk for microbiologically-defined BV when compared to TLR10 sufficient women. (C) In the validation cohort, women with TLR10 TC/CC genotype (“deficient”) are at increased risk for clinically-defined BV after adjusting for race (adjusted odds ratio, aOR = 3.11, 95% confidence interval = [1.07, 9.05], p = 0.04). There is no statistically significant difference in risk of microbiologically-defined BV between TLR10 sufficient and deficient women.

We hypothesized that these two SNPs may have an additive effect on risk of clinically-defined BV. We compared women having neither, one, or both minor alleles of TLR10 rs11096955 and rs4129009 in the discovery cohort. We found that women with one minor allele were at 2.93-fold increased risk of clinically-defined BV (95% CI [2.10, 3.76], $p = 0.01$), while women with both minor alleles were not at any statistically significant increased risk of BV compared to women with both wild-type/sufficient alleles. These results suggest that rs11096955 and rs11096957—which are in complete linkage disequilibrium—may have greater effect on TLR10 function than rs4129009.

Because TLR10 rs11096955 and rs4129009 minor allele frequencies vary depending on ancestry^{70,80}, we performed stratified analyses in African-Americans and Caucasians. Although haplotype structure differed in each population (Appendix B), the patterns we detected in the larger cohort were reproduced in both Caucasians and African-Americans; diminished sample size within each subcohort decreases our ability to detect differences between TLR10 deficient and sufficient study participants (discovery cohort analysis in Figure 3.3; validation cohort analysis in Figure 3.4 [rs11096955] and Figure 3.5 [rs4129009]). Correlations between TLR10 deficiency and BV were preserved in regression analyses using race as a covariate, although the relationship between TLR10 rs11096955 TG/GG (deficiency) and BV was no longer below our statistical significance threshold (adjusted hazard ratio = 1.99, 95% CI [0.095, 4.37], $p = 0.09$).

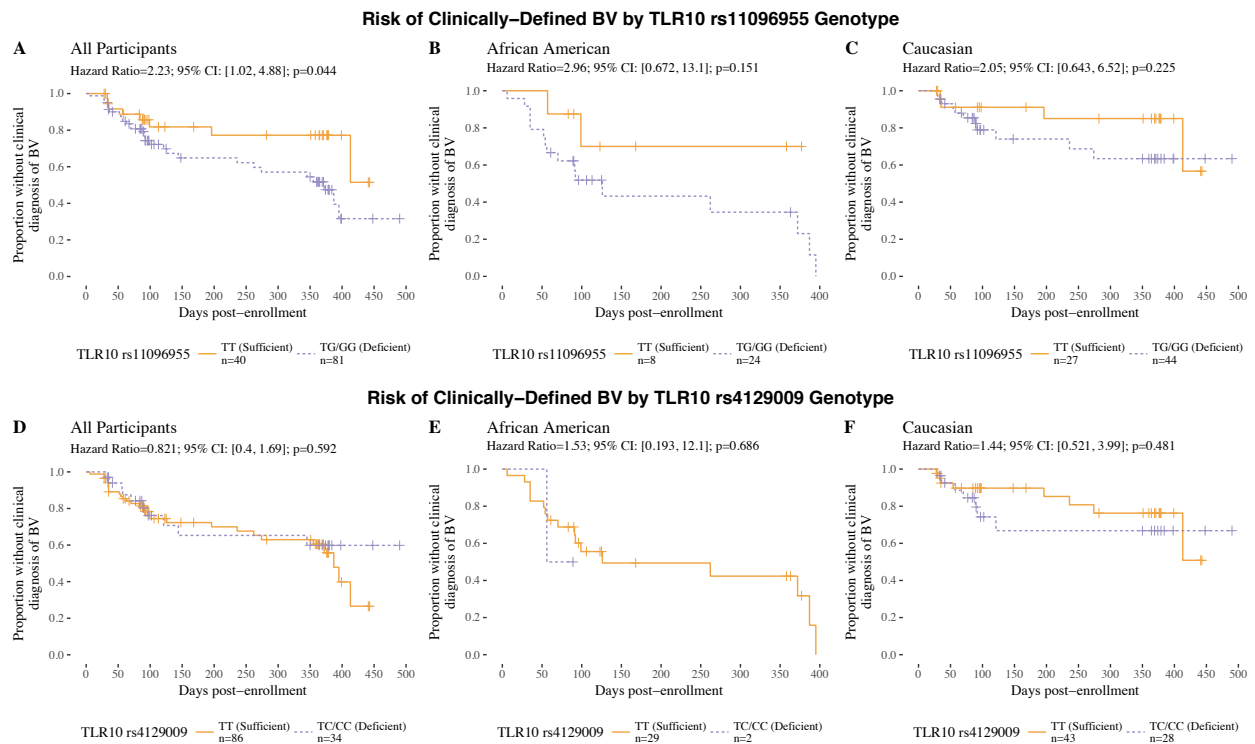


Figure 3.3. Race-stratified analysis TLR10 deficiency and clinically-defined BV in the discovery cohort

(A)– (C) Results of stratified analysis of TLR10 rs11096955 TG/GG (deficient) versus TT (sufficient). (A) Unadjusted analysis of whole cohort, demonstrating statistically significant relationship between TLR10 rs11096955 TG/GG (deficient) and increased risk of clinically-defined BV (B) Among African-Americans, the relationship between TLR10 rs11096955 and BV is preserved, but no longer reaches statistical significance. (C) Similarly, among Caucasians, the relationship between TLR10 rs11096955 and BV is also preserved

(D)-(F) Results of stratified analysis of TLR10 rs4129009 TC/CC (deficient) versus TT (sufficient). (D) No relationship between TLR10 rs4129009 and BV, using data from the whole cohort. (E) The lack of relationship between TLR10 rs4129009 and BV is recapitulated in the African-American sub-cohort. (F) Similarly, among Caucasians, the relationship between TLR10 rs11096955 and BV is also preserved

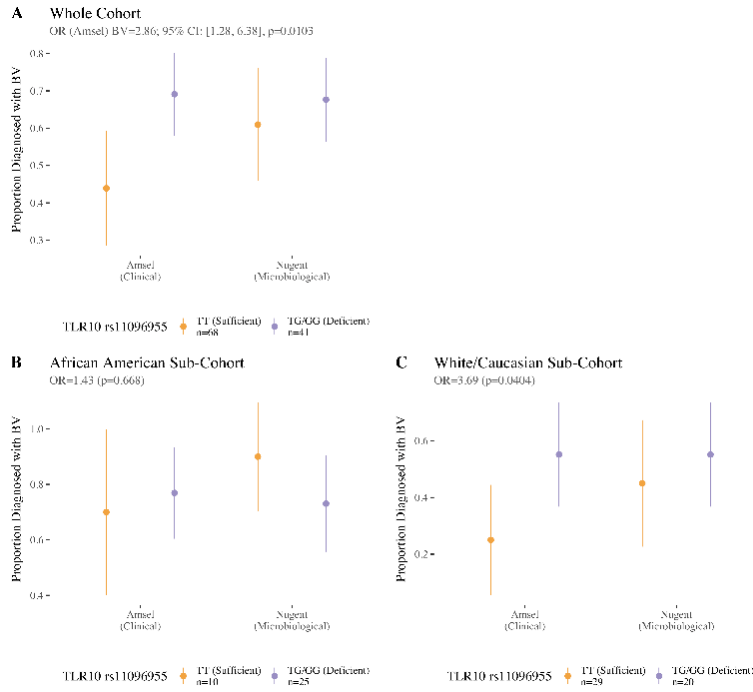


Figure 3.4. Race-stratified analysis TLR10 rs1106955 in the validation cohort

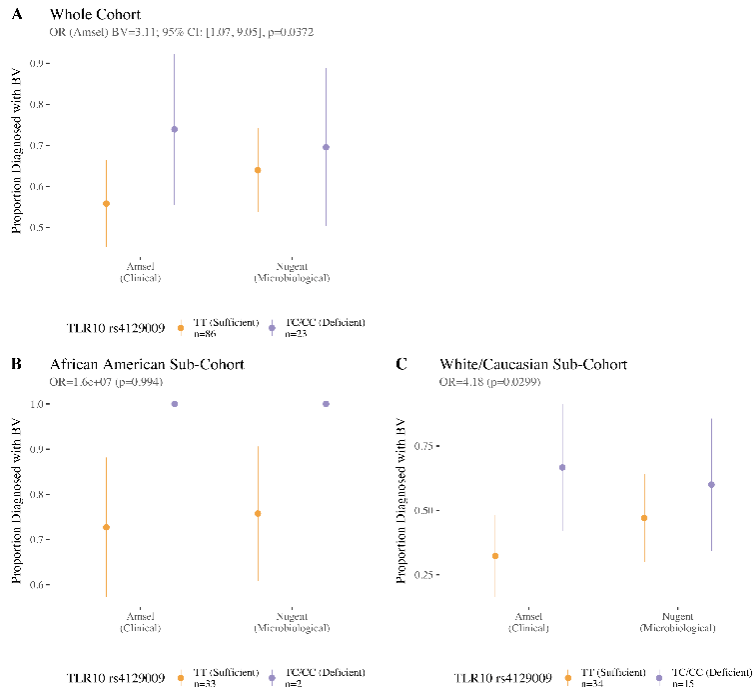


Figure 3.5. Race-stratified analysis TLR10 rs4129009 in the validation cohort

3.3.2 TLR10 deficiency is associated with reduced *Lactobacillus* colonization

Contrary to our hypothesis that increased inflammation would aid in the clearance of BVAB, there were no significant differences in BVAB colonization between those with or without TLR10 deficiency in either cohort. However, in both discovery and validation cohorts, TLR10 deficiency was associated with reduced *Lactobacillus* colonization (Figure 3.6, Figure 3.8).

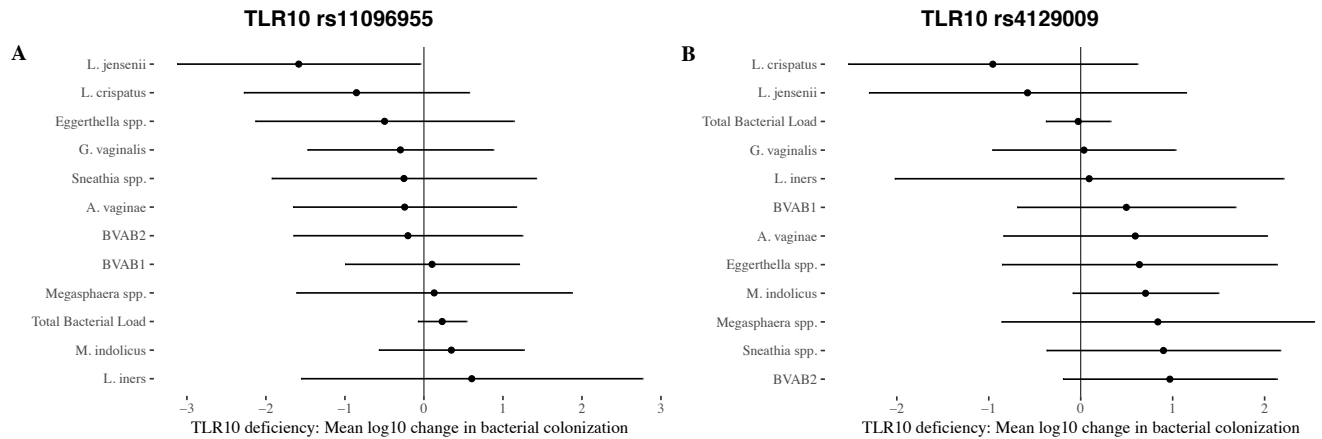


Figure 3.6. TLR10 deficiency is associated with disruptions in *Lactobacillus* colonization Results of mixed effects linear modeling of quantity of vaginal bacteria as a function of TLR10 deficiency (with study participant-level random effects to control for repeated measurements).

Dot/point represents effect size estimate; line corresponds to 95% confidence interval. Comparisons made between TLR10 deficient study participants and matched TLR10 sufficient controls. (A) Women with TLR10 rs11096955 genotype TG/GG have significantly higher quantity of *L. jensenii* and trend towards higher overall bacterial load. (B) Women with TLR10 rs4129009 genotype TC/CC trend towards lower *L. crispatus* and *L. jensenii* colonization.

Compared to matched controls in the discovery cohort (Appendix B), TLR10 SNP rs11096955 TG/GG (deficiency) was on average associated with 1.58- \log_{10} lower absolute

concentration of *L. jensenii* (Figure 3.7A-B, 95% CI: [-3.12, -0.05], $p = 0.04$) and a trend towards higher total bacterial load (Figure 3.7C-D, 95% CI: [-0.077, 0.538]; $p = 0.13$).

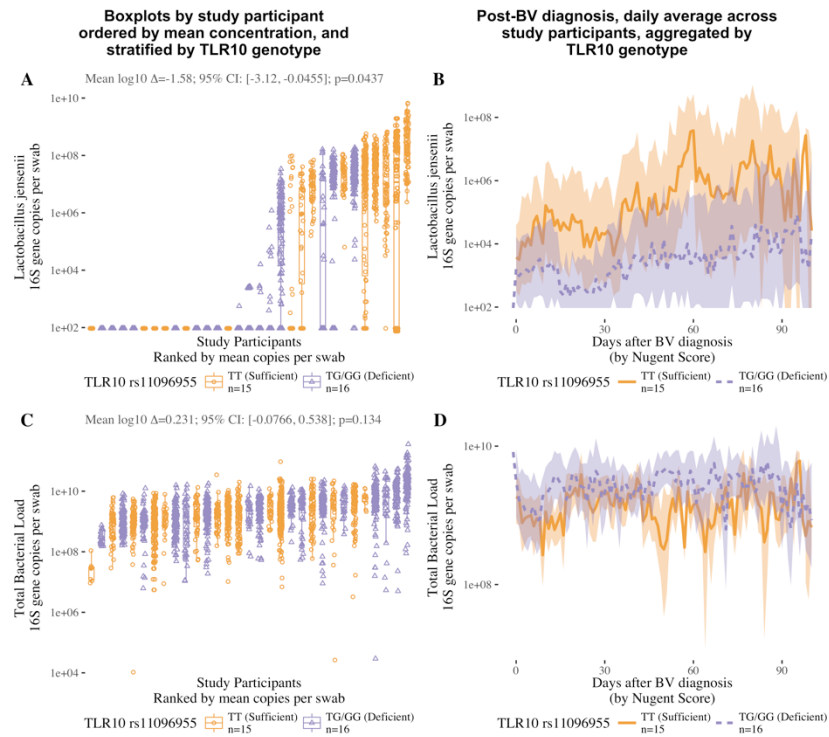


Figure 3.7. TLR10 rs11096955 TG/GG is associated with lower *L. jensenii* concentration and higher total bacterial load

Colonization across time in TLR10 deficient cases and matched TLR10 sufficient controls, divided into separate boxplots with overlaid scatterplots for each study participant to show within-subject variability (A, C), and depicted as a daily, moving average aggregated by TLR10 deficiency versus sufficiency (B, D, shading depicts 95% bootstrapped confidence interval). (A) Women with TLR10 deficiency have an average of 1.58- \log_{10} fewer *Lactobacillus jensenii* 16S rRNA copies per swab (absolute concentration) than matched TLR10 sufficient controls. (B) After Nugent-defined BV diagnosis, women with TLR10 deficiency have an average level of colonization with *L. jensenii* that is consistently below TLR10 sufficient women. (C) Women with TLR10 deficiency trend towards higher absolute concentration of bacteria, with an average of 0.231- \log_{10} more 16S rRNA gene copies per swab than matched TLR10 sufficient controls. (D) After diagnosis with BV, women with TLR10 deficiency have an average total bacterial load that consistently higher than the average total bacterial load in TLR10 sufficient women.

In the validation cohort (Appendix C), TLR10 SNP rs11096955 TG/GG (deficiency) was associated with a 0.807- \log_{10} lower absolute concentration of *L. gasseri* (Figure 3.8A, $p = 0.07$).

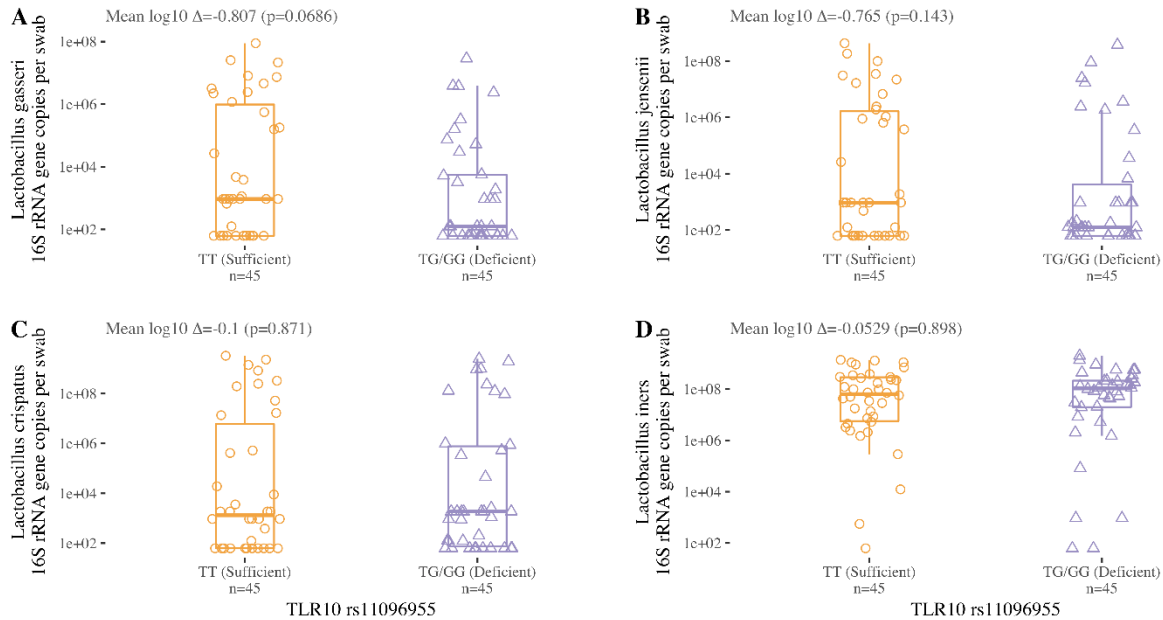
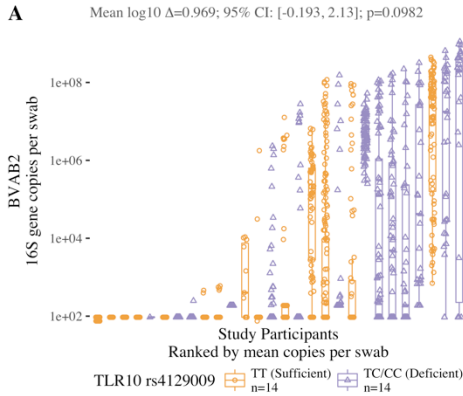


Figure 3.8. In validation cohort, with rs11096955 TG/GG genotype trend towards lower *Lactobacillus gasseri* concentration

Boxplots with overlaid scatterplots depicting distribution of cross-sectionally measured *Lactobacillus* concentration (each point represents a different study participant). (A) On average, women with TLR10 rs11096955 TG/GG genotype (“deficient”) had 0.807- \log_{10} *L. gasseri* rRNA gene copies per swab in comparison to TLR10 sufficient women ($p = 0.07$). No differences were seen between TLR10 deficient and sufficient women in (B) *L. jensenii*, (C) *L. crispatus*, or (D) *L. iners* rRNA gene copies per swab.

In the discovery cohort, TLR10 rs4129009 TC/CC (deficient) cases were matched to TT (sufficient) controls; however, deficient genotypes had slightly increased risk for BV (Appendix B). With this caveat, women with TLR10 rs4129009 TC/CC genotype showed a trend towards higher concentrations of BVAB2 and *Mageeibacillus indolicus* (BVAB3; Figure 3.6, Figure 3.9A-D). Similar to TLR10 rs11096955 TG/GG (deficient), women with TLR10 rs4129009 TC/CC were estimated to have lower *L. jensenii* and *L. crispatus* concentrations (Fig. 2B, Figure 3.9E-H). There were no differences in colonization by SNP rs4129009 genotype in the validation cohort (data not shown). Together, these data suggest that TLR10 may be involved in preventing clinical signs of BV and maintaining robust *Lactobacillus* colonization.

