Surfactant Protein D Reverses the Gene Signature of Transepithelial HIV-1 Passage and Restricts the Viral Transfer Across the Vaginal Barrier

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Effective prophylactic strategy against the current epidemic of sexually transmitted HIV-1 infection requires understanding of the innate gatekeeping mechanisms at the genital mucosa. Surfactant protein D (SP-D), a member of the collectin family of proteins naturally present in the vaginal tract, is a potential HIV-1 entry inhibitor at the cellular level. Human EpiVaginal tissues compartmentalized in culture inserts were apically exposed to HIV-1 and/or a recombinant fragment of human SP-D (rfhSP-D) and viral passage was assessed in the basal chamber containing mononuclear leukocytes. To map the gene signature facilitating or resisting the transepithelial viral transfer, microarray analysis of the HIV-1 challenged EpiVaginal tissues was performed in the absence or presence of rfhSP-D. Mucosal biocompatibility of rfhSP-D was assessed ex vivo and in the standard rabbit vaginal irritation model. The passage of virus through the EpiVaginal tissues toward the underlying target cells was associated with a global epithelial gene signature including differential regulation of genes primarily involved in inflammation, tight junctions and cytoskeletal framework. RfhSP-D significantly inhibited HIV-1 transfer across the vaginal tissues and was associated with a significant reversal of virus induced epithelial gene signature. Pro-inflammatory NF-κB and mTOR transcripts were significantly downregulated, while expression of the tight junctions and cytoskeletal genes was upheld. In the absence of virus, rfhSP-D directly interacted with the EpiVaginal tissues and upregulated expression of genes related to structural stability of the cell and epithelial integrity. There was no increment in the viral acquisition by the PBMCs present in basal chambers wherein, the EpiVaginal tissues in apical chambers were treated with rfhSP-D. The effective concentrations of rfhSP-D had no effect on lactobacilli, epithelial barrier integrity and were safe on repeated applications onto the rabbit vaginal mucosa. This pre-clinical safety data, coupled with its efficacy of restricting viral passage via reversal of virus-induced gene expression of the vaginal barrier, make a strong argument for clinical trials of rfhSP-D as a topical anti-HIV microbicide.

Keywords: surfactant protein D, HIV-1, vaginal, microarray, chemokines, microbicide
INTRODUCTION

A clear majority of the HIV-1 infections are due to heterosexual contact; more than 50% of HIV-1 infected individuals are women and most children living with HIV-1 today are infected via mother-to-child transmission (1). Thus, an effective vaginal microbicide for the prevention of sexual transmission of HIV-1 to women will have a huge impact on limiting the HIV epidemic and its devastating consequences for both adults and children. Despite this well-perceived need of intervention and the efforts made to date in understanding the vaginal mucosal barrier (2–4), the development of a safe and effective topical vaginal microbicide has several technical challenges (5–7). Clinical trials involving most of the promising candidates showed reduced efficacy as they adversely affected the vaginal milieu (7). Evaluation of microbicides in vivo using SIV-macaque and humanized mouse models comes at a high cost and the findings may only be an extrapolation to HIV-1 transmission in humans (7). A serious limitation is lack of an appropriate ex vivo model for the evaluation of efficacy of potential compounds on the viral passage across the vaginal barrier to the target immune cells (8–11). The ex vivo model should also assess compatibility of the candidate molecules with the mucosal integrity and barrier function including the colonization with healthy vaginal microbiome.

Of special interest for pharmaceutical development are candidate microbicides that would regulate vaginal innate immune responses with minimal adverse effects on the physiology (12, 13). Collectins are a group of secreted, antimicrobial pattern recognition proteins in the female reproductive tract (14–17). Surfactant Protein D (SP-D) is one such collectin expressed by the epithelium, lining the vaginal tract (18). Previously, we have demonstrated that a recombinant fragment of human SP-D (rfhSP-D) containing homotrimeric neck and C-type lectin domains binds to HIV-1 envelope glycoprotein gp120, and inhibits viral entry and replication in target immune cells (19). Beyond its pattern recognition capability, SP-D interacts with various immune cells, maintains Th1/Th2 balance in the lungs and induces immune quiescence (20, 21). By virtue of its natural presence in the vaginal tract, broad anti-microbial activity and immune-regulatory functions, SP-D is a unique microbicide candidate. Importantly, anti-HIV-1 activity of rfhSP-D was intact in physiological fluids like vaginal lavage and seminal plasma which comprise of diverse enzymes, pH and inhibitors (19).

In this study, we assessed the effect of rfhSP-D on the interactions of vaginal epithelial tissues and HIV-1 using a rational scheme for ex vivo microbicidal testing. The scheme is designed to resemble sexual transmission of the virus and comprises of bioengineered vaginal tissues, immune cells and clinical isolates of Lactobacillus. In our model, HIV-1 traverses through the intact, multi-layered vaginal epithelium toward the underlying mononuclear leukocytes. We report, for the first time, a “gatekeeping” gene signature of bioengineered human tissues induced upon HIV-1 exposure. In this model, rfhSP-D showed no adverse effects on the vaginal barrier, concomitant with a significant impediment of viral movement to the activated PBMCs in the basal compartment. Epithelial transcriptome revealed reversal of HIV-1 induced differential expression of genes associated with the cytoskeleton, inflammation and barrier integrity. A range of preclinical assays confirmed safety of rfhSP-D for vaginal application at the similar concentrations it restricted viral transfer ex vivo, and thus, establishing it as a promising anti-HIV-1 vaginal microbicide.

MATERIALS AND METHODS

Human Cell Lines

Well-characterized and immortalized human vaginal (Vk2/E6E7, ATCC® CRL-2616™), endocervical (End1/E6E7, ATCC® CRL-2615™), and ectocervical (Ect1/E6E7, ATCC® CRL-2614™) cell lines developed by Dr. Raina Fichorova (22), were cultured in antibiotic-free keratinocyte serum-free medium (KSFМ), supplemented with 50 µg/ml bovine pituitary extract, 0.1 ng/ml epidermal growth factor (Gibco, Invitrogen, USA), and 0.4 mM CaCl2 (Fisher Scientific, USA). These cell lines are known to retain their physiological characteristics and are useful models for various female reproductive tract infections, including HIV-1 (22–26).

Vaginal Bioengineered Tissue (EpiVaginal Tissue)

Twenty four EpiVaginal™ (VEC-100™) tissues and medium were purchased from MatTek (Ashland, MA, USA). These tissues are derived from primary human ectocervical/vaginal epithelial cells, and possess characteristics comparable to that of the normal tissues of origin (26, 27).

Clinical Lactobacillus Isolates

Lactobacillus crispatus isolates were obtained from vaginal swab samples of healthy women participating in a vaginal microbiota research study at the Brigham and Women’s Hospital (Boston, MA, USA) (6). Lactobacillus fermentum spp mucosae (TRF#36), Lactobacillus gasseri (TRF#8), and Lactobacillus salivarius (TRF#30) were a kind gift from Prof. GP Talwar, the Talwar Research Foundation (New Delhi, India) (28).

Preparation of rfhSP-D

A recombinant fragment of human SP-D (rfhSP-D), composed of trimeric neck and lectin domains along with 8 Gly-X-Y repeats, was expressed in E. coli, purified and characterized, as described previously (19, 29, 30). The endotoxin level in the rfhSP-D preparations was determined using the QCL-1000 Limulus amebocyte lysate system (BioWhittaker Inc., USA). The endotoxin concentration in the various preparations ranged between 2.8 and 5.1 pg/µg of rfhSP-D. Controls of various experiments were spiked by adding equivalent amounts of LPS (Sigma-Aldrich, USA).

