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# Antimicrobial Peptides in Cervicovaginal Lavage of Women Married to HIV Sero-Reactive Men are not Associated with Resistance to HIV but Modulate Mucosal Pro-Inflammatory Response

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## Abstract

**Objectives**: It is critical to understand the HIV transmission dynamics in women, who are married to HIV sero-positive men and either go into HIV concordant relationship (WCR) or remain HIV discordant (WDR) over a period of time. Antimicrobial peptides (AMPs) are known to possess anti-HIV activities and may offer protection against acquisition of HIV. We investigated if AMP expression is associated with HIV sero-negative status of the WDR population, and how the co-regulated innate immune responses at the genital mucosa differ between WDR and WCR individuals in eastern-India.

**Methods**: Levels of human beta-defensins, human neutrophil peptides 1-3 and IL-8 were measured by ELISA in the cervicovaginal lavarge (CVL) collected from WCR and WDR individuals. Moreover, we studied the activation of TLR signalling pathways and TNF-alpha induction in THP-1 cells by the microbial components of CVLs and their modulation by pre-treatment of the cells with recombinant AMPs.

**Results**: Antimicrobial peptide expression in the CVLs showed no significant association with resistance to HIV infection. We observed a shift toward significant co-regulated expression of hBD2 and hBD3 ($r=0.52$, $p<0.0001$) and hBD3 and IL-8 ($r=-0.26$, $p=0.03$) in the WCR group compared with the WDR ($r=0.30$, $p=0.13$ for hBD2 and hBD3; $r=-0.15$, $p=0.44$ for hBD3 and IL-8) population. We also found that hBD3 inhibited the pro-inflammatory immune responses induced by CVL, perhaps through the inhibition of TLR4 and TLR1/2 signalling. This was in agreement with the negative correlation between hBD3 and IL-8 expression in the WCR population.

**Conclusions**: Previous studies identified different microbial composition of CVLs between women living with and without HIV. This might result in a difference in the co-regulated expression of AMPs and inflammatory mediators between the WCR and WDR populations. hBD3 may be critical to control inflammation at the cervico-vaginal mucosa, indicating its novel therapeutic potential in HIV infection.

**Keywords:** HIV; WCR; WDR; CVL; AMPs; Toll like receptor

## Introduction

Four out of every 1000 heterosexually married couples in India live in HIV discordant relationship [1]. It is therefore important to understand the transmission dynamics in these couples. It is also important to note that the sexual route of HIV transmission is less efficient compared to other routes, such as injecting drug use or transfusion-mediated transmission [2,3]. Most studies have focused on the factors facilitating HIV transmission in sexually active population, while little is known about the protective biological determinants. Innate immunity has been a subject of interest in this regard. However, data is scarce from Indian population on what could be the factors that may protect married heterosexual women from getting infected with HIV from their husbands, which is the current prevailing HIV epidemiologic situation in India.

Female reproductive tract produces and secretes a wide spectrum of cytokines, chemokines and antimicrobial molecules from the epithelial and immune cells [4]. They have been shown by investigators to offer protection against HIV transmission. Examples of such response include up-regulation of certain chemokines, such as RANTES, MIP-1 alpha and MIP-1 beta, expression of anti-HIV antibodies and cytotoxic T-cell responses [5-7]. A previous cohort study reported that enhanced beta chemokines expression was associated with resistance to HIV infection in Indian women living in HIV discordant relationship [8]. Antimicrobial peptides (AMPs), such as human elafin/traffin, beta defensins2 (hBD2), human neutrophil peptide 1-3(hNP1-3) and α-Defensin 5, lysozyme, lactoferrin and secretory leukoprotease inhibitor (SLPI) may inhibit HIV infection [9-14], although their relations to HIV transmission appear to be complex. A study involving a cohort of sex workers in Keniya identified that hNP and LL37 levels were associated with increased risk of HIV acquisition, although these patients were infected with other sexually transmitted diseases [15,16]. However, the aforementioned studies did not examine the interplay between HIV infection and AMPs in heterosexually-married couples living in HIV concordant or discordant relationships.

It is currently unclear if AMP expression is associated with higher levels of resistance to HIV infection in heterosexually-married Indian population. A larger question is how mucosal innate immune responses

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differ between the women who live in marital relations with HIV sero-reactive men and either go in a HIV concordant relationship (WCR) or remain HIV discordant (WDR) over a period of time. This forms the basis of our present work.

We had access to a cohort of heterosexual married couples as part of a larger epidemiological study [17] approved by the Institutional Ethics Committee of NICED. Our study participants were a sub-population of this investigation. AMPs in the cervicovaginal lavages (CVLs) of women participants of the current study were assayed to describe the difference, if any, between HIV sero-reactive wives and those not infected with HIV. We also addressed the complex relations between cervicovaginal inflammation and the expression of antimicrobial peptides.

## Material and Methods

### Collection and processing of CVLs

We pre-informed the willing participants to abstain from non-protective sex at least 3 days prior to clinical examination and specimen collection. CVLs were collected from non-menstruating women as described previously [18-20]. Ten millilitre of PBS was instilled into the vaginal cavity with the stream directed toward the external os of the cervix and the fluid was aspirated after it was allowed to pool in the posterior fornix. Secretions were centrifuged at 10,000 g for 5 min to pellet down the cellular fraction and the supernatants were stored in aliquots in a -80°C freezer for future estimation of human β-defensins 1, 2 and 3 (hBD1, 2, 3) and human neutrophilic peptide 1–3 (hNP1-3).

### Reagents

**Cell culture:** Human monocytic cell line THP-1 were obtained from ATCC and were grown in suspension in RPMI 1640 medium supplemented with 10% FBS and 0.5 mM 2-mercaptoethanol. To differentiate into macrophages, THP-1 cells at a density of $5 \times 10^5$ cells/ml were treated with 100 nM phorbol 12-myrisate 13-acetate (PMA) for 24 h. Plastic-adherent cells were further cultured in complete RPMI 1640 medium for additional 24 h before the experiment. Human cervical epithelial cell line HeLa was cultured in DMEM supplemented with 10% FBS. Cells were stimulated with recombinant hBD1, hBD2, hBD3 and hNP1-3 (Cat no: D9565, D9690, SRP4524 and D2043, respectively; all purchased from Sigma) or toll-like receptor (TLR) ligands purchased from InVivogen. According to the manufacturer, hBD3 was free from LPS contamination.