Boxplots by study participant ordered by mean concentration, and stratified by TLR10 genotype



Post-BV diagnosis, daily average across study participants, aggregated by TLR10 genotype

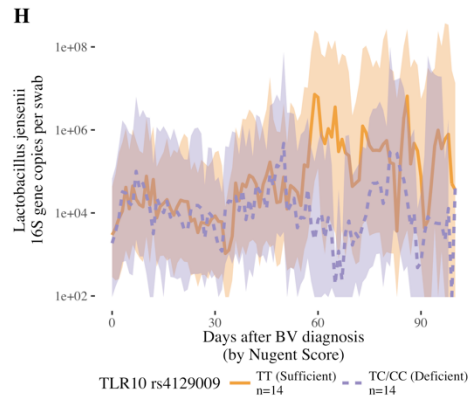
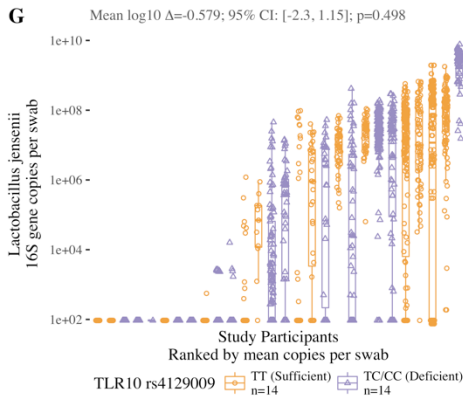
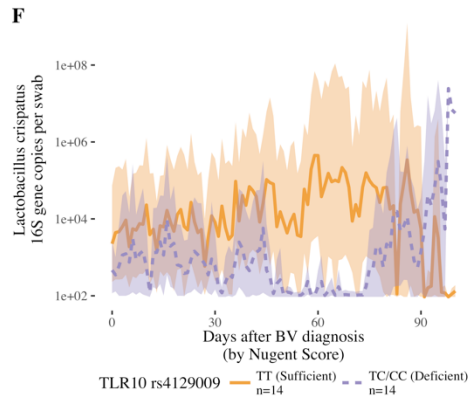
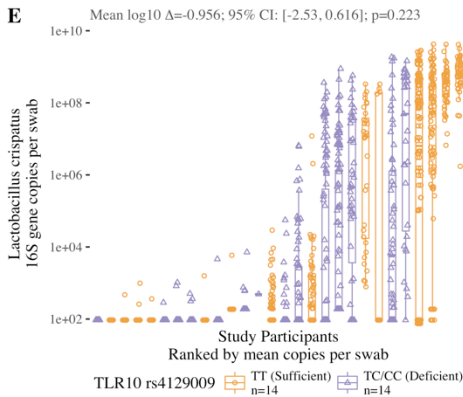
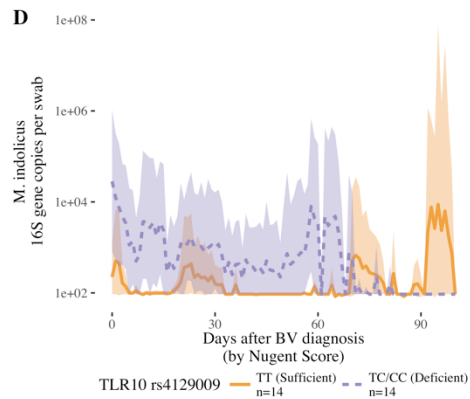
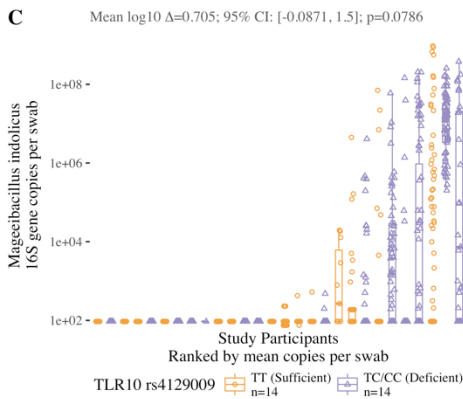
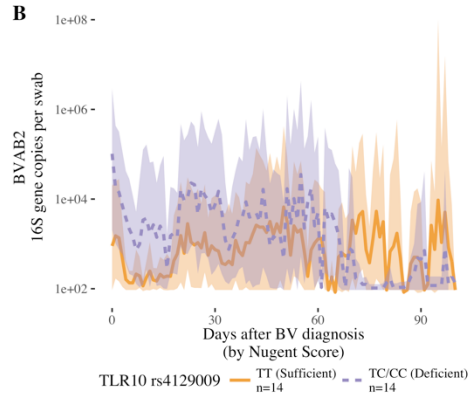


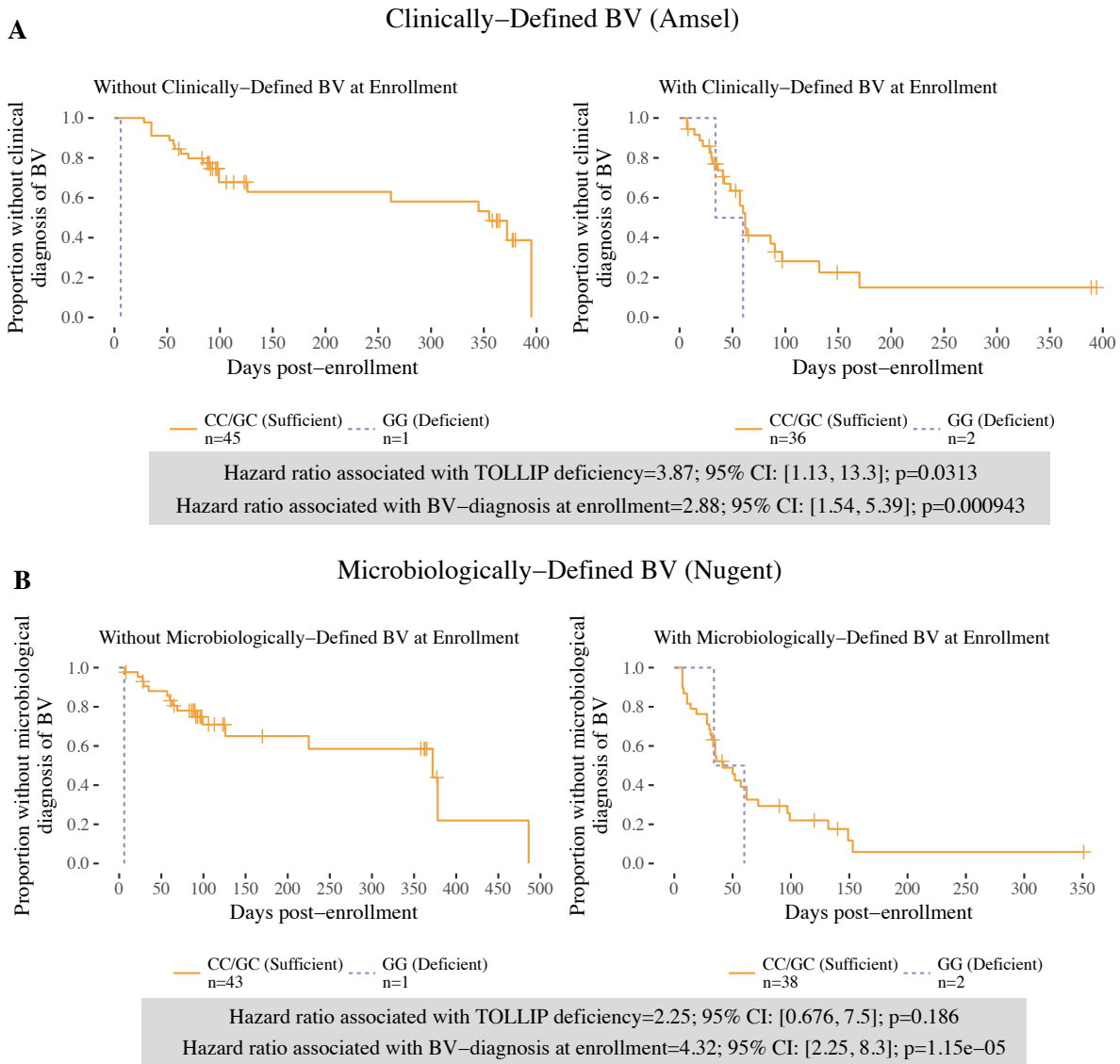
Figure 3.9. TLR10 rs4129009 TC/CC is associated with a trend towards higher concentration of BVAB2, *M. indolicus*

Colonization across time in TLR10 deficient cases and matched TLR10 sufficient controls, divided into separate boxplots with overlaid scatterplots for each study participant to show within-subject variability (A, C, E, G), and depicted as a daily, moving average aggregated by TLR10 deficiency versus sufficiency (B, D, F, H; shading depicts 95% bootstrapped confidence interval). (A) TLR10 deficient women show a trend towards 0.97- \log_{10} more BVAB2 16S rRNA copies per swab (95% CI: [-0.19, 2.13], $p = 0.10$) in comparison to TLR10 sufficient participants. (B) After Nugent-defined BV diagnosis, average colonization with BVAB2 remains consistently higher in TLR10 deficient women, until approximately 60 days post-diagnosis. (C) Individuals with TLR10 deficiency have 0.71- \log_{10} more *M. indolicus* (BVAB3) 16S rRNA copies per swab (95% CI: [-0.09, 1.50], $p = 0.08$). (D) After diagnosis of microbiologically-defined BV, women with TLR10 deficiency are colonized with a greater average quantity of *M. indolicus* (BVAB3). (E) Women with TLR10 deficiency exhibit a trend towards 0.96- \log_{10} fewer *Lactobacillus crispatus* 16S rRNA copies per swab (95% CI: [-2.53, 0.62], $p = 0.22$). (F) After Nugent-defined BV diagnosis, women with TLR10 deficiency have average *L. crispatus* colonization below that of their TLR10 sufficient counterparts, with these differences fading after approximately 75 days. (G) Women with TLR10 deficiency exhibit a trend towards 0.58- \log_{10} fewer *L. jensenii* 16S rRNA copies per swab than matched TLR10 sufficient controls (95% CI: [-2.30, 1.15], $p = 0.50$). (H) Although TLR10 sufficient and deficient women have similar average *L. jensenii* concentration immediately following BV diagnosis, after approximately 60 days, TLR10 deficient women show a trend towards lower concentrations.

3.4 TOLL INTERACTING PROTEIN (TOLLIP)

3.4.1 *TOLLIP deficiency is associated with increased risk of clinically-defined BV and lower colonization with BVAB in the discovery cohort*

Only three TOLLIP deficient women had available longitudinal data in the discovery cohort, all of whom were African-American. Thus, we performed an exploratory analysis of TOLLIP deficiency, BV, and colonization with BVAB, including only observations collected from non-Caucasian study participants. TOLLIP deficiency was associated with increased risk of clinical, but not microbiological BV (Figure 3.10). Of two TOLLIP deficient, BV-positive women in the longitudinal cohort, both were diagnosed with recurrent BV within 60 days of enrollment. The TOLLIP deficient, BV-negative woman without BV at enrollment developed incident BV six days after enrollment. Taken in aggregate, controlling for BV-diagnosis at enrollment, TOLLIP deficiency was associated with 3.87-fold increased risk of clinically-defined BV (95% CI: [1.13, 13.3]; $p = 0.03$). There were no significant differences in risk of clinical or microbiological BV associated with TOLLIP in the validation cohort (data not shown).



Abbreviations: BV=bacterial vaginosis; CI=confidence interval; TOLLIP=Toll-interacting protein

Figure 3.10. TOLLIP deficiency is associated with increased risk of clinically-defined BV (A) In the discovery cohort, women with TOLLIP deficiency are at 3.87-fold increased risk of developing clinically-defined BV, controlling for clinically-defined BV status at enrollment. (B) No significant differences exist in risk of microbiological (Nugent) BV, controlling for diagnosis at enrollment.

3.4.2 *TOLLIP* deficiency is associated with decreased colonization with BVAB in the discovery cohort

In a comparison of vaginal bacterial colonization between *TOLLIP* deficient and matched controls (Appendix B), we found that *TOLLIP* deficient women were colonized with significantly less *Megasphaera* spp. (mean difference $\log_{10} = -3.19$; $p = 0.03$; Figure 3.13A), *A. vaginae* (mean $\Delta\log_{10} = -2.24$; $p = 0.04$; Figure 3.11B), and *G. vaginalis* (mean $\Delta\log_{10} = -1.45$; $p = 0.048$; Figure 3.11C). Additionally, women with *TOLLIP* deficiency trended towards lower colonization with BVAB1, *Sneathia* spp., BVAB2, *Mageeibacillus indolicus* (BVAB3), and *Eggerthella* spp. (Figure 3.12).

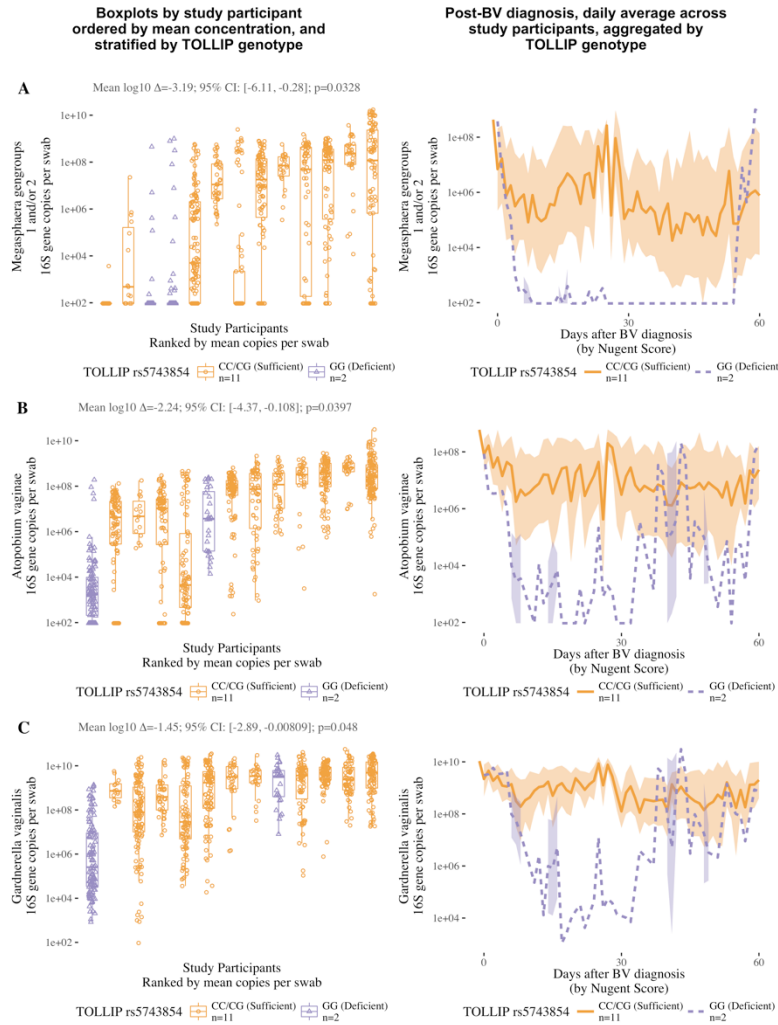


Figure 3.11. TOLLIP deficiency is associated with lower colonization with BVAB in the discovery cohort

(A)-(C) Colonization across time in TOLLIP deficient cases and matched TOLLIP sufficient controls, divided into separate boxplots with overlaid scatterplots for each study participant to show within-subject variability (left), and depicted as a daily, moving average aggregated by TOLLIP deficiency versus sufficiency (right, shading depicts 95% bootstrapped confidence interval). (A) TOLLIP deficient women have 3.19- \log_{10} fewer *Megasphaera* types 1 and/or 2 16S rRNA gene copies per swab than matched TOLLIP sufficient controls ($p = 0.03$). (B) TOLLIP deficient women have an average of 2.24- \log_{10} fewer *Atopobium vaginae* 16S rRNA gene copies per swab. (C) TOLLIP deficient women have an average of 1.45- \log_{10} fewer *Gardnerella vaginalis* 16S rRNA gene copies per swab.

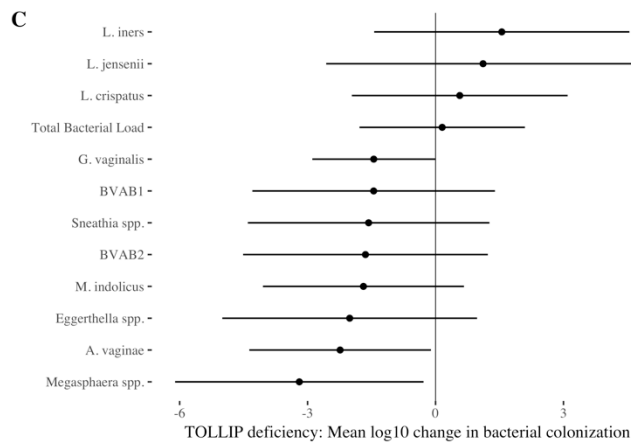


Figure 3.12. Differences in bacterial colonization associated with TOLLIP deficiency
 In addition to being associated with lower *Megasphaera* spp., *A. vaginae*, and *G. vaginalis*, TOLLIP deficient women in the discovery cohort demonstrated trends towards lower BVAB1, *Sneathia* spp., BVAB2, *Mageeibacillus indolicus* (BVAB3), and *Eggerthella* spp. concentrations.

In the validation cohort (Appendix C), women with TOLLIP deficiency had lower concentration of *L. gasseri* (mean $\Delta\log_{10} = -0.954$; $p = 0.02$; Figure 3.13). Concentration of other bacterial species was not significantly different between TOLLIP deficient and sufficient women in the cross-sectional cohort (data not shown).

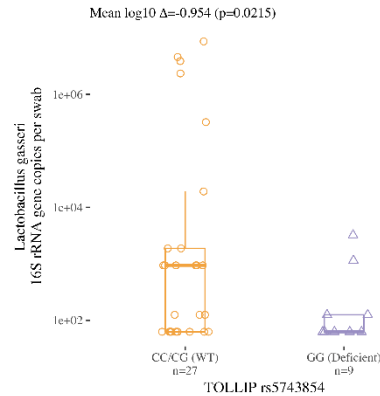


Figure 3.13. TOLLIP deficiency is associated with lower *L. gasseri* concentration in validation cohort

3.5 DISCUSSION

Although BV can be treated successfully using topical or systemic antibiotics, it recurs frequently^{18,40}, disproportionately affects women of African ancestry¹, and is a risk factor for preterm labor^{10–12}, HIV/STI transmission and acquisition^{4,5,7}, and other negative sequelae^{13–15}. It remains unclear why colonization with BVAB results in the appearance of clinical signs in some women, while others remain asymptomatic. Here, we demonstrate that a loss of negative regulation of innate immunity by TLR10 or TOLLIP (demonstrated to result in increased inflammation^{72,74,79}) is associated with *increased* clinically defined BV (Amsel) but not microbiologically defined BV (Nugent), and furthermore can result in altered vaginal bacterial colonization.

To our knowledge, this is the first report of a significant association of TLR10 or TOLLIP in the lower reproductive tract. We also demonstrate a relationship between functionally characterized SNPs in TOLLIP and TLR10, and risk of BV or alteration of vaginal bacterial colonization. Because homozygotes of the minor allele of TOLLIP rs5743854 are rare

among non-African ancestry populations^{70,80}, future studies could focus on the effect of this SNP in large, longitudinal cohorts of women of African ancestry.

Although we find a significant relationship between TLR10 rs11096955, BV, and *Lactobacillus* colonization, TLR10 rs11096955 is in complete linkage disequilibrium with TLR10 rs11096957, and tightly linked to rs4129009; additional studies are needed to determine which of these SNPs cause TLR10 deficiency.

Although we found TOLLIP deficiency to be associated with increased risk of clinically-defined BV, we also found TOLLIP deficiency to be associated with *decreased* risk of colonization with *Megasphaera* spp., *A. vaginae*, and *G. vaginalis*. Giving these seemingly contradictory results, we investigated which of Amsel's clinical signs were more or less common in participants who were diagnosed by Amsel's criteria but had a Nugent score that was not concordant with BV (Table 3.1). Surprisingly, high vaginal fluid pH was reported in all such episodes in both discovery and validation cohorts. Presence of clue cells (>20% per high-powered field) was also commonly identified in discovery and validation cohorts. These data may suggest that hyper-inflammatory host response may lead to decreased colonization with *Lactobacillus* spp., especially those lactobacilli that robustly produce lactic acid. For instance, deficient negative regulation of TLR signaling may also lead to upregulation of cell surface molecules that are used for bacterial adhesion, thereby leading to the presence of clue cells. The presence of amine odor and discharge were more variably identified, which may indicate that these are driven by bacterial metabolism. Indeed, metabolomic analysis of vaginal fluid from women with and without BV indicates that BV bacterial communities are the likely source of amine odor⁵⁷.

Table 3.1. Clinical signs present during instances of discordant BV diagnosis (diagnosed clinically, without microbiological confirmation)

<i>Discovery cohort</i>		
	Amsel's Positive, Nugent Score < 7 N = 16	
	Present	Absent
Vaginal fluid pH > 4.5	16	0
>20% Clue Cells per HPF ^a	15	1
Amine odor	13	3
Thin, homogeneous discharge	11	5

<i>Validation cohort</i>		
	Amsel's Positive, Nugent Score < 7 N = 4	
	Present	Absent
Vaginal fluid pH > 4.5	4	0
>20% Clue Cells per HPF ^a	3	1
Amine odor	3	1
Thin, homogeneous discharge	4	0

^a HPF = high powered field

Chapter 4. TOLL-LIKE RECEPTOR-4 IN THE LOWER FEMALE GENITAL TRACT AND BACTERIAL VAGINOSIS

Adapted from ⁸¹

4.1 INTRODUCTION

Toll-like receptor 4 (TLR4), in complex with its co-receptor MD2, binds lipopolysaccharide (LPS) present on the outer membrane of Gram-negative bacteria, and orchestrates the innate immune response⁸²⁻⁸⁶. TLR4, when in its complete and membrane-bound form (mTLR4), regulates inflammation via both MyD88 and TRIF-dependent pathways (reviewed in ⁸⁷). Myeloid cells co-express membrane-bound CD14 (mCD14), which results in increased sensitivity by efficiently delivering LPS to the mTLR4-MD2 complex and mediates endocytosis of TLR4 after LPS binding (reviewed in ⁸⁷). Myeloid cells also produce soluble CD14 (sCD14), which sensitizes cells that do not express mCD14 to LPS^{88,89}. In mice, an mRNA splice variant composed of only the extracellular domain of TLR4 was shown to encode a soluble product (sTLR4) capable of inhibiting inflammatory response to LPS⁹⁰. Multiple mRNA isoforms have been identified in humans as well, including one that appears to encode sTLR4⁹⁰; sTLR4 has also been detected by ELISA in human saliva, plasma, and amniotic fluid⁹¹⁻⁹³. Recombinant, soluble, human TLR4 has been shown to inhibit inflammatory responses to LPS^{94,95}.

There is considerable debate about the role TLR4 plays in bacterial vaginosis (BV). TLR4 appears to be expressed in very low levels by the vaginal epithelium⁹⁶⁻⁹⁸, and may only be present in the endocervix^{98,99} in a soluble form that is released into the endocervical mucus⁹⁷.

Pro-inflammatory responses resulting from LPS agonism of mTLR4 may result in clearance of Gram-negative BV-associated bacteria (BVAB). Alternatively, by attenuating the inflammatory response to Gram-negative BVAB, sTLR4 may contribute to homeostatic conditions that foster robust colonization with *Lactobacillus* spp. while also preventing the release of host cell products related to inflammation that could be used as a nutrient source for BVAB and could lead to clinical signs of BV. This alternative hypothesis is consistent with recent findings correlating deficiency in TLR10, a negative regulator of TLR signaling, with increased risk of clinically-defined BV and lower colonization with *L. jensenii*⁶³. However, while sTLR4 has been described in amniotic fluid⁹², there are no published studies (to our knowledge) exploring the presence or role of sTLR4 in the vagina.

TLR4 single nucleotide polymorphisms (SNPs) rs4986790 and rs4986791 are functionally characterized non-synonymous mutations in the extracellular LPS-binding domain of TLR4¹⁰⁰. TLR4 rs4986790 causes a mutation adjacent to the TLR4-MD2 binding interface (299D>G), and is in close linkage disequilibrium with TLR4 SNP rs4986791 (399T>I)^{70,80}. Previous structural studies demonstrated that TLR4 possessing both rs4986790 and rs986791 rare variants have diminished capacity to bind monophosphoryl lipid A, a “weak” ligand, without any detectable changes in binding affinity of *E. coli* lipid A¹⁰¹. Earlier work by the same group identified SNP rs4986790 as the mutation causing structural changes in TLR4¹⁰². Together, these studies suggest that these alleles impact function of both mTLR4 and its negative regulator, sTLR4.