Assessment of the Expression of SP-D in Human Vaginal Cells (VK2/E6E7) and Cervicovaginal Lavage (CVL)

To assess the presence of SP-D in CVL, total protein was precipitated using chilled acetone; 25 µg total protein was loaded per well and subjected to 12% SDS-PAGE under
reducing conditions and then electrophoretically transferred to a nitrocellulose membrane for immuno-blotting. Mouse monoclonal anti-human SP-D antibody (Abcam, UK) was used at a dilution of 1:500, whereas, rabbit polyclonal anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP) was used at a dilution of 1:1,000 (Dako). Detection was done using chemiluminescent detection kit (Amersham Biosciences, Piscataway, NJ). For immunostaining, Vk2/E6E7 cells were grown on cover slips, probed with the mouse monoclonal anti-human SP-D antibody (Abcam) and further detected with anti-mouse Phycoerythrin-conjugates (Molecular Probes). Nuclei were counterstained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich), the coverslips were mounted in Vectashield (Vector Laboratories) and visualized under a confocal microscope (Zeiss, Germany). To determine transcript levels of SP-D, total RNA was extracted using Trizol (Invitrogen) from Vk2/E6E7 cells; 3 μg of total RNA was reverse transcribed into cDNA using Superscript III first strand synthesis kit (Invitrogen) and subjected to PCR (Veriti Machine, Applied Biosystems). Primers for SP-D, SP-A2, and 18S were designed using NCBI Primer BLAST Software (Supplementary Table 1). The resultant PCR products were electrophoresed on a 2% agarose gel at 100V on electrophoresis. The bands were detected via ethidium bromide under UV light.

**Ex vivo Model of Vaginal HIV-1 Transmission**

In order to mimic vaginal transmission of HIV-1, a novel ex vivo model was developed using EpiVaginal tissues (Figure 1A). Upon delivery, EpiVaginal tissues were acclimatized in the medium overnight. Blood (from non-autologous donors) was subjected to Ficoll separation and PBMCs were isolated. PBMCs were activated for 48 h using rhIL-2 (100 U/ml) (Sigma-Aldrich) and PHA (5 μg/ml) in the RPMI 1640 medium (Fisher Scientific) containing 10% FBS and 0.5% antibiotic solution (Gibco, Invitrogen). Activated PBMCs (10⁵) were seeded in a 12-well plate as target cells for further replication of migrated virions. In a fresh tissue culture plate, inserts containing EpiVaginal tissues were placed in each well. In our previous study, we have reported the anti-HIV activity of purified native human SP-D, rfhSP-D and another variant of recombinant fragment of SP-D, lacking eight triplets of collagen repeats (delta-rfhSP-D) (19). Although delta-rfhSP-D showed anti-HIV activity in the TzMirb reporter assays (IC₅₀ 43.282 ± 10.76 μg/ml) it was 3-fold less potent than the native human SP-D and rfhSP-D (IC₅₀ of rfhSP-D with various viral isolates and target cell types ranged between 6.726 ± 0.63 and 13.676 ± 3.37 μg/ml) (19). In another assay (as described in the section “Viability MTT assay”), we evaluated the effect of various concentrations of rfhSP-D (1.562–100 μg/ml) on the viability of vaginal epithelial cells and used the maximal tolerated dose of rfhSP-D (100 μg/ml) in the ex vivo model. The physiological concentration of free SP-D in various body fluids ranges from 0.5 to ~3 μg/ml (31, 32). In view of the ability of SP-D to bind several molecules, such as immunoglobulins, fatty acids and nucleic acids, its total physiological concentration is expected to be much higher (33–35). Apical tissues were treated with rfhSP-D (100 μg/ml) or a synthetic analog of *Mycoplasma fermentans* lipopeptide macrophage activating lipopeptide 2 (MALP-2) (Alexis Biochemicals, USA) (25 nM) for 20 min before inoculation with 100 TCID₅₀ R5 tropic HIV-1R-M-CSF. After 24 h incubation, apical and basal supernatants were collected for determining levels of immune mediators. Basal supernatants were used to determine HIV-1 p24 Ag by ELISA, as per manufacturer’s instructions (R&D Systems).

**Microarray Gene Expression Analysis**

The microarray data, described in this study, has been deposited in the NCBI Gene Expression Omnibus (GEO) under the GEO series accession number GSE107478.

**RNA Isolation**

Total RNA was extracted using TRIZOL® Reagent (Invitrogen); RNA quantity and quality were determined using Nanodrop® spectrophotometer (NanoDrop Technologies, Wilmington, DE). Targets were prepared using the Illumina RNA amplification kit (Ambion, Austin, TX). cRNA was synthesized from 200 ng of the total RNA followed by amplification and labeling steps. Amplified biotin-labeled cRNA was hybridized to the Illumina Human HT12 V6 bead chip. Illumina Bead Studio was used to extract the raw data from the bead chip. Raw data was Quantile normalized and baseline transformation was carried out to obtain median of all samples using GeneSpring GX 12.5 software (Agilent Technologies Inc, Santa Clara, USA).

**Statistical Analysis and Differentially Expressed Genes**

Differentially expressed probe sets (genes) in the treated cells in comparison to the untreated cells were identified by applying Volcano Plot using a fold-change threshold (absolute fold-change >1.5). A statistically significant “t-test” “P-value” threshold was adjusted for false discovery rate of <0.001. Statistically significantly enriched transcripts with a “P-value” adjusted for false discovery rate of <0.05, based on the hyper-geometric distribution test corresponding to differentially expressed genes, were determined using the Student’s “t-test” along with Benjamini Hochberg FDR test. Unsupervised hierarchical clustering of the differentially expressed genes following treatment in comparison to the untreated cells was performed using Euclidian algorithm with Centroid linkage rule to identify gene clusters whose expression levels were significantly reproduced across the replicates.

**Biological Pathways and Gene Ontology Enrichment Analysis**

Differentially expressed gene list was subjected to a biological significance analysis by GOElite tool. A total of 21,887 protein coding genes were used as the background and the differentially expressed gene list was used as query. Database of GeneOntology categories, Wikipathways, KEGG Pathways, Pathway Commons, Pheno Ontology, Diseases, Protein Domains, Transcription factor targets, and tissue expression were configured for significance analysis. Each query list was subjected to the “Over-representation Analysis” against each of the above databases.
Z score and permutation or Fisher’s Exact Test p-value were calculated to assess over-representation of the enriched biological categories.

Biological Analysis Network Modeling of Differential Regulome

Enriched biological categories, along with the differentially expressed genes, were used as input for BridgeIsland Software (Bionivid, Bangalore, India) for identifying the key edges that connect genes with biological categories. Statistical scores from differential expression and biological analysis were used as attributes to visualize the network. Output of BridgeIsland Software was used as input to Cytoscape V 2.8. Circular layout and yFiles algorithm were used to visualize the network that encompasses biological categories. Further to this core network, all the differentially expressed genes were colored based on their fold change to reflect the rhSP-D treatment induced differential regulome.