**Luciferase assay:** HeLa cells were engineered to express MD2 and CD14 (pFLAG-CMV1-hMD2; Addgene plasmid#13028 and pcDNA-CD14; Addgene plasmid#13645 were kind gifts from Doug Golenbock) along with NF-kB-luciferase reporter. Cells were seeded at a density of $2 \times 10^5$ cells per well into 24-well tissue culture plates 16 h before transfection. Twenty four hours post-transfection, cells were stimulated with 1 μg/ml of various agonists for 8h and luciferase reporter activities were measured with the Dual-Luciferase reporter assay system (Promega) using the constitutive low-level expression of *Renilla luciferase* as a transfection control.

**ELISA:** AMP concentrations in the CVL samples were measured by commercially available ELISA kits for hBD1 (cat no: 100-240-BD1), hBD2 (cat no: 100-250-BD2), hBD3 (cat no: 100-260-BD3) and hNP1-3 (cat no: 100-270-NP1) purchased from Alpha Diagonistics, USA following the manufacturer's protocols. IL-8 and TNF-alpha ELISA kits were purchased from Ebiosciences. Expression of AMPs and cytokines were normalised on the basis total proteins present in the CVLs.

**RNA isolation and real time PCR:** Total RNA was extracted from the treated cells using Trizol reagent (GIBCO) following the manufacturer's protocol. RNA (1.5 μg) was reverse transcribed into cDNA with oligo-dT primers in a 20 μl reaction volume for 50 min at 42°C. The reaction was terminated by incubating at 72°C for 10 min. Real-time Sybr green PCR assays were performed in a Step one plus PCR Systems (Applied Biosystems Inc) using 100 ηg cDNA as template in a 12 μl reaction volume to measure relative mRNA abundance of target genes (Interferon-beta - forward primer, 5' CCAGGGGAAAACTCATGAGC 3' and reverse primer, 5' AGCAATTGTCCAGTCCCAGA 3'; GAPDH - forward primer, 5' GAGAACGGGAAGCTTGTCATC 3' and reverse primer, 5'CATGACGAACATGGGGGCATC 3'). The thermal cycling conditions included 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 62°C for 30 s.

**Western blotting:** Cells were lysed in NP-40 lysis buffer (50mM Tris, pH 8.0, 150mM NaCl, 1.0% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1mM vanadate, 100mM molybdate, 20mM sodium fluoride, and protease inhibitor cocktail). All the reagents were purchased from Sigma. Total cell lysates were resolved in an 8–12% SDS PAGE and the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were incubated with blocking buffer (3% BSA in TBS added with 0.05% Tween 20) followed by probing with anti-phospho-SYK, anti-phospho-TBK, anti–phosphor JNK anti-phosphor-ERK, anti-phosphor-P38, anti-phospho-PKA, anti-phospho-CREB, anti-PKA and anti-IkBalpha (all from Cell Signalling Technology), or anti-tubulin and anti-CREB (Santacruz Biotechnology) antibodies. Immune complexes were detected after probing with Horseradish peroxidase–conjugated secondary antibodies (Thermo Pierce) using Supersignal West pico Chemiluminescence Western blotting detection system (Pierce) and visualized by autoradiography.

### Statistical analysis

Since the values representing the concentrations of AMPs and IL-8 in the CVLs were not distributed normally, we compared the concentrations of each peptide in the HIV sero-reactive and sero-negative groups using Man-whitney U test. Association between continuous variables was calculated by Spearman's rank correlation test using GraphPad InStat version 3.0a (GraphPad Software, San Diego, CA). p value of $<0.05$ was considered statistically significant.

## Results

### Study participants

**Socio-demographic and health profile of the study participants:** The mean age of wives was 30 years (median 31 years; SD ± 6 years), with HIV sero-positive group (mean 30 years; median 28 years; SD ± 7 years) being younger than sero-negatives mean 32 years; median 33 years; SD ± 6 years). However, this difference did not reach statistical significance (p=0.26). Among the husbands of the female participants, 87 (94%) were detected positive for HIV, while all the sero-negative wives were in HIV discordant relationship with their husbands. Socio-demographic and health attributes, compared between the wives, with and without HIV infection, are depicted in Table 1. The mean plasma viral load of husbands or wives living with HIV did not differ significantly from their counterparts (79700.5 vs 52726.2, copies per milliliter respectively; p=0.94). Median CD4 count of HIV sero-reactive wives was 414 (range 87-947). Mean plasma viral load of HIV sero-reactive wives was 26124.9 copies per milliliter and 34% (23/67) of them were on ART.

Genital examination revealed that among the husbands 8% (7/93) had genital ulcers and 1% (1/93) had anal ulcers, while another 8%

| | HIV sero-status of wives | | p-value* |
|---|---|---|---|
| | Reactive (67) n (%) | Non-reactive (26) n (%) | |
| **Information on marriage and health** | | | |
| Mean duration of marriage in years (sd; median) | 11.7 (± 7.2; 12) | 15 (± 6.8; 14) | 0.04 |
| **Husbands' parameters** | | | |
| **Husbands on ART** | | | |
| Yes | 42 (67) | 21 (33) | 0.09 |
| No | 25 (83) | 5 (17) | |
| **Husbands HSV2 sero-reactive** | | | |
| Yes | 44 (79) | 12 (21) | 0.08 |
| No | 23 (62) | 14 (38) | |
| **Husbands' sexual practices in past year** | | | |
| Never used condoms | 11 (69) | 5 (31) | 0.79 |
| Infrequent condom use | 9 (75) | 3 (25) | |
| Always used condoms | 43 (74) | 15 (26) | |
| Did not have intercourse | 4 (57) | 3 (43) | |
| **Wives' parameters** | | | |
| **Wives HSV2 sero-reactive** | | | |
| Yes | 40 (78) | 11 (22) | 0.13 |
| No | 27 (64) | 15 (36) | |
| **Wives having bacterial vaginosis** | | | |
| Yes | 17 (81) | 4 (19) | 0.3 |
| No | 50 (69) | 22 (31) | |
| * Mann-Whitney test for continuous variables and Chi-Square for categorical variables. %=Row percentages | | | |

**Table 1:** Attributes compared across HIV sero-status of wives (N=93).

(7/93) had genital warts. Urethral discharge of pus and anal molluscum was present in 1% husbands each, while none had anal warts. A relatively greater proportion (13%) of husbands (12/93) had inguinal bubo and/or painful scrotal swelling. Vaginal discharge was present in 65% (60/93) of the wives. Ano-rectal discharge was observed in 4% (4/93), and vulvo-vaginal ulcers and inguinal bubo, each, was present in 2% (2/93) of the wives. Anal molluscum was observed in a single (1%) wife. None of the wives had genital warts, warts on the cervico-vaginal wall, genital molluscum, anal ulcers or anal warts.