Previous studies have examined the relationship between these SNPs and risk of BV⁴⁸⁻⁵⁰. Although these SNPs are closely linked, two studies examining prevalent BV in pregnant women found opposite results: Genc et al.⁴⁹ found an *increased* risk of BV associated with TLR4

deficiency resulting from the minor allele of rs4986790 while Goepfert et al.⁵⁰ found a *decreased* risk of BV associated with the TLR4 deficiency resulting from minor allele of rs4986791. Genc et al. used culture methods to assess vaginal colonization with BVAB and found a trend towards increased isolation rates and quantities of vaginal Gram-negative *Prevotella*, *Bacteroides*, and *Porphyromonas* species in TLR4 deficient women, suggesting that pro-inflammatory responses resulting from mTLR4 agonism are important for the clearance of these Gram-negative BVAB. However, in the same study, the authors found statistically significantly more *G. vaginalis*—a Gram-positive BVAB. This surprising “off target” effect raises the possibility that TLR4 deficiency in the vagina may be realized mainly in the form of deficient sTLR4 negative regulation of LPS response. However, in the absence of more comprehensive data about vaginal colonization to correlate with the presence of sTLR4, the balance between mTLR4 and sTLR4 in BV pathogenesis remains unknown.

Here, we replicate previously-identified associations between TLR4 deficiency and increased risk of BV⁴⁹ and explore the mechanisms underlying this correlation. In TLR4 sufficient women—but not TLR4 deficient women—we find a protective effect of endocervical ectopy. Furthermore, we demonstrate significantly higher overall bacterial load, higher concentrations of Gram-positive BVABs (*Gardnerella vaginalis*, *Atopobium vaginae*), and a significantly less in *Lactobacillus jensenii* concentration associated with TLR4 deficiency. We find sTLR4 in cervical and vaginal secretions and find higher concentration of sTLR4 present in women with endocervical ectopy and women sampled during the proliferative phase of the menstrual cycle. Together, these data are consistent with a model by which sTLR4 functions as a key *negative* regulator of innate immune response to LPS, decreasing the appearance of

clinically-identified signs of BV, promoting *Lactobacillus* colonization, and restricting production of inflammatory host cell products that may promote the growth of BVAB.

4.2 MATERIALS AND METHODS

4.2.1 Cohort enrollment

In addition to the enrollment procedures described in Chapter 1, in Seattle-based discovery and validation cohorts, date of last menstrual period and contraceptive use were collected by study participant self-report using a computer-assisted survey instrument. Study clinicians performed speculum examination of the vagina and uterine cervix, and noted the presence of endocervical ectopy (none, 25%, 50%, 75%, 100%). In addition, at the time of enrollment in the Seattle validation cohort, cytobrushes were inserted approximately 1 cm into the endocervical canal, rotated two full turns, then withdrawn. The cytobrush was then placed into a 15 mL conical tube with 2 mL phosphate buffered saline, and then mixed with vortex at moderate speed for 2 minutes. The tubes were briefly centrifuged and the cytobrush removed. The conical tube was then centrifuged (10 minutes, 800 x g). Supernatant was harvested and frozen at -80C.

As a second validation cohort, residual, stored vaginal swabs were obtained from a sub-cohort women enrolled in a nationally-representative study of vaginal colonization with *Clostridium sordellii*¹⁰³. From this larger study, we selected samples from non-pregnant, Caucasian women reporting no antibiotic usage during the last 4 weeks, reporting either use of oral contraceptive pills or non-hormonal contraception. Women with Nugent score 4-6 (“intermediate” microbiota) were excluded to focus this study on women with and without BV. Vaginal fluid pH was measured by study clinicians; participants in this study were assessed for BV by Nugent score.

4.2.2 *Selection and genotyping of single nucleotide polymorphisms*

TLR4 rs4986790 and rs4986791 genotypes were determined in the Seattle-based discovery and validation cohorts as well as the second validation cohort using commercially-available TaqMan genotyping assays (Thermo Fisher, Waltham, MA), as described in Chapter 1⁶³. Because these SNPs are in a high degree of linkage disequilibrium, and rs4986790 has been shown to cause structural changes that would affect binding of MD2/LPS to TLR4¹⁰², we focused our analysis on this SNP alone. Data were analyzed using a dominant model, based on previously published data¹⁰⁰.

4.2.3 *Quantification of TLR4, LBP, and MD2*

Participants were stratified by Nugent diagnosis and TLR4 deficiency and randomly selected for TLR4, LBP, and MD2 measurement. To measure protein concentrations within vaginal fluid (swab preparation supernatant from Seattle discovery cohort) and endocervical cytobrush samples (from Seattle validation cohort), supernatants were thawed to room temperature, centrifuged (20 minutes, 1000 x g), and aliquoted to minimize freeze-thaw cycling. Aliquots were warmed to room temperature (per manufacturer's instruction) before being assayed.

To measure sTLR4 produced by endocervical cells *in vitro*, primary cells were plated in 24-well plates and incubated at 37C for 72 hours before supernatants were collected. Supernatants were centrifuged at 1000 x g for 20 minutes (per manufacturers' protocol) to remove cellular material.

TLR4 concentration was determined using LSBio TLR4 sandwich chemiluminescent immunoassay (CLIA) (catalog number LS-F29972). MD2 and lipopeptide binding protein (LBP) concentrations were determined by sandwich ELISA (LifeSpan BioSciences, catalog number

LS-F7426; R&D Systems, catalog number DY870-05). For all assays, samples below the level of detection for the assay were imputed with half the level of detection.

4.2.4 *Statistical methods*

Statistical analysis of TLR4 deficiency, BV risk, and bacterial colonization in the Seattle-based discovery and validation cohorts are described in Chapter 1.

Our primary analysis of cross-sectional data obtained from the nationally-representative validation cohort involved a comparison of BV prevalence (assessed by Nugent score) by TLR4 deficiency status. As a secondary analysis, we used a Mann-Whitney U test to compare vaginal fluid pH between TLR4 deficient and sufficient study participants (as a surrogate for clinically-defined BV). sTLR4 concentration in endocervical cytobrush supernatants were compared between population subgroups using Mann-Whitney U tests.

4.3 RESULTS

4.3.1 *Cohort characteristics*

Demographics of the Seattle-based discovery and validation cohorts are explored in Chapter 2. The second validation cohort is described in Table 4.1, and was composed of N=377 non-Hispanic, Caucasian women, ages 18-45. Consistent with prior data published by others⁵⁴, women without BV (diagnosed by Nugent score) were more likely to report use of hormonal contraception (42% versus 27% of women with BV).

Table 4.1. Second Validation Cohort Demographics

	BV-Negative [†] N = 295		BV-Positive [†] N = 82		Total N=377		p _{χ²}
	n	%	n	%	n	%	
<i>Age</i>							
18-29	168	57%	41	50%	209	55%	0.44
30-39	84	28%	25	30%	109	29%	
40-45	43	15%	16	20%	59	16%	
<i>Hormonal Contraception</i>							
Yes	124	42%	22	27%	146	39%	0.04
No	160	54%	57	70%	217	58%	
Refuse to report	11	4%	3	4%	14	4%	

[†] Bacterial vaginosis assessed by Nugent score (7-10).

4.3.2 *TLR4 deficiency is associated with increased risk of clinical BV*

We hypothesized that TLR4 deficiency is associated with increased risk of both clinical and microbiological BV. In the discovery cohort, the minor allele frequency of rs4986790 was 0.16 (Table 4.2).

Table 4.2. Discovery Cohort Allele Frequencies

	TLR4 rs4986790				Tot.	p _{χ²} [§]
	<i>Suf.</i> [†]	<i>Def.</i> [†]		NR [‡]		
	AA	AG	GG			
<i>All</i>						
Total	138	42	9	25	214	
<i>Race</i>						
AIAN/NHPI [¶]	5	1	0	1	7	0.33
Asian	3	0	0	1	4	
African-American	37	17	5	15	74	
Caucasian	80	21	4	6	111	
Other	12	3	0	1	16	
Two or more races	1	0	0	1	2	
<i>Age</i>						
18-30	70	16	5	11	102	0.53
31-40	37	15	2	3	57	
41-50	29	9	2	10	50	
Refuse to report	2	2	0	1	5	
<i>Hormonal Contraception</i>						
Yes	51	11	1	9	72	0.08
No	86	31	8	16	141	
Refuse to report	1	0	0	0	1	
<i>History of BV</i>						
Yes	89	34	7	18	148	0.02
No	45	6	2	6	59	
Refuse to report	4	2	0	1	7	

[†]Genotype associated with TLR4 "sufficiency" (Suf.) or "deficiency" (Def.).

[‡]Genotype was unable to be resolved or was not obtained.

[§]P-value comparing demographics in TLR4 sufficient versus deficient.

[¶]AIAN/NHPI = American Indian, Alaska Native, Native Hawaiian, or Pacific Islander.

Individuals with TLR4 deficiency were at 4.18-fold increased risk for clinically diagnosed BV in the Seattle-based discovery cohort (Figure 4.1A, 95% CI: [2.10, 9.63], $p < 0.001$). No differences in risk of microbiological BV were detected (Figure 4.1B). Because the minor allele of rs4986790 is more common in a European ancestry background, we performed an analysis of TLR4 deficiency and clinically-defined BV adjusting for race. In this adjusted analysis, TLR4 deficiency continued to be associated with increased risk for BV (adjusted hazard ratio = 3.99, 95% CI: [1.76, 8.42], $p < 0.001$), while Caucasian race was associated with a trend towards decreased risk of BV (adjusted hazard ratio = 0.50, 95% CI: [0.23, 1.03], $p = 0.06$).

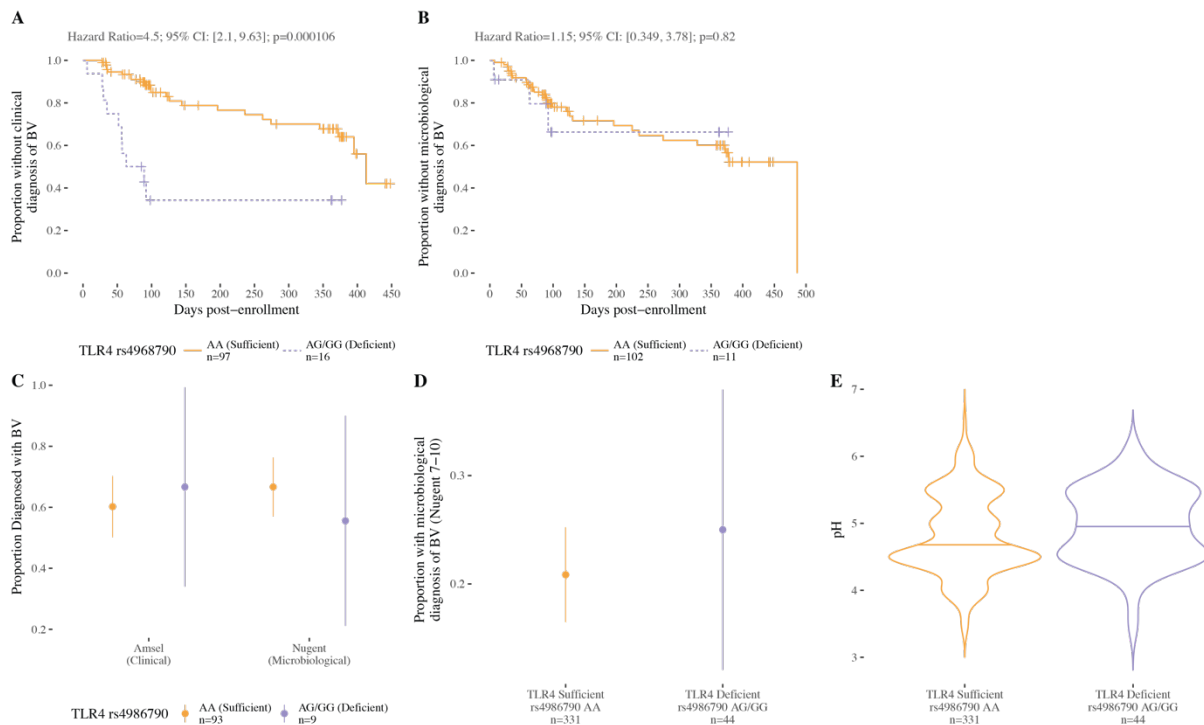


Figure 4.1. TLR4 deficiency conferred by rs4986790 AG/GG is associated with increased risk of clinical but not microbiological BV

(A) Using Cox proportional hazards regression, TLR4 deficient women demonstrate a 4.5-fold increased risk of clinical BV in the discovery cohort. (B) TLR4 deficiency does not modify risk of microbiological BV in the discovery cohort. (C) In the Seattle-based validation cohort, women with TLR4 deficiency have similar risk of BV diagnosed clinically by Amsel’s criteria (left) and microbiologically by Nugent score (right). (D) In the second validation cohort, TLR4 sufficient (orange, right) and TLR4 deficient (purple, right) women have similar risk of microbiologically-defined BV. (E) Violin plot depicting distribution of vaginal fluid pH in TLR4 sufficient (orange, left) and TLR4 deficient (purple, right) women in second validation cohort. Horizontal lines represent median value for each group; width of “violin” indicates frequency of pH measurement. Vaginal fluid pH trends higher in TLR4 deficient women ($p = 0.056$, Mann-Whitney).

In the Seattle-based validation cohort, TLR4 SNP rs4986790 was not associated with risk of clinical (odds ratio = 1.31, 95% CI: [0.31, 5.57], $p = 0.72$) or microbiological (odds ratio = 0.65, 95% CI: [0.16, 5.58], $p = 0.54$) diagnosis of BV (Figure 4.1C); adjusting for race did not substantively change the results of this analysis (data not shown). With a sample size of only $N=111$, statistical power in this cohort was limiting. Thus, we examined the relationship between TLR4 deficiency and BV in a third validation cohort of $N=377$ non-pregnant, Caucasian women (Table 4.3).

Table 4.3. Second Validation Cohort Allele Frequencies

	TLR4 rs4986790				Tot.	$p_{\chi^2}^{\S}$
	<i>Suf.</i> [†]	<i>Def.</i> [†]		NR [‡]		
	AA	AG	GG			
<i>All</i>	331	44	0	2	377	
Total						
<i>Age</i>						
18-30	188	21	0	0	209	0.16
31-40	90	18	0	1	109	
41-50	53	5	0	1	59	
<i>Hormonal Contraception</i>						
Yes	131	15	0	0	217	0.77
No	188	27	0	2	146	
Refuse to report	12	2	0	0	14	

[†]Genotype associated with TLR4 "sufficiency" (Suf.) or "deficiency" (Def.).

[‡]Genotype was unable to be resolved or was not obtained.

[§]P-value comparing demographics in TLR4 sufficient versus deficient.

Similar to the results found in the discovery cohort, TLR4 deficiency was not associated with risk of microbiologically-defined BV (Figure 4.1D, RR = 1.20, 95% CI [0.60, 1.95], p = 0.56). While presence of BV was not assessed by clinicians in this study, pH—the most sensitive indicator of presence of BV¹⁰⁴—was measured. TLR4 deficient women trended towards higher pH, but these results did not fall below our statistical significance threshold (Figure 4.1E, p = 0.06, Mann-Whitney U). Our ability to only detect statistically significant differences in risk of clinically-defined BV in the large, longitudinal discovery cohort of women at high risk for BV may indicate the effect size of TLR4 deficiency may be more modest in the general population. However, our results suggest that TLR4 deficiency may place women at increased risk for clinically-defined BV.

4.3.3 *TLR4 deficiency modifies the effect of endocervical ectopy*

We next hypothesized that the increase in TLR4-expressing tissue associated with cervical ectopy in the cervicovaginal niche would decrease risk of BV only in women with fully functional TLR4 (i.e. TLR4 SNP rs4986790 AA). As expected, the presence of cervical ectopy at enrollment ($\geq 25\%$) was associated with a trend towards protection from clinical BV, but only in women with functional TLR4 (Figure 4.2A, hazard ratio associated with ectopy in TLR4 sufficient women = 0.22, 95% CI [0.03, 1.63], p = 0.14). Among TLR4 deficient women, the presence of cervical ectopy had no effect on risk of clinical BV (Figure 4.2A, hazard ratio associated with ectopy in TLR4 deficient women = 0.90, 95% CI [0.23, 3.51], p = 0.88). To check for potential confounding between TLR4 deficiency and endocervical ectopy, we performed an additional analysis to confirm that TLR4 deficient women had similar prevalence of endocervical ectopy as TLR4 sufficient women at enrollment (25.5% versus 17.4%, p = 0.22, Chi-Square).

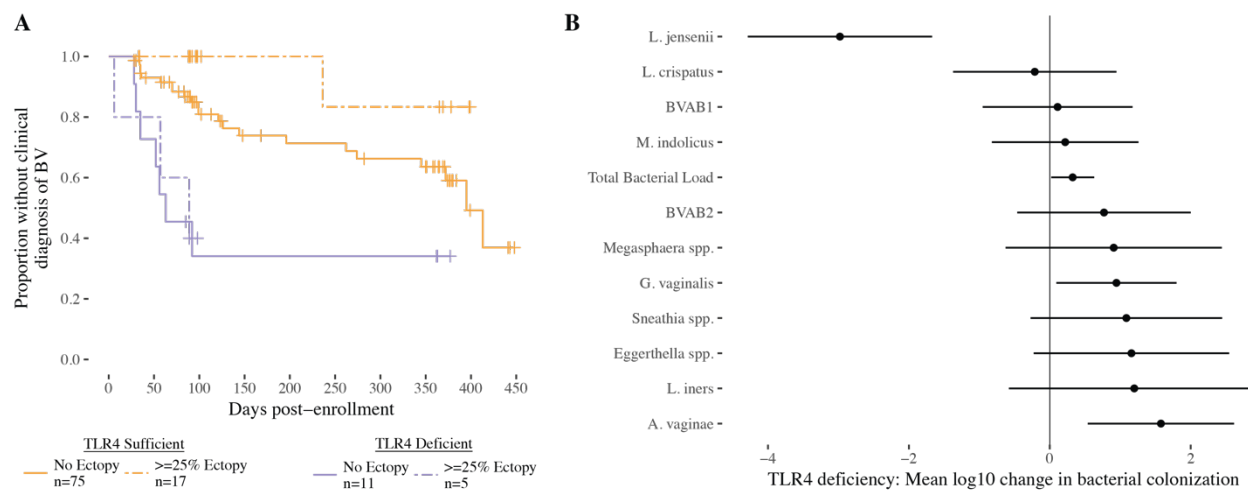


Figure 4.2. Endocervical ectopy and vaginal bacterial colonization in TLR4 sufficient and deficient study participants

(A) Presence of endocervical ectopy (indicated by dotted line) is associated with a non-significant trend towards decreased risk of BV in TLR4 sufficient women (orange, HR = 0.22, 95% CI: [0.03, 1.63], $p = 0.14$) but not in TLR4 deficient women (purple, HR = 0.90, 95% CI: [0.23, 3.51], $p = 0.88$). (B) Estimated difference in 16S rRNA gene copies per swab associated with TLR4 deficiency (point), with 95% confidence interval (horizontal line). Vertical line marks zero, or no difference in bacterial concentration associated with TLR4 deficiency. 95% confidence intervals not intersecting this vertical line are concordant with statistically significant difference in the indicated species. Women with TLR4 deficiency have statistically significantly higher concentration of BV-associated bacteria (BVAB) *Atopobium vaginae*, *Gardnerella vaginalis*, and lower quantity of *Lactobacillus jensenii*. Total bacterial load is also significantly increased in women with TLR4 deficiency.

4.3.4 *TLR4 deficiency is associated with higher concentrations of A. vaginae, G. vaginalis, and total bacterial load*

We hypothesized that dampened innate immune responses to LPS associated with mTLR4 deficiency would be associated with higher colonization with Gram-negative BVAB. To test this hypothesis, we examined colonization with BVAB and lactobacilli in the discovery cohort (Figure 4.2B).

In the discovery cohort, we found a trend towards higher concentrations of *Sneathia* spp. in TLR4 deficient women (Figure 4.3A, mean log₁₀ difference = 1.09 16S copies per swab; 95% CI: [-0.27, 2.45], p = 0.11) in comparison to matched TLR4 sufficient controls (Appendix B). No other Gram-negative bacteria were assessed by quantitative PCR in the discovery cohort.

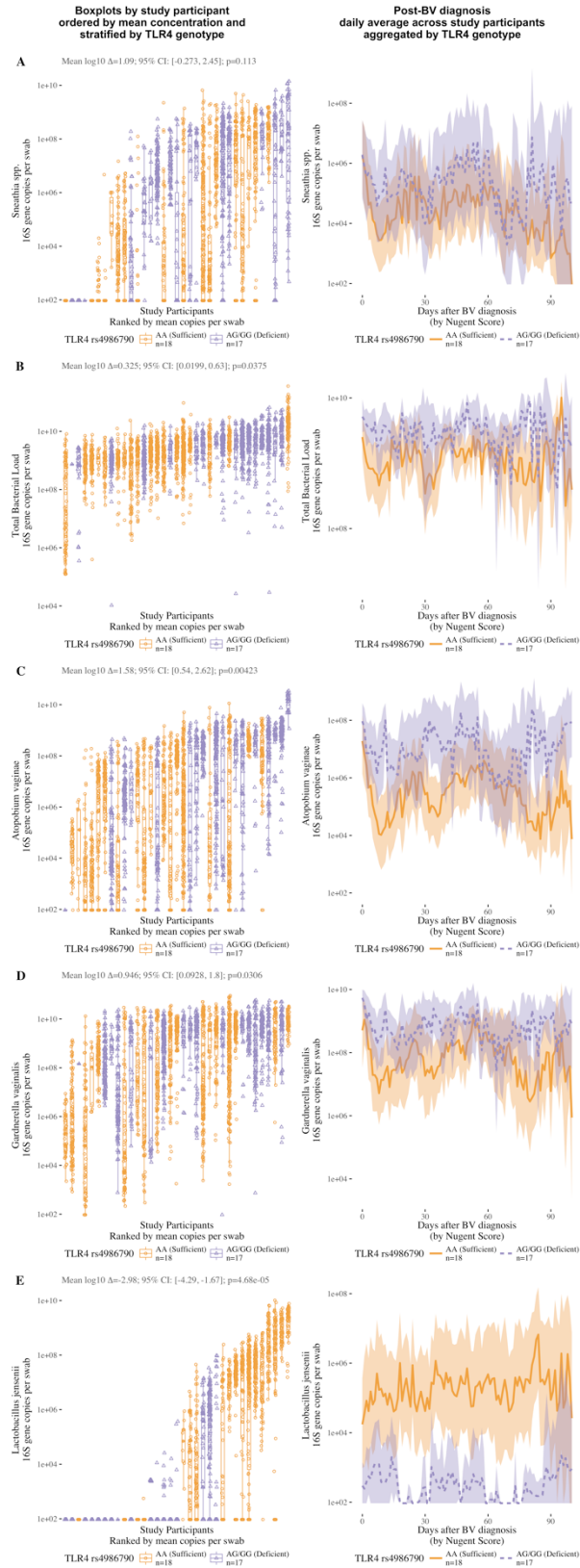


Figure 4.3. TLR4 deficiency is associated with higher concentration of total bacteria, *A. vaginae*, *G. vaginalis*, and lower concentration of *L. jensenii*

Colonization across time in TLR4 deficient cases and matched TLR4 sufficient controls, divided into separate boxplots with overlaid scatterplots for each study participant to show within-subject variability (left), and depicted as a daily, moving average aggregated by TLR4 deficiency versus sufficiency (right, shading depicts 95% bootstrapped confidence interval). (A) Species-specific quantitative PCR assays for the 16S rRNA gene demonstrate a trend of higher *Sneathia* spp. colonization in TLR4 deficient women (mean 1.09 log₁₀, p = 0.11). (B) Using a broad-range 16S rRNA gene qPCR assay for the measurement of total bacterial load, women with TLR4 deficiency had a higher bacterial concentration of 0.325-log₁₀ 16S copies per swab (p = 0.04). (C) Women with TLR4 deficiency demonstrated 1.58-log₁₀ more *Atopobium vaginae* 16S rRNA gene copies per swab (mean, p = 0.004) and (D) 0.946-log₁₀ more *Gardnerella vaginalis* 16S rRNA gene copies per swab (mean, p = 0.03). (E) *Lactobacillus jensenii* colonization was significantly lower in women with TLR4 deficiency (-2.98 mean log₁₀ 16S rRNA gene copies per swab, p<0.0001).