Validation by Real Time RT-PCR

Since, EpiVaginal tissues (of ectocervical origin) used in the assays were sufficient enough for microarray analysis, we carried out the validation of microarray data using an ectocervical cell line (Ect1/E6E7) under conditions similar to the ex vivo model of HIV-1 transmission (same MOI). Cells were seeded in a 96-well plate, grown up to confluence and then treated with rhSP-D (100 µg/ml) for 20 min, before inoculation with 100 TCID<sub>50</sub> R5 tropic HIV-1JR-CSF at 37°C for 24 h. Total RNA was isolated using Trizol (Invitrogen) and the quality of RNA was assessed by nano-spectrophotometry and the nucleotide: protein ratio (260:280) was determined. 1–3 µg of RNA was reverse transcribed into cDNA using Superscript III first strand synthesis kit (Invitrogen). The resulting cDNA was used for real time PCR via the Bio-Rad CFX96 TouchTM real-time PCR detection system using the iQTM SYBR Green Supermix (Bio-Rad, USA). 18s RNA was used as the housekeeping control. Primers were designed using NCBI Primer BLAST Software. Primer sequences and conditions are provided in the Supplementary Table 1.

mRNA Levels of Tight Junction Proteins in EpiVaginal Tissues After HIV-1 Challenge

In order to determine the status of the vaginal barrier after the viral challenge, transcripts from EpiVaginal tissues for the tight junction proteins viz. Claudin 2, 3, 4, 5, and occludin were quantified using real time qPCR. Owing to the limited EpiVaginal tissue, the qPCR analysis was not extended to quantitation of protein levels. Primers sequences were synthesized (Sigma-Aldrich) as reported previously (36). Primer sequences and conditions are provided in the Supplementary Table 1.
Susceptibility of PBMCs to HIV-1 Acquisition

HIV-1 is known to replicate faster in the activated human PBMCs (37). We have shown previously that rfhSP-D rhSP-D does not alter the activation alter the activation of PBMCs and leads to induction of quiescence in the activated PBMCs (21). The present assay was designed to specifically determine the impact of supernatants from rhSP-D treated EpiVaginal tissues on the activation status and viral acquisition of PBMCs. Non-activated PBMCs (10^5) were seeded in a 12-well plate and apical regions of the culture inserts containing EpiVaginal tissues were treated with rhSP-D (100 μg/ml), MALP-2 (25 nM), or left untreated for 24 h. Following incubation, basal PBMCs were collected and challenged with 100 TCID50 HIV-1 for 4 h to assess the rate of HIV-1 acquisition (Figure 1B). PBMCs were washed and cultured further in RPMI 1640 medium containing 10% FBS and 1% antibiotic solution for 7 days, and HIV-1 p24 levels in culture supernatants were measured. Viability of PBMCs was evaluated at the end of the assay (data not shown).

MTT Viability Assay

To assess the likely effect of rfhSP-D on cell viability, MTT assay was performed on Vk2/E6E7 and Ect1/E6E7 cell monolayers. Cells were seeded in a 96-well plate, grown up to confluence and then treated with a range of rfhSP-D concentrations at 37°C for 24 h. Culture supernatants were then collected for measuring immune mediators. Cells were treated with 1 × MTT containing KSFM and incubated overnight; 0.04 N acidified isopropanol was added to the cells to dissolve the formazan crystals. This color intensity, read at OD570, is directly proportional to the number of viable cells, as measured by a Victor2 counter with Wallac 2.01 software (PerkinElmer Life Sciences, USA) using a reference wavelength at 630 nm. The OD of untreated (medium alone) control cells was considered as 100%; percent viability of rfhSP-D treated cells was calculated as compared to untreated control.

NF-κB Luciferase Assay

End1/E6E7 immortalized epithelial cells were transfected with pHTS-NF-κB firefly luciferase reporter vector (Biomyx Technology, USA) using a gene-juice transfection protocol (38). Cells were seeded in a 96-well plate, grown until confluent monolayers and treated with indicated concentrations of rfhSP-D for 24 h at 37°C. A synthetic analog of viral double-stranded RNA, Poly (I:C) (10 μg/ml) (InvivoGen, USA), a TLR3 agonist, and MALP-2 (25 nM), a TLR2/6 agonist, were used as positive controls. After incubation, the supernatant was removed, cells were lysed in GloLysis buffer, and activation of luciferase was determined using a Bright-Glo luciferase assay system (Promega, USA). Luminescence signal was quantified via a Victor2 1420 multi-label microplate counter with Wallac 2.01 software (PerkinElmer Life Sciences).

Assay for Toxicity to Lactobacillus

Direct toxicity assay on vaginal lactobacilli was performed using a colorimetric assay as described previously (39). TRF#8, TRF#30, TRF#36, and Lactobacillus crispatus LC223 were grown in the Lactobacillus MRS Broth (HiMedia™ Laboratories). Bacterial density was adjusted to an OD670 of 0.06, corresponding to a 0.5 McFarlands turbidity standard or ca. 10^8 CFU/ml. RfhSP-D was plated at the appropriate concentrations into a 96-well round bottom plates in a volume of 100 μl, and the diluted Lactobacillus spp were added in a volume of 100 μl. Commercially available penicillin-streptomycin solution (Gibco, Invitrogen) at a maximal test concentration of 1.25 U/ml and 1.25 μg/ml respectively) was used as a positive control for toxicity. Plates were incubated in an orbital shaker at 35°C under anaerobic conditions using AnaeroPack system (PML Microbiologicals, Wilsonville, OR) for 24 h. Bacterial growth was determined by measurement of the OD590 using a Victor2 counter with Wallac 2.01 software (PerkinElmer Life Sciences) (40).

Lactobacilli-Epithelial Colonization Assay

Colonization of epithelial cells by lactobacilli in presence of rfhSP-D was assayed as described earlier (6). Briefly, the Lactobacillus crispatus isolate, suspended in antibiotic-free KSFM (2.2 × 10^6 CFU/cm^2), was added to confluent epithelial surfaces Vk2/E6E7 and End1/NF-κB cells (10:1 ratio) and allowed to adhere on the epithelial monolayer; unbound bacteria were washed off by two washes of sterile Dulbecco’s phosphate-buffered saline (PBS) (Invitrogen). To the bacteria-epithelial cell co-culture, indicated concentrations of rfhSP-D, Poly (I:C) or MALP-2 was added to each well. Supernatants were collected after 24 h to measure immune mediators. Vk2/E6E7 epithelial cells were washed twice with sterile PBS and examined for viability by MTT (data not shown) and colony forming unit (CFU) assays. End1/NF-κB co-culture plate was used to evaluate NF-κB activation.

Colony Forming Units (CFU) Counts

Viable bacteria associated with Vk2/E6E7 monolayers were measured by CFU counts after 24 h of epithelial colonization followed by 24 h exposure to rfhSP-D (6). To enumerate cell-associated bacteria, epithelial cells were washed with cold PBS and hypotonically lysed in ice-cold HyPure water for 15 min, followed by adjustment of osmolality with PBS (2X) (Fisher Scientific). Bacteria collected were plated on Brucella anaerobic agar with 5% sheep blood (Becton, Dickinson and Company, USA) as per the standardized protocol, and incubated in an anaerobic chamber (Coy Laboratory Products, USA) (10% hydrogen, 10% carbon dioxide, and 80% nitrogen) at 35°C for up to 72 h (until colonies were formed), followed by visual counting of CFU.

Quantitation of Immune Mediators

Culture supernatants from the EpiVaginal tissues (apical), PBMCs (basal) and Vk2/E6E7 (with or without bacterial co-culture) were collected separately from various experiments. A custom designed 3-plex assay for GRO-α (CXCL1), MIP-3α (CCL20), and RANTES (CCL5) was used via Multiplex Electro-chemiluminescence (Meso Scale Discovery, USA) as per manufacturer’s instructions.

Rabbit Vaginal Irritation (RVI) Model Treatment

Young adult reproductive age nulliparous Belgium white rabbits (5–8 months old, body weight 2.2 kg ± 20%) (n = 5 per group)
were divided into rhSP-D, placebo and SDS (positive control) groups. The aqueous gel formulation was prepared by dissolving methyl paraben 0.18% (w/v) and propyl paraben 0.02% (w/v) in heated glycerin 8.6% (v/v). Hydroxyethyl cellulose 2.5% (w/v) was added and dispersed to form an organic phase. Citric acid 1.0% (w/v) was dissolved in purified water alone or aqueous solutions of rhSP-D (100 µg/ml) or 1% SDS. The pH was adjusted to 4.4, and the solution was clarified by passage through a 0.22 µm filter. Aqueous and organic phases were mixed and stirred well before use. Various gel formulations (1 ml) were administered intra-vaginally to their respective groups using an insulin syringe (without a needle) daily for 10 consecutive days.

