Anti-HIV-1 Activity of Elafin Is More Potent than Its Precursor's, Trappin-2, in Genital Epithelial Cells

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Cervicovaginal lavage fluid (CVL) is a natural source of anti-HIV-1 factors; however, molecular characterization of the anti-HIV-1 activity of CVL remains elusive. In this study, we confirmed that CVLs from HIV-1-resistant (HIV-R) compared to HIV-1-susceptible (HIV-S) commercial sex workers (CSWs) contain significantly larger amounts of serine antiprotease trappin-2 (Tr) and its processed form, elafin (E). We assessed anti-HIV-1 activity of CVLs of CSWs and recombinant E and Tr on genital epithelial cells (ECs) that possess (TZM-bl) or lack (HEC-1A) canonical HIV-1 receptors. Our results showed that immunodepletion of 30% of Tr/E from CVL accounted for up to 60% of total anti-HIV-1 activity of CVL. Knockdown of endogenous Tr/E in HEC-1A cells resulted in significantly increased shedding of infectious R5 and X4 HIV-1. Pretreatment of R5, but not X4 HIV-1, with either Tr or E led to inhibition of HIV-1 infection of TZM-bl cells. Interestingly, when either HIV-1 or cells lacking canonical HIV-1 receptors were pretreated with Tr or E, HIV-1 attachment and transcytosis were significantly reduced, and decreased attachment was not associated with altered expression of syndecan-1 or CXCR4. Determination of 50% inhibitory concentrations (IC₅₀) of Tr and E anti-HIV-1 activity indicated that E is ~130 times more potent than its precursor, Tr, despite their equipotent antiprotease activities. This study provides the first experimental evidence that (i) Tr and E are among the principal anti-HIV-1 molecules of CVL; (ii) Tr and E affect cell attachment and transcytosis of HIV-1; (iii) E is more efficient than Tr regarding anti-HIV-1 activity; and (iv) the anti-HIV-1 effect of Tr and E is contextual.

Heterosexual transmission of human immunodeficiency virus type 1 (HIV-1) via mucosal surfaces propels the HIV-1 pandemic (81). Evidence indicates that women represent more than half of newly HIV-infected adults worldwide (75). The continued spread of HIV-1, the lack of effective vaccines, and increased female susceptibility to the infection underscore the urgent need for alternative approaches for HIV-1 control, particularly within the female genital tract (FGT).

Female genital epithelial cells (ECs) play a crucial role in HIV-1 infection, as their recognition and passing, or sequestering, of the virus can predetermine the magnitude of innate immune responses and the fate of potential HIV-1 target cells residing in the submucosa. Since the role of dysregulated HIV-driven mucosal innate responses, as well as chronic immune activation, and the lack of efficient control of HIV-1 are becoming more accepted, so is the need for novel factors and approaches to keep inflammation and the virus at bay.

The interaction between viral envelope glycoprotein 120 (gp120) and canonical HIV-1 cell-surface receptors CD4 and CCR5/CXCR4 is important for HIV-1 binding/entry into cells (8). While the majority of HIV-1-susceptible cells, including TZM-bl cells, a derivative from HeLa cervical ECs, express CD4, CCR5, and CXCR4 (54, 79), primary genital ECs express only CXCR4 and CCR5 (41), but not CD4 (8). However, both cell types remain permissive to HIV-1 attachment/entry and transmigration (8, 41), indicating that HIV-1 can utilize alternative cellular molecules for these purposes. Indeed, primary genital ECs and the endometrial epithelial cell line HEC-1A express a number of alternative receptors, including proteoglycans (PG) with heparan sulfate (HSPG) or chondroitin sulfate (CSPG) chains (8, 46), as well as galactosylceramide (GalCer) (24–25, 85), and mannose receptors (74).

Among HSPGs, syndecans, and not glypicans, were shown to be more important for HIV-1 attachment/entry into various target cells, such as macrophages (62), T cells (9), and genital ECs (7, 59). Furthermore, enzymatic removal of heparan sulfate moieties significantly reduced HIV-1 attachment/entry into target cells, as was shown for syndecan-1, which accounted for at least 60% of attached HIV-1 to HEC-1A cells (59). These observations confirm the importance of syndecans in HIV-1–cell encounters.