**Antimicrobial peptide concentrations were not significantly different in CVLs of WCR and WDR women from Eastern India:** We evaluated the expression of AMPs, such as, hBD1, hBD2, hBD3 and hNP1-3 by ELISA in CVLs of WCR and WDR women. Our intent was to investigate how the innate immune response of FGT as a mucosal organ differs between the two groups of women rather than to evaluate the immune responses in specific compartments of the female genital tract (FGT). hNP1-3 were the most abundant AMPs in the CVLs. However, the median concentrations of hNP1-3 in the two groups of women did not differ significantly (5.75 ng/ml vs 9.24 ng/ml, p=0.18). Box plot representation of non-parametric test in this regard is shown in Figure 1. In addition, median concentrations of hBD1 (3255 pg/ml vs 5501 pg/ml, p=0.10), hBD2 (202 pg/ml vs 144 pg/ml, p=0.45) and hBD3 (236 pg/ml vs 221.5 pg/ml, p=0.34) were also not different between the WCR and WDR groups.

**Levels of hBD3 were positively correlated with hBD2, but negatively correlated with hBD1 in the CVLs of WCR and WDR women:** We investigated if the correlation between the expressions of AMPs changed because of HIV infection. To this end, we evaluated the association between any two AMPs by Spearman's rank correlation test. The results showed that hBD3 and hBD2 expressions were

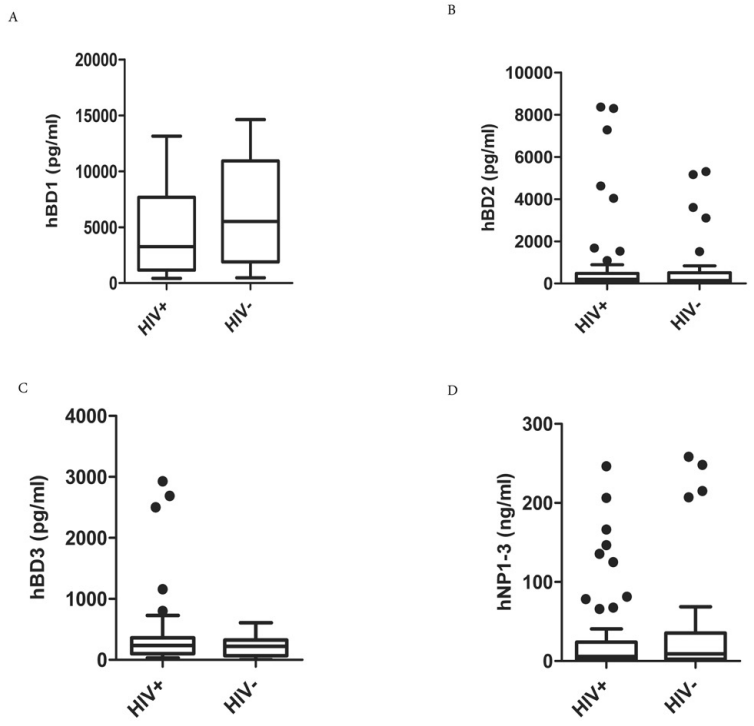


**Figure 1:** Comparison of antimicrobial peptide concentrations in CVLs WCR and WDR women.
Antimicrobial peptides level in CVLs were measured by ELISA and comparison between WCR (HIV+) and WDR (HIV-) women were done by Man-Whitney U test.

positively correlated in WCR group (r=0.52, p<0.0001) (Figure 2B). However, the correlation between the CVL levels of hBD2 and hBD3 in the WCR group was higher than that in the WDR group(r=0.30, p=0.13). HIV-associated microbial infections, such as other STIs and pro-inflammatory cytokines (e.g., TNF-alpha, IL-1β) might influence the higher co-regulated expression of hBDs in the female genital tract,

