CD101 Immunoglobulin-like Variants and Cytokine Profiles in the Female Genital Tract: A Confirmatory and Exploratory Analysis with Vaginal Swab Samples from a large cohort of African HIV-1 serodifferent couples

Alene Chang

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Committee:

Jairam Lingappa

Romel Mackelprang

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Abstract

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Alene Chang

Chair of the Supervisory Committee:

Jairam Lingappa,

Department of Global Health, Medicine, and Pediatrics

Variants in the Ig-like domain of CD101 are associated with an increased risk of heterosexually acquired HIV infection. The mechanism through which CD101 influences HIV susceptibility is still unclear. HIV-1 primarily gains access to the host through the genital mucosa after sexual exposure, and analysis of epidemiological and tissue-specific inflammatory factors are critical. We used existing vaginal swab samples and epidemiological data from the Partners PrEP study from HIV-seronegative women (N=89) in heterosexual couples. Genotypes for 12 *CD101* SNPs and 28 soluble immune factors were assayed. Based on 4 candidate cytokines from previous work, and to conduct an exploratory analysis on the other cytokines, we ran multivariable linear regression models (frequency of condomless sex, BV, DMPA, and PrEP as covariates) to compare log10 cytokines and chemokine levels to the number of Ig-like variants (copies of alleles). We did not find any significant cytokines at the $\alpha < 0.05$ for the confirmatory analysis, nor did we find any significant cytokines at the FDR $\alpha < 0.05$ level in our exploratory analysis.

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Introduction

1.1 Our understanding of HIV

Our understanding of HIV transmission and susceptibility has evolved through the last several decades. There are a variety of behavioral, biologic, and social factors that are determinants of HIV sexual transmission risk. These include frequency of unprotected sex, number of partners[1], genital HIV-1 RNA level of the sexual partner living with HIV[2,3] (PLWH), male circumcision status[4], and the genital inflammatory environment[5,6]. However, even accounting for these factors, studies in a variety of observational cohorts have shown that some individuals despite documented exposure to HIV-1 appear to be more resistant to HIV-1 infection[7].

1.2 CD101

Following the discovery of the protective deletion variant CCR5-delta32[8], there has been a lot of interest in identifying host genetic factors that influence HIV-1 susceptibility[9]. Rare or intermediate frequency genetic variants are not well studied in HIV-1, and further understanding of host genetic factors could provide new paths to HIV prevention and offer new opportunities for intervention. However, there are numerous challenges to having the statistical power to identify rare variants. Recently, Drs. Lingappa and Mackelprang's team at the University of Washington (UW) used data and samples from follow-up of African HIV-serodifferent heterosexual couples to screen for rare and intermediate frequency variants in highly HIV-exposed partners living without HIV-1 (PLWoH). They used an extreme exposure phenotype case-control study design to sample PLWoH for whole genome sequencing (WGS)[10]. They compared functional single nucleotide variants (SNV) with MAF<0.125 in highly HIV-exposed HIV-seronegative individuals to lower exposed HIV-seroconverters. Using a variant burden

analysis (RVT1)[11] they found that having any of five low-frequency/rare variants in the Ig-like domain of *CD101* was associated with an increased risk of HIV infection (HR = 4.3, $p=6.3 \times 10^{-5}$; $p_{corr}=1.9 \times 10^{-4}$); whereas having any of four *CD101* variants in the cytoplasmic domain were not[12]. These candidate *CD101* Ig-like variants are present in 20%–25% of East Africans which suggests the potential for significant impact on HIV-1 susceptibility in this population.

CD101 encodes a transmembrane protein expressed on the surface of T cells, monocytes, and dendritic cells. Regulatory T-cell (Treg) expressing higher levels of reference *CD101* were more effective at suppressing activated T cells[13]. *CD101* plays a key role in CD4 Treg potency. Presence of these Ig-like variants in *CD101* is associated with increased levels of activated T cells and increased CD4 and CD8 T cells producing proinflammatory cytokines in circulation. Regulatory T cells also show reduced ability to suppress effector T cell responses which may also contribute to the proinflammatory environment observed. These mutations in *CD101* Ig-like domains may increase HIV acquisition risk through heightened baseline T-cell inflammation[14]. In a simian immunodeficiency virus (SIV) model for HIV, *CD101*-expressing CD4 cells were depleted following infection. This depletion is associated with a higher viral burden and increased inflammatory cytokine levels[15].