Sensing of HIV-1 through pattern recognition receptors (PRRs) initiates a series of signaling events that lead to the secretion of not only inflammatory (51, 59) but also endogenous innate protective factors with a broad spectrum of antimicrobial activity (15, 63, 83), including against HIV-1 (19, 68, 77). Recently, increased levels of serine antiproteases trappin-2 (Tr) and elafin (E) were found in cervicovaginal lavages (CVLs) from highly exposed HIV-resistant (HIV-R) commercial sex workers (CSWs) in Nairobi, Kenya (30). Despite repeated HIV-1 exposures, these women have no signs of infection, but show the presence of HIV-specific and potent immune responses both systemically (32) and mucosally (34), suggesting that the local milieu in their genital tract and continuous viral exposure (33) somehow prevent the virus from crossing the epithelial barrier and establishing productive infec-

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tion. These findings also indicate that we are still far from having a clear understanding of HIV-1 transmission, pathogenesis, and correlates of protection at mucosal surfaces, and more studies in such cohorts could generate insightful findings.

The better-known members of the whey acidic protein (WAP) family are E and its precursor, Tr (Tr/E), which are structurally related to secretory leukocyte protease inhibitor (SLPI) and the 20-kDa prostate stromal protein (ps20) (6). An evolutionarily conserved four-disulfide core (FDC) structure, also called a WAP or a WAP FDC (WFDC) domain, is shared among WAP family members. The WAP domain is rich in cysteine residues that stabilize four disulfide bonds involved in protease inhibition (73). However, not all WAP-containing proteins are antiproteases, as shown for ps20 (6), eppin (86), mouse SWAM1 and SWAM2 proteins (23), and omwaprins from snake venom (49). Tr is secreted as a cationic unglycosylated (95-amino-acid [aa]) 9.9-kDa protein. The proteolytic cleavage of the N-terminal cementin domain of Tr liberates the C-terminal 57-residue with the WAP domain (5.9 kDa) as a soluble protein, elafin (E) (4, 21, 65). In the N terminus of Tr there is a transglutaminase substrate-binding domain (TSBD) with four repeated motifs containing the consensus sequence Gly-Gln-Asp-Pro-Val-Lys (50). The N terminus of E also contains one such motif (4), hence allowing both Tr and E to be covalently linked to extracellular matrix proteins like heparin by a tissue transglutaminase, most likely contributing to wound healing (22). Often the term “E” is used interchangeably to denote either Tr or E, making it unclear which protein is referred to in a text.

Tr and E are pleiotropic molecules in nature with ascribed antiproteolytic, immunomodulatory, and antimicrobial properties (13, 48, 60). Tr and E exhibit inhibitory properties against human neutrophil elastase (HNE), proteinase 3, and endogenous vascular elastase (12). Tr and E were also shown to inhibit the growth of a number of fungal as well as Gram-positive and Gram-negative bacterial pathogens (48, 70), but sparing *Lactobacillus* (14). It is becoming increasingly clear that together with SLPI, Tr and E exhibit more than just an antiprotease function. In addition to antimicrobial activity, in their arsenal, Tr and E also possess multifaceted immunomodulatory properties that target binding, recognition, and mounting of innate inflammatory responses against bacterial and viral antigens (13, 43, 60). Both Tr/E and SLPI were shown to reduce activation of NF- κ B and AP-1 by altering I κ B activation (27) and proteosomal degradation (10), respectively, in response to inflammatory and bacterial stimulation. More recent studies also reported immunomodulatory properties of Tr/E, either through dampening or promoting inflammation (10, 71) and priming of the immune system (43, 57). We also recently found that recombinant adenovirus (Ad)-augmented Tr/E mediated increased poly(I-C)-driven antiviral protection that was associated with reduced proinflammatory factors, lower expression of RIG-I and MDA5, and attenuated NF- κ B activation (13). Tr and E have been identified in both serum and mucosal secretions (35). Various tissues, including skin, colon, placenta, lungs, the FGT, as well as cells like neutrophils, macrophages, and epithelial cells (36–37, 45, 53, 64, 78) were also shown to express Tr/E. *In vitro*, together with keratinocytes, bronchial, alveolar, and genital ECs were demonstrated to constitutively produce small amounts of Tr/E (55, 76), unlike SLPI (82). In contrast, Tr/E secretion is greatly induced in response to inflammatory stimuli, such as tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-