Under an alternative model, in which sTLR4 negative regulation plays a more central role than pro-inflammatory mTLR4 responses in the lower female genital tract, we would predict that the effects of mTLR4/sTLR4 deficiency would affect colonization with Gram positives *and* Gram negatives. Total concentration of vaginal bacteria was significantly higher in TLR4 deficient women (Figure 4.3B, mean log₁₀ difference = 0.33 16S copies per swab, 95% CI: [0.02, 0.63], p = 0.04). Additionally, commonly identified BVAB were more abundant in TLR4 deficient women: *A. vaginae* (Figure 4.3C, mean log₁₀ difference = 1.58 16S copies per swab, 95% CI: [0.54, 2.62], p = 0.004) and *G. vaginalis* (Figure 4.3D, mean difference log₁₀ difference = 0.946 16S copies per swab, 95% CI: [0.09, 1.80], p = 0.03). Higher concentration of BVAB were accompanied by a dramatically lower concentration of *L. jensenii* (Figure 4.3E, mean log₁₀ difference = -2.98, 16S copies per swab, 95% CI: [-4.29, -1.67], p < 0.0001). Furthermore, TLR4 deficient women exhibited trends towards higher concentrations of other Gram-positive BVAB, including BVAB2, *Eggerthella* spp., and *L. iners* (Figure 4.2B). Together, the lack of statistically significant difference in *Sneathia* spp. and significantly higher concentrations of Gram-positive BVAB suggest the regulatory role of sTLR4 outweighs the LPS pro-inflammatory response of mTLR4.

4.3.5 Soluble TLR4 (sTLR4) is higher in women with cervical ectopy

To our knowledge, sTLR4 has not been reported in cervical mucus or vaginal fluid. To test whether sTLR4 is present *in vivo*, we measured sTLR4 in a stratified random sample of n=62 endocervical cytobrush supernatants collected from women in the Seattle-based discovery cohort. sTLR4 was above the level of detection in all endocervical cytobrush samples assayed. sTLR4 concentrations did not differ by hormonal contraceptive status (Figure 4.4A), or BV status (diagnosed microbiologically using Nugent score in Figure 4.4B, or clinically using

Amsel's criteria in Figure 4.4C). Furthermore, Gram-negative bacteria in vaginal fluid (*Prevotella amnii* in Figure 4.4D, *Prevotella timonensis* in Figure 4.4E, and *Sneathia* spp. in Figure 4.4F) did not correlate with endocervical sTLR4 concentration.

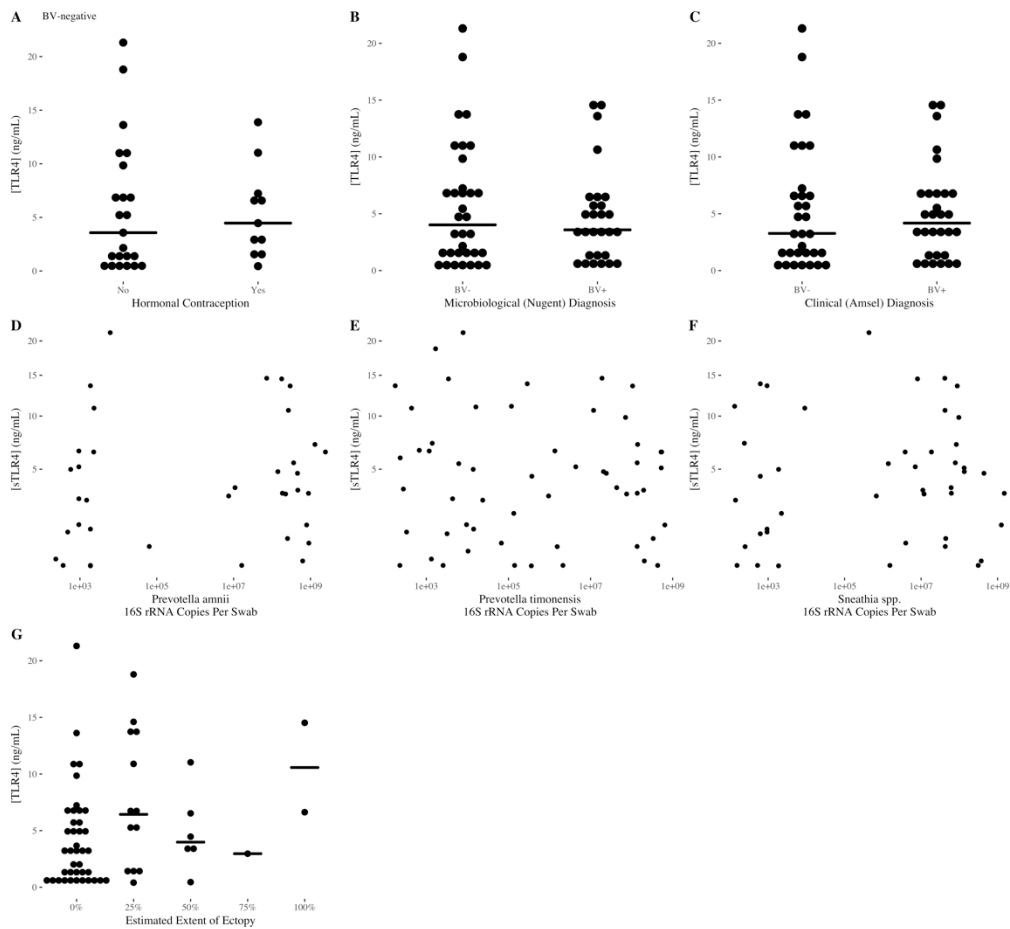


Figure 4.4. In the Seattle-based validation cohort, sTLR4 concentration in endocervical cytobrush supernatants does not correlate with hormonal contraception use, TLR4 rs4986790 genotype, BV status, or concentration of Gram-negative BVAB

(A) Hormonal contraceptive status does not appear to associate with sTLR4 concentration in the endocervix in a sub-sample of women without BV at enrollment. (B) BV diagnosis by Nugent score does not appear to affect sTLR4 concentration. (C) BV diagnosis by Amsel’s criteria does not appear to affect sTLR4 concentration. (D)-(F) sTLR4 concentration does not appear to associate with concentration of Gram-negative vaginal bacteria: (D) *Prevotella amnii*, (E) *Prevotella timonensis*, and (F) *Sneathia* spp. (G) sTLR4 concentrations in endocervical cytobrush supernatants do not increase with increasing degrees of ectopy. When grouped together, presence of $\geq 25\%$ endocervical ectopy is associated with increased sTLR4 concentration (Figure 4.5B).

We examined whether TLR4 rs4986790 genotype correlated with expression levels of sTLR4 and found trends towards higher concentration of sTLR4 in BV-negative women with TLR4 deficiency (Figure 4.5A, $p = 0.13$, Mann-Whitney U), and lower concentration of sTLR4 in BV-positive women with TLR4 deficiency (Figure 4.5A, $p = 0.06$, Mann-Whitney U).

We hypothesized that decreased risk of clinical BV in women with TLR4 sufficiency and cervical ectopy is moderated by increased sTLR4 concentrations occurring in women with cervical ectopy. While increasing degrees of endocervical ectopy was not associated with the presence of increased concentrations of sTLR4 (Figure 4.4G), women who were found to have >25% endocervical ectopy on speculum exam had significantly increased sTLR4 concentration in endocervical mucus (Figure 4.5B, $p < 0.0001$, Mann-Whitney U).

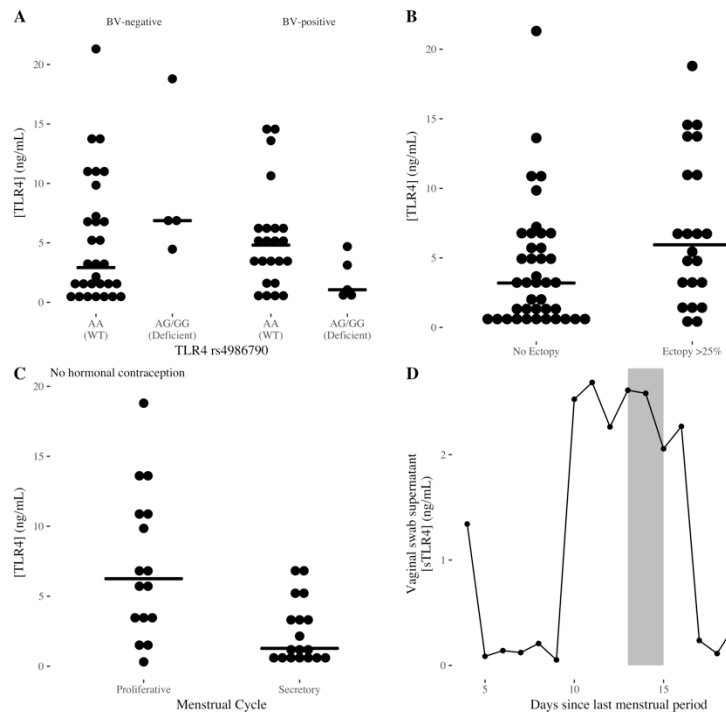


Figure 4.5. Soluble TLR4 is modified by presence of cervical ectopy and menstrual cycle phase

(A) TLR4 deficient women without Nugent diagnosis of BV (left) have similar sTLR4 concentrations compared to TLR4 sufficient women ($p = 0.13$) while women with Nugent diagnosis of BV (right) and TLR4 deficiency (SNP rs4986790 AG/GG) may have lower concentrations of sTLR4 ($p = 0.06$). (B) Dotplots of sTLR4 concentration in endocervical cytobrush supernatants in women without and with endocervical ectopy (group medians indicated by horizontal line). Women with cervical ectopy have significantly higher concentrations of sTLR4 ($p < 0.0001$, Mann-Whitney U). (C) Dotplots of sTLR4 concentration in endocervical cytobrush supernatants by menstrual cycle phase (group medians indicated by horizontal line). Among women not on hormonal contraception, those reported being within 14 days of their last menstrual period (LMP; proliferative phase) had significantly higher concentration of sTLR4 than those greater than 14 days since LMP (secretory phase) ($p = 0.002$, Mann-Whitney U). (D) sTLR4 was found in vaginal swab supernatants from a single study participant, with highest concentrations occurring around the time of ovulation (10-16 days after the start of last menstrual period, gray shading indicates 14 day since last menstrual period).

4.3.6 *sTLR4 is significantly higher in women sampled during proliferative phase of menstrual cycle*

Estradiol has been shown to inhibit proinflammatory responses mediated through IL-1 β ¹⁰⁵ and diminish response to LPS and polyI:C¹⁰⁶ in a uterine epithelial cell culture model. Additionally, higher colonization with *Lactobacillus* spp. and has been observed during the proliferative phase of the menstrual cycle¹⁰⁷. Thus, we hypothesized that sTLR4 concentrations would be highest during the proliferative phase of the menstrual cycle. To test this hypothesis, we selected the subset of study participants not reporting use of hormonal contraceptives to eliminate variability introduced by exogenous hormones. Consistent with our hypothesis, we found that women sampled during the proliferative phase (reporting less than 14 days since last menstrual period [LMP]) had significantly higher concentration of sTLR4 in endocervical mucus when compared to women sampled during the secretory phase (reporting 15 or more days since LMP, Figure 4.5C, $p = 0.002$, Mann-Whitney U).

We measured sTLR4 concentrations in daily vaginal swabs collected from a single study participant during the course of one menstrual cycle (days 4-21, Figure 4.5D). This study participant was never diagnosed with BV by Amsel's criteria or Nugent score during their study enrollment, and did not report use of hormonal contraceptives. Strikingly, sTLR4 concentrations reached their highest levels in the time period when ovulation would be expected to occur, during days 10-16 of the menstrual cycle.

These results suggest that the relationship between TLR4 deficiency and BV is not mediated through differences in quantity of sTLR4 expressed. Furthermore, it appears that endocervical sTLR4 concentration is not a function of Gram-negative bacteria present in the vagina and is instead modulated hormonally.

4.4 DISCUSSION

In this chapter, we reproduce previously published associations between TLR4 deficiency (associated with rs4986790 AG/GG) and increased risk of BV in a discovery cohort. We explore mechanisms that may underlie this relationship, including the effect of exposure of TLR4-expressing tissue to the vaginal compartment (in the form of endocervical ectopy), and differences in bacterial colonization associated with TLR4 deficiency. TLR4 sufficient women with endocervical ectopy trend towards decreased risk of clinical BV; this trend does not exist in women with TLR4 deficiency. These results suggest that TLR4 expressed by endocervical epithelial cells—whether in its membrane-bound or soluble form—must be fully functional to protect against the development of BV. Additionally, we find differences in bacterial colonization associated with TLR4 deficiency that are not specific to Gram-negative BVAB: *G. vaginalis*, *A. vaginae*, and total bacterial load were all higher in women with TLR4 deficiency. Our use of species-specific qPCR to measure concentrations of BVAB and common vaginal lactobacilli comprises the most detailed exploration to date of the role of TLR4 in the vaginal microbiota. Finally, we demonstrate the novel finding that sTLR4 is present in cervical mucus and vaginal fluid, with significant increases in sTLR4 during the proliferative phase of the menstrual cycle and in women with endocervical ectopy. Future studies could further elucidate the role of sTLR4 in modulating lower genital innate immune response, susceptibility to BV and BV-related sequelae such as HIV infection and pre-term birth.

Although we are unable to validate the relationship between TLR4 deficiency and increased risk of BV at the widely-accepted $p < 0.05$ level, the relationship between TLR4 deficiency and increased vaginal pH (the component of Amsel's criteria that is most sensitive for the presence of BV¹⁰⁴) was validated with $p = 0.06$ in a second cohort. Although the second

validation cohort was large (N=377), there were no participants who were homozygous for the minor allele of rs4986790, which would have led to a less profound phenotypic difference between TLR4 sufficiency and deficiency. While the discovery cohort was composed of women at high risk for BV, the second validation cohort was intended to reflect the general population of reproductive-age women. The less profound difference in BV risk between TLR4 sufficient and deficient women in the second validation cohort strongly suggests that susceptibility to BV is driven by both host factors and exposure to BVAB.

This study uses a standard but relatively crude measure of endocervical ectopy; future studies could use colposcopy or digital, image-based measurement. While we were able to detect sTLR4 and LBP in cervical mucus, we were unable to detect MD2 by ELISA. This may be due to MD2 being bound by TLR4, thereby obscuring the MD2 antigen recognized by our ELISA antibodies. Alternatively, MD2 concentration could be below limit of detection for our assay. Future studies could use Western blot or other methods for the detection of MD2. Although we are able to demonstrate a correlation between TLR4 deficiency, increased risk of BV, and increased colonization with BVAB, we are unable to show causation. Additional mechanistic studies could be undertaken, for instance using an *in vitro* system capturing the complex interactions between epithelium and immune cells.

The data we present here suggest that TLR4 may have a heterogeneous role in the lower female genital tract mucosa. Cells expressing substantive TLR4 are localized to the endocervix: endocervical epithelial cells produce and secrete sTLR4 into the endocervical mucus, and neutrophils and macrophages express mTLR4. However, inflammation involving leukocytic infiltrate is not a hallmark of BV pathogenesis¹⁰⁷. Thus, we believe that the effect of sTLR4—detectable even in dilute, vaginal swab supernatant—outweighs the pro-inflammatory responses

associated with mTLR4. Thus, sTLR4 may represent one mechanism by which lower genital tract response to LPS is moderated. We propose a model whereby sTLR4 promotes tolerance of Gram-negative organisms in the vagina, which in turn *preserves* an anti-inflammatory environment beneficial to homeostatic colonization with *Lactobacillus* spp, and prevents the appearance of inflammation-related host products benefiting the growth of BVAB. We believe that increases in Gram-positive BVABs and total bacterial load associated with TLR4 deficiency are indicative of hyper-responsiveness to LPS, augmenting a pro-inflammatory phenotype that may benefit BVAB growth and promote the appearance of the clinical signs of BV.

Chapter 5. GENETIC VARIATION IN TOLL-LIKE RECEPTOR-5 AND COLONIZATION WITH FLAGELLATED BACTERIAL VAGINOSIS-ASSOCIATED BACTERIA

Adapted from: ¹⁰⁸

5.1 INTRODUCTION

Bacterial vaginosis (BV) is a common vaginal dysbiosis, involving a bacterial community shift from one composed of a few species of lactobacilli to a diverse community of anaerobic and facultative bacteria with increased species richness and evenness¹⁰⁹. Nugent score is the “gold standard” for diagnosing bacterial vaginosis (BV), involving examination of Gram stained vaginal fluid by an experienced microscopist who identifies bacterial morphotypes associated with BV or with its absence¹⁶. While all Nugent scores between 7-10 are defined as bacterial vaginosis, Nugent scores of 9 or 10 are associated with the presence of “*Mobiluncus* morphotypes,” which appear as curved, Gram-negative rods. Vaginal *Mobiluncus* spp. have been observed closely associated with host epithelial cells, with flagellar structures identified using electron microscopy¹¹⁰ and motility observed on wet mount^{111,112}. Using molecular methods, our group found that many of these bacterial cells that were being identified as *Mobiluncus* spp. on Gram stains were actually BVAB1¹⁰⁹, an uncultivated member of the Clostridium phylum¹¹³ with high specificity for BV²².

More recently, our group used HiC to de-convolute reads from shotgun metagenomic sequencing to acquire a closed genome for BVAB1¹¹⁴. These data revealed five genes encoding flagellin—a virulence factor previously unassociated with BVAB1. Furthermore, studies

undertaken by our group have revealed that *Mobiluncus* spp. and BVAB1 are associated with spontaneous pre-term birth¹⁰, raising the possibility that these flagellated species are able to ascend into the upper reproductive tract. Although the involvement of flagellated organisms in BV has been established for over thirty years^{111,112,115}, vaginal innate immune responses to flagellin have not been explored.

TLR5 responds to conserved regions located in the N- and C-terminals of the flagellin monomer^{116–119}. TLR5 is strongly expressed by vaginal, ectocervical, and endocervical epithelium^{99,97}. A dominant-negative stop codon mutation in TLR5 (TLR5 “deficiency”) abolishes TLR5 signaling¹²⁰, and is associated with increased susceptibility to infection with some flagellated organisms^{120–122}. TLR5 deficiency is associated with *increased* risk of ulcerative colitis in an Indian cohort¹²³ but also with *decreased* prevalence of Crohn’s disease in an Ashkenazi Jewish subpopulation of a US-based study¹²⁴, suggesting a complex relationship between TLR5-mediated immune responses and the microbiota.

We hypothesized that TLR5 deficiency would be associated with increased risk of clinically- and microbiologically-defined BV, as well as increased colonization with flagellated BV-associated bacteria—*M. curtisii*, *M. mulieris*, and BVAB1. Contrary to our hypothesis, we found no relationship between TLR5 deficiency and bacterial vaginosis, and that TLR5 deficiency is associated with *decreased* colonization with *M. mulieris* and BVAB1, but not associated with differences in *M. curtisii* colonization. We probed this relationship further, finding that heat-inactivated *M. mulieris* and cervicovaginal fluid from women colonized with BVAB1 stimulate a TLR5-specific innate immune response, whereas heat-inactivated *M. curtisii* does not. Differences in flagellin composition among bacteria may explain differences in TLR5 responses noted here.

5.2 MATERIALS AND METHODS

5.2.1 *Cohort enrollment*

In addition to the discovery (Cohort 1) and validation cohorts (Cohort 2) used for genetic analyses (described in Chapter 1), a third clinical cohort (Cohort 3) was enrolled between April 2011 and July 2013 from the Public Health Seattle-King County (PHSKC) STD Clinic. In Cohort 2 and Cohort 3, study clinicians collected cervicovaginal lavage (CVL) by inserting 5 ml sterile saline then using a pipette to transfer the saline into a 15 mL conical tube. The resultant fluid was divided into two 15 ml conical tubes and centrifuged for 10 minutes at 800 x g. The supernatant was separated into 2 cryovial tubes and frozen at -80°C. After arrival in the lab from the clinic, CVL supernatants were thawed, spun down at 1000 x g for 20 minutes (to remove any residual human cells), and aliquoted. Single aliquots were thawed for downstream experiments.

5.2.2 *SNP genotyping*

TLR5 rs5744168 was genotyped using a commercially available TaqMan assay (Thermo Fisher, Waltham, MA). The discovery cohort (Cohort 1) assay was run on Fluidigm 48.48 chip. The validation cohort (Cohort 2) was run on Fluidigm 96.96 chip. Genotypes that could not be resolved on those chips were run as single-SNP assays using StepOnePlus (Applied Biosystems, Foster City, CA). Dominant model was chosen for analysis based on prior published data¹²⁰.

5.2.3 *DNA extraction and quantitative PCR*

DNA was extracted using commercially-available bacterial DNA extraction kits, and quantitative PCR (qPCR) was performed as described elsewhere⁶³. Specific for this study, a multiplex qPCR assay to quantify *Mobiluncus mulieris* and *Mobiluncus curtisii* was created, using forward primer Mobi1007F, 5'-CTTACCAAGGCTTGACATACA-3', reverse primer

Mobi1088R, 5'-ACCACCTGTACACCACC-3', and species-specific probes Mmuli1034_1054, 5'-VIC-CATGCCAGAGATGGTGTGG-MGB-NFQ-3', and Mcurt1034_1054, 5'-FAM-TGGTTCCAGAGATGGGCCAG-MGB-NFQ-3. Pre-cycling conditions were: 50°C for 2 minutes followed by 95°C for 20 seconds, followed by 45 cycles of: 95°C for 2 seconds, 57°C for 20 seconds, 72°C for 20 seconds.