1.2.1 Host immunologic changes as risk factors for HIV-1 acquisition

Given the role of *CD101* in regulation of inflammation, it is important to examine-how *CD101* genotype influences soluble responses in the genital mucosa, while accounting for other potential confounding factors that affect inflammation.

Several factors may influence the inflammatory environment in the genital mucosa and potentially confound the relationship between *CD101* variants and cytokine levels. The frequency of unprotected sex can modify the inflammatory pathway – HIV-1 may stimulate host response factors through pathogen recognition pathways – *in vitro* studies have shown that primary human epithelial cells up-regulate TNFα, IL-6, IL-10 IL-8, IL-1β, GRO, TNFβ, and IL-1α in response to infectious R5 type HIV-1[16,17]. Likewise, exposure to seminal plasma has been shown to be involved in inflammatory responses, activating the NF-kB signaling pathway in vaginal cells[18]. Damage to the mucosal lining of the female genital tract is also associated with acquisition of HIV infection[19]. Bacterial vaginosis (BV), the most common cause of vaginal complaints brought to medical attention and a risk factor for acquiring HIV-1, has been demonstrated to influence pro-inflammatory cytokine concentrations[20]. In one study of African women, elimination of BV was associated with reducedlevels of pro-inflammatory cytokines in genital secretions of IL-1ß, IL-8, and RANTES[21,22].

Hormonal changes across the menstrual cycle can also influence the inflammatory environment, leading to fluctuations in cervicovaginal immunity through the menstrual cycle[23]. Previous observational studies observed a change in cervicovaginal cytokines associated with depot-medroxyprogesterone acetate (DMPA), a form of hormonal birth control, but more recent work has not shown adverse changes in inflammation or risk of STIs[24]. Oral PrEP usage has also been shown to alter genital mucosal immune activation in women with HSV-2 but has not been shown to significantly alter cytokine levels[25,26].

In a recent study, genital softcup samples were tested for 71 cytokines and chemokines. Women with one or more vs. no *CD101* Ig-like variants were associated with significant reduction in levels of LIF, G-CSF, as well as IL-1a, PDGF-AB/BB, RANTES, Eotaxin-2, Eotaxin-3, IL-16,

IL-33, and TPO. This suggests a mechanism for how these variants impact HIV risk, as RANTES, IL-16 and LIF have been implicated in HIV inhibitory activity and G-CSF, PDGF-AB/BB, IL-33 and LIF facilitate tissue homeostasis and repair[27].

1.3 The purpose of this study

Women are disproportionately affected by the HIV epidemic worldwide– as of 2024, 53% of all people living with HIV (PLWH) were women and girls[28]. Genital inflammation is a significant biological correlate for HIV-acquisition risk[29]. HIV-1 primarily gains access to the host through the genital mucosa after sexual exposure, and analysis of epidemiological and tissue-specific inflammatory factors can aid in characterizing susceptibility to infection. Increased inflammation can amplify the number of HIV-susceptible cells, underscoring the need to characterize the immune response in the genital mucosa—the primary site of entry for sexual transmission of HIV-1—to further our understanding of HIV-1 acquisition[30].

However, the pathways through which *CD101* influences HIV susceptibility have not yet been fully described. We seek to expand the scope of literature by integrating host genetic variation with behavioral and epidemiological context. Studies have found reduced production of pro-inflammatory cytokines and chemokines in women that resisted HIV infection but did not establish whether pre-existing responses were protective against HIV-1 transmission[31,32].

It is important to examine how *CD101* influences the genital mucosal environment and to assess relative levels of pro-inflammatory and anti-inflammatory cytokines in those with and without functional variants. Confounding of the host genetic component with other etiological factors such as BV, DMPA, oral PrEP, and frequency of unprotected sex should also be considered.