Necropsy was done on day 11 following euthanasia. The vaginal tissues were collected in formalin and processed for making paraffin blocks. 5µm ribbon of paraffin bearing sections were made using a microtome and collected on poly-L-lysine-coated glass slides.

RVI Scoring
RVI scoring of hematoxylin-eosin stained tissue slides was blinded. Briefly, the tissues were scored from 0 to 4 for epithelial damage (0 = normal, 1 = flattening, 2 = metaplasia, 3 = erosion, and 4 = ulceration) and leukocyte infiltration, edema and congestion (0 = absent, 1 = minimal, 3 = moderate, 4 = marked). At least three sections of vaginal tissues (both proximal and distal) of each animal were assessed for each of the above four parameters. Total score of each animal was calculated and was averaged with number of sections analyzed. Standard RVI method suggests that a total score from 1 to 4 is to be considered as minimal irritation, 5–8 as mild irritation, 9–11 as moderate irritation and 12–13 as marked irritation (41).

Statistical Analysis
Student t-test, One-way analysis of variance (ANOVA; Bonferroni or Dunnett’s multiple-comparison analyses) was performed using GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, CA). p-value of <0.05 was considered significant.

RESULTS
Global Gene Signature of HIV-1 Challenged EpiVaginal Tissues: Clues to Early Events During Vaginal Transmission

Figure 1 depicts the experimental design used in the study to map the transcriptome of EpiVaginal tissues under different conditions. We report here, for the first time, a compendium of genes that were differentially expressed in EpiVaginal tissues when challenged with HIV-1 alone (355), HIV-1 in presence of rhSP-D (518), or rhSP-D alone (185) (Supplementary Figures S1, S2, S4). For the identification of differentially expressed genes, data was subjected to unsupervised hierarchical clustering using Pearson Uncentered algorithm with average linkage rule using Cluster 3.0 software. The resultant cluster was visualized using Tree View software. It revealed distinct patterns of upregulated and downregulated genes following treatments and indicated significant reproducibility within the replicates (Supplementary Figures S1, S2, S4). The microarray data was validated by evaluation of transcript expression by real time RT-PCR for six randomly selected, differentially expressed genes by HIV-1 and rhSP-D to represent the three functional categories (Figures 2B, 4D). Gene regulatory network analysis of the three interactions revealed involvement of several biological processes and pathways. Differentially expressed genes, along with the pathways, were subjected to regulatory network modeling, that resulting in the identification of key genes that act as bridges (involved in more than one process) and islands (which are specific to a process) (Figures 2A, 4B, C, 6B; Supplementary Figure S3). We have focused on three important processes involved in HIV-1 transmission, which are cell-cell interaction and barrier integrity, innate immune response, and cell survival. The networks were refined further to comprise the most relevant genes (Figures 4B, C, 6B).

HIV-1 induces a cytokine/chemokine storm at the mucosal sites (42) that facilitate the viral entry and transmission. HIV-1 challenged EpiVaginal tissues showed an upregulation of transcripts of cytokines and chemokines, such as IL-32, CCL20, αα, and CXCL9. Transcripts of other pro-inflammatory genes, such as MYD88, ADAM17, TNFSF14, IL-1R2, HLA-F, CD58, PKN2, and STX3 were also upregulated. A significant upregulation of PSMB10, executor caspases CASP7 and CASP1 of the inflammasome is suggestive of pyroptosis. However, a few inflammation-related genes MMP9, MUC-1, SERPINE1, TGF-α, TMEM173 were downregulated.

Interestingly, a group of interferon-inducible guanylate-binding proteins (GBP1, GBP2, and GBP5) were upregulated, suggesting that the vaginal epithelium attempts to mount an antiviral response. TRIM21, another interferon-inducible gene, was also found to be upregulated along with the interferon-inducible transcription factors, such as IRF1, ATF3, BATF2, and CREB1.

A likely breach in the vaginal barrier after HIV-1 exposure is evident by alterations in several genes encoding for proteins of plasma membrane, cytoskeletal framework and gap junction. Actin cytoskeleton rearrangement (CNN3), integral plasma membrane proteins (Cavin-1, STX6), microtubules and cytoplasmic dynactin binding (DCTN1), extracellular matrix glycoprotein (LAMB1), cell-cell recognition and signaling molecules (MSN, CD44) were all downregulated. Gap junction proteins, GJα1 and GJB6, were also downregulated. TFF2, which protects the mucosa from injury or insults, stabilizes the mucus layer and aids healing of the epithelium, was...
HIV-1 Traverses Through EpiVaginal Tissue and rfhSP-D Impedes This Movement

The ex vivo model (Figure 1A). Mimics several aspects of vaginal transmission of HIV-1. The reconstructed, multi-layered EpiVaginal explants in the upper chamber serve as the first line of protection. The activated PBMCs present in the lower chamber serve as targets for the viral particles that traversed through the EpiVaginal tissues. In this model, along with HIV-1, rfhSP-D or both, we also used MALP-2 as a positive control for inflammation.

At 24h, HIV-1 p24 Ag was detected in the supernatants of basal chambers. A higher level of p24 Ag detected in the supernatants, when the vaginal tissues were challenged with HIV-1 in presence of MALP-2 (>1.6-fold higher than control; [Medium alone]), suggested that more virions migrated to the basal chamber (though could not attain statistical significance). RfhSP-D significantly reduced the viral transfer and only one-fifth (20 ± 2.6%) of the HIV-1 p24 Ag level was detected in the basal PBMCs supernatants, as compared to the HIV-1 alone (100%) (Figure 4A).

RfhSP-D Reverses the HIV-1-Induced Gene Signature: Decoding the Protective Response

To recognize the gene signature that illustrates inhibition of vaginal transfer of HIV-1, we analyzed transcriptome of rfhSP-D treated HIV-1 challenged EpiVaginal tissues in the
apical chamber (Supplementary Figure S2). We observed a remarkable reversal of gene expression associated with gap junction proteins, plasma membrane and cytoskeletal framework of the cell. Several genes including GJA1, GJB6, CAV1, CAV2, LAMBI, ACTN1, DBN1, and DCTN1 were upregulated upon rfhSP-D treatment, which were otherwise downregulated by HIV-1 (Supplementary Table S2; Figures 4B,C). Maintenance of vaginal barrier integrity by rfhSP-D was evident from upregulation of NECTIN1 and CD44 along with gap junction genes. Interestingly, DBN1, ACTN1, NECTIN1, and CD44 have been shown to act as anti-viral or entry inhibitors (Table 1). Inflammation is the primary reason for epithelium breakage and compromised vaginal barrier. RfhSP-D reversed HIV-1 induced inflammatory genes, such as ADAM17, MYD88, SMAD3, SMAD6, CD58, CCL20, TRIM21, and SMARC1. NF-κB and mTOR, the two master regulators of inflammation, were also downregulated, suggesting induction of a state of quiescence within the EpiVaginal tissues. A few anti-inflammatory genes were upregulated; IL-20, BCL-3, NME1, NME2, CHEK1, and CDKN1C. SOCS2 and SOCS3 were selectively upregulated, suggesting rfhSP-D mediated dampening of pro-inflammatory cytokine production. TGF-β pathway seems to be relevant in vaginal transfer of virus since several genes (SMAD3, SMAD6, TSC1, EID2, TGF-β-1,1, TGF-βR, and TGFA) of this pathway were altered by HIV-1 and reversed by rfhSP-D (Table 1; Figures 4C,D).