1 β), lipopolysaccharide (LPS), and neutrophil elastase (55, 61, 69). Interestingly, we and others have recently found that Tr/E secretion from the genital ECs is induced in response to a mimic of a viral double-stranded RNA (dsRNA), poly(I-C) (20; A. G. Drannik, K. Nag, X.-D. Yao, B. M. Henrick, J.-M. Sallenave, and K. L. Rosenthal, submitted for publication), hence confirming a significant role of Tr/E in antiviral immune inflammatory responses in the FGT mucosa.

In addition to observations by Iqbal et al., (30), further reports on antiviral activities of Tr/E emerged recently (13; Drannik et al., submitted), and the direct anti-HIV-1 effect of E was suggested as a protective mechanism against HIV-1 (20). Together with Tr/E, SLPI and ps20 have also been implicated in HIV-1 infection. While SLPI was shown to inhibit infection of HIV-1 permissive cells (39, 56), in contrast, ps20 enhanced HIV-1 infectivity and susceptibility of target cells (1). These observations suggest that a balance in SLPI and ps20, as well as other host molecules with documented anti- and pro-HIV-1 activities, might be an integral part in determining host's susceptibility to HIV-1 infection and should be considered in designing anti-HIV-1 interventions.

CVL comprises cell secretions from the FGT and contains a wide range of antimicrobial molecules. Some of these factors, including lactoferrin, α and β defensins, MIP-1 α , MIP1- β , MIP-3 α , SLPI, Tr/E, SDF-1 α , and RANTES have been shown to possess anti-HIV-1 activity and directly contribute to CVL's natural anti-HIV-1 activity, as reviewed by Hickey et al. (28). Significantly higher levels of Tr/E identified in CVLs of HIV-R compared to HIV-S CSWs suggested an important role of Tr/E in HIV-1 resistance in these subjects. Although Tr and E have been shown to be anti-HIV-1 factors, there is no clear understanding of how important Tr and E are pertaining to the total anti-HIV-1 capacity of CVL nor whether both Tr and E are equipotent in this activity. There is also no clear understanding whether the anti-HIV-1 effects of Tr/E depend on cellular environment. In this study, we have systemically addressed these issues and provide fundamental information regarding anti-HIV-1 activity of Tr and E.

MATERIALS AND METHODS

Study participants. Women within the cohort of Pumwani commercial sex workers (CSWs) from Nairobi, Kenya, which represents an ongoing, open cohort with participants enrolled between years 1989 and 2009, were enrolled during scheduled biannual resurveys. Within the cohort, women that remained HIV negative for at least 7 years of follow-up, as assessed by both serology and reverse transcriptase PCR (RT-PCR), and who were clinically healthy and free of concomitant sexually transmitted infections (STIs) as well as remained active in sex work, were considered HIV resistant (HIV-R) (17). Study participants who were HIV-uninfected CSWs, but had been followed up for less than 7 years, were defined as HIV susceptible (HIV-S). All of the participants in the cohort had similar socioeconomic and genetic backgrounds. As part of the resurvey, these women are routinely screened for the signs of coexisting STIs (*Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Haemophilus ducreyi*, syphilis, and bacterial vaginosis). No CSWs from the cohort that were enrolled in this study were found to have these coexisting STIs. Study protocols were approved by ethics review boards at the Universities of Nairobi, Manitoba, and McMaster. All participants provided signed, informed consent.

Isolation of mucosal samples. Cervicovaginal lavage fluid (CVL) specimens were generated by washing the ectocervix and aspirating lavages from the posterior fornix as described before (30). In total, 43 samples were collected in this study (HIV-S, $n = 23$; HIV-R, $n = 20$). Specimens were stored at -70°C until further analysis.