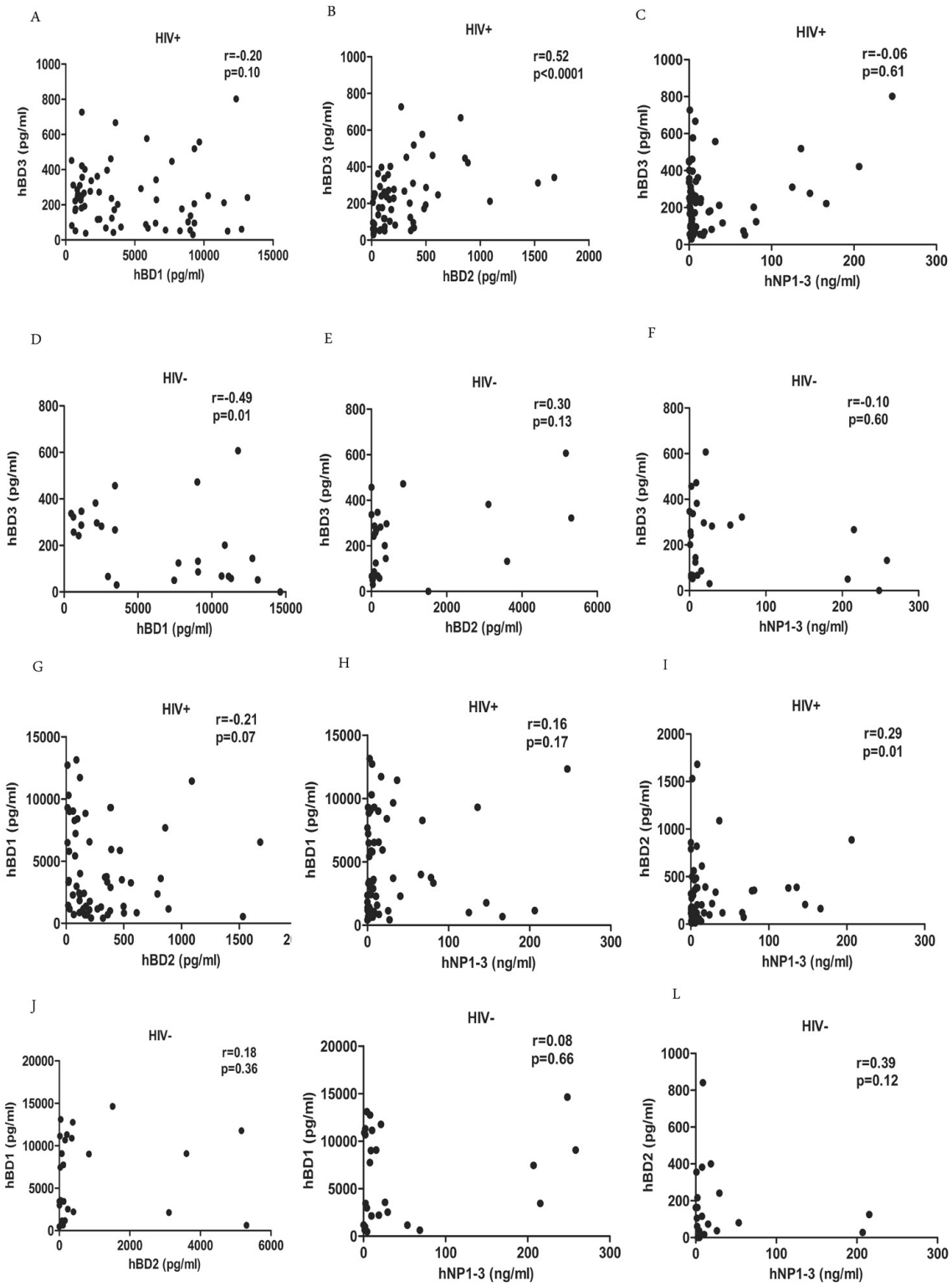


**Figure 2:** Correlation among CVL levels of antimicrobial peptide in WCR (HIV+) and WDR (HIV-) women.
Co regulated expression of different antimicrobial peptides in CVL of WCR (HIV+) and WDR (HIV-) women were analysed by using Spearman's rank correlation test.

suggesting a common regulatory mechanism for their expression [21-23]. In contrast, CVL levels of hBD1 and hBD3 correlated negatively in both groups of women (WDR, r= -0.49, p=0.01; WCR, r = -0.20, p= 0.10) (Figures 2A,2D). An inverse correlation between the above AMPs in the placenta of HIV positive women were also reported by an earlier study [24]. We speculated that there might be functional inhibition between hBD3 and hBD1. However, we did not experimentally validate our hypothesis.

**Comparison between pro-inflammatory chemokine IL-8 expression levels in the CVLs of WCR and WDR women and their association with antimicrobial peptide expression:** We evaluated how the expression of inflammatory chemokines in the CVLs differed between the WCR and WDR individuals and if this was linked to AMP expression. To address this issue, we measured the IL-8 protein in the CVLs by ELISA and found that its median concentrations in the above groups of women were not significantly different (333 pg/ml vs 264 pg/ml, p=0.38; Figure 3A). Next, we checked the association between the expression of individual antimicrobial peptides and IL-8. We observed positive correlation between hNP1-3 and IL-8 levels (Figures 3H,3I), while hBD3 expression was inversely correlated with IL-8 (r=-0.26, p=0.03; Figure 3F) in WCR, but not in WDR(r=-0.15, p=0.44) women.

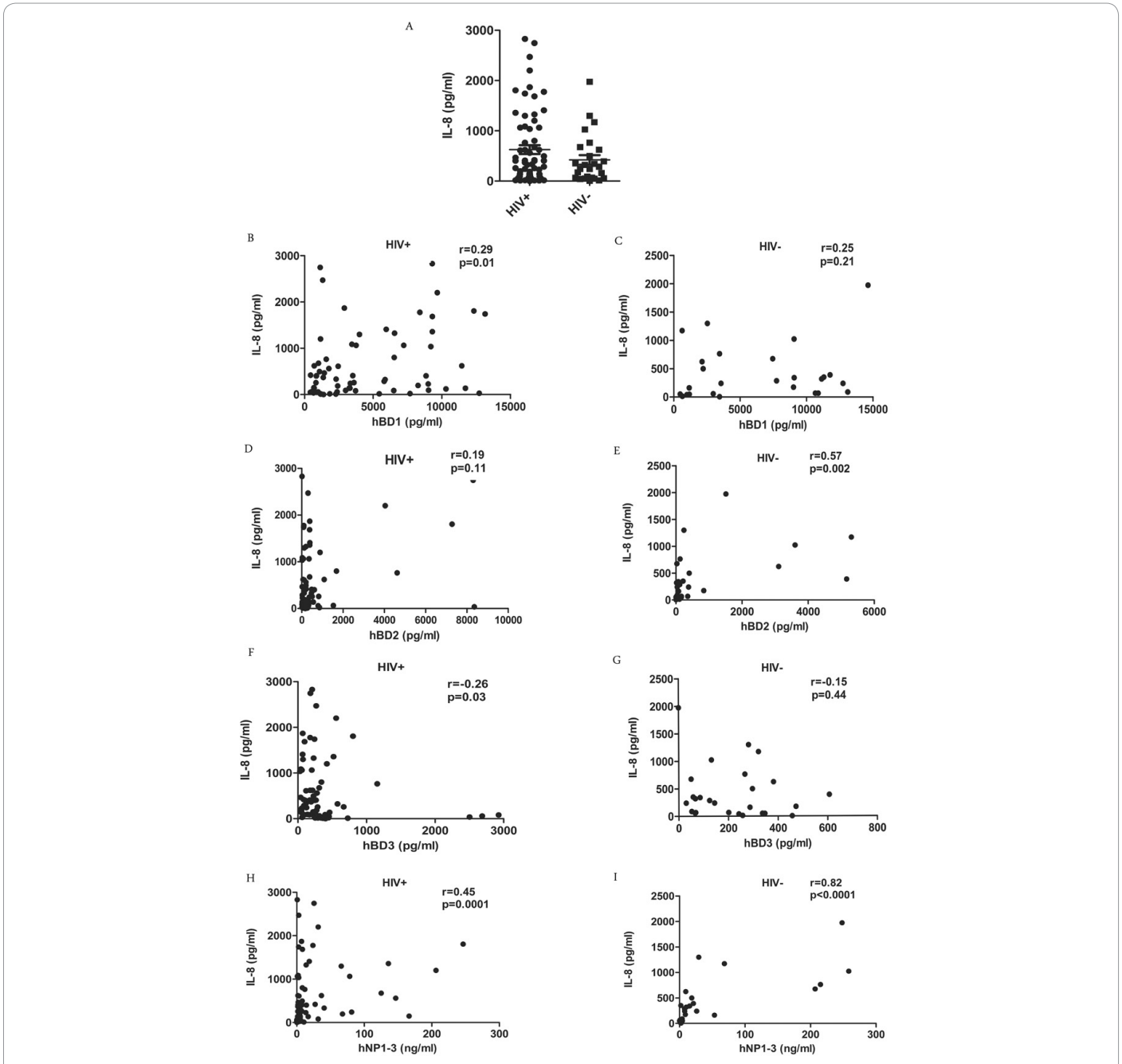


**Figure 3:** Comparison of IL-8 levels in CVLs between HIV+ WCR and WDR HIV- women.
CVL expressions of IL-8 were measured by ELISA and compared using the nonparametric Mann-Whitney U test. The association between the concentrations of IL-8 and antimicrobial peptides were calculated using Spearman's rank correlation test.