We identified some pro-inflammatory genes that were upregulated in rfhSP-D treated HIV-challenged EpiVaginal tissues, such as CD40, ILK, ESR1, EGF, FGFR2, HIPK2, SOD1, MAP3K1, IFI16, NOD2, IL-1B, HTRA1, EDNRA. Interestingly, there was a downregulation of the intrinsic SP-D gene expression that suggested a mitigation of inflammatory response of vaginal tissues in presence of rfhSP-D (Figures 4C,D). HIV-1 challenged EpiVaginal tissues showed an upregulation of SFTP D (gene encoding SP-D protein) transcript (Figure 4C) whereas, rfhSP-D pretreatment followed by HIV-1 challenge reverted the HIV-1 induced upregulation of rfhSP-D (Figure 4D). rfhSP-D treatment alone did not alter the expression of native SP-D transcript (Figure 6B).

HIV-1 Induced Downregulation of Tight Junction Gene Expression Is Rescued by RfhSP-D

HIV-1 is known to downregulate tight junction proteins in order to traverse through the weakened vaginal barrier (36). We assessed the status of claudins and occludin in the EpiVaginal tissues. HIV-1 challenge led to a significant decrease in the transcript levels of tight junction proteins that were further downregulated when simultaneously treated with MALP-2 (Figures 5A–E). RfhSP-D countered the HIV-1 induced downregulation of transcripts of claudin 2, 3, 5 and occludin, except claudin 4, suggesting a reduced damage to the vaginal integrity (Figures 5A–E).

RfhSP-D Does Not Enhance the Susceptibility of Target Cells to HIV-1 Acquisition

Another experimental setup was designed to interrogate whether rfhSP-D, on its own, caused inflammation within EpiVaginal tissues (in the absence of HIV-1 challenge), which in turn increased susceptibility of target cells (Figure 1B). PBMCs in the basal chamber of rfhSP-D treated tissues showed no significant increase in the acquisition of HIV-1 while the inflammatory MALP-2 treated tissues showed increased p24 levels on day 6 (Figure 6A).

RfhSP-D Treatment Strengthened Vaginal Barrier: SP-D a Natural Vaginal Host Defense Molecule

Alterations in the transcripts of EpiVaginal tissues induced by rfhSP-D were also identified by microarray analysis. Of the total 185 genes differentially regulated, 103 were upregulated and 82 were downregulated (Supplementary Figure S4). Upregulation of CAV1 and CAV2, along with Laminins (LAMA3, LAMB1, LAMC1, and LAMC2), which are essential for formation and function of the basement membrane, was suggestive of a strengthened mucosal barrier. Collagen transcripts (COL4A4, COL5A1, COL5A2 and COL7A1, COL17A1), important structural components of basement membranes, were also upregulated. With an integral role in adhesion of the epithelium...
FIGURE 4 | rhSP-D impedes viral movement across the EpVaginal tissue barrier and reverses HIV-1 induced gene signature: (A) Determination of HIV-1 p24 Ag by ELISA in supernatants from basal chambers at 24h. Data represents mean ± S. D of three sets. *indicates statistical significance *p < 0.05 relative to medium alone.

(Continued)
to extracellular matrix, integrins α3, α6, and β4, were upregulated by rfhSP-D (Figure 6B; Supplementary Figure S5A). Specific upregulation of genes related to structural stability of the cell and epithelial integrity suggested that rfhSP-D strengthened the local tissue architecture. Consistent with its established anti-inflammatory role, rfhSP-D downregulated genes that promote inflammatory signals, such as GBP2, IRF1, ATF3, CREB1, IGFBP2, and IGFBP7.

SP-D is synthesized by the human vaginal epithelial cells and its uterine expression is hormone regulated (17). We detected SP-D in the vaginal lavage of normal cycling women (Supplementary Figure S5A). Vaginal epithelial cells (Vk2/E6E7) also showed transcripts of SP-D (Supplementary Figure S5B). In addition, confocal microscopy revealed that SP-D protein was being produced by Vk2/E6E7 and could be localized in the cytoplasm (Supplementary Figure S5C). With its natural presence in the vaginal tract, its role as a pattern recognition protein and in strengthening of the vaginal barrier as evident from gene expression studies, SP-D seems to be vital as the first line of defense at the vaginal surfaces.

**rfhSP-D Has No Adverse Effect on Cell Viability and NF-κB Translocation**

In addition to the ex vivo efficacy of rfhSP-D as an inhibitor of the vaginal transmission of HIV-1, it was pertinent to evaluate the safety of rfhSP-D application on the vaginal surface. As a first step, we assessed its effect on the viability and inflammation of vaginal and ectocervical cells. Within the concentration range of 1.562–100 µg/ml and a duration of 24 h treatment, the viability of vaginal and ectocervical cells was unaltered (Figure 7A). NF-κB activation is a prerequisite for inflammation and breach of vaginal barrier providing access to HIV-1 entry. Hence, to determine the effect of rfhSP-D on the NF-κB activation, we used an endocervical cell line (End1/E6E7) transfected with pHTS–NF-κB firefly luciferase reporter. None of the indicated rfhSP-D concentrations induced NF-κB activation, whereas MALP-2 and poly I:C, agonists of TLR-2/6 and TLR3 respectively, led to a significant activation (Figure 7B). Furthermore, rfhSP-D did not cause any alteration in the levels of anti-inflammatory immune mediators, such as interleukin-1 receptor antagonist (IL-1RA), secretary leukocyte protease inhibitor (SLPI) and elafin, which are known to maintain vaginal homeostasis (data not shown).

**RfhSP-D Does Not Adversely Affect Vaginal Lactobacilli**

*Lactobacilli*, as vaginal commensals, are integral to the female reproductive tract. A direct toxicity assay revealed that rfhSP-D did not adversely affect viability of the clinical isolates of *Lactobacilli* (TRF #8, TRF #30, TRF#36 and *Lactobacillus crispatus* LC223) (Figure 8A). Lactic acid produced by *Lactobacilli* contributes to vaginal defense and any alteration in its production would enhance susceptibility to pathogens including HIV-1 (43). The pH of the supernatant from cultures treated with rfhSP-D was acidic like untreated controls. As expected, Pen-Strep reduced the viability of *Lactobacilli* and the supernatant showed a significantly higher pH (toward neutral) (Figure 8B).

**rfhSP-D Does Not Interfere With Vaginal Epithelium-Lactobacilli Interaction**

Since the vaginal microflora tightly controls the epithelial immune functions in a species- and strain-specific manner, any interference from topically applied microbicides or potential anti-HIV-1 agents may prove detrimental. Thus, we employed vaginal *Lactobacilli* colonization model that mimics in vivo conditions (6). In the co-culture conditions, rfhSP-D treatment did not lead to any reduction in CFU counts (Figure 8C).

Epithelial interaction with commensals leads to enhanced inflammation in a regulated manner; when exacerbated, it enhances susceptibility to HIV-1 and when calmed, it compromises immunity. Hence, we assessed the effect of rfhSP-D on NF-κB induction in this co-culture model. Importantly, NF-κB levels were not affected across all the tested concentrations of rfhSP-D (Figure 8D). Poly I:C and MALP-2 did show an exaggerated NF-κB activity. Further, rfhSP-D did not significantly alter the levels of chemokines, such as RANTES, GRO-α, MIP-3α, corroborating no adverse effect on vaginal immune physiology (Figures 9A–C).

**Repeated Application of rfhSP-D on Rabbit Vaginal Surface Does Not Induce Inflammation**

Rabbits with repeated vaginal application of 1% SDS (positive controls) showed rupturing of the epithelial barrier and hemorrhage, whereas, rfhSP-D and placebo groups showed no signs of inflammation (Figures 10A–C). As per the RVI scoring, vaginal sections of rfhSP-D and placebo groups showed none or minimal irritation. The total sum of RVI scoring was 2.98 ± 0.6 for the rfhSP-D group and was not significantly different from the RVI score of 2.54 ± 0.3 for the placebo group, whereas, 1% SDS showed a moderate inflammation score of 9.7 ± 1.01, indicating gross toxicity (Figure 10D).