Reagents and cell lines. Poly(I:C) (Sigma-Aldrich, Oakville, Ontario, Canada) was reconstituted in the phosphate-buffered saline (PBS). HEC-1A and TZM-bl (JC53-BL) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in McCoy's 5A medium, modified (Invitrogen Life Technologies, Burlington, Ontario, Canada), and Dulbecco's modified Eagle's medium (DMEM), respectively, supplemented with 10% fetal bovine serum, 1% HEPES (Invitrogen Life Technologies), 1% L-glutamine (Invitrogen Life Technologies), and 1% penicillin-streptomycin (Sigma-Aldrich, Oakville, Ontario, Canada) at 37°C in 5% CO₂.

Trappin-2 and elafin proteins for *in vitro* experiments. Two preparations of Tr and E proteins were tested *in vitro*: preparation 1 was commercial human recombinant 6× His-trappin-2 (Tr) (with a C-terminus six-His tag) (R&D Systems, Burlington, Ontario, Canada) (65), and preparation 2 was commercial human recombinant elafin (E) (with no tag) HC4011 (Hycult Biotech, Uden, Netherlands).

Depletion of Tr/E from CVL samples. Specific antibodies against Tr/E (anti-Tr/E) (AF1747, goat IgG; R&D Systems) and against E (anti-E) (HM2063, mouse IgG2b; Hycult Biotech) were chemically cross-linked with cyanogen bromide (CNBr) beads, as per the supplier's protocol (Sigma-Aldrich). Free antibodies were removed by repeated (8×) washing of the beads with low-pH (acetate buffer, pH 4) and high-pH (bicarbonate buffer, pH 9) buffers (total washing, $8 \times 2 = 16$) at 4°C and confirmed by spectrometric analyses of the respective washings. The beads were reconstituted in PBS and subjected for depletion experiments. Control CNBr beads were also prepared in the same fashion without addition of any antibody and were used for preparing mock-depleted sample. The samples were treated with control beads for 2 h at room temperature in the presence of protease inhibitor cocktail (Roche, Mississauga, Ontario, Canada). The supernatants were collected after centrifugation at $3,000 \times g$ for 5 min at 4°C and considered precleared or mock samples. The precleared samples were added to the antibody-conjugated (anti-E-CNBr and anti-Tr/E-CNBr) beads and allowed to mix overnight at 4°C to deplete relevant target proteins from the precleared samples (mock). The E- and Tr/E-depleted samples were collected after centrifugation at $3,000 \times g$ for 5 min at 4°C as supernatants and subjected to functional assay. The depletion of the relevant target proteins from the samples by the antibody-conjugated anti-E-CNBr- and anti-Tr/E-CNBr-labeled beads was confirmed by Western blot (WB) analysis. The depletion efficiency of the beads was further evaluated by specific for Tr/E enzyme-linked immunosorbent assay (ELISA). CVL samples were further concentrated by the Amicon Ultra-15 filtration system (Millipore, Billerica, MA).

WB analysis. Tr/E- or mock-depleted pooled and concentrated CVLs from HIV-R CSWs received 1× protease inhibitor (Roche, Mississauga, Ontario, Canada), and protein was quantified using a standard method. Following a standard protocol, protein (15 µg) was electrophoresed on a 4 to 15% gradient denaturing SDS-PAGE gel (Bio-Rad Laboratories), transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, Arlington Heights, IL), and detected with primary anti-human Tr/E TRAB20 (Hycult Biotech) antibodies (1:500). After incubation with horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Laboratories), blots were visualized using a SuperSignal West Femto substrate kit (Thermo Scientific, Rockford, IL).