5.2.4 Amino acid sequence analysis of flagellin from BVAB1, *M. curtisii*, *M. mulieris*

BVAB1 FlaA amino acid sequences were acquired from Sycuro et al. 2018¹¹⁴. *Mobiluncus mulieris* (strains: 35239, 35243, 28-1, FB024-16, FDAARGOS_303) and *Mobiluncus curtisii* (strains: 35242, 43063, 51333) genes annotated as “flagellin” were obtained from the PATRIC database¹²⁵, in addition FliC sequences from *P. aeruginosa*, and *S. enterica* serovar Typhimurium, and FlaA from *L. monocytogenes*. Sequences were aligned using the MUSCLE algorithm implemented in Geneious¹²⁶. After further inspection, *M. mulieris* FDAARGOS_303 was excluded from the analysis as it contains 16 genes annotated as *flaA* in its genome and was thus an outlier among the other *M. mulieris* whole genome sequences. The amino acid sequences aligning to the previously described 10-amino acid TLR5 recognition sequences present in canonical agonists (*Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhimurium, and *Listeria monocytogenes*) were identified as the probable recognition sequences of BVAB1 and *Mobiluncus* spp.. The gross alignments were compared to assess sequence length and presence of conserved regions D0, D1, D2, and D3 (reviewed in ¹²⁷).

5.2.5 Preparation of heat-inactivated *M. curtisii* and *M. mulieris*

M. curtisii ATCC 35241 and *M. mulieris* UPII 28-I were first grown on Brucella agar with 5% blood (supplemented with hemin and vitamin K; Hardy Diagnostics, Santa Maria, CA)

and were passaged twice before use in experiments. A loop of bacterial cells scraped from the plate was suspended in fresh PYGmod broth and used to inoculate disposable culture tubes containing 5mL PYGmod¹²⁸. Bacteria were incubated for 72 hours at 37°C (without shaking) in an anaerobe chamber (Anaerobe Systems, Morgan Hill, CA), after which time cells were pelleted, washed 3 times, resuspended in 3mL PBS, and heat treated at 65°C for 30 minutes. Heat inactivated cells were aliquoted and stored at -20°C until use.

5.2.6 *Identification of bacteria and cervicovaginal lavage supernatants capable of stimulating TLR5 response*

HEK Blue Null1 (Invivogen, San Diego, CA; cat. no. hkb-null1, lot no. 39-01-hkbnull1) and HEK Blue hTLR5 (Invivogen, San Diego, CA; cat. no. hkb-htlr5, lot no. 38-01-hkbhtlr5) cells were grown according to manufacturer's protocol. These cell lines were derived from HEK293 cells and have each has been transfected with a plasmid encoding a NF-kB-driven secretory embryonic alkaline phosphatase (SEAP) reporter. Per the manufacturer's package insert, HEK Blue hTLR5 expression is 20- to 100-times the endogenous hTLR5 expression in HEK293 cells.

For each biological replicate, cells were thawed from frozen stocks (passage number 3-5), washed in fresh DMEM (Gibco, Thermo Fisher, Waltham, MA), and plated on a 65mm tissue culture treated dishes in 5-6 mL media containing selective antibiotics. The following day, cells were passaged to 150mm dishes and incubated with selective antibiotics (Null1: Zeocin; hTLR5: Zeocin and Blasticidin; Invivogen, San Diego, CA) until 50-60% confluent. Media was replaced every day to ensure effective concentrations of selective antibiotics. Before use in an experiment, cells were washed with fresh PBS, then scraped in 2-3mL PBS, resuspended, and counted. Concentrated cells in PBS were resuspended in fresh HEK-Blue Detection media (Invivogen,

San Diego, CA; cat. no. hb-det2) at an approximate concentration of 140,000 cells/mL. Each plate included purified FlaA from *Bacillus subtilis* (Invivogen, San Diego, CA; cat. No. tlr1-pbsfla) as a positive control, and PBS (Gibco/Thermo Fisher, Waltham, MA) as a negative control. Each experiment included three technical replicates per condition. Optical density at 620 nm (OD₆₂₀) was measured using a VersaMax microplate reader (Molecular Devices, San Jose, CA).

As noted in the manufacturer's product notes, any exogenous source of NF- κ B activation will produce a SEAP-response in both HEK Blue Null1 and HEK Blue hTLR5 cells. These include cytokines such as TNF α , or ligands for any endogenously expressed pattern recognition receptors (TLR3, TLR5, and NOD1).

5.2.7 *Measurement of IL-8, IL-6, and IL-1 β in vaginal fluid*

A subset of 13 TLR5 deficient and 2 TLR5 sufficient study participants from the validation cohort were selected for vaginal fluid cytokine/chemokine measurement. Cervicovaginal lavage supernatants were aliquoted and assayed for a panel of cytokines using the Luminex platform (Austin, TX). We focused our analyses on IL-8, IL-6, and IL-1 β as these were the only cytokines/chemokines that were detectable in a majority of samples. Any samples that were undetectable were imputed at half the level of detection.

5.2.8 *Statistical analysis*

In addition to the statistical analysis procedures described in Chapter 1, non-parametric, Mann-Whitney tests were used to do pairwise tests in HEK reporter cell experiments. Within cell types (HEK Null1 versus HEK hTLR5), comparisons were made between each experimental condition and the negative control. Where there were significant differences between the

experimental condition and negative control, a between cell type comparison was made. Cytokine concentrations were compared between TLR5 deficient and sufficient participants using Mann-Whitney nonparametric tests.

5.3 RESULTS

5.3.1 *Cohort characteristics*

TLR5 deficiency did not correlate with any potential confounders of BV risk, including race, age, hormonal contraception, and self-reported history of BV (Table 5.1, discovery cohort; Table 5.2, validation cohort). In the discovery cohort, minor allele frequency (MAF) was 0.145; in the validation cohort, MAF was 0.036.

Table 5.1. Discovery Cohort Allele Frequency

TLR5 rs5744168						
	<i>Suf.</i>	<i>Def.</i>				
	CC	CT	TT	NR [†]	Tot.	χ^2 [‡]
All						
Total	139	47	4	24	214	
<i>Race</i>						
AIAN/NHPI	6	1	0	0	7	0.45
Asian	2	1	0	1	4	
African-American	47	12	1	14	74	
Caucasian	72	28	3	8	111	
Other	12	4	0	0	16	
Two or more races	0	1	0	1	2	
<i>Age</i>						
18-30	66	22	2	12	102	0.97
31-40	39	15	0	3	57	
41-50	30	10	2	8	50	
Refuse to report	4	0	0	1	5	
<i>Hormonal Contraception</i>						
Yes	45	16	2	9	72	0.86
No	93	31	2	15	141	
Refuse to report	1	0	0	0	1	
<i>History of BV</i>						
Yes	98	31	4	15	148	0.62
No	35	16	0	8	59	
Refuse to report	6	0	0	1	7	

[†]Genotype was unable to be resolved or was not obtained.

[‡]P-value comparing demographics in TLR5 sufficient versus deficient.

Table 5.2. Validation Cohort Allele Frequency

	TLR5 rs5744168				Tot.	$p_{\chi^2}^{\ddagger}$
	<i>Suf.</i>	<i>Def.</i>		NR [†]		
	CC	CT	TT			
All						
Total	103	8	0	0	111	
<i>Race</i>						
AIAN/NHPI	2	0	0	0	2	0.63
Asian	6	0	0	0	6	
African-American	35	1	0	0	36	
Caucasian	44	6	0	0	50	
Other	10	4	0	0	14	
Two or more races	3	0	0	0	3	
<i>Age</i>						
18-30	61	3	0	0	64	0.23
31-40	17	4	0	0	21	
41-50	15	1	0	0	16	
51-60	7	0	0	0	7	
Refuse to report	3	0	0	0	3	
<i>Hormonal Contraception</i>						
Yes	23	0	0	0	23	0.27
No	75	8	0	0	83	
Refuse to report	5	0	0	0	5	
<i>History of BV</i>						
Yes	67	7	0	0	74	0.40
No	34	1	0	0	35	
Refuse to report	2	0	0	0	2	

[†]Genotype was unable to be resolved or was not obtained.

[‡]P-value comparing demographics in TLR5 sufficient versus deficient.

5.3.2 *TLR5 deficiency is not associated with risk of clinically- or microbiologically-defined BV*

We hypothesized that TLR5 deficiency would be associated with increased risk of both clinically- and microbiologically-defined BV. Contrary to our hypothesis, TLR5 deficiency was not associated with risk of BV by either definition (Figure 5.1A-B).

To explore whether TLR5 deficiency could specifically impact colonization with *Mobiluncus* morphotypes identified in Nugent scoring of Gram stains, we performed an exploratory analysis examining whether TLR5 deficiency was associated with increased risk of women having a vaginal fluid Nugent score of 9 or 10 (which is associated with “*Mobiluncus* morphotypes”). Strikingly, in the discovery cohort, TLR5 deficiency appeared to be *protective* against the appearance of *Mobiluncus* morphotypes on vaginal Gram stains (Figure 5.1C). In a survival analysis examining time to first Nugent score of 9-10, no TLR5 deficient women were observed at a clinic visit with associated Nugent score of 9-10 (9,048 person-days of follow-up time), compared with 14 Nugent scores of 9-10 observed in TLR5 sufficient women (22,335 person-days follow-up time; $p = 0.02$, log-rank test). Nugent scores of 9-10 were less prevalent in the validation cohort participants with TLR5 deficiency (Figure 5.1E) but the difference was not statistically significant ($p = 0.59$).

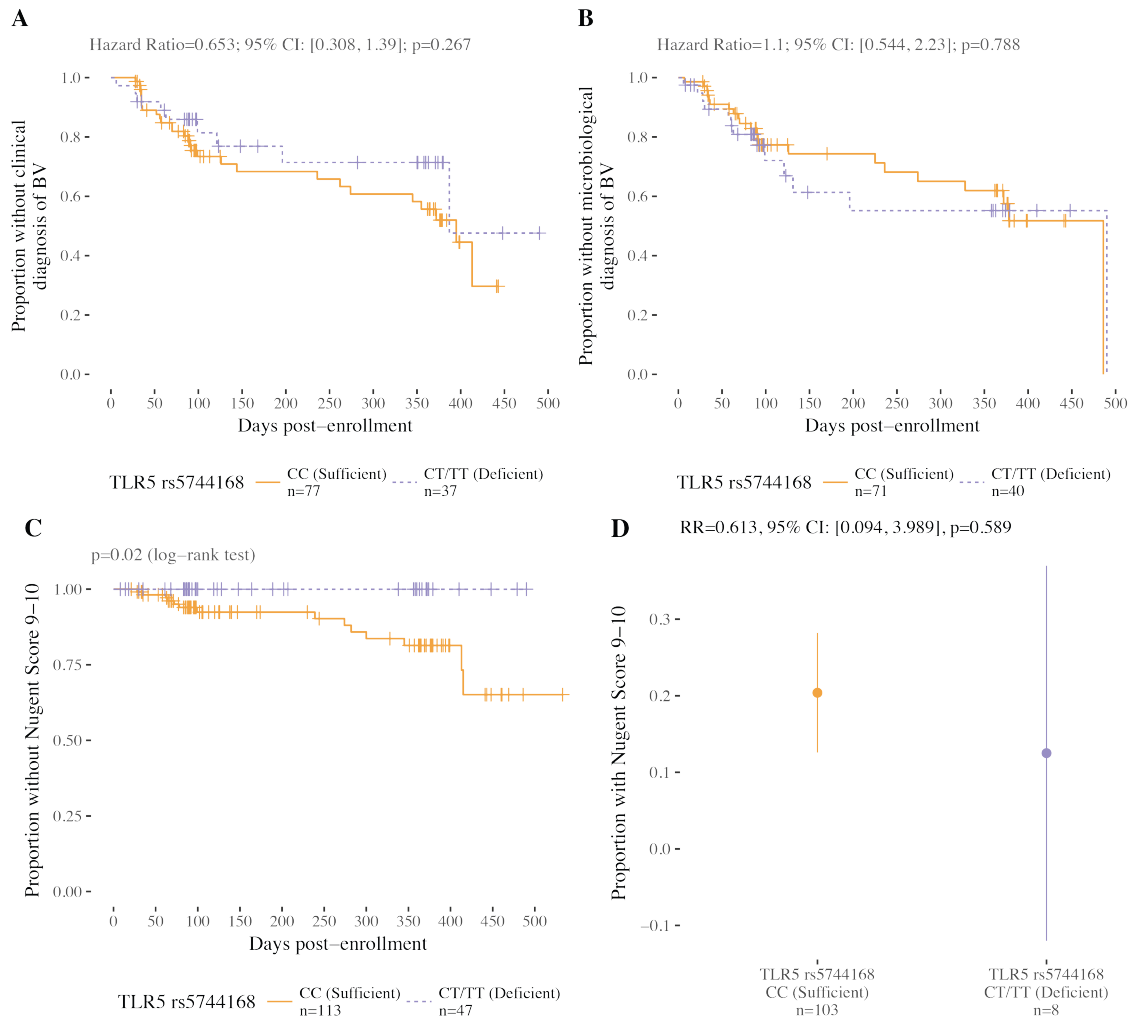


Figure 5.1. TLR5 deficiency is associated with increased risk of Nugent score 9-10 in a longitudinal, discovery cohort

(A)-(C) Survival curves and results of Cox proportional hazards regression comparing time to first clinic-visit associated episode of BV among TLR5 deficient (purple dashed line) versus TLR5 sufficient (orange solid line) women in the discovery cohort (A) TLR5 deficient and sufficient women are at similar risk of clinically-defined BV (Amsel's). (B) TLR5 deficient women are at similar risk of microbiologically-defined BV (Nugent) compared to TLR5 sufficient women. (C) Survival curve depicting proportion without clinic-associated Nugent score of 9-10. Of 47 TLR5 deficient women, none were observed to have Nugent scores of 9-10 at a follow-up clinic visit, compared to 14 of 113 TLR5 sufficient women. (D) TLR5 deficient women in the validation cohort show no difference in prevalence of Nugent scores of 9-10.

5.3.3 *TLR5 deficiency is associated with decreased colonization with BVAB1 and M. mulieris, but not M. curtisii*

While our original hypothesis posited that concentrations of BVAB1 and *Mobiluncus* spp. are increased in TLR5 deficient women, the lower incidence and prevalence of *Mobiluncus* morphotypes by Gram stain suggested the opposite. To probe this further, BVAB1 colonization was measured using quantitative PCR (qPCR) in the discovery cohort and assessed in matched TLR5 deficient cases and TLR5 sufficient controls (Appendix B). Surprisingly, we found that BVAB1 concentrations were indeed significantly *lower* in women with TLR5 deficiency (Figure 5.2A-B). This correlation was replicated in the validation cohort (Figure 5.2C).

In matched TLR5 deficient cases and TLR5 sufficient controls in the validation cohort (Appendix C), TLR5 deficient women had significantly lower concentration of *M. mulieris* (Figure 5.2D) and no significant differences in *M. curtisii* (Figure 5.2E). Concentrations of *Mobiluncus* spp. were not assessed in the discovery cohort.

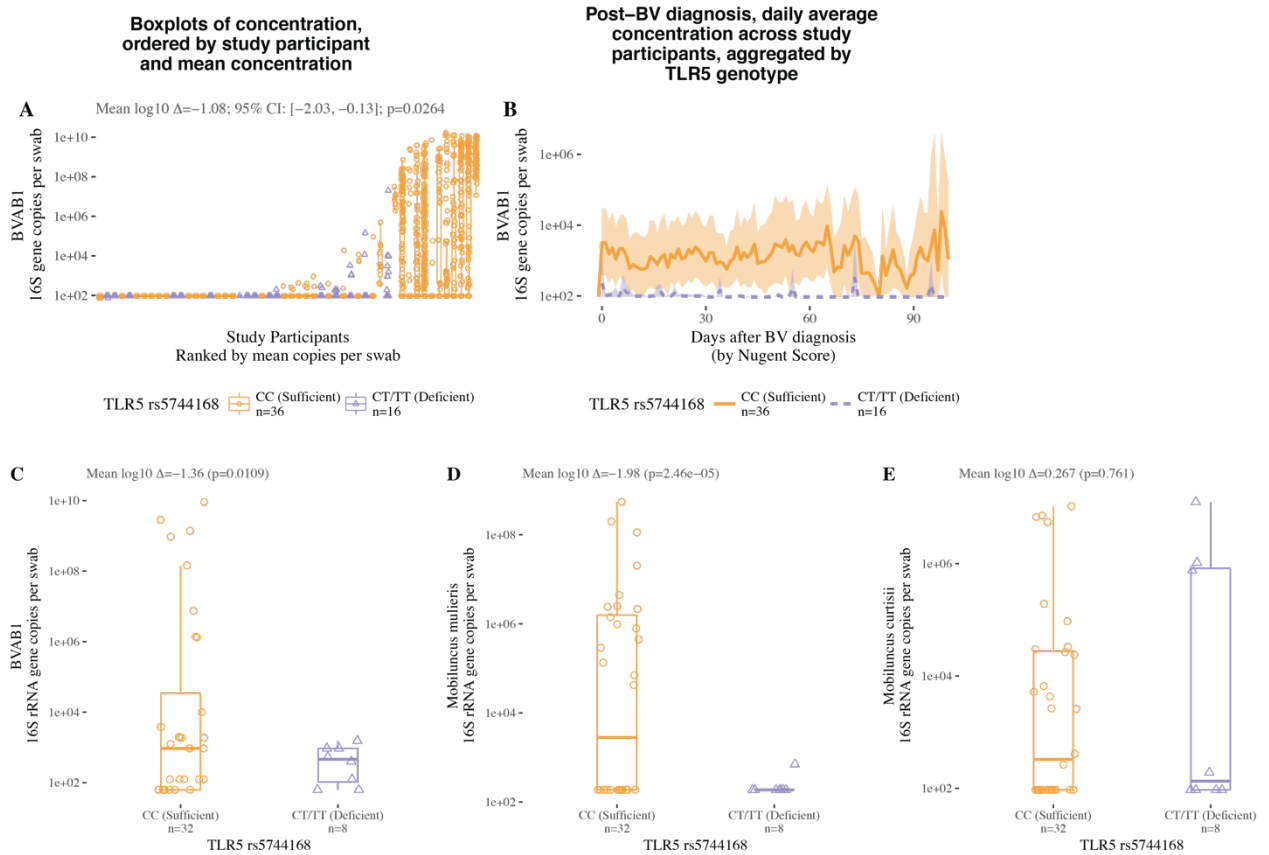


Figure 5.2. TLR5 deficiency is associated with lower colonization with BVAB1, *M. mulieris* (A) Discovery cohort colonization with BVAB1 in TLR5 deficient cases and matched TLR5 sufficient controls, divided into separate boxplots with overlaid scatterplots for each study participant to show within-subject variability. Women with TLR5 deficiency had 1.08- \log_{10} fewer BVAB1 16S rRNA gene copies per swab in comparison to matched controls (mean, $p = 0.02$). (B) Discovery cohort colonization with BVAB1 in TLR5 deficient cases and matched TLR5 sufficient controls, depicted as a daily, moving average aggregated by TLR5 deficiency versus sufficiency (shading depicts 95% bootstrapped confidence interval). At most time points after BV diagnosis, the confidence intervals of mean colonization with BVAB1 in TLR5 deficient and sufficient participants do not overlap. (C) In the validation cohort, TLR5 deficient women have an average of 1.36- \log_{10} fewer BVAB1 16S rRNA gene copies per swab in comparison to TLR5 sufficient women, and (D) 1.98- \log_{10} fewer *M. mulieris* 16S rRNA gene copies per swab. (E) In the validation cohort, TLR5 sufficient and deficient women had similar colonization with *M. curtisii*.

5.3.4 Predicting TLR5 agonism based on FlaA amino acid sequences

The data presented thus far suggest that TLR5 deficiency protects against colonization with BVAB1 and *M. mulieris* (but not *M. curtisii*), which led us to investigate the flagellin amino acid sequences (FlaA) encoded by these species. We gathered FlaA amino acid sequences, aligned them using MUSCLE, and assessed these alignments grossly for the appearance of conserved and variable domains. These sequences were compared to canonical TLR5 agonists¹²⁹ and sequences from flagellated bacteria that are known to escape TLR5 recognition¹¹⁷.

We first noted differences in the number of *flaA* genes encoded by each organism. BVAB1 possesses 5 distinct *flaA* genes, of which four have high amino acid sequence homology¹¹⁴. *M. curtisii* isolates consistently possessed two *flaA* genes each, while *M. mulieris* genomes contained between three (strain ATCC 35239) and six (strains FB024-16, 28-1) *flaA* genes. As depicted in Figure 5.3A, FlaA from *Mobiluncus* spp. were highly variable. Both *M. curtisii* and *M. mulieris* encode a potentially novel FlaA sequence with “disordered domain” attached to the highly conserved N-terminal. The hypervariable, D2 and D3 regions—which are bound by antibodies to the flagellar filament—varied in length as well.

We then analyzed a 10-amino acid sequence in the N-terminal conserved domain that is highly conserved across flagellated species and predicted to bind TLR5¹²⁹. Within this 10-amino acid sequence, we examined three key amino acids, shown by Smith et al.¹¹⁶ to be important for both TLR5 recognition and flagellar motility. Based on our epidemiological findings, we predicted that BVAB1 and *M. mulieris* would have flagellin amino acid sequences with key recognition and motility residues conserved, while *M. curtisii* would have at least one key amino acid substitution.

Consistent with our hypothesis, these three, key amino acids we examined were conserved in both isoforms of BVAB1 FlaA as well as all *M. mulieris* isolates (Figure 5.3B). Thus, we predicted that BVAB1 and *M. mulieris* would stimulate a TLR5 response. *M. curtisii* isolates consistently had a single Q>K substitution in the last amino acid of the TLR5 recognition sequence (Figure 5.3B). However, most species that escape TLR5 recognition possess substitutions in 2-3 out of 3 key residues; thus, we predicted incomplete abrogation of TLR5 agonism resulting from stimulation with *M. curtisii* FlaA.