**DISCUSSION**

Inflammation and breach of mucosal barrier are the two major events that render the “gatekeeping mechanisms” ineffective.
| Gene name | HIV-1 (FC) | rhfSP-D + HIV-1 (FC) | Functions | Ref. | Role in HIV | Ref. |
|-----------|------------|----------------------|-----------|------|-------------|------|
| **INFLAMMATION** | | | | | | |
| ADAM17    | Up (1.51)  | Down (−1.16)         | A protease critical in cleavage of TNF-α and other inflammatory proteins to active form. Important in diverse cellular processes such as proliferation, migration, cell adhesion | PMID: 20184396 | Nef activates and shuttles activated ADAM17 into exosomes | PMID: 23317503 |
|           |            |                      |           |      | Exosomal Nef and ADAM17 activates quiescent CD4+ T lymphocytes via TNF-α | PMC4178784 |
| MMP9      | Down (−3.69) | Up (3.75)            | Proteolytic enzyme, degrades extracellular matrix. | PMID: 12540195 | Induced by Tat in astrocytes | PMC2679334 |
|           |            |                      |           |      | Uregulated by gp120 in vaginal epithelial cell line | PMC3222676 |
| MYD88     | Up (1.82)  | Down (−1.26)         | Universal adapter protein downstream of TLRs (except TLR 3) to activate the transcription factor NF-κB | PMID: 18064347 | HIV-1 Tat Activates both the MyD88 and TRIF Pathways To Induce TNF-α and IL-10 in Monocytes | PMID: 27053552 |
| RIPK1     | Up (1.29)  | Down (−1.95)         | Serine/threonine kinase that regulate a variety of cellular processes such as cell death and innate immune responses to viral and bacterial infection, induces necroptosis | PMID: 19524512 | Cleaved by HIV proteases and modulate cellular response | PMC4546280 |
|           |            |                      |           |      | | |
| CDS8      | Up (1.64)  | Down (−1.25)         | Interaction between CD2 and its counterreceptor, CD58 (LFA-3) aids in T cell-APC cell contact | PMID: 10380930 | Engagement of CDS8 enhances HIV-1 replication in monocytic cells | PMID: 8656013 |
| TFF2      | Up (1.99)  | Down (−2.11)         | Secreted into the mucus layer where it stabilizes the mucin gel layer and stimulates migration of epithelial cells. Upregulated in chronic inflammation | PMID: 19064997 | – | – |
| SERPINE1  | Down (−2.04) | Up (2.92)            | An inhibitor of fibrinolysis, high concentrations of the gene product are associated with thrombophilia | PMID: 24669362 | Monocytes from asymptomatic viremic HIV(+) individuals show increased PAI-1 (SERPINE1) | PMID: 22815948 |
| CCL20     | Up (2.94)  | Down (−2.61)         | Responsible for the chemo-attraction of iDCs, effector/memory B cells and T cells. High specificity for CCR6 | PMID: 27617163 | Attracting key immune cells, including Th17 cells and dendritic cells, to sites of infection and propagating the virus to other sites of the body | PMID: 28005525 |
| TRIM21    | Up (3.29)  | No change (1.08)      | Intracellular antibody effector in the intracellular antibody-mediated proteolysis pathway. Directs the virions to the proteasome. | PMID: 21045130 | Chimeric restriction factor TRIM21–CypA provides highly potent protection against HIV-1 without loss of normal innate immune TRIM activity | PMID: 22909012 |
| SOCS2     | Down (−1.2) | Up (1.63)            | Down-regulation of cytokine signaling | PMID: 12208853 | Tat impaired the IFN γ - receptor signaling pathway at the level of STAT1 activation, via Tat-dependent induction of suppressor of cytokine signaling-2 (SOCS-2) activity | PMID: 19279332 |

(Continued)
| Gene name | HIV-1 (FC) | rfhSP-D + HIV-1 (FC) | Functions | Ref. | Role in HIV | Ref. |
|-----------|------------|----------------------|-----------|------|-------------|------|
| SOCS3     | No change  | Up (1.09)            | Down-regulation of cytokine signaling | PMID: 9202125 | Protein levels were lower in CD4 (+) T cells of HIV-infected patients than in healthy controls. Suppressed Th17 levels correlate with elevated SOCS3 expression in CD4 T cells during acute simian immunodeficiency virus infection | PMID: 21337543 |
|           |            | (1.9)                |           | PMID: 9430658 |                                        | PMID: 23596301 |
|           |            |                      |           | PMID: 9857039 |                                        |                  |
| NOS3      | Up (1.18)  | Down (−1.14)         | Major determinant of vascular tone and blood pressure | PMID: 7514568 | Nitrile oxide inhibits HIV tat-induced NF-κB activation | PMID: 10393859 |
| PYCARD    | Down (−1.64) | Up (2.47)          | Involved in NLRP3 induced inflammasome. Responsible for cleavage of pro-caspase 1 | PMID: 20303873 | Involved caspase-1 dependent pyroptosis of HIV infected CD4 T cells | PMC4047036 |
| SMARCD1   | Down (−1.95) | Up (2.45)          | Part of SWI/SNF complexes that regulate gene activity of chromatin remodeling, may act as tumor suppressor | PMCID: PMCS406539 | Role in HIV-1 assembly, interaction between Nef and INI1/SMARCB1 augments replicability of HIV-1 in resting PBMCs facilitate Tat-mediated HIV-1 transcription | PMID: 27558426 |
|           |            |                      |           | PMID: 25559666 |                                        | PMID: 16889688 |
| CREB1     | Up (1.69)  | Down (−2.04)         | CREB family of transcription factors consists of cAMP-responsive activators including CREB, cAMP response element modulator, and activating transcription factor | PMID: 10872467 | Tat utilizes CREB to promote IL-10 production, although the significance of this regarding HIV pathogenesis is not entirely clear. IL-10 can inhibit HIV-1 replication in monocytes and macrophages | PMID: 7527449 |
| RIPK3     | Down (−1.28) | Up (1.64)          | Serine/threonine kinases that regulate a cellular processes such as cell death and innate immune responses to viral and bacterial infection, induces necroptosis | PMID: 19524512 | Not cleaved by HIV proteases and modulate cellular response | PMC4546280 |
| SOD1      | Down (−1.30) | Up (1.23)          | Enzyme attaches (binds) to molecules of copper and zinc to break down toxic, charged oxygen molecules called superoxide radicals. | PMID: 7901908 | SOD1 prevents gp120 and Tat elicited reactive oxygen species (ROS) and rescued neuron apoptosis | PMID: 17396361 |
| TGFBR2    | Up (1.21)  | Down (−1.7)         | TGF-β mediates its actions through heteroregic kinase receptor complex consisting of TGF receptors of type 1 and 2 | PMID: 1333888 | Increased expression upon Tat treatment of epithelial cells | PMID: 15857508 |
| TGFA      | Up (1.15)  | Down (−1.48)         | Exerts several effects on target cells, such as neovascularization promotion and mitogenic signaling. | PMID: 9242560 | Significant rise in chronic HIV type 1 infection | PMID: 27268396 |
| SMAD6     | Down (−1.37) | Up (1.59)          | Smad6 inhibits signaling by the TGF-beta superfamily | PMID: 9335505 | Down-regulated after Tat treatment of U937 macrophages | PMID: 16282533 |
| STX3      | Up (2.01)  | Down (−2.04)         | Potentially involved in secretion of IL-6 from dendritic cells following activation of TLRs | PMID: 25674084 | Depletion of STX3 reduced HCMV production | PMID: 25583387 |
| Gene name | HIV-1 (FC) | rfhSP-D + HIV-1 (FC) | Functions | Ref. | Role in HIV | Ref. |
|-----------|------------|----------------------|-----------|------|-------------|------|
| XRCC2     | Up (2.33)  | Down (−2.38)         | DNA repair protein binding to double stranded breaks | PMID: 10227297 | Suppression of retroviral infection by XRCC2 | PMID: 15297876 |
| CYTOSKELETON AND CELL-CELL INTERACTION AND INTEGRITY | | | | | | |
| GJA1      | Down (−3.07) | Up (3.39) | Involved in intercellular communication (GJIC) between cells to regulate cell death, proliferation, and differentiation. Involved in inflammation | PMID: 28546458 | Blocking of HIV entry through CD44-hyaluronic acid interactions. | PMID: 25155464 |
| CD44      | Down (−2.42) | Up (5.25) | Cell-surface glycoprotein involved in cell–cell interactions, cell adhesion and migration | PMID: 16982844 | Cav-1 Tat induced alterations of tight junction protein. Cav-1 mediated uptake via langerin restricts HIV-1 infectivity | PMID: 18667611 |
| CAV1      | Down (−1.89) | Up (2.53) | Cav-1 is enriched in caveolae, involved in endocytosis, signal transduction. Role in innate immune defense, and it regulates macrophage cytokine production and signaling | PMID: 23454155 | Similar to Cav-1 and also inhibits cell proliferation, migration and invasion | – |
| CAV2      | Down (−1.22) | Up (2.48) | Similar to Cav-1 and also inhibits cell proliferation, migration and invasion | PMID: 28416666 | DBN1 suppresses dynamin-mediated endocytosis via interaction with cortactin. DBN1 restricts the entry of viruses into host cells and more broadly to function as a crucial negative regulator of diverse dynamin-dependent endocytic pathways | PMID: 23926103 |
| DBN1      | Down (−1.86) | Up (1.74) | DBN1 suppresses dynamin-mediated endocytosis via interaction with cortactin. DBN1 restricts the entry of viruses into host cells and more broadly to function as a crucial negative regulator of diverse dynamin-dependent endocytic pathways | PMID: 28392352 | Nectin cell adhesion molecule, plays role in organization of adheren junctions and tight junction | PMID: 24586397 |
| NECTIN1   | No change (1.04) | Up (2.94) | Nectin cell adhesion molecule, plays role in organization of adheren junctions and tight junction | PMID: 21835307 | Inhibit the replication of HIV-1 in cultured cord blood mononuclear cells and chronically HIV-infected U937 cells | PMID: 7576911 |
| IGFBP3    | No change (−1.05) | Up (2.11) | Binds IGF-I and IGF-II with relatively low affinity, and belongs to a subfamily of low-affinity IGFBPs. It also stimulates prostacyclin production and cell adhesion. | PMID: 26312134 | α-Actinin regulates the immune synapse formation and is required for efficient T cell activation. silencing of either EWI-2 or α-actinin-4 increased cell infectivity. Regulation of the actin cytoskeleton at T cell immune and virological synapses | PMID: 22689882 |
| ACTN1     | No change (−1.01) | Up (1.34) | Major actin cross-linking proteins found in virtually all cell types as a cytoskeleton. | PMID: 19944606 | Gap junction channels shutdown under inflammatory conditions, including viral diseases. | PMC4774036 |
| GJB6      | Down (−1.95) | Up (2.87) | Gap junctions allow the transport of ions and metabolites between the cytoplasm of adjacent cells | | | | |