HIV stock preparation. The laboratory-adapted IIIB (X4-tropic) HIV-1 strain HIV-1_{IIIB} was prepared in peripheral blood mononuclear cells (PBMCs), and the ADA (R5-tropic) HIV-1 strain HIV-1_{ADA} was prepared in adherent macrophages purified from human PBMCs as described elsewhere (31). Supernatants of infected cells were collected, concentrated using 30-K Amicon filter tubes (Millipore, Billerica, MA), and assayed for p24 using an HIV-1-p24 ELISA kit according to the manufacturer's protocol. A dose of 10 ng/ml of p24 was used for transcytosis, immunofluorescence, and small interfering RNA (siRNA) experiments. When the median (50%) tissue culture infectious doses (TCID₅₀) for each final stock were determined, using the Reed-Muench method in TZM-bl cells (5.00×10^3 for HIV-1_{IIIB} and 2.77×10^4 for the HIV-1_{ADA} stock),

100 TCID₅₀ of each HIV-1 stock was used in HIV-1 attachment and TZM-bl infection assays.

TZM-bl infection assay. The TZM-bl cells used in this study express CD4, CXCR4, and CCR5 as well as *tat*-responsive luciferase and β -galactosidase reporter genes (31, 47, 54, 79). Cells (2.5×10^4 cells per well) were seeded in a 96-well plate the day before. One hundred TCID₅₀ of cell-free HIV-1_{ADA} and HIV-1_{IIIB} strains was incubated with various concentrations of all Tr or E proteins for 1 h at 37°C, in triplicate, before addition of protein and HIV mixtures onto untreated cells. Alternatively, protein-pretreated cells were repeatedly washed (4×) with PBS before receiving virus. Untreated cells with medium alone served as a negative control. For experiments with CVLs, lavage samples were diluted 1:4 and incubated with 100 TCID₅₀ of the cell-free HIV-1_{ADA} and HIV-1_{IIIB} strains for 1 h at 37°C, in triplicate, before addition onto TZM-bl cells. Viral infection was carried out over a period of 48 h and in the presence of 25 µg/ml diethylaminoethyl dextran (Sigma-Aldrich) per well. Luciferase production was developed by Bright-Glo reagents (Promega, Sunnyvale, CA) according to the supplier's protocol. Luminescence was read in the Veritas microplate luminometer (Promega) and is presented as relative light units (RLU).

HIV-1 attachment assay. HIV-1 attachment was determined as described in reference 38, but with minor modifications. Briefly, HEC-1A cells were grown to confluence in a 96-well plate. Cell-free HIV-1 (100 TCID₅₀ of HIV-1_{IIIB} and HIV-1_{ADA}) or cells were incubated either with medium or increasing doses of Tr or E at 37°C for 1 h. Each sample was performed in triplicate. After 1 h of incubation, cells were repeatedly (4×) washed with PBS, and HIV-1 or medium was added. Cells that were not initially pretreated with the proteins received either medium, virus alone, or virus preincubated with the proteins. Cells that were initially pretreated with the proteins received virus alone. After an additional 1 h 30 min of incubation, unattached virus was removed, and cells were repeatedly washed (4×) and lysed (1% Triton X-100 for 45 min at 37°C). Cell lysates were harvested and centrifuged at $11,000 \times g$ for 5 min. The amount of HIV-1 p24 associated with cell lysates was evaluated using an HIV-p24 ELISA according to the supplier's protocol.

HIV-1 transcytosis assay. Transcytosis of cell-free HIV-1 through the HEC-1A monolayer was performed as described before (31), with minor modifications. Briefly, HEC-1A cells were seeded at 1×10^5 cells per insert and grown as a tight polarized monolayer on a permeable polycarbonate support (0.4-µm-pore-diameter membrane tissue culture inserts; BD Falcon, Mississauga, Canada) for a minimum of 3 days, changing media every other day. Cells that had reached a transepithelial resistance (TER) of 350 to 470 Ω/cm² (EVOM; World Precision Instruments, Sarasota, FL) across monolayers, which was significantly higher than the background levels of <190 Ω/cm² in inserts with no cells, were considered confluent and used in the experiments. Cells were pretreated apically with various doses of Tr or E for 1 h before addition of 10 ng p24 of a cell-free HIV-1 inoculum for subsequent culturing with recombinant proteins for an additional 8 h at 37°C, at which point, virus transcytosis to the basolateral compartment was assessed. Supernatants from the basolateral compartment were collected and concentrated in 30-K Amicon microcentrifugation tubes (Millipore) and quantitatively analyzed by testing β -galactosidase activity of TZM-bl cells, resulting in cells turning blue upon HIV-1 infection. Data are expressed as percentages of infectious particles compared to a positive control or virus alone.