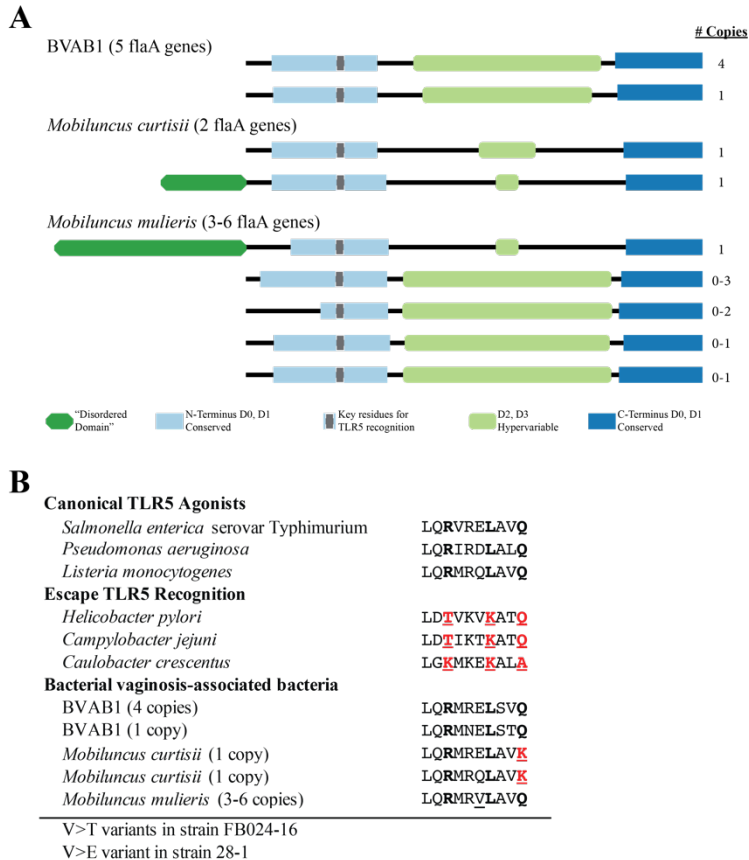


Figure 5.3. Alignment of FlaA amino acid sequences reveals diversity among BVAB (A) Schematic depicting alignments of FlaA amino acid sequences from BVAB1 (1 genome), *M. curtisii* (3), and *M. mulieris* (4). Hypervariable domains denoted by green; blue represents conserved regions. Width of box is proportional to number of amino acids in each domain. Grey box highlights location of ten amino acid sequence within N-terminal D0/D1 previously found to be highly conserved and necessary for TLR5 recognition. These alignments revealed a novel “disordered domain” attached to the N-terminal of *M. curtisii* (112aa) and *M. mulieris* (252aa) FlaA sequences. While the C-terminal D0 and D1 domains were relatively conserved across species, size of D2 and D3 hypervariable region ranged from 29aa to 273aa. N-terminal D0 and D1 domains ranged from 88aa to 150aa. (B) Detail of ten amino acid sequence recognized by TLR5, with key amino acid residues highlighted in bold. Underlined residues are departures from the sequence in canonical TLR5 agonists; residues in red are mutations found to be important for both TLR5 recognition and flagellar motility. FlaA from BVAB1 and *M. mulieris* have no mutations in key residues and are anticipated to agonize TLR5. Q>K mutation in 10th amino acid in *M. curtisii* may result in decreased TLR5 agonsim.

5.3.5 *Heat-inactivated M. mulieris stimulates a TLR5-mediated immune response, but heat-inactivated M. curtisii does not*

We tested our sequence-based predictions of TLR5 agonism using heat inactivated *M. curtisii* ATCC 35241 and *M. mulieris* UPII 28-I in co-culture with HEK Blue Null1 and HEK Blue hTLR5 reporter cells (Invivogen, San Diego, CA). Even though HEK cells were inoculated with the equivalent of approximately 10^6 CFU of *M. curtisii* (MOI = 79) or 10^3 CFU of *M. mulieris* (MOI < 1), we consistently found that *M. mulieris* produced a TLR5-specific NF- κ B inflammatory response, while *M. curtisii* did not. Results shown are the aggregate of three biological replicates, with these assays being performed on different days (Figure 5.4).

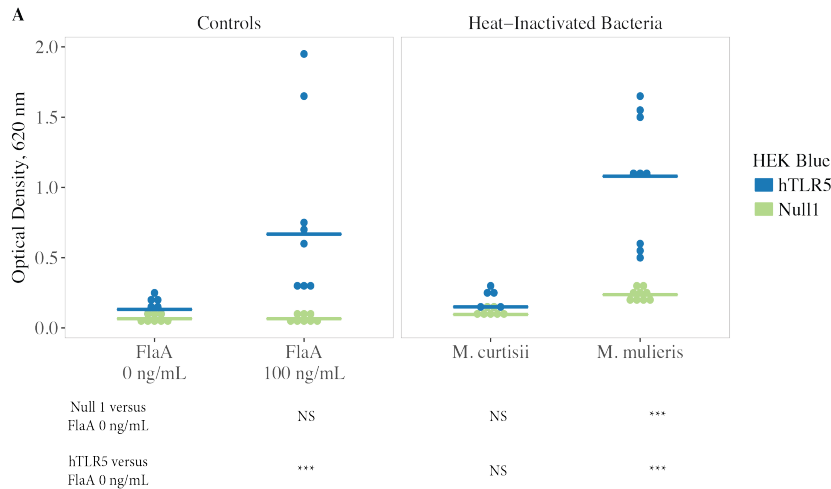


Figure 5.4. TLR5 responses associated with heat-inactivated *M. mulieris*, but not *M. curtisii* HEK293 cells transfected with a plasmid containing a NF- κ B-driven secretory, embryonic alkaline phosphatase (SEAP) reporter alone (HEK Blue Null1) or in addition to a plasmid encoding hTLR5 (HEK Blue hTLR5) were acquired from Invivogen (San Diego, CA). Heat-inactivated *M. mulieris* stimulates a TLR5-specific inflammatory response, while heat-inactivated *M. curtisii* does not stimulate TLR5. Positive control, flagellin from *Bacillus subtilis* (FlaA); negative control, PBS.

5.3.6 *Cervicovaginal lavage fluid from women highly colonized with BVAB1 stimulate a TLR5-dependent immune response*

Unlike *Mobiliuncus*, BVAB1 has not (to our knowledge) been grown in pure culture. Thus, we obtained cervicovaginal lavage (CVL) supernatant from women in the validation cohort colonized with varying degrees of BVAB1, *M. curtisii*, and *M. mulieris* colonization. HEK Blue Null1 and hTLR5 cells were incubated with CVL supernatant and examined for NF- κ B activation. Study participant C2D was highly colonized with *M. curtisii* without high quantities of *M. mulieris* or BVAB1 present and CVL from this participant did *not* stimulate a TLR5-modulated immune response (Figure 5.5). Study participants C2A, C2B, and C2C were highly colonized with BVAB1 and CVLs from these study participants stimulated a TLR5-specific NF- κ B inflammatory response (Figure 5.5). Results shown are the aggregate of three biological replicates, with assays done on different days.

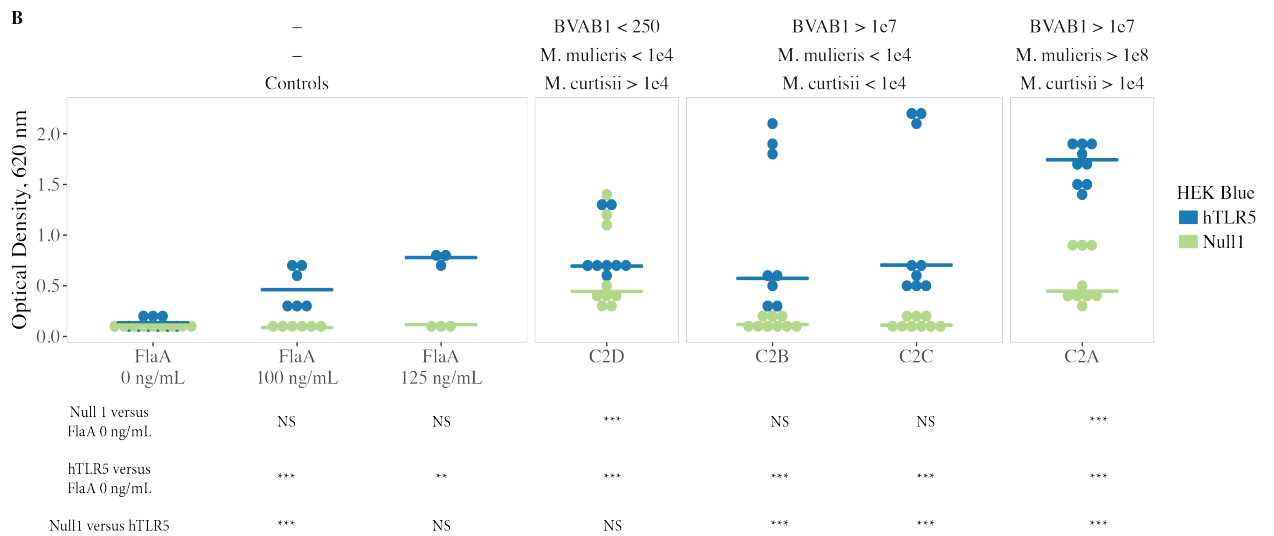


Figure 5.5. TLR5 responses associated with vaginal fluid from women colonized with BVAB1 HEK293 cells transfected with a plasmid containing a NF- κ B-driven secretory, embryonic alkaline phosphatase (SEAP) reporter alone (HEK Blue Null1) or in addition to a plasmid encoding hTLR5 (HEK Blue hTLR5) were acquired from Invivogen (San Diego, CA). Cervicovaginal lavage fluid (CVL) from women highly colonized with *M. curtisii* ($>10^4$ 16S rRNA gene copies per swab)--in the absence of colonization with BVAB1 (<250 16S rRNA gene copies per swab) and *M. mulieris* ($<10^4$ 16S rRNA gene copies per swab)--stimulates an inflammatory response through NF- κ B in both hTLR5 over-expressing and Null1 cell lines. CVL from women with high quantities of BVAB1 ($>10^7$ 16S rRNA gene copies per swab) and *M. mulieris* ($>10^8$ 16S rRNA gene copies per swab) stimulate inflammation through NF- κ B in cells that express hTLR5. CVL from women highly colonized with only BVAB1 ($>10^7$ 16S rRNA gene copies per swab) stimulated inflammation through NF- κ B in hTLR5 overexpressing cells, and to a lesser extent in Null1 cells.

In order to verify these results, we identified study participants who were highly colonized with BVAB1 from a third cohort. CVL from these participants was used to stimulate HEK reporter cells as describe above. Again, CVL from women highly colonized with BVAB1 and *M. mulieris*—with less than 10^5 *M. curtisii* 16S rRNA gene copies per swab—stimulated a TLR5-mediated NF-kB response. When greater than 10^5 *M. curtisii* 16S rRNA gene copies per swab was present, an NF-kB response was stimulated, but it was again not specifically mediated through TLR5 (Figure 5.6). Results shown are the aggregate of two biological replicates, performed on different days.

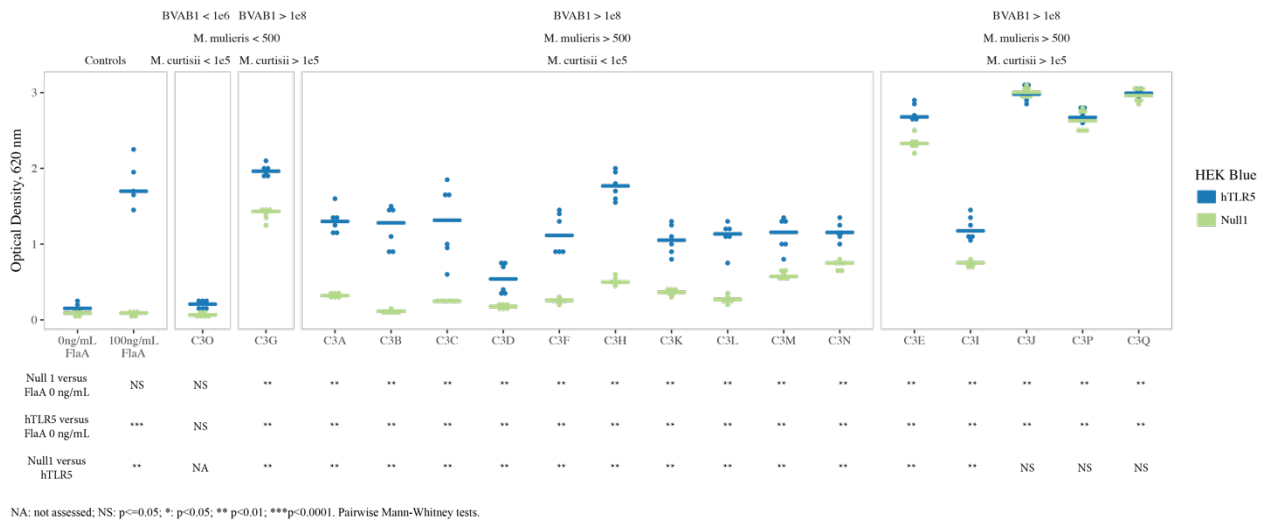


Figure 5.6. Vaginal fluid from Cohort 3 participants highly colonized with BVAB1 stimulate TLR5-dependent inflammatory responses
Cervicovaginal lavage fluid (CVL) from women highly colonized with BVAB1 ($> 10^8$ 16S rRNA gene copies per swab)—in the absence of substantial colonization with *M. curtisii*--stimulates a TLR5-mediated inflammatory response. In the presence of *M. curtisii*, a non-TLR5-specific response is stimulated.

5.3.7 Increased IL-8 in TLR5 deficiency

As a corollary to our HEK reporter cell assay results, we predicted increased pro-inflammatory cytokines in vaginal fluid of TLR5 sufficient women colonized with BVAB1 and *M. mulieris*. To test this prediction, we selected a subset of participants from the validation cohort (13 TLR5 sufficient, 2 TLR5 deficient) and assayed cervicovaginal lavage fluid for IL-8, IL-6, and IL-1 β . Of the participants selected, 8 of 13 TLR5 sufficient and 1 of 2 TLR5 deficient women were BV-positive by Nugent criteria; all reported Caucasian race, and none reported use of hormonal contraception.

Surprisingly, we found significantly *increased* IL-8 in vaginal fluid from TLR5 deficient women (Figure 5.7A, $p = 0.02$, Mann-Whitney), as well as trends towards increased IL-6 (Figure 5.7B, $p = 0.15$) and IL-1 β (Figure 5.7C, $p = 0.07$). We examined concentrations of BVAB1 (Figure 5.7D), *M. mulieris* (Figure 5.7E), and *M. curtisii* (Figure 5.7F) in relation to IL-8 concentration. Examining TLR5 sufficient participants only, we used linear regression to estimate the effect of a one- \log_{10} increase in flagellated BVAB concentration (16S rRNA gene copies per swab) on \log_{10} IL-8 concentration. We found non-statistically significant, negative correlations between both BVAB1 and *M. mulieris* and IL-8; *M. curtisii* was not correlated with IL-8 concentration. Together, these data suggest that BVAB1 and *M. mulieris* elicit a TLR5 response but may attenuate host inflammation *in vivo*.

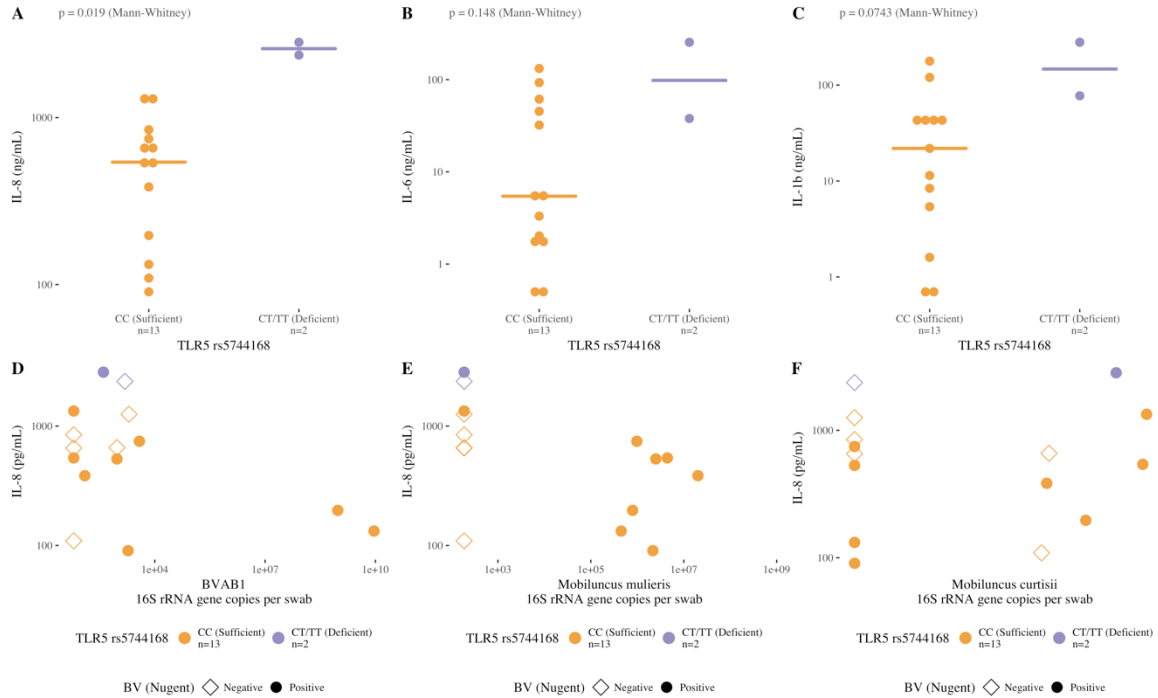


Figure 5.7. Trend towards increased concentrations of vaginal fluid inflammatory cytokines in TLR5 deficiency

(A)-(C) A subset of validation cohort cervicovaginal lavage supernatants were assayed by ELISA for IL-8 (A), IL-6 (B), and IL-1b (C) concentration (shown on y-axis on log₁₀ scale). (A) In the validation cohort, TLR5 deficient women demonstrate a significant increase in IL-8 concentration compared to TLR5 sufficient participants ($p = 0.02$, Mann-Whitney). (B) TLR5 deficient participants demonstrate a trend towards increased concentrations of IL-6 ($p = 0.15$, Mann-Whitney) and (C) IL-1b ($p = 0.07$, Mann-Whitney). (D)-(F) Relationship between vaginal concentration of flagellated BVAB (x-axis, log₁₀ scale) and vaginal IL-8 concentration (y-axis, log₁₀ scale). Solid circles represent data from a woman with BV; hollow diamonds represent data from women without BV (by Nugent score). Linear regression used to estimate effect of one-log₁₀ increase in flagellated BVAB concentration on log₁₀ IL-8 concentration in TLR5 sufficient participants only. Observations from TLR5 deficient participants plotted for reference. (D) BVAB1 concentration (16S rRNA copies per swab) is associated with a trend towards lower IL-8 ($-0.067 \log_{10} \text{IL-8 pg/mL}$, 95% CI: $[-0.14, 0.01]$, $p = 0.11$). (E) *M. mulieris* concentration (16S rRNA copies per swab) is associated with a trend towards lower IL-8 ($-0.073 \log_{10} \text{IL-8 pg/mL}$, 95% CI: $[-0.17, 0.03]$, $p = 0.18$). (F) *M. curtisii* is not associated with IL-8 concentration ($0.07 \log_{10} \text{IL-8 pg/mL}$, 95% CI: $[-0.95, 1.08]$, $p = 0.9$).

5.4 DISCUSSION

Although it has been over three decades since flagellated bacteria were observed in vaginal fluid from women with BV^{111,115,110}, the role of innate immune recognition of flagellin by TLR5 has never been explored in this syndrome. Here, we demonstrated an epidemiological association between genetically-encoded TLR5 deficiency and *decreased* risk of Nugent scores of 9-10 (associated with the presence of *Mobiluncus* morphotypes on Gram stain)¹⁶. Although we did not replicate this result in our validation cohort, our validation cohort had limited sample size with only eight TLR5 deficient participants. Exploring how TLR5 deficiency affects colonization, we found *lower* colonization with BVAB1 and *M. mulieris* in discovery cohort women with TLR5 deficiency, without any differences in *M. curtisii* colonization detected. We explored the mechanisms underlying this surprising relationship and found that while FlaA proteins encoded by BVAB1 appear very similar to other TLR5 agonists, FlaA proteins encoded by *Mobiluncus* spp. are highly variable, including a FlaA predicted to have a large, N-terminal “disordered domain” not found on canonical TLR5 agonists. We demonstrated robust TLR5 agonism resulting from stimulation with heat-inactivated *M. mulieris*, but not *M. curtisii*. Because BVAB1 cannot be grown in pure culture, we used CVL supernatants from women colonized with varying concentrations of BVAB1 and found that CVL from women highly colonized with BVAB1 stimulates a TLR5-dependent NF- κ B inflammatory response. Lastly, we explored IL-8 concentration in TLR5 sufficient women colonized with TLR5 agonizing species (BVAB1, *M. mulieris*) and surprisingly found a trend towards *lower* IL-8 concentration associated with the presence of these TLR5 agonists. However, our examination of IL-8 was limited by sample size; future studies could examine the relationship between BVAB1, *Mobiluncus* spp. and IL-8 concentrations in larger cohorts.

These data are consistent with a model whereby *M. mulieris* and BVAB1 require a host, inflammation-associated product of epithelial cell TLR5 agonism in order to robustly colonize. We predict that *M. mulieris* and BVAB1 stimulate TLR5, provoking a TLR5-mediated, NF- κ B-dependent pro-inflammatory response, which creates a niche for these species to occupy. This inflammatory response may decrease availability of host nutrients used by vaginal lactobacilli (e.g. glycogen or metal ions, such as Mn^{2+} , Mg^{2+}) or cause the direct elimination of lactobacilli (via release of host antimicrobial proteins, for example). *Mobiluncus* spp. have been localized in close association with host epithelial cells by electron microscopy¹¹⁰, suggesting that these flagellated bacteria are able to attach to host cells. Innate immune response to flagellin could facilitate the creation of binding sites used by BVAB1 or *M. mulieris*--this could occur in the form of upregulation or post-translational modifications to host extracellular proteins. Alternatively, in light of the reproductive imperative to prevent infection of the uterus, TLR5 agonism could lead to changes in mucin production in the cervix to reinforce a mechanical barrier to invasion; mucin could be nutrient source for these bacteria.

While TLR5-mediated immune response to flagellated bacteria appears to support robust vaginal colonization with some species, it is unknown whether this response results in protection from ascending infection with these bacteria. This question could be examined in cohort studies of pelvic inflammatory disease or preterm birth. In addition, future studies could take a multi-omics approach to examine how flagellin stimulation of lower genital tract mucosa changes gene expression, as well as the proteome, glycome, and metabolome. Lastly, innate immune response to flagellated organisms is mediated by TLR5, which recognizes extracellular flagellin, and NLRC4, which recognizes cytosolic flagellin¹³⁰. Here, we focus on TLR5, as NLRC4 does not have any well-characterized, common SNPs. As data on NLRC4 genetic variation emerges,

future studies could examine whether NLRC4 plays a role in vaginal colonization with flagellated organisms.

Curved, motile, Gram-negative rods were described in the discharge of a patient with postpartum endometritis over a century ago¹³¹ and more recently, our group identified colonization with BVAB1 to be a risk factor for spontaneous pre-term birth¹⁰, suggesting that these flagellated organisms found in vagina are able to ascend to the uterus. We hypothesize that innate immune detection of flagellated organisms in the lower genital tract developed as an evolutionarily-driven defense against ascending infection—especially during pregnancy when these bacteria may be associated with preterm labor. Indeed, the robustness of this response has led to the proposed use of vaginally-delivered, purified flagellin as an adjuvant for therapeutic vaccine delivery¹³². The vaginal niche is frequently exposed to gut microbes, many of which possess flagellin genes. Although we find preterm labor associated with the flagellated BVAB we discuss here, intestinal bacteria are rarely implicated in preterm labor¹³³, perhaps indicating that this heightened defense against flagellated organisms in the vagina may have some adaptive advantages despite the potentially significant drawbacks we have uncovered in the analysis we present here.