Evaluation of these complex mechanisms between exposure and host factors requires examination of well-characterized HIV-1 uninfected individuals in serodifferent relationships where the mucosal environment can be studied with epidemiological context in the presence of quantifiedpr exposure to HIV-1.

1.4 Goals of Analysis

We sought to characterize the association between the number of copies of *CD101* Ig-like variants with mucosal cytokines using an existing dataset of cytokines present in female genital tract secretions evaluated in a cohort of African participants. In a study conducted with genital softcup samples, four of the 28 cytokines that we assayed were previously found to be associated with our genotype of interest and the other cytokines were not found to be significantly associated with *CD101* genotype. We applied updated covariate adjustments to assess the association within a larger epidemiological context.

We had the following aims:

Aim 1: To replicate prior findings and quantify the effect of *CD101* Ig-like variants on four cytokines (IL-1a, G-CSF, RANTES, IL-33) previously identified as significantly reduced in individuals with *CD101* Ig-like variants. Adjust for factors affecting the genital mucosal environment by incorporating epidemiological factors into the model

Aim 2: Examine the association between 24 cytokines and chemokines and characterize the inflammatory environment through an exploratory analysis on the full cytokine panel with statistical correction (FDR) with *CD101* Ig-like variant copies. Adjust for factors affecting the genital mucosal environment by incorporating epidemiological factors into the model

1.5 Hypothesis

Based on the mechanism of *CD101* regulating inflammation and affecting HIV-1 susceptibility that we expected to observe significant effects on genital cytokine levels and significant associations between *CD101* Ig-like variants and changes in the soluble inflammatory environment (cytokines/chemokines) in the CVT.

Hypothesis 1: Results will recapitulate those of a candidate cytokine analysis, seeing a significant reduction in levels of IL-1a, RANTES, IL-10, IL-33.

Hypothesis 2: *CD101* Ig-like variants will be associated with increased pro-inflammatory cytokines after adjusting for genital mucosal inflammatory factors.

Hypothesis 3: *CD101* Ig-like variants will be associated with a reduction in HIV-inhibiting cytokines in the genital mucosa after adjusting for genital mucosal inflammatory factors.

2. Methods

2.1 Data Source

The Mucosal Environment and HIV Prevention (MEHP) study used existing genital samples and epidemiological data from the Partners PrEP study, a longitudinal, multisite, phase 3, randomized, double-blind placebo-controlled trial of preexposure prophylaxis (PrEP) to prevent HIV-1 acquisition among HIV-uninfected partners in HIV-1–serodifferent couples.

For the MEHP study Tandem MS and cytokine bead arrays (Milliplex) were performed on archived vaginal swabs from HIV-1 uninfected samples. A subset of women also had vaginal and cervical biopsies evaluated by immunohistology for CD4+/CCR5+ cells and gene expression microarrays to assess specific inflammatory mediators.

2.2 Sample Selection

The MEHP grant proposal had several aims, all with study samples assayed for genital cytokines and chemokines. However, due to a high proportion of out-of-range data in other samples, we selected the group with the highest level of completeness.

The samples used for this study came from Aim 1b of the MEHP grant proposal. This sample set prioritized completeness of samples and participants who had multiple sample types available. Samples were collected for both vaginal and cervical biopsies in RNAlater for microarray studies; vaginal and cervical swabs at the same visit for proteomics; and serum at the same visit for DMPA, EE2 and P4. Additional vaginal and cervical biopsies were added from exit visits of women randomized to PrEP with persistently high drug adherence through the study and who have both cervical and vaginal swabs available for proteomics, and serum for DMPA, EE2 and P4 analysis. To address my aims, I used the cytokine data on vaginal swabs from Aim 1b

participants, due to their high sample completeness and completeness of cytokine data (100%). This included a total of N=20 women on the placebo arm and N=70 individuals that were in the PrEP arm[33]. One individual from this set was missing genotyping data. A total of n = 89 participants were included in the final analysis, each with complete cytokine, genotyping, and epidemiological data (Table 1).