FC—Fold Change.
leading to HIV-1 transmission. The present study establishes a recombinant fragment of human SP-D (rfhSP-D) as a candidate microbicide, which remarkably inhibited HIV-1 transfer in an ex vivo model comprising of multi-layered vaginal mucosal tissue. We also report the gene expression profile of HIV-1 challenged EpiVaginal tissues. RfhSP-D specifically reversed the infection-promoting gene signature induced by the virus, thereby, maintaining the integrity of vaginal epithelium and suppress the pro-inflammatory milieu. Furthermore, in vitro and in vivo safety studies implied that the rfhSP-D is safe for mucosal application at the concentrations that to restrict viral passage.

Transcriptome snapshot of the EpiVaginal tissues upon HIV-1 challenge revealed an inflammatory response comprising of chemokines, cytokines and components of inflammasome. Upregulation of these genes would act in sync, contributing to a generalized local inflammation in the vaginal epithelium. Fanibunda et al. (44) reported global gene expression in a monolayer of vaginal epithelial cell line (Vk2/E6E7) challenged with HIV-1 recombinant gp120 protein with a predominant induction of immunomodulatory processes and proteases. Following HIV-1 exposure, primary genital epithelial cell cultures showed enhanced proinflammatory cytokines (e.g., TNF-α and IL-6) and disruption of tight junctions, such as claudins, occluding, and ZO-1, leading to a compromised barrier (36, 45). Barouch et al. demonstrated that 24 h post-vaginal SIV challenge, the host lacked expression of the antiviral restriction factors and the response comprised of NLRX1 and TGF-α which incapacitated a strong anti-viral response (46). Consistent with the previous reports, the ex vivo model of human vaginal tissues showed pro-inflammatory response on viral challenge. Alongwith, it showed upregulation of host restriction factors, such as guanylate-binding proteins (GBP1, GBP2, GBP5), TRIM21 and other IFN-inducible genes. GBP5 has been recently reported as a host restriction factor in virus-challenged macrophages (47). Although, not proven in the context of HIV-1, TRIM21 is known to obstruct the incoming antibody-opsonized non-enveloped virions and efficiently mediate post-entry neutralization and innate immune signaling (48, 49). Being effective intracellularly, it is possible to hypothesize that these restriction factors may prevent further movement of the transcytosed virions (50). HIV-1 can also pass freely through the intercellular gaps in the vaginal epithelium. We observed a dramatic downregulation of several genes of the plasma membrane and cytoskeleton framework along with downregulation of tight junction proteins (claudins and occludin) induced by the virus. Although the EpiVaginal tissue attempts to mount an interferon response, the excessive inflammation and a disturbance in cellular functions weaken the epithelial barrier and provide a gateway to the underlying...
target cells (Figure 11A). There are several compelling evidence of interaction of HIV-1 with the vaginal epithelial cells via TLR2 and TLR4 (46), gp340 (51), syndecans (52), and human mannose receptor (53). These receptors, when engaged with PAMPs, initiate an inflammatory cascade. In our model, MALP-2 that activated the TLR2/6 inflammatory axis, synergizes with HIV-1 to further reduce the expression of tight junction proteins and enhances chemokine secretion, reiterating their crucial role in HIV transmission.

The gene signature of EpiVaginal tissues, induced by HIV-1, reflected key mechanisms for viral movement across the multilayered epithelium resulting in its acquisition by the underlying PBMCs. RhSP-D showed a remarkable ability to restrict viral movement (though not a complete blockade in the experimental conditions). Previously, we and others have shown that rhSP-D (as well as native SP-D) potently binds to HIV-1 gp120, leading to agglutination and inhibition of infectivity of target cells (19, 54). It can, therefore, be considered that interaction of trimeric rhSP-D with HIV-1 plausibly results in large complexes that are unable to travel through the tight vaginal barrier. Moreover, since HIV-1 envelope protein gp120 primarily makes the first contact with the epithelium, restriction of this interaction by rhSP-D may also contribute to a shift from HIV-1 induced gene signature. The presence of a fraction of the virions in the basal chamber of rhSP-D treated EpiVaginal tissues indicated interaction of the HIV-1 with the EpiVaginal tissues (although it was significantly reduced). In addition, evident from the differential gene expression of the rhSP-D treated EpiVaginal tissues, rhSP-D directly interacted with the vaginal epithelial cells and thus strengthened
the barrier with upregulated expression of cytoskeleton-related genes. Taken together, these observations suggest that rhSP-D was able to contain the HIV-1 induced changes in gene expression of the EpiVaginal tissues. Significant upregulation of transcripts for several tight junction proteins in the HIV-1 challenged EpiVaginal tissues in presence of rhSP-D validated this hypothesis. The two key pro-inflammatory players, NF-κB and mTOR (55, 56), were significantly downregulated, suggesting...
likely inhibition of the sequential steps of HIV-1 transmission. Notably, the Guanylate binding proteins (GBPs) were either upregulated or unaltered, suggesting that rfhSP-D facilitated the protective response mounted by the EpiVaginal tissue against HIV-1 (Figure 11B). We have recently reported DC-SIGN as a novel receptor of SP-D (using rfhSP-D). A tripartite engagement between DC-SIGN, rfhSP-D and gp120 significantly inhibited transfer of HIV-1 from DC-SIGN to the PBMCs (57). This finding may hold immense importance in vaginal transmission of HIV-1, since DC-SIGN on dendritic cells acts as “Trojan horse” that captures HIV-1 in the mucosa and facilitates its transport to secondary lymphoid organs rich in CD4+T cells followed by trans-infection (58).