The “disordered domain” present at the N-terminal of some copies of *M. mulieris* and *M. curtisii* flagellin genes may constitute a novel mechanism for escaping TLR5 recognition, shielding the conserved N- and C-terminal regions from innate immune recognition by NLRC4 and TLR5. The *flaA* genes containing this “disordered domain” may be differentially expressed depending on stage of colonization. For example, because this bulky “disordered domain” may interfere with stacking of flagellin monomers (and thus, would inhibit motility), this particular *flaA* gene may only be expressed after the bacteria has attached to a host cell. Future studies

could examine sigma factors associated with upregulation of these novel *flaA* genes to elucidate under which growth contexts these genes are expressed.

Our data suggest that the condition defined clinically and microbiologically as BV is not caused by the presence of *flaA* in BVAB1 or *M. mulieris* and its interaction with TLR5. However, vaginal colonization with BVAB1 and *M. mulieris* may be dependent on signaling of TLR5 through a functional ligand of FlaA.

Because TLR5 deficiency appears to protect against colonization with specific BVAB but does not prevent the appearance of clinically- or microbiologically-defined BV, it is unlikely that treatment with TLR5 antagonists would be an effective preventive measure against BV, possibly related to the heterogeneity in the microbiology of BV. However, it is possible that TLR5 antagonism could protect against BV caused by BVAB1 dominant communities. The data presented here suggest that BV involving BVAB1 and/or *M. mulieris* may have a distinct inflammatory phenotype differentiating it from BV not involving these species. These observations open new avenues for understanding how specific vaginal bacteria interact with the host to produce adverse health outcomes in women.

Chapter 6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1 UPDATED MODEL OF BV PATHOGENESIS

The findings described in this dissertation suggest that innate immune responses may promote clinical signs of BV and colonization with BVAB (results summarized in Table 6.1). The clearest examples of this are described in Chapter 3, in which I find that deficient negative regulation of innate immune response by TLR10 results in increased risk of clinically-defined BV, and in Chapter 5, where I find TLR5 deficiency correlated with lower colonization with flagellated BVAB (*M. mulieris* and BVAB1). In Chapter 4, I posit a pleiotropic role of TLR4 in the lower genital tract--pro-inflammatory in its membrane-bound form and as a soluble mediator of LPS response. This model of TLR4 activity in the lower genital tract is based on the changes in bacterial colonization I observed in TLR4 deficient women, who were colonized with increased quantity of Gram-positive BVAB and significantly lower quantities of *L. jensenii*.

Table 6.1. Summary of findings

Deficiency in Toll-like Receptor/Negative Regulator:			
Toll-like Receptor/ Negative Regulator	Association with BV	Association with BVAB	Association with lactobacilli
<i>Chapter 2</i>			
TLR1	N.S.	N.S.	N.S.
TLR6	N.S.	N.S.	↑ <i>L. iners</i> ↓ <i>L. jensenii</i> (Discovery cohort only)
<i>Chapter 3</i>			
TLR10	↑ risk of clinically-defined BV (Confirmed in both cohorts)	N.S.	↓ <i>L. jensenii</i> (Discovery cohort) ↓ <i>L. gasseri</i> (trend) (Validation cohort)
TOLLIP	↑ risk of clinically-defined BV (Discovery cohort only)	↓ <i>Megasphaera</i> spp. ↓ <i>Atopobium vaginae</i> ↓ <i>Gardnerella vaginalis</i> (Discovery cohort only)	N.S.
<i>Chapter 4</i>			
TLR4	↑ risk of clinically-defined BV (Discovery cohort only)	↑ <i>Atopobium vaginae</i> ↑ <i>Gardnerella vaginalis</i> (Discovery cohort only)	↓ <i>L. jensenii</i> (Discovery cohort only)
<i>Chapter 5</i>			
TLR5	N.S.	↓ BVAB1 (Confirmed in both cohorts) ↓ <i>M. mulieris</i> (Assessed in validation cohort only)	N.A.

N.S. = No statistically significant association

N.A. = Not assessed

My analyses showed associations between TLR10, TOLLIP, and TLR4 deficiencies and increased risk of BV in the discovery cohort. The relationship between TLR10 and BV was also confirmed in the validation cohort. TLR10, TOLLIP, and TLR4 deficiencies were *not* correlated with risk of microbiologically-defined BV, despite high correlation between these clinical and microbiological definitions of BV. I hypothesize that hyper-inflammatory responses associated with TLR10, TOLLIP, and TLR4 deficiencies may predispose to some of the clinical signs of BV. In particular, inflammatory response may: decrease availability of nutrients needed by lactobacilli, or may result in direct antagonism against lactobacilli, which would raise vaginal fluid pH; influence mucin composition and the appearance of thin, homogeneous discharge associated with BV; or, result in upregulation or posttranslational modifications of extracellular proteins that are used as binding sites by BVAB, which may increase frequency of “clue cells.” Elements of this response could also aid in niche creation for BVAB, connecting increased risk of clinically-defined BV to increased colonization with specific species of BVAB.

We observed lower concentrations of commensal lactobacilli in women with TLR10, TLR4, and TLR6 deficiency. In particular, we observed significantly lower *L. jensenii* colonization in the discovery cohort when these SNPs were present, suggesting that *L. jensenii* may be acutely sensitive to the presence of inflammation. While both *L. crispatus* and *L. jensenii* are associated with the absence of BV, only *L. jensenii* concentration is statistically significantly lower in the presence of TLR10, TLR4, and TLR6 deficiency. Our group has previously demonstrated a negative correlation between *L. jensenii* colonization and cervicitis⁹. The results presented here suggest that *L. jensenii* may be poorly adapted to colonize in the presence of vaginal or cervical inflammation. Furthermore, our findings suggest that Gram-positive cell products may act as TLR6 or TLR10 ligands, consistent with previous findings¹³⁴. An

inflammation-mediated decrease in *L. jensenii* colonization would also be consistent with an innate immune response being beneficial to BVAB niche creation.

Based on the results presented in this dissertation, I propose the following, updated model of BV pathogenesis (schematized in Figure 6.1): (A) BV-associated bacteria (BVAB) present pathogen-associated molecular patterns (PAMPs) to (B) Toll-like receptors (TLRs) located on the surface of host cells. TLRs mediate the host pro-inflammatory response (C). Downstream effects of this pro-inflammatory response aid niche creation for BVAB (D), which results in higher concentrations of BVAB and lower *L. jensenii*, *L. crispatus*, and *L. gasseri* concentrations (E).

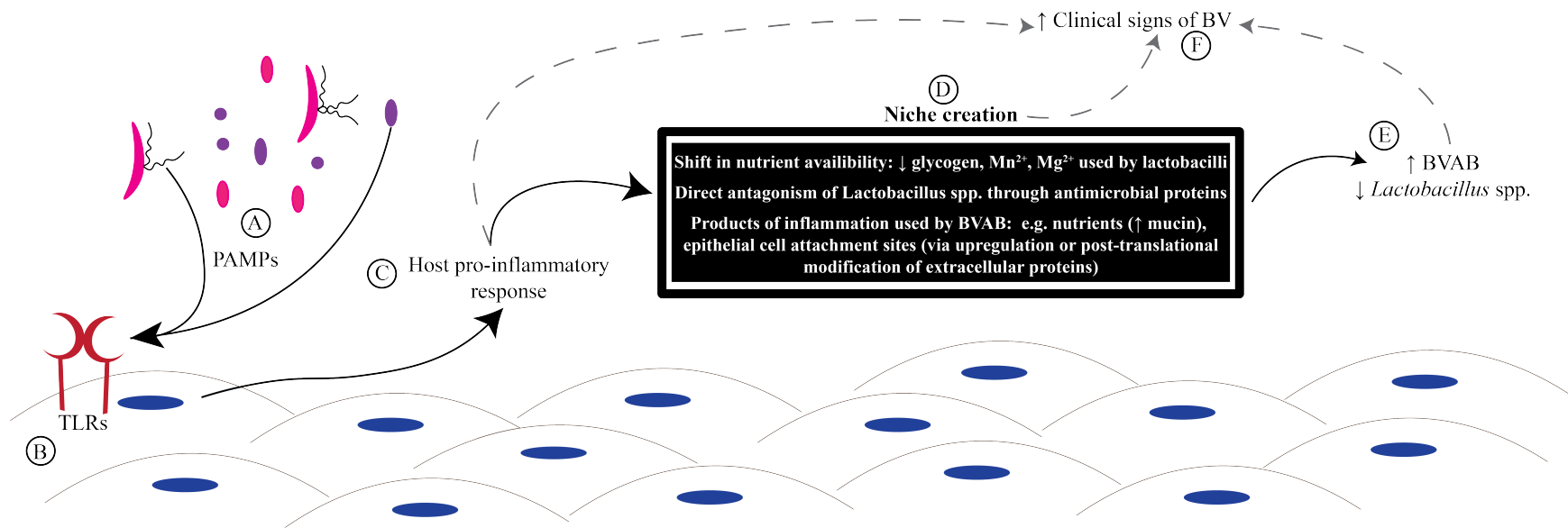


Figure 6.1. Updated model of BV pathogenesis

TOLLIP deficiency was correlated with both *increased* risk of BV and *lower* concentrations of *Megasphaera* spp., *A. vaginae*, and *G. vaginalis*. These results seem to suggest that clinical signs and symptoms can be present in women predisposed to hyperinflammation with or without the full complement of bacteria that would yield a Nugent score concordant with BV. Such a vaginal bacterial community may be lacking some of the microbes that are more specific to BV (like *Megasphaera* or *Mobiluncus* spp.). Because these were results from a small number of TOLLIP deficient cases and TOLLIP sufficient controls, I have depicted this possible connection in Figure 6.1 as dashed, grey lines linking “host pro-inflammatory response” and “niche creation” directly to clinical signs of BV (F).

6.2 RELATIONSHIP TO OTHER STUDIES OF THE VAGINAL MICROBIOTA

Our data are consistent with a model of BV pathogenesis requiring not only exposure to BVAB *but also* a host mucosal milieu that is susceptible to colonization and overgrowth with BVAB. This model provides a unified framework for the pathogenesis of clinically-defined BV. Known risk factors such as sexual intercourse without barrier protection, an increased number of sex acts, and number of lifetime sexual partners increase the likelihood of inoculation. Other risk factors for BV may influence immune response to vaginal microbes. For example, a recent study by Si et al.¹³⁵ demonstrated heritability of the vaginal microbiota and a link between obesity and risk of BV in a cohort of twins. Obesity is associated with chronic inflammation¹³⁶, which may influence vaginal mucosal response to BVABs, increasing susceptibility to BV. Other risk factors, such as smoking¹, vitamin D deficiency³⁵, chronic stress¹³⁷, and coinfection with HSV¹³⁸ may also have an inflammation-mediated impact on the vaginal microbiota and clinical signs of BV.

6.3 STRENGTHS AND LIMITATIONS

The hypothesis-driven approach taken in this dissertation focuses on a select number of functionally characterized SNPs in candidate genes that I believe have high likelihood of impacting the innate immune response to vaginal bacteria, and I test these hypotheses in two independent cohorts. Although not all findings from the discovery cohort are confirmed in the validation cohort, the main findings we present here are based on the longitudinal, discovery cohort, which has more statistical power and incorporates data on an individual's propensity toward BV and BVAB colonization over time. Furthermore, I was able to detect differences in the absolute concentrations of vaginal bacteria using species-specific quantitative PCR, which may be more informative than relative abundance data generated in most microbiome studies. To my knowledge, this dissertation comprises the most comprehensive study of the relationship between Toll-like receptor genetic variation and vaginal colonization with individual species of bacteria undertaken to date.

Because TLR10 has no known ligand, I was unable to follow up these results with further mechanistic studies. Because TLR4 and TLR5 are very well characterized, relationships between these TLRs, BVAB colonization, and BV could be explored to shed light on underlying mechanisms. In the case of TLR4, I demonstrated the presence of soluble TLR4 in vaginal and cervical fluids. The role of sTLR4, a soluble mediator of LPS response, has not been explored in other studies of innate immunity in the lower female genital tract. The data shown in Chapter 4 suggest that sTLR4 works in conjunction with mTLR4 and CD14 to mediate the inflammatory response to Gram-negative organisms. To explore the surprising relationship between TLR5 deficiency and lower colonization with BVAB1 and *M. mulieris*, I explored FlaA sequences encoded by flagellated BVAB, tested samples containing FlaA from these organisms for TLR5

agonism, and demonstrated a negative correlation between BVAB1 and *M. mulieris* concentration and vaginal IL-8 concentration.

There are several limitations to our study. First, neither animal nor *in vitro* systems sufficiently recapitulate the human urogenital microbiota and immune response. Thus, we rely on data generated from observational studies of human subjects and are unable to demonstrate causality. Although we observed statistically significant relationships between some TLR/TOLLIP genotypes conferring deficiencies and clinically-defined BV, as well as shifts in vaginal bacterial colonization, for many of these SNPs there was less than 100% penetrance. In addition to the innate immune components I have explored in this thesis, there are myriad genes involved in regulating innate and adaptive immune response to vaginal bacteria. Future studies could take a hypothesis-generating approach to discover other genes, and networks of genes, that could affect susceptibility to BV and drive colonization with vaginal bacteria. Furthermore, offsetting mutations to the SNPs examined here may be responsible for some variability in our results. Finally, epigenetically-driven “trained immunity”¹³⁹ may promote more robust responses to BVAB in women who have previously been exposed, potentially contributing to high recurrence rates among women with BV. Because many of these SNPs are relatively rare, examining the effect of various SNPs working in tandem would likely require the enrollment of very large cohorts to detect synergistic and compensating effects of various mutations.

6.4 QUESTIONS REMAINING AND FUTURE DIRECTIONS

Our data do not explore the relationship of these SNPs to inflammatory markers in the vagina. In the absence of an animal or *in vitro* model that captures the host-microbe interactions occurring in the lower female genital tract, clinical studies could examine kinetics of chemokine and cytokine concentrations in vaginal fluid after a confirmed inoculation event (e.g. intercourse

with partner who is colonized with BVAB). Based on the data explored in this dissertation, I predict women with hyperresponsive innate immune genotypes (TLR10 deficiency, TOLLIP deficiency, TLR4 deficiency, and TLR5 sufficiency) would have: higher chemokine/cytokine concentrations following the inoculation event, (2) higher levels of BVAB colonization, and (3) higher risk of clinically-defined BV after inoculation event.

TOLLIP deficiency was associated with *increased* risk of clinically-defined BV in the discovery cohort and with *lower* colonization with *Megasphaera* spp., *A. vaginae*, and *G. vaginalis*. This result raises the possibility that TOLLIP-moderated TLR-responses (primarily TLRs 1, 2, 4, 5, and 6) are helpful to clear some BVAB, but also results in the increased appearance of clinical signs of BV. Future studies could examine these genes, and could account for interactions between innate immune genetic variants. Such an undertaking would require the enrollment of cohorts of substantial size, as some of these genetic variants are relatively rare.

This work highlights the important interplay of host immunity and microbial colonization in shaping risk of disease and clinical manifestations. Innate immune genetic variation may influence the risk of BV-related sequelae, both independently and synergistically with microbes. The role of innate immune genetic variation in regulating the pro-inflammatory phenotype of endocervical leukocytes could be examined in the context of cervicitis, pre-term labor, and pelvic inflammatory disease. An improved mechanistic understanding of innate immune genetic variation in the female reproductive tract could potentially open new avenues of therapy for the treatment and prevention of BV, as well as other obstetric and gynecological conditions.

These results suggest that assessment of women's susceptibility or resistance to inflammation may be crucial in the prevention and treatment of vaginal dysbiosis. Personalized medicine approaches with the goal of sustained cure and reduction of BV-related sequelae could

be explored in future studies. For example, patients could be assessed for baseline risk factors, *including* their genetic susceptibility to inflammation, exploring both periodic, presumptive treatment for BV and modulation of inflammation to reduce risk of BV and negative obstetric and gynecologic sequelae.

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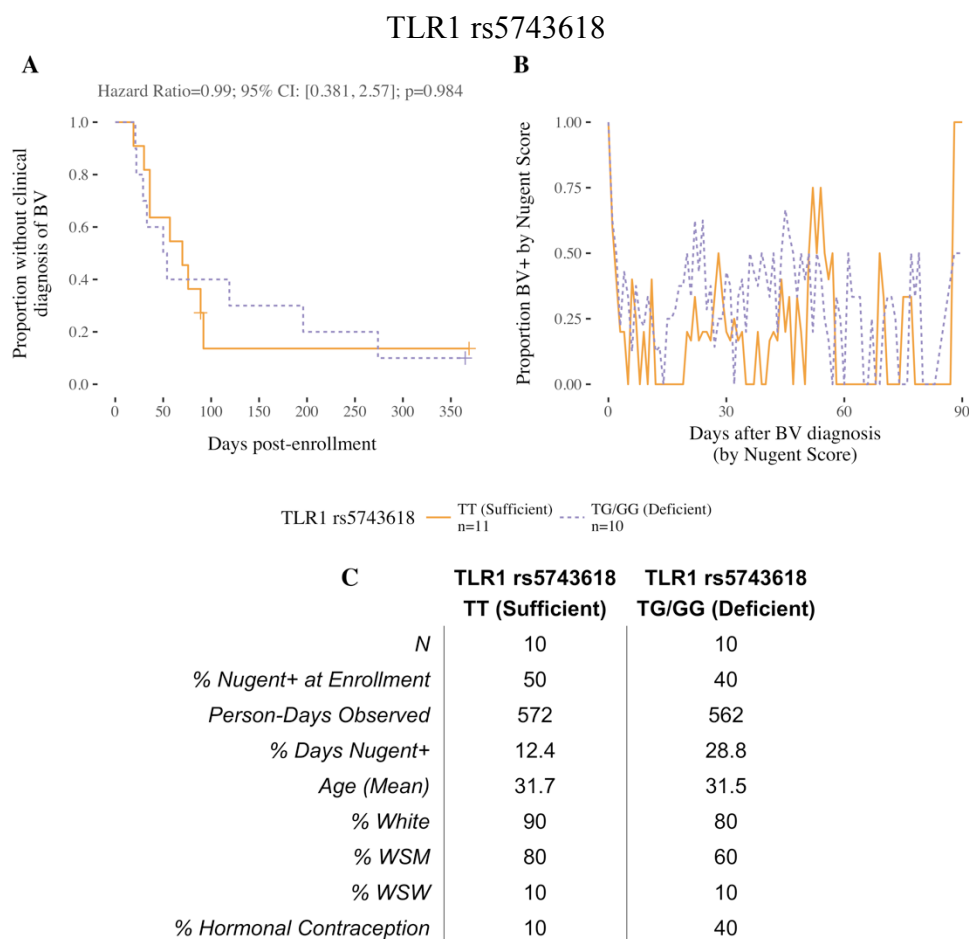
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APPENDIX A: MINOR ALLELE FREQUENCIES OF SINGLE NUCLEOTIDE POLYMORPHISMS EXAMINED IN THIS STUDY

Gene	SNP RS Number	NT Change	AA Change	Sufficient Genotype	Deficient Genotype	Citation	Minor Allele Frequency (MAF)			Thermo Fisher Cat No. 4351379 Assay ID
							1000 Genomes	Discovery Cohort	Validation Cohort	
<i>Chapter 2</i>										
TLR1	rs5743618	T>G	I>S	TT/TG	GG	Hawn et al. 2007 ⁶⁸	0.199	0.487	0.486	NA
TLR6	rs3821985	G>C	Synonymous	GG/GC	CC	Shey et al. 2010 ⁶⁹	0.445	0.412	0.431	C_25615369_10
<i>Chapter 3</i>										
TLR10	rs11096955 [†]	T>G	I>L	TT	TG/GG	Oosting et al. 2014 ⁷⁴ , Stappers et al. 2015 ⁷²	0.500	0.445	0.413	C___309086_10
TLR10	rs11096957 [†]	T>G	N>H	TT	TG/GG	Oosting et al. 2014 ⁷⁴ , Stappers et al. 2015 ⁷²	0.500	0.445	0.413	C___309088_10
TLR10	rs4129009	T>C	I>L	TT	TC/CC	Oosting et al. 2014 ⁷⁴ , Stappers et al. 2015 ⁷²	0.147	0.140	0.124	C___309084_10
TOLLIP	rs5743854	C>G	Synonymous	CC/CG	GG	Shah et al. 2017 ⁷⁹	0.261	0.153	0.220	C_11281491_20
<i>Chapter 4</i>										
TLR4	rs4986790	A>G	D>G	AA	AG/GG	Arbour et al. 2000 ¹⁰⁰	0.060	0.159	0.049	C_11722238_20
<i>Chapter 5</i>										
TLR5	rs5744168	C>T	R>STOP	CC	CT/TT	Hawn et al. 2003 ¹²⁰	0.050	0.145	0.036	C_25608804_10

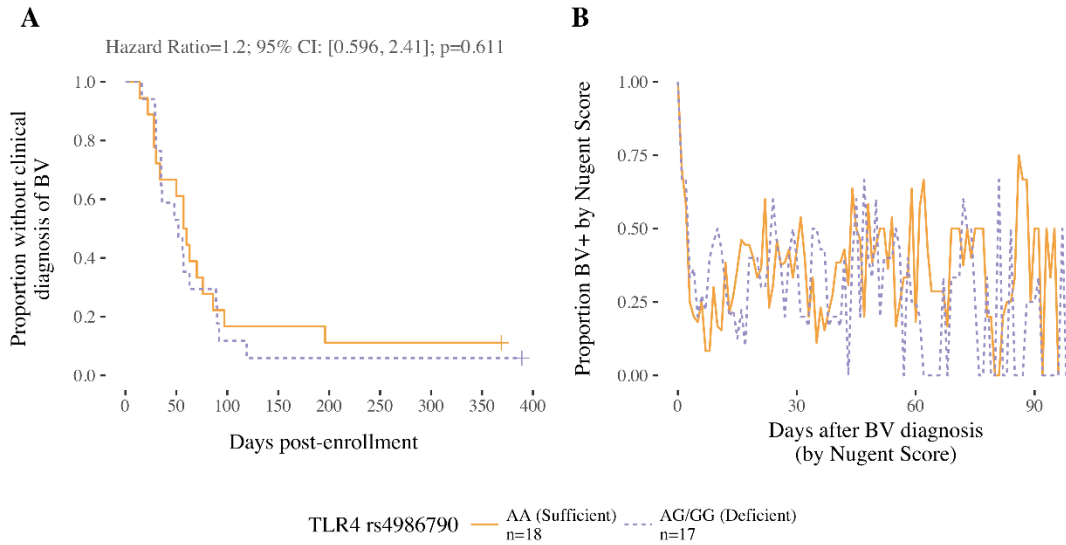
[†]These SNPs are in complete linkage disequilibrium

APPENDIX B: ASSESSING THE ADEQUACY OF MATCHING TLR/TOLLIP DEFICIENT CASES AND SUFFICIENT CONTROLS IN THE DISCOVERY COHORT



(A) Survival curves depicting time to first diagnosis with BV by Amsel’s criteria, stratified by TLR1 rs5743618 genotype. There are no significant differences in time to first BV diagnosis between TLR1 sufficient controls and TLR1 deficient controls. (B) Daily average proportion of women who are BV-positive by Nugent score, stratified by TLR1 rs5743618 genotype, starting at the time of first Nugent diagnosis of BV, and following up to 100 days post-diagnosis. (C) Demographic characteristics of TLR1 sufficient and deficient women from longitudinal cohort included in analysis of bacterial colonization. TLR1 deficient women are slightly less likely to have BV at enrollment, and are less likely to report white race, having male partners, and are more likely to report hormonal contraception.