2.3 Study Samples

In this study our goal was to evaluate the relationship of *CD101* variants with the inflammatory environment. The sample data includes genotyping on 13 *CD101* SNPs collated from OpenArray, MIP, and iSelect assays. and Bio-plex assay data on 28 cytokines. Demographic and epidemiological data were collected at sample collection, including age, BV status (by Nugent score), hormonal contraception use (measured DMPA with mass spec), proportion of unprotected sex, and PrEP use on all women in the Partners PrEP Genital Mucosal Study. Using the cytokine data from these participants, we ran comparisons on the cytokine environment in the genital mucosa for participants using a dosage variant analysis for number of copies of alleles of Ig-like variants. We performed a replication analysis on four cytokines that were shown to have significant association with *CD101* Ig-like variants in previous work[34]. an exploratory analysis to determine whether there is an association between *CD101* variants and pro-inflammatory cytokines.

Participants were initially selected for the sample in a factorial design to increase power and assess the effects of BV and DMPA on the genital environment. Hormonal contraceptives have been shown to alter the genital environment, which was initially a concern in our study design

due to previous studies indicating a link to HIV-1 risk[35]. However, subsequent findings from the ECHO study have demonstrated that the HIV risk associated with DMPA use is lower than previously anticipated. We will account for other factors previously described in our analysis that could affect the inflammatory environment, controlling for presence of bacterial vaginosis, PrEP use, and frequency of unprotected sex.

2.4 Data Processing

Processing Cytokine Data

For consistency in cytokine data, we decided on standardized methods to pre-process cytokines. Cytokines can range from very small to very large concentrations and so necessitate the log 10-transformation of the concentrations. Data falling below the detection limits would be replaced with a value randomly sampled between from the minimum observed value to ½ of the smallest observed value. Cytokines on the outer limit of detection, would be replaced with the largest observed value for that cytokine. For cytokines tested with high rates of missing values. If 20% or more of the data is out of range or missing, the data will be made categorical (0 for undetected; 1 for detected). Any duplication would be dealt with by selecting the non-censored value or the lowest %CV value. Cytokines are part of the inflammatory milieu and are therefore interdependent. To account for multiple testing error and dependence, we used Benjamini-Hochberg false discovery rate for all exploratory cytokines[36]. FDR was not used for cytokines from the previous candidate analysis.

Cytokines were first processed for the MEHP Aim 1a sample. For 24 of the 28 cytokines in the analysis, more than 20% of the data was out of range or missing. In contrast, the MEHP Aim 1b sample had 100% of the cytokine data values.

Processing Genotype Data

We combined the OpenArray, iSelect, and MIP *CD101* genotype data by SNP and participant ID. These values were imputed into a binarized variable to compare those who had variants in the Ig-like category were compared to with no Ig-like variants.

Processing Epidemiological data

The following variables were accounted for as covariates in the linear regression model:
BV was included as a 3-level factor by Nugent score (Neg = [0,3]; Pos = [7,10]; Intermediate = (3,7)). DMPA in serum as assayed with mass spectrometry (detectable vs undetectable), frequency of unprotected sex (as a count measure), and the placebo vs non-placebo arm of the PrEP trial.

We generated descriptive statistics to report on epidemiological variables for the cohort (Table 1).

2.5 Statistical Analysis

2.3.1 Replication Analysis

We performed a multivariable linear regression using standard packages in R on $\log_{10}$-transformed cytokine concentrations. We used our dosage variable for Ig-like alleles as our predictor and included the frequency of unprotected sex, BV, DMPA, and PrEP as covariates. For the candidate cytokines we did not use Benjamini-Hochberg FDR because these have been previously shown to be significant [37].

2.3.2 Exploratory Analysis

We performed a multivariable linear regression using standard packages in R on log_{10}-transformed cytokine concentrations. We used our dosage variable for Ig-like alleles as our predictor and included the frequency of unprotected sex, BV, DMPA, and PrEP as covariates. False discovery rate was then used to account for multiple testing error and the interdependence of cytokines.

3. Results

3.1 Candidate Cytokine Analysis

We modeled the association between *CD101* Ig-like variant status and log_{10}-transformed mucosal cytokine concentrations, adjusting for BV status, hormonal contraceptive status (DMPA), PrEP use, and frequency of unprotected sex. Due to limited variation (identical values in 88 of the 89 participants) in the observed concentration of IL-33, model estimates were unstable and not interpretable (β=1.4e-16). The other cytokines from the candidate analysis (IL-1a, RANTES and G-CSF) were not significantly associated with *CD101* Ig-like variants in our model.