**Antimicrobial peptides inhibit TLR4-, TLR2- and CVL-induced proinflammatory responses *in vitro:*** Next, we assessed if cervico-vaginally expressed antimicrobial peptides have anti-inflammatory properties, in addition to their microbicidal functions. To address this issue, we performed *in vitro* studies with recombinant antimicrobial peptides, which possess similar functions as their natural counterparts [25]. Microbes carry evolutionary conserved structures like LPS, Peptidiglycan, DNA and flagellin, which induce pro-inflammatory response by activating host innate immune arm through the engagement of toll-like receptors (TLRs). TLR2 and TLR4 are most frequently involved host receptors in the induction of pro-inflammatory responses by microbial stimulation. We pre-treated human monocytic cell line THP-1-derived macrophages with one of the recombinant antimicrobial peptides at a time followed by LPS (ligand for TLR4) or PAM3 (Pam3CSK4; synthetic ligand for TLR2/1). We found that only hBD3 inhibited both LPS- and PAM3-induced TNF-alpha and IL-6 expression (Figures 4A,4B) as well as IFN-beta induction by LPS (Figure 4D). We evaluated the dose response of hBD3 on TNF-alpha expression and found that a dose of 10 µg/ml was required for the maximum effects (supplementary Figure 1). This dose of hBD3 did not induce TNF-alpha expression in THP-1 cells or exert any cytotoxic effects (supplementary Figure 1G,1F) and was used for all subsequent experiments. Since we had earlier observed that expression of one AMP was associated with the expression of the others, we checked whether anti-inflammatory properties of hBD3 were influenced by other CVL-expressed AMPs. To this end, we pre-treated THP-1 cells with different combinations of AMPs followed by LPS. We found that the inhibitory role of hBD3 on LPS-induced TNF-alpha expression was not influenced by other AMPs (Figure 4C).

Bacterial colonization of the cervico-vaginal tract may be different in HIV-infected and non-infected populations [26]. Microbial recognition by the host generally occurs via the TLRs. This results

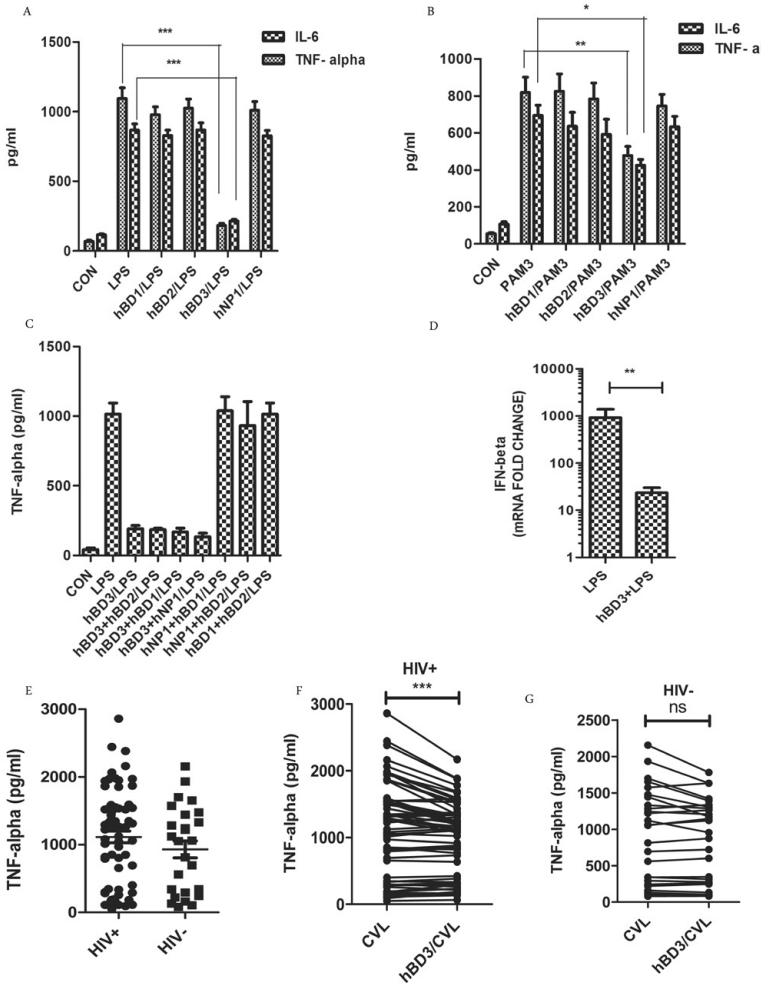


**Figure 4:** Antimicrobial peptides inhibit TLR4, TLR1/2 and CVL induced proinflammatory response *in vitro.*
(A-C) THP-1 cells were pre-stimulated with different antimicrobial peptides (10ug/ml), either alone or in combinations for 1 hour followed by LPS (200 ng/ml) or PAM3csk (100 ng/ml) treatment for 12 hours. TNF-alpha and IL-6 were measured in the culture supernatants by ELISA. Data represent mean +/- SD of two independent experiments. (D) Relative expressions of IFN beta mRNA (GAPDH normalized) was measured by RT-qPCR after 3 hrs of LPS treatment (mean +/- SD values representatives of two independent experiments). Significant differences comparing with or without addition of antimicrobial peptide were assessed by nonparametric Mann-Whitney U test and are indicated by *$P$< 0.05, **$P$ <0.01, and***$P$ <0.001. (E) THP 1 cell-derived macrophages were stimulated with CVLs from WCR (HIV+) and WDR( HIV-) women (10% of culture volume) for 24 hrs in the presence of gentamycin (50ug/ml). TNF-alpha were measured in the culture supernatants by ELISA and analysed by nonparametric Mann-Whitney U test: (WCR=67, WDR=26). (F, G) THP-1 cells were pre-stimulated with hBD3 (10 ug/ml), for 1 hour followed by CVLs from either WCR (F) or WDR (G) individuals; P value was calculated by the Wilcoxon matched-pairs test.