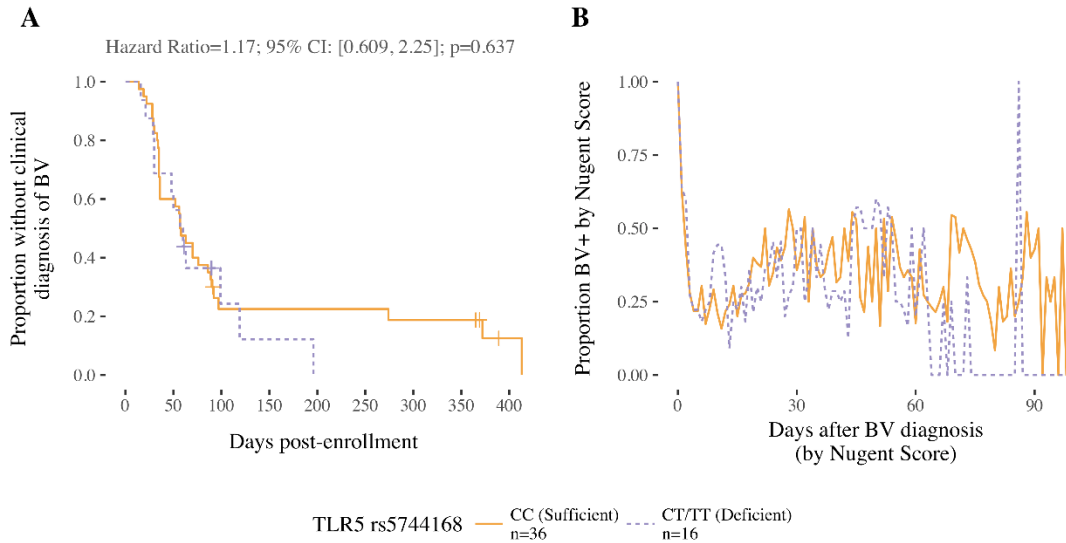
TLR4 rs4986790



	TLR4 rs4986790	
	AA (Sufficient) n=18	AG/GG (Deficient) n=17
<i>N</i>	18	17
<i>% Nugent+ at Enrollment</i>	83.3	88.2
<i>Person-Days Observed</i>	1270	890
<i>% Days Nugent+</i>	25.2	20.6
<i>Age (Mean)</i>	29.9	29.8
<i>% White</i>	44.4	47.1
<i>% WSM</i>	50	70.6
<i>% WSW</i>	22.2	23.5
<i>% Hormonal Contraception</i>	27.8	23.5

(A) Survival curves depicting time to first diagnosis with BV by Amsel’s criteria, stratified by TLR4 rs4986790 genotype. There are no significant differences in time to first BV diagnosis between TLR4 sufficient controls and TLR4 deficient controls. (B) Daily average proportion of women who are BV-positive by Nugent score, stratified by TLR4 rs4986790 genotype, starting at the time of first Nugent diagnosis of BV, and following up to 100 days post-diagnosis. (C) Demographic characteristics of TLR4 sufficient and deficient women from longitudinal cohort included in analysis of bacterial colonization. Groups appear balanced for major risks of BV diagnosis, including BV diagnosis at enrollment, age, race, and sexual behaviors (women who have sex with men [WSM], women who have sex with women [WSW]), and hormonal contraceptive status.

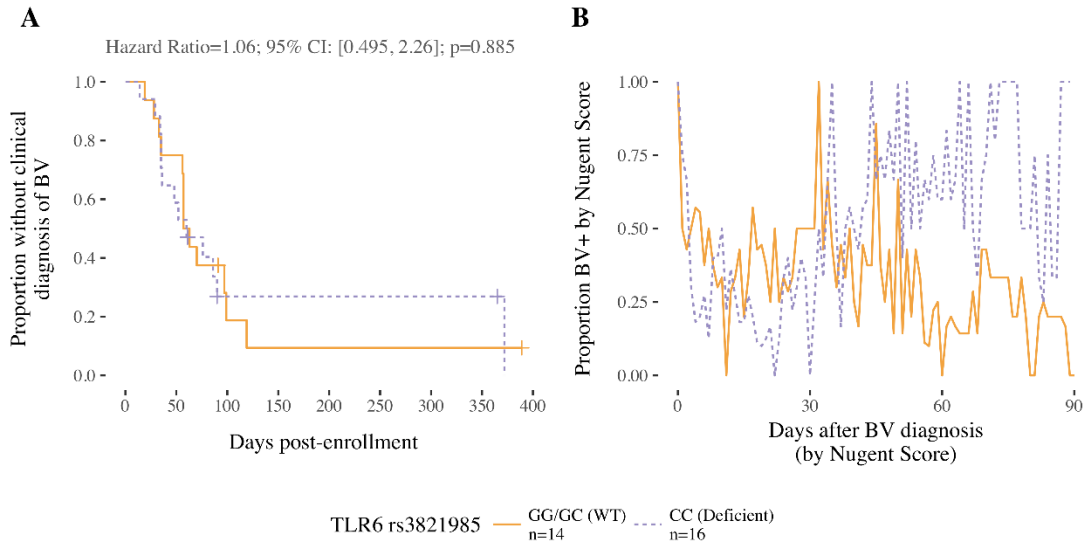
TLR5 rs5744168



	TLR5 rs5744168	
	CC (Sufficient) n=36	CT/TT (Deficient) n=16
<i>N</i>	36	16
<i>% Nugent+ at Enrollment</i>	66.7	56.2
<i>Person-Days Observed</i>	2650	929
<i>% Days Nugent+</i>	22.1	22.1
<i>Age (Mean)</i>	31.6	30.4
<i>% White</i>	36.1	56.2
<i>% WSM</i>	52.8	62.5
<i>% WSW</i>	16.7	25
<i>% Hormonal Contraception</i>	36.1	37.5

TLR5 deficient cases and TLR5 deficient controls were matched on Nugent diagnosis at enrollment, age, race, sexual preference, and hormonal contraception use in a 2:1 ratio. (A) Survival curves depicting time to first diagnosis with BV by Amsel’s criteria, stratified by TLR5 deficiency. There are no significant differences in time to first BV diagnosis between TLR5 sufficient controls and TLR5 deficient cases. (B) Daily average proportion of women who are BV-positive by Nugent score, stratified by TLR5 genotype, starting at the time of first Nugent diagnosis of BV, and following up to 100 days post-diagnosis. (C) Demographic characteristics of TLR5 sufficient and deficient women from longitudinal cohort included in analysis of bacterial colonization. Groups appear balanced for major risks of BV diagnosis, including BV diagnosis at enrollment, age, race, and sexual behaviors (women who have sex with men [WSM], women who have sex with women [WSW]), and hormonal contraceptive status.

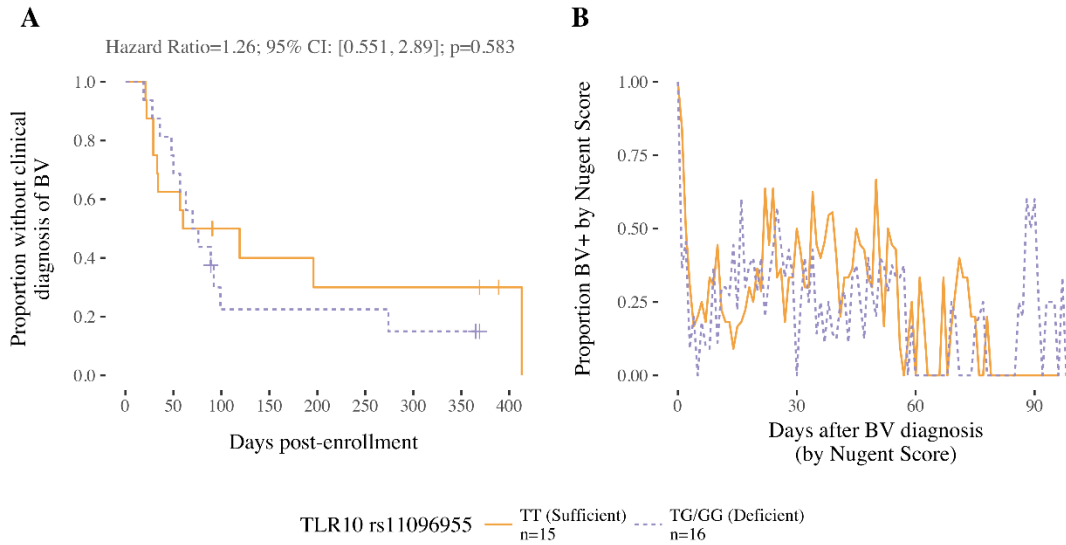
TLR6 rs3821985



	TLR6 rs3821985 GG/GC (WT)	TLR6 rs3821985 CC (Deficient)
<i>N</i>	14	16
<i>% Nugent+ at Enrollment</i>	78.6	62.5
<i>Person-Days Observed</i>	973	692
<i>% Days Nugent+</i>	22.4	34.7
<i>Age (Mean)</i>	30.9	31.8
<i>% White</i>	7.14	6.25
<i>% WSM</i>	35.7	37.5
<i>% WSW</i>	14.3	6.25
<i>% Hormonal Contraception</i>	28.6	56.2

(A) Survival curves depicting time to first diagnosis with BV by Amsel’s criteria, stratified by TLR6 rs3821985 genotype. There are no significant differences in time to first BV diagnosis between TLR6 sufficient controls and TLR6 deficient controls. (B) Daily average proportion of women who are BV-positive by Nugent score, stratified by TLR6 rs3821985 genotype, starting at the time of first Nugent diagnosis of BV, and following up to 90 days post-diagnosis. (C) Demographic characteristics of TLR6 sufficient and deficient women from longitudinal cohort included in analysis of bacterial colonization. TLR6 deficient women are slightly less likely to have report having sex with women [WSW], and are more likely to report hormonal contraception.

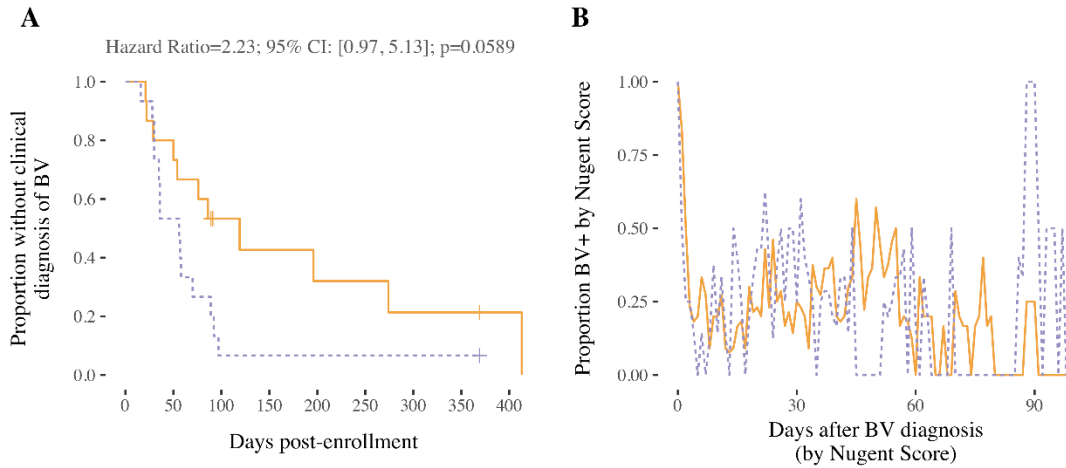
TLR10 rs11096955/rs11096957



	TLR10 rs11096955	
	TT (Sufficient) n=15	TG/GG (Deficient) n=16
<i>N</i>	15	16
<i>% Nugent+ at Enrollment</i>	46.7	50
<i>Person-Days Observed</i>	911	947
<i>% Days Nugent+</i>	25.7	17.1
<i>Age (Mean)</i>	30.1	29.6
<i>% White</i>	46.7	56.2
<i>% WSM</i>	46.7	93.8
<i>% WSW</i>	20	6.25
<i>% Hormonal Contraception</i>	33.3	31.2

(A) Survival curves depicting time to first diagnosis with BV by Amsel’s criteria, stratified by TLR10 rs1106955 genotype. There are no significant differences in time to first BV diagnosis between TLR10 sufficient controls and TLR10 deficient cases. (B) Daily average proportion of women who are BV-positive by Nugent score, stratified by TLR10 rs1106955 genotype, starting at the time of first Nugent diagnosis of BV, and following up to 100 days post-diagnosis. (C) Demographic characteristics of TLR10 sufficient and deficient women from longitudinal cohort included in analysis of bacterial colonization. Groups appear balanced for major risks of BV diagnosis, including BV diagnosis at enrollment, age, race, and sexual behaviors (women who have sex with men [WSM], women who have sex with women [WSW]), and hormonal contraceptive status.

TLR10 rs4129009

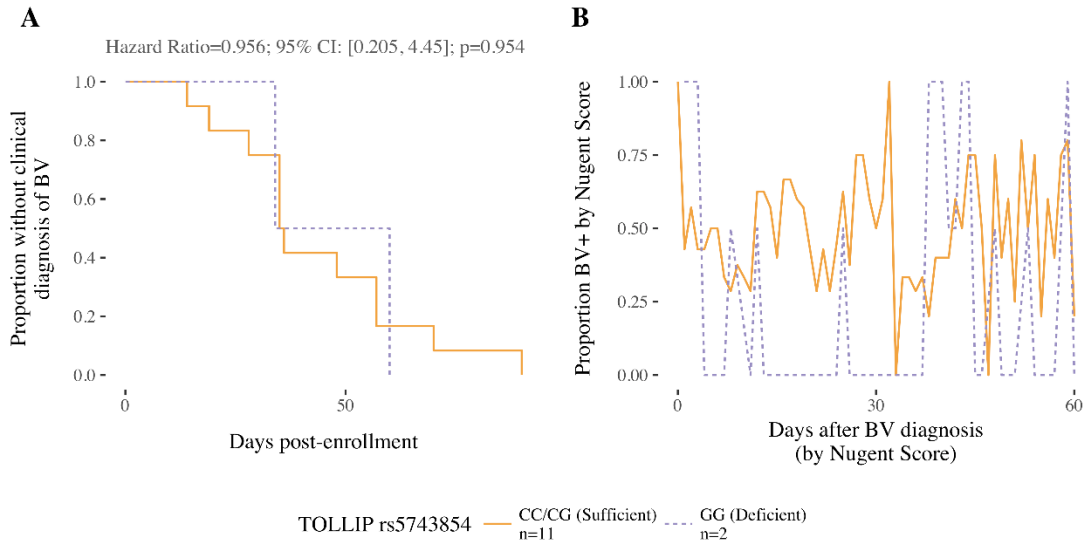


TLR10 rs4129009 — TT (Sufficient) n=14 TC/CC (Deficient) n=14

C	TLR10 rs4129009 TT (Sufficient)	TLR10 rs4129009 TC/CC (Deficient)
<i>N</i>	14	14
<i>% Nugent+ at Enrollment</i>	42.9	71.4
<i>Person-Days Observed</i>	942	831
<i>% Days Nugent+</i>	22	14.4
<i>Age (Mean)</i>	32.2	31.1
<i>% White</i>	85.7	85.7
<i>% WSM</i>	57.1	71.4
<i>% WSW</i>	21.4	35.7
<i>% Hormonal Contraception</i>	35.7	35.7

(A) Survival curves depicting time to first diagnosis with BV by Amsel’s criteria, stratified by TLR10 rs4129009 genotype. TLR10 deficient women included in the matched case-control analysis of vaginal bacterial colonization trended towards increased risk of BV (hazard ratio [HR] of 2.23, $p = 0.06$) (B) Daily average proportion of women who are BV-positive by Nugent score, stratified by TLR10 rs4129009 genotype, starting at the time of first Nugent diagnosis of BV, and following up to 100 days post-diagnosis. (C) Demographic characteristics of TLR10 sufficient and deficient women from longitudinal cohort included in analysis of bacterial colonization. Groups appear balanced for some major risks of BV diagnosis, including age, race, and hormonal contraceptive status. However, women in the TLR10 deficient group were more frequently diagnosed with BV by Nugent score at enrollment and were more likely to report sexual behavior (women who have sex with men [WSM], women who have sex with women [WSW]).

TOLLIP rs5743854



	TOLLIP rs5743854 CC/CG (Sufficient)	TOLLIP rs5743854 GG (Deficient)
<i>N</i>	11	2
<i>% Nugent+ at Enrollment</i>	90.9	100
<i>Person-Days Observed</i>	474	68
<i>% Days Nugent+</i>	35.4	23.5
<i>Age (Mean)</i>	33	33
<i>% Black</i>	100	100
<i>% WSM</i>	45.5	100
<i>% WSW</i>	0	0
<i>% Hormonal Contraception</i>	27.3	50

(A) Survival curves depicting time to first diagnosis with BV by Amsel’s criteria, stratified by TOLLIP rs5743854 genotype. There are no significant differences in time to first BV diagnosis between TOLLIP sufficient controls and TOLLIP deficient controls. (B) Daily average proportion of women who are BV-positive by Nugent score, stratified by TOLLIP rs5743854 genotype, starting at the time of first Nugent diagnosis of BV, and following up to 60 days post-diagnosis. (C) Demographic characteristics of TOLLIP sufficient and deficient women from longitudinal cohort included in analysis of bacterial colonization. Groups appear balanced for major risks of BV diagnosis, including BV diagnosis at enrollment, age, and race. However, while 100% of TOLLIP deficient study participants report sex with men (WSM), only 45.5% of TOLLIP sufficient controls report sex with men (WSW). Additionally, 50% of TOLLIP deficient cases report hormonal contraception use, compared to 27.3% of TOLLIP sufficient controls.

APPENDIX C: ASSESSING THE ADEQUACY OF MATCHING TLR/TOLLIP DEFICIENT CASES AND SUFFICIENT CONTROLS IN THE VALIDATION COHORT

	TLR5 rs5744168		TLR10 rs11096955		TLR10 rs4129009		TOLLIP rs5743854	
	CT/TT	CC	TG/GG	TT	CT/CC	TT	GG	CC/CG
	Deficient (Cases)	Sufficient (Controls)	Deficient (Cases)	Sufficient (Controls)	Deficient (Cases)	Sufficient (Controls)	Deficient (Cases)	Sufficient (Controls)
N	8	32	38	38	22	22	9	27
% Nugent 7-10	†	†	57.9%	55.3%	68.2%	54.6%	77.8%	85.2%
Age (years, average)	33.9	33.1	32.5	31.3	29.4	30.7	38.2	33.7
% White	75.0%	75.0%	50.0%	47.4%	68.2%	72.7%	11.1%	11.1%
% Hormonal contraception	0.0%	0.0%	23.7%	23.7%	27.3%	31.8%	11.1%	14.8%
% Vaginal douching (ever)	75.0%	50.0%	44.7%	50.0%	40.9%	36.4%	77.8%	59.3%

† TLR5 deficiency was not found to be related to risk of BV, thus TLR5 deficient study participants were not matched on microbiologically-defined BV status.

APPENDIX D: QUANTITATIVE PCR ASSAYS

Target	Forward Primer	Reverse Primer	Probes	PCR Conditions
TLR1 rs5743618	>1673F_hTLR5 5' -CCCGG AAAGT TATAG AGGAA CCCT- 3'	>1956R_hTLR1 5' -CTTCA CCCAG AAAGA ATCGT GCC-3'	>hTLR1_1805T 5' -VIC-CAGAT CCAAG TAGAT GCAGA G-MBG- NFQ-3' >hTLR1_1805G 5' -FAM-AGATC CAAGT AGCTG CAGAG-MGB-NFQ- 3'	60C for 30 sec 95C for 1 min <i>40 Cycles:</i> 95C for 6 sec 57C for 20 sec 72C for 25 sec 60C for 30sec
<i>M. curtisii</i> <i>M. mulieris</i> (multiplexed)	>Mobi1007F 5' -CTTAC CAAGG CTGA CATAC A-3'	>Mobi1088R 5' -ACCAC CTGTA CACCA CC-3'	>Mmuli1034_1054 5' -VIC-CATGC CAGAG ATGGT GTGG-MGB-NFQ-3' >Mcurt1034_1054 5' -FAM-TGGTT CCAGA GATGG GCCAG-MGB-NFQ-3'	50C for 2 min 95C for 20sec <i>45 Cycles:</i> 95C for 2 sec 57C for 20 sec 72C for 20 sec

VITA

Born in Houston, Tex., Erin dela Cruz moved to Orange County, Calif. at the age of three. She graduated from Dana Hills High School in Dana Point, Calif. in 2002. Erin attended the University of California, Berkeley, where she double-majored in Applied Mathematics and Public Health and obtained Bachelor of Arts degree with honors in 2006. Erin joined the RAND Corporation as a Research Assistant, staffing projects under the direct supervision and mentorship of RAND Health social scientists, Drs. Cheryl Damberg, Katherine Watkins, Sarah Hunter, and Karen Chan-Osilla. While at RAND, Erin's projects included: the creation of tools for comparison of health policy interventions, studies of patient safety program implementation, investigation of quality of care impact related to value-based payment strategies, and evaluation of brief interventions for co-occurring depression and substance use disorder. With the encouragement of her administrative supervisor and mentor, Dr. Stuart Olmsted, Erin applied to Medical Scientist Training Programs (MSTP) in 2010, joining the University of Washington (UW) MSTP in 2011. Committed to understanding how human contexts influence disease risk and fascinated by the emerging microbiome field, Erin joined the Molecular and Cellular Biology graduate program and the laboratory of Dr. David Fredricks in 2013. There, she developed a project examining the relationships between innate immune genetic variation, risk of BV, and colonization with vaginal bacteria. After graduation from the UW MSTP, Erin hopes to continue to study host factors underlying infections disease risks as a scientist in the Centers for Disease Control.