3.2 Exploratory Cytokine Analysis

For our exploratory analysis we did not find any cytokines to meet a false discovery rate (FDR) threshold of $q < 0.05$. However, we did see effect size estimates across several cytokines aligned closely with the candidate analysis and biological expectations (Table 2). TNF-α showed a large positive effect estimate ($\beta = 0.17$, 95% CI [0.03, 0.31], $p = 0.023$), suggesting increased levels in

those with *CD101* Ig-like variants. IFN-γ also had a positive association with genotype (β = 0.11, 95% CI [0.01, 0.21], p = 0.034).

4. Discussion

This study aimed to replicate the findings of the Kinga Study candidate cytokine analysis. We could not accomplish this due to the lack of significance for three out of four of the cytokines (IL-1a, RANTES, and G-CSF). Upon examining the observed concentrations for IL-33 we found that concentrations were uniform across all but one participant (who was in the exposed group with *CD101* Ig-like variants). The lack of variation or normal distribution of cytokines led to extremely small beta estimates and inflated the significance level. Repeating this analysis on a larger more diverse sample may elucidate the effect of *CD101*. One factor to consider between the Vick study and this replication is the differing sample collection method. The Kinga Study used softcup samples, while this analysis used vaginal Dacron swabs. SoftCup samples have been shown to be more sensitive for measuring the association between cytokine and chemokine levels than vulvovaginal swabs, especially for cytokines at lower concentrations. This may be more effective for assessing the potential association between cytokine and chemokine levels and *CD101*[38]. We were unable to use a large portion of the sample set due to data being below the lower limit of detection. This uncaptured range of cytokine data could fill in the gaps in our analysis in future work.

This study also aimed to examine associations between cytokines and *CD101* Ig-like variants. We were able to accomplish this with our model and identified two cytokines with large effect sizes that were significant before accounting for false discovery rate. While these are not statistically significant with FDR, we can see general trends in the elevation of pro-inflammatory

cytokines that fit with our proposed mechanism of action for *CD101*. We did not expect to see associations in the other cytokines as they did not show significant association in prior work.

Our results showed a positive effect TNF-α that was significant before FDR. Although these results must be taken with a grain of salt due to multiple testing bias, it is worthwhile to think about the potential implications of an association with *CD101*. While our study was with HIV-1 seronegative individuals, there are some studies that show that TNF- α has an immunological role in people living with HIV. TNF-α is an important pro-inflammatory cytokine and often elevated in people infected with HIV-1[39]. *In vitro* studies demonstrate enhanced transcription of HIV-1[40]. In those with untreated primary HIV-1 infection, high TNF-α increased risk of reaching a CD4+ T-cell count of <500 cells/mm^3 [41].

We also observed a positive association in IFN-γ that was significant before FDR. This result is also not significant but fits into the trend of increased levels of pro-inflammatory cytokines. High levels of circulating IFN-γ plays a role in the course of HIV-infection. IFN-γ also induces the production of proinflammatory cytokines and chemokines on epithelial cells and upregulates innate defense mechanisms[42].

These results may support the hypothesis that *CD101* elevates pro-inflammatory cytokines, increasing background inflammation, as a mechanism to increase susceptibility to HIV-1 infection.

5. Conclusion

5.1 Limitations

Due to the structure of the study sample, we had to adjust for BV, DMPA, and PrEP use. Although these factors are biologically relevant to genital inflammation, adding so many additional factors to the analysis for a relatively small sample does run the risk of overfitting our model. Several of the analytes also had very low variance and as such the results of the model were un-interpretable.

For this thesis work, we could not use the samples from Aim 1a due to the additional work required to clean the data – standards and standard curves were not reliable across plates and could not be recovered at the time of this project. Out-of-range values make up >20% of the data for 24 out of 28 cytokines. This means that we are effectively randomizing the data through our imputation, resulting in uninformative results that are not true null effects. However, this data may be more of use in characterizing the genital mucosal due to the more extreme values. Future work may include using Belysa® Software to compare standard curves between plates to help generate observed concentrations for out of range values. This is a larger sample set, which n=240 participants with a wide distribution of observed cytokines and if incorporated into this analysis would help to increase statistical power.