in the activation of NF-κB pathway, leading to the induction of pro-inflammatory molecules, such as TNF-alpha. We analyzed TNF-alpha expression to test the hypothesis whether TLR signalling pathways activated by CVLs from the HIV WCR and WDR women differ. To this end, we stimulated human THP-1 cell-derived macrophages, which express TLRs 1, 2, 4 and 6 with CVLs. Stimulation with WDR and WCR CVLs induced 1057 pg/ml (median, range=80–2156 pg/ml) and 1261 pg/ml (median, range=50–2,860 pg/ml), respectively of TNF-alpha. However, the difference was not statistically significant (p=0.28, Mann-Whitney U test) (Figure 4E). Next, to investigate if hBD3 could inhibit CVL-associated pro-inflammatory responses, we pre-treated THP-1 cells with hBD3 followed by WDR and WCR CVLs in the presence gentamycin (50 ug/ml) to inhibit CVL-derived microbial growth. Pre-treatment with hBD3 significantly suppressed TNF-alpha expression (p=0.0001, Wilcoxon matched-pairs test) by WCR individuals, but not by WDR women (p=0.26, Wilcoxon matched-pairs test) (Figure 4F,4G), suggesting that TLR signalling pathways activated by the CVLs of the above two groups might be different.

**hBD3 inhibits TLR4 signalling pathway:** Next, we checked the anti-inflammatory properties of hBD3 in the epithelial cells. For this purpose, we engineered HeLa cells, a cervical cancer cell line, to express a luciferase reporter gene driven by the NF-kB consensus binding sequences. Since these cells do not respond to LPS due to the lack of the co-receptor molecules, CD14 and MD2, we over-expressed them from plasmids before the cells were stimulated with LPS. Pre-treatment with different antimicrobial peptides showed that only hBD3 inhibited LPS-induced Nf-kB-luciferase activities in the Hela cells, further confirming its anti-inflammatory properties (Figure 5A).

It is known that downstream of TLR4, LPS activates both MyD88- and TRIF-dependent pathways. While MyD88-dependent signalling initiates from the cell surface TLR4 receptor and activate NF-kB and MAP kinases, TRIF-dependent pathways regulate IFN-beta production after endosomally-localized TLR4 is activated. CD14-mediated activation of SYK regulates endosomal localization of TLR4, while inhibitors may abrogate the activation of either or both of the MyD88 and TRIF signaling pathways by LPS. We observed that hBD3 abrogated LPS-induced activation of NF-kB and MAP kinases, as evidenced by the lack of phosphorylation of MAPKs and failure of IKB-degradation (Figure 5B). In addition, it inhibited TBK activation by blocking activation of SYK (Figure 5C).

**Protein kinase A (PKA)-dependent pathways are partly involved in hBD3-mediated inhibition of LPS signalling:** hBD3 induces cyclic AMP (cAMP) by activating melanocortin receptor [27], while cAMP-activated PKA was reported to inhibit LPS-induced TNF-alpha expression [28] .To investigate if PKA was responsible for the inhibitory role of hBD3, we blocked PKA activation by pre-treating the cells with H89, a commercially available potent PKA inhibitor. The results showed partial reversal of the inhibitory effects of hBD3 on TNF-alpha and IL-6 expression by LPS, but no alteration of IFN-beta production (Figures 6A-6C). To further confirm the role of PKA, we directly evaluated its activation by hBD3, and also the activation of its downstream target CREB. The results showed activation of both

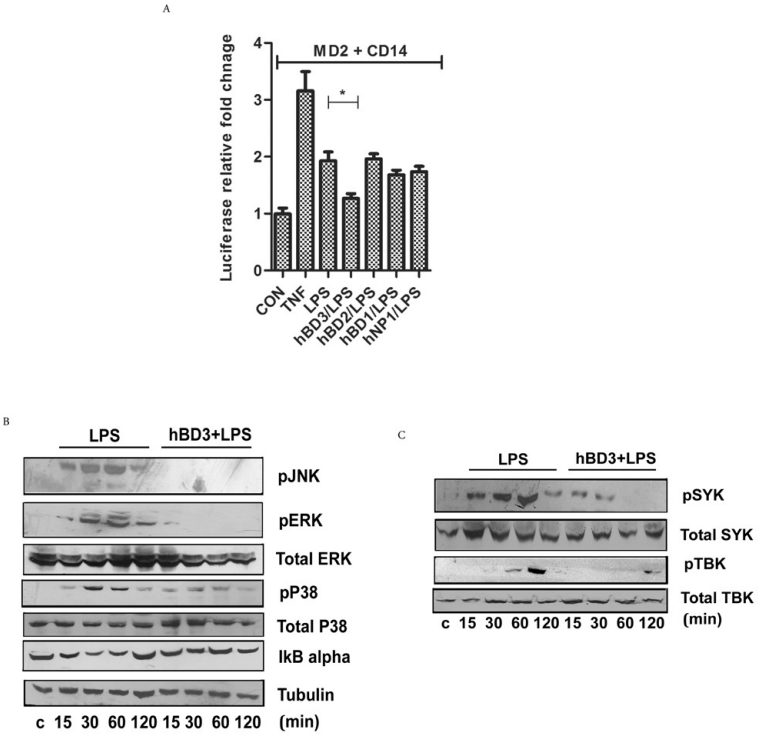


**Figure 5:** Antimicrobial peptides inhibit TLR4 signalling pathways.
(A) Hela cells were transfected with NF-kB-luciferase construct along with CD14 and MD2. Renila lucifearse was used as transfection control. Cells were pre-treated with different anti microbial peptides (10ug/ml) for 1 hour and luciferase assay was performed after 8 hours of LPS treatment. Graph shows fold changes of firefly luciferase activities compared to the unstimulated cells and normalized against renilla luciferase activities (data represent mean ± SD values of two independent experiments analysed by Mann-Whitney U test). (B, C) THP1 cells were stimulated with LPS (200ng/ml) with or without pre-treatment of hBD3 (10ug/ml) for 1hr. Activation of MAPKs, SYK and TBK was analyzed by the phosphorylation of the kinases and NF-kB activation was studied by the degradation of IkBα using western blots. Data are representative of one of the three independent experiments.