SP-D has been shown to potently inhibit the infectivity of other enveloped viruses, such as Influenza A Virus (IAV) (59) and Respiratory Syncytial Virus (RSV) (60), concomitant with induction of an anti-inflammatory environment by interacting with mucosal epithelial and immune cells. This unique property has made rfhSP-D a viable therapeutic option for cystic fibrosis, neonatal lung disease and smoking-induced emphysema (61). RfhSP-D seems to have a similar role against HIV-1 at the vaginal interface. While rfhSP-D limits viral access, it also induces a state of immune quiescence in the vaginal tissues. There is a direct correlation of immune quiescence at the mucosal sites, with resistance to HIV-1 acquisition in serodiscordant women (62). It would be worth exploring the clinical significance of the candidate genes associated with restricted transmission identified in the present study in the highly exposed seronegative women.

An anti-HIV molecule can be effective as a microbicide only if it retains its anti-viral activity without inducing immune activation. Several candidates have failed in the clinical trials due to inflammation caused to the epithelial and target cells, leading to enhanced susceptibility to the virus. Our model revealed that treatment with rfhSP-D did not induce any aberrant inflammatory response by EpiVaginal tissues and did not lead to activation of PBMCs (target cells), and thus, minimized the likelihood of viral transfer and acquisition. In contrast, MALP-2 showed increased activation and susceptibility of PBMCs to the virus, confirming the appropriateness of the model for the evaluation microbicides (63).

To save time and resources, an extensive characterization of candidate prophylactics is warranted before testing their efficacy in vivo. Therefore, we subjected rfhSP-D to a series of safety evaluations. RfhSP-D was well-tolerated by human vaginal and ectocervical cells; even at the highest concentration (100 µg/ml), no apparent alterations in the viability of vaginal epithelial cells or inflammation were observed. Similarly, rfhSP-D did not adversely affect the growth of lactobacilli or acid production. However, in the vagina, the epithelial cells and microflora together determine the vaginal health. Vaginal microflora is critical in regulating the epithelial innate immune response. To accurately replicate the in vivo condition, we tested safety of rfhSP-D in an epithelial-bacterial colonization model (6).

As is the case for a successful microbicide candidate, rfhSP-D did not affect lactobacilli counts, NF-kB activation and chemokine levels in the co-culture. Although SP-D potently
FIGURE 10 | Rabbit Vaginal Irritation (RVI) model demonstrates intact integrity of mucosal barrier on repeated application of rfhSP-D gel. H&E staining of vaginal sections of rabbits (n = 5/group) treated with (A) placebo gel (B) rfhSP-D (100 µg/ml) gel and (C) 1% SDS gel (positive control) daily for 10 consecutive days. Sections from 1% SDS gel treated rabbits showed inflamed epithelial barrier with significant infiltration of polymorphonuclear cells (PMNs) (depicted by “red arrows”) and hemorrhage (depicted by the “red asterisk”). Black arrow heads depict epithelial membrane with minimal infiltration of PMNs in the “placebo gel” and “rfhSP-D (100 µg/mL) gel” in (A,B). Magnification 10× (D) RVI score of the rfhSP-D treated group was not significantly different from the placebo group. At least three sections of vaginal tissues (both proximal and distal) of each animal (blinded) were scored from 0 to 4 for epithelial damage (0 = normal, 1 = flattening, 2 = metaplasia, 3 = erosion, and 4 = ulceration) and leukocyte infiltration, edema and congestion (0 = absent, 1 = minimal, 3 = moderate, 4 = marked). Total score of each animal was calculated and was averaged with number of sections analyzed. A total score from 1 to 4 is to be considered as minimal irritation, 5–8 as mild irritation, 9–11 as moderate irritation, and 12–13 as marked irritation. *p < 0.05 was considered statistically significant.

FIGURE 11 | A schematic model illustrating effects of HIV-1 and rfhSP-D on EpiVaginal tissues. (A) The intact epithelium seems to be breached after HIV-1 exposure. Alterations in the genes encoding tight junction proteins, cytoskeleton and those contributing to inflammation are plausibly the critical events in HIV-1 transmission through the multi-layered tissue. (B) rfhSP-D potently binds to HIV-1 and interacts with EpiVaginal tissues, reverses HIV-1 induced gene signature, and inhibits HIV-1 transmission.
inhibits reproductive tract pathogens, such as Chlamydia (64) and Candida (65), we report the SP-D-commensal interaction for the first time. Further investigations may ascertain molecular determinants that define the ability of SP-D to differentiate between vaginal pathogens and commensals. One plausible reason could be evolution of tolerance of the vaginal microflora in the presence of SP-D and other anti-microbial proteins and peptides naturally secreted in the vagina (66). SP-D is naturally expressed and secreted by the human vaginal epithelial cells. Therefore, it was expected that repeated application of rhSP-D may not harm the vaginal surface. There were no evident histological signs of mucosal toxicity in the rabbit vagina, suggesting that rhSP-D is well-tolerated in vivo.

In summary, we demonstrate the transcriptional gene expression signatures of EpiVaginal tissues in response to HIV-1. An ex vivo model of vaginal transmission of HIV-1 was developed that revealed novel genes and features of HIV-1 transmission, and offers a highly reproducible, cost-effective, non-animal model to study efficacy of candidate microbicides. Importantly, rhSP-D emerges as a potent anti-HIV-1 microbicide candidate, and the results provide a strong argument for its further evaluation in non-human primate models.

ETHICS STATEMENT

Cervicovaginal lavage (CVL) was collected from the normal cycling females with an approval from the Institutional Ethics Committee for Clinical Studies, ICMR-NIRRH (Project No. 148/2008). Blood (n = 5) was collected from healthy donors (as determined by clinical examination) at the Department of Pathology, Brigham and Women’s Hospital, Boston, MA, under the Partners Healthcare IRB approval (2003P002150). Written informed consent was obtained from each participant. The rabbit vaginal irritation study was approved by the "Institutional Animal Ethics Committee (IAEC)," ICMR-NIRRH, Mumbai (Project No. 08/2012). The IAEC has been recognized by the central organization "Committee for the Purpose & Supervision of Experiments on Animals (CPCSEA)." We strictly adhered to the CPCSEA protocols and guidelines for animal care during the animal experimentation.

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AUTHOR CONTRIBUTIONS

HP conceived the study, designed, performed and analyzed the experiments, and wrote the paper. KK carried out the RVI model study. KK, GT, and SR carried out the primer designing and Real-time RT-PCR validation of the gene expression. HY conducted the cell viability assessment, recruitment of study participants, and data analysis. PC and MV carried out the microarray analysis, pathway analysis, and presentation. UK provided rhSP-D for the study and critical suggestions for the manuscript. TM conceived and co-ordinated the study, procured the intra-mural grant and ICMR-Medical Innovation Fund support, mediated the clinical collaboration, defended the protocol for IEC approval, analyzed the data, and edited the paper. RF conceived and co-ordinated the study, facilitated HP’s experimentation at BWH, analyzed the data, and edited the paper. All authors reviewed the results and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00264/full#supplementary-material
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