5.2 Clinical Implications

The prevalence of *CD101* variation in the population also has significant clinical implications – 20-25% of East Africans may have one or more variants in the seven Ig-like domains associated

with increased HIV-1 acquisition risk. Identifying the mechanism that links *CD101* to the immune milieu is a crucial step to understanding how host inflammation influences sexual acquisition of HIV-1

5.3 Summary

Although our findings did not replicate previously observed associations, it does contribute to the broader knowledge of inflammation in the genital mucosa. This also broadens our understanding of the genital mucosal environment and offers an avenue for development of crucial cytokine therapies or assessments of background inflammation that may impact HIV-1 susceptibility.

Tables and Figures

Table 1: Sample Demographics

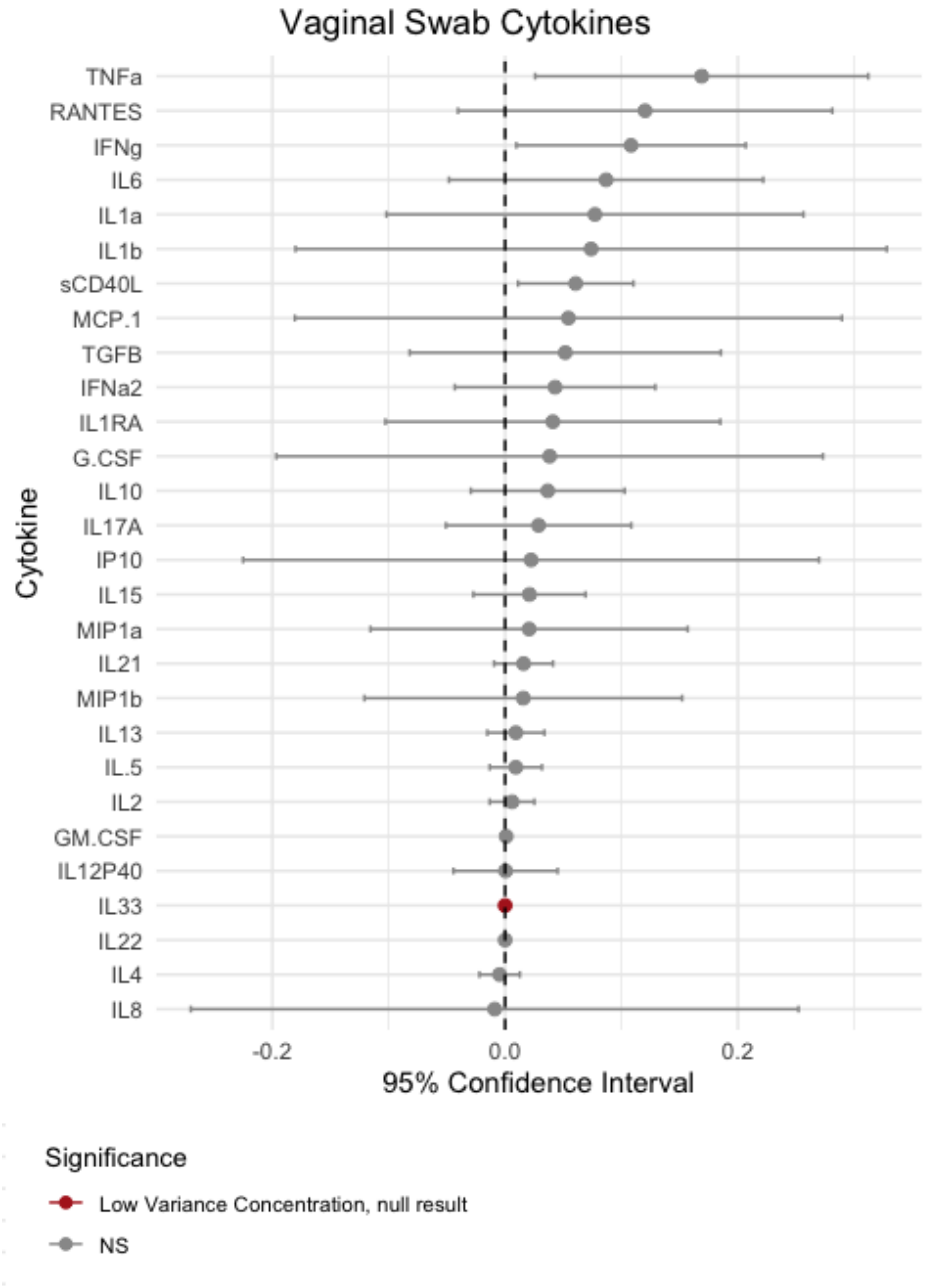
Characteristic	≥1 Ig-like Variant N = 33[1]	No Ig-like Variant N = 56[1]	Overall N = 89[1]
DMPA			
Neg	24 (73%)	31 (55%)	55 (62%)
NotKnown	1 (3.0%)	4 (7.1%)	5 (5.6%)
Pos	8 (24%)	21 (38%)	29 (33%)
BV			
-	22 (79%)	38 (81%)	60 (80%)
+	3 (11%)	3 (6.4%)	6 (8.0%)
Intermediate	3 (11%)	6 (13%)	9 (12%)
PrEP			
placebo	7 (21%)	12 (21%)	19 (21%)
post.prep	15 (45%)	23 (41%)	38 (43%)
prep	11 (33%)	21 (38%)	32 (36%)
Age	37.3 (6.3)	37.5 (6.2)	37.5 (6.2)