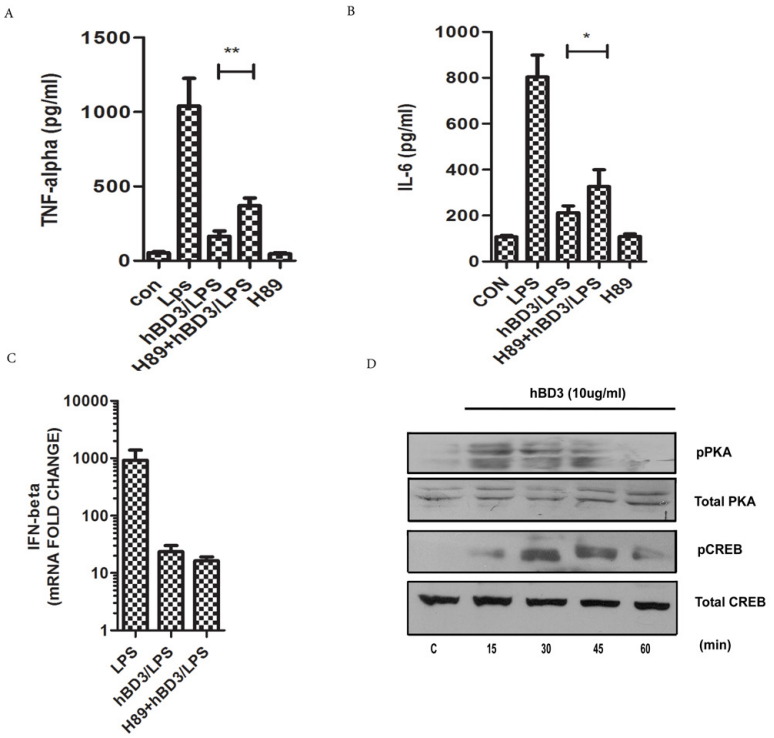


**Figure 6:** PKA dependent pathways are partially involved in hBD3 mediated inhibition of LPS signalling.
THP1 cells were pretreated with hBD3 with or without H89, a PKA inhibitor (10μM) and hBD3 (10ug/ml) together or hBD3 alone for 1hr before the addition of LPS (200ng/ml). (A, B ) IL-6 and TNF-alpha were measured by ELISA 12hrs after LPS treatment. (C) IFN-beta expression was analyzed after 2hrs of LPS stimulation by Real time PCR and normalized against GAPDH expression (Mean ± SD of at least two independent experiments). (D, E) THP1 cells were treated with hBD3 (10μg/ml) for the indicated time points and phosphorylation of PKA and CREB was analysed by Western blots. Significant differences comparing with or without addition of H89 inhibitor and hBD3 were assessed by nonparametric Mann-Whitney U test and are indicated by **P*<.05, ***P*<.01.

PKA and CREB by hBD3 treatment (Figure 6D). However, cAMP-PKA pathways were only partially involved in mediating the inhibitory role of hBD3 on LPS-induced proinflammatory cytokines expression and additional mechanisms might co-exist.

## Discussion

We found that the expressions of AMPs in the CVL were not different between the WCR and WDR individuals recruited from West Bengal, an Eastern Indian State. hNP1-3 was the most abundant AMP found in the CVL samples collected from these groups of women living in either HIV concordant or HIV discordant relationship with their husbands. However, median expression of hBD1 and hNP1-3 tended to be higher in the HIV WDR individuals compared with their WCR counterparts. Expression of hBD2 and hBD3 remained low irrespective of the HIV status of the women. We found positive correlation between hBD2 and hBD3 in the CVLs of WCR population, whereas expression of hBD1 and hBD3 was negatively correlated in the study population. In addition, there were significant inverse correlations between IL-8 and hBD3 levels in the CVLs of the WCR group. Finally, hBD3 inhibited the activation of TLR4 and TLR1/2 signalling and the induction of TNF-alpha production by the CVLs of WCR women.

Analysis of married women living in HIV concordant and discordant relations may identify therapeutically relevant host factors, which inhibit HIV infection in the latter group. A research group from Western India studied the issue of innate immunity in HIV sero-negative female sex workers (FSWs) who had enhanced vulnerability to HIV infection due to their occupational character. They found increased activation state of the NK cells and plasmacytoid dendritic cells (pDCs) in the above Indian cohort [29] .

Earlier studies reported hNP1-3 as the most abundantly expressed AMPs in the CVLs of a cohort of sex workers in Keniya [15]. We also found that hNP1-3 levels were higher than other AMPs in our study population. However, no significant difference in the levels between the two groups of women was detected despite the evidence for anti-HIV functions of hNP1-3 and the expectations that the levels might be lower in the HIV-infected population. One possible explanation may be the proneness of HIV-infected individuals to infection, which may elevate hNP1-3 levels [30,31]. However, we did not address this issue in the present study.

Since our expression data suggest that hNP1-3 levels tend to be higher in the HIV discordant women (Figure 1D), it is tempting to speculate that increased concentrations of hNP1-3 in these individuals may contribute to their apparent resistance to HIV infection. Previous studies showed association of hNP1-3 levels with decreased chances of mother to child transmission of HIV [32]. It remains to be examined involving larger samples of population if such association reaches statistical significance for heterosexual couples as well. However, hBD2 and hBD3 levels were lower than those of hBD1 and hNP1-3 and similar concentrations were found for the HIV concordant and discordant women in our study population. Researchers in the past had shown that hBD2 and hBD3 can inhibit R5 and X4 HIV infection in a dose-dependent manner [33], although the doses were higher than the physiological concentration found in the CVL. Our data indicate that hBD2 and hBD3 may not be associated with HIV discordance in the married Indian women.

We found higher correlation of hBD3 expression with hBD2, than other AMPs. This indicates the sharing of common signalling mechanism for the induction of hBD2 and hBD3 [23]. Published reports suggest that hBD2 and hBD3 expression depends on either microbial stimulation or inflammatory cytokines, a finding consistent with our observation. In our study, the negative correlation between hBD1 and hBD3 expression was more in the discordant group, which might result from a shift in the microbial composition or hBD3 induction by inflammatory cytokines. A negative correlation between hBD1 and hBD3 was also reported in the placentas from HIV-1-infecetd mothers [24]. Thus, co-regulated antimicrobial peptide expression differed between the two groups of Indian women, although the absolute levels of individual peptides were quite similar.