[1] n (%); Mean (SD)

Table 2: Linear regression results for association of soluble factors with *CD101* Ig-like variants sorted by P-Value

ANALYTE	ESTIMATE	SE	95% CI LOW	95% CI HIGH	P-VALUE	FDR P
IL22	1.50e-19	6.14e-20	2.92e-20	2.70e-19	0.017	0.162
IL33	1.41e-16	5.79e-17	2.76e-17	2.55e-16	0.017	Confirmatory
SCD40L	0.061	0.025	0.011	0.11	0.019	0.162
TNFA	0.169	0.073	0.026	0.312	0.023	0.162
IFNG	0.108	0.05	0.01	0.207	0.034	0.192
RANTES	0.12	0.082	-0.041	0.281	0.146	Confirmatory
IL6	0.087	0.069	-0.048	0.222	0.211	0.779
IL21	0.016	0.013	-0.009	0.041	0.223	0.779
IL10	0.037	0.034	-0.029	0.103	0.28	0.792
IFNA2	0.043	0.044	-0.043	0.129	0.331	0.792
GM.CSF	9.59e-04	0.001	-0.001	0.003	0.386	0.792
IL15	0.021	0.025	-0.027	0.069	0.397	0.792
IL1A	0.077	0.091	-0.102	0.256	0.401	Confirmatory
IL.5	0.009	0.011	-0.013	0.032	0.422	0.792
TGFB	0.052	0.068	-0.082	0.185	0.45	0.792
IL13	0.009	0.013	-0.015	0.034	0.462	0.792
IL17A	0.029	0.041	-0.051	0.108	0.481	0.792
IL2	0.006	0.01	-0.013	0.025	0.542	0.797
IL1B	0.074	0.13	-0.18	0.328	0.57	0.797
IL1RA	0.041	0.073	-0.103	0.185	0.578	0.797
IL4	-0.005	0.009	-0.022	0.013	0.598	0.797
MCP.1	0.054	0.12	-0.181	0.289	0.651	0.829
G.CSF	0.038	0.12	-0.196	0.273	0.75	Confirmatory
MIP1A	0.021	0.07	-0.116	0.157	0.767	0.895
MIP1B	0.016	0.07	-0.121	0.152	0.823	0.922
IP10	0.022	0.126	-0.225	0.27	0.86	0.926
IL8	-0.009	0.133	-0.27	0.252	0.947	0.982
IL12P40	3.82e-04	0.023	-0.044	0.045	0.987	0.987

Figure 1: Forest Plot of Results



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