We measured an abundantly-expressed pro-inflammatory chemokine, IL-8 in the CVLs and found no significant difference between the WCR and WDR women. This is consistent with the previous reports [19]. However, wide dispersion of IL-8 among HIV-infected women of our study population indicates immune dysregulation. Other pro-inflammatory cytokines, such as TNF-alpha and IL-6 were undetectable, due to either low levels of expression or dilution by the large volume of PBS (10 ml) used for collecting CVLs in our study. Several regulatory cytokines/chemokines may influence the levels of AMPs in the CVLs. Madhuri thakar et al. has identified association between higher TGF-beta and lower AMP (Elafin and SLPI) levels at the genital mucosal surface of HIV-infected Indian women [34]. We found that IL-8 expression was positively correlated with hNP1-3, but inversely correlated with hBD3 expression in the HIV-infected study population. These findings indicate interdependent regulatory mechanisms for cytokines/chemokines and AMP expression. Considering the potent antimicrobial effects of hBD3, an inverse correlation of its expression with IL-8 suggests that higher hBD3 levels might lead to increased killing of pathogens [35] and lower pro-inflammatory microbial loads, leading to lower IL-8 secretion by the epithelium or immune cells. Other possibility is that hBD3 directly interfered with the signalling pathways responsible for IL-8 expression.

We addressed by cell-culture assays the complex relations between hBD3 and inflammatory response in the WCR women. The purpose was to evaluate the anti-inflammatory properties of the AMPs expressed in the CVLs. Microbial components like LPS, peptidoglycan, teichoic acid, DNA and HIV structural protein gp120 may induce pro-inflammatory cytokines/chemokines after being recognized by the pattern recognition receptors including TLRs expressed by vaginal epithelial and immune cells [36]. We used widely-expressed microbial components, LPS (TLR4 ligand), PAM3 (Pam3CSK4; a synthetic lignad for the activation of TLR1/2 signalling), bacterial DNA (ligand for TLR9) and flagellin (ligand for TLR5) to evaluate anti-inflammatory properties of the AMPs expressed in CVLs. Among the AMPs studied by us, hBD3 inhibited both LPS- and PAM3-induced signalling in THP-1-derived macrophages and TLR4 signalling in cervical epithelial cells. Previous reports also suggested that hBD3 may inhibit TLR4 signalling [37]. We observed that a concentration of 1μg/ml of hBD3 is capable to inhibit LPS-induced TNF-alpha expression, while a higher dose (10 μg/ml) is required for the maximum effects (supplementary Figures 1A,1C). This may correlate with hBD3 levels in the FGT mucosa. That our measured concentration of hBD3 in the CVLs was much lower could be explained by the fact that the mucosal secretions were significantly diluted in the CVLs, which were collected after instillation of 10 ml of PBS into the FGT. Moreover, hBD3 is further induced during genital infection, frequently found in women infected with HIV that would correspond to the experimental context of suppression of TLR4 activation *in vitro*. Earlier studies reported that mucosal hBD3 concentration may reach as high as 100 ug/ml [33].

We examined the stimulatory capacity of CVLs from WCR and WDR women and whether hBD3 could inhibit CVL-associated inflammatory response in addition to direct killing of microbes. We were unable to find any significant difference between the inflammatory responses to CVLs from the two groups of women. Stimulatory capacity of CVLs may be attributed to the activation of host pattern recognition receptors, such as TLRs by the molecules derived from the vaginal microbial flora, which comprises of several species of lactobacilli [38]. A difference in the vaginal flora may lead to differential activation of the TLR signalling pathways and different cytokine/chemokine production [39]. Klebanoff et al. (1999) have shown that *L. crispatus*, which is present in the normal vaginal flora, induces TNF-alpha secretion and activation of NF κB [40]. We found that hBD3 significantly inhibited TNF-alpha expression induced by CVLs (from WCR) induced. Debra Mares et al. has shown that bacterial ligand(s) present in the vaginal tract can stimulate TLR2 [41]. Another study by Mirmonsef P et al. [42] has shown that CVLs from bacterial vaginosis (BV) [18], a condition frequently associated with HIV infection, can induce TLR4- and TLR2-dependent inflammatory signals. They found that neutralizing antibody to TLR2 only slightly reduced stimulation of the myeloid cell lines, while antibody to TLR4 reduced IL-8 production by 30%–60% [42]. Recently, researchers have noted that HIV-1 gp120 induces TLR2- and TLR4-mediated innate immune activation in human female genital epithelium [43]. These observations indicate that hBD3 may inhibit CVL-induced proinflammatory response by inhibiting TLR 1/2 and TLR4 signalling pathways.

It is known that binding of LPS to TLR4 activates both MyD88 and TRIF dependent pathways. Our findings of hBD3-mediated inhibition of LPS-activated pathways are consistent with the previous reports [44]. This may be partially achieved through PKA-mediated inhibition of NF-kB activation or CREB-mediated sequestering of p300 and CBP, which are required for optimal NF-kB activation [45]. Detail mechanism of hBD3-mediated inhibition of TLR4 and TLR1/2 signalling is outside the scope of this paper and requires further elaborative study.

Our study found no significant relations between the levels of four AMPs in the CVL and HIV infection status of married Indian women whose husbands were living with HIV. However, co-regulated expression of AMPs differed between WCR and WDR women. The underlying mechanisms of the above phenomenon and its relations to HIV infection remain unclear. We also found an inverse relation between IL-8 and hBD3 levels in the CVL of HIV-infected women. IL-8 is critical for inflammatory response that confers protection against infection. However, a tight regulation of inflammation is required to prevent host damage due to excessive inflammation. Whether hBD3 plays a deciding role in the above regulation needs to be further investigated.

Our data indicate that AMPs may inhibit LPS and PAM3 signalling. Chronically HIV-infected individuals and SIV-infected rhesus macaques exhibit increased levels of circulating LPS. Specifically, HIV-infected patient with <200 CD4 cells/μl shows significantly higher level of LPS than uninfected individuals [46]. *In vitro* exposure to microbial and viral PAMPs may cause activation-induced apoptosis of CD4+ and CD8+ T cells [47]. LPS-mediated persistent activation of immune system is one of the major contributing factors to progression to AIDS. How systemic expression of imunomodulatory antimicrobial peptides (such as LL37, hBD3) associate with persistent immune activation still remains a matter of investigation. Although hBD3 is predominantly

expressed at the mucosal surfaces, it was also found in the systemic circulation. Our preliminary data indicate systemic presence of hBD3 in the serum of HIV infected patients with wide variations of its levels. Whether immunomodulatory AMPs determine the state of immune activation in HIV-infected patients and the therapeutic potential of AMPs to delay progression to AIDS merit detail investigation.

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