MTT viability assay. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Biotium, Inc., Hayward, CA) was used as per the manufacturer's instructions to determine viability of HEC-1A cells and was described elsewhere (52).

ELISAs. CVLs and cell-free supernatants were stored at -70°C until assayed for human Tr/E with an ELISA DuoSet kit (R&D Systems) according to the supplier's protocol. Human SLPI, LL37, and lactoferrin were quantified by specific ELISA kits (Hycult Biotech, Uden, Netherlands). The cutoff limits were as follows: Tr/E, 31.25 pg/ml; SLPI, 20 pg/ml; LL37, 0.1 ng/ml; and lactoferrin, 0.4 ng/ml. p24 was detected by HIV-p24 ELISA as per the supplier's protocol (HIV-1 p24 antigen capture assay; Advanced

BioScience Laboratories, Inc., Kensington, MD) with a cutoff limit of 3.1 pg/ml. Analytes were quantified based on standard curves obtained using a Tecan Safire ELISA reader (MTX Lab Systems, Inc.).

Tr/E knockdown by RNA interference. Small interfering (siRNA) molecules (Invitrogen Life Technologies) (GenBank accession no. NM_002638) were used to specifically knock down human Tr/E. The siRNA molecules were within positions 67 to 420 within a single ORF through the following sequence starting from position 202, CCCGUUAA AGGACAAGUUU. For nontargeting siRNA, an RNAi-negative control (medium GC content), 12935-300 (Invitrogen Life Technologies), was used. Transfections of siRNA (8 pmol) were done using Lipofectamine RNAiMAX (Invitrogen Life Technologies) and Opti-MEM I reduced serum medium (Invitrogen Life Technologies) as per the supplier's instructions. HEC-1A cells (3×10^4 in a 100- μ l total volume of complete growth medium) were transfected in a 96-well BD Falcon culture plate (BD Biosciences) for 48 h before addition of 25 μ g/ml of poly(I-C) or 10 ng of p24 of HIV-1_{IIIB} and HIV-1_{ADA} strains. Knockdown efficiency was monitored using Tr/E ELISA.

FACS analyses. For fluorescence-activated cell sorter (FACS) analyses, cells were pretreated with either medium alone or containing 1 μ g/ml of Tr or E for 1 h, followed by extensive washing and exposure to cell-free HIV-1 (100 TCID₅₀ of HIV-1_{IIIB} and HIV-1_{ADA}) for 1 h 30 min. After additional washing, cells were detached with a cell stripper (CellGro; Mediatech, Inc., Manassas, VA), and 1 million cells were incubated with antibodies (1 μ g) in 100 μ l of PBS containing 0.5% bovine serum albumin (Sigma-Aldrich) for 30 min on ice. The anti-human CXCR4 (306513; clone 12G5) and anti-human syndecan 1 (CD138) (352307; clone DL-101) antibodies with their corresponding isotype controls were obtained from BioLegend (San Diego, CA). Cells were washed and fixed in 2% paraformaldehyde overnight before being analyzed using a BD FACSCanto flow cytometer (BD Pharmingen). The data obtained were analyzed using FlowJo 9.0.1 software (TreeStar).

Protease inhibition assay. Antiprotease activity of Tr and E was assessed by their ability to inhibit human neutrophil elastase (HNE) (Sigma-Aldrich) activity and measured by applying the elastase-specific chromogenic substrate and measuring the change in optical density at 405 nm (OD₄₀₅), as was described elsewhere (71). The assay was performed in a 96-well plate by combining Tr or E protein (final volume, 10 μ l/well) or diluent alone with 50 ng of purified HNE or diluent alone (negative control) at 10 μ l/well and incubating the mixture for 30 min at 37°C. Subsequently, 50 μ l of HNE substrate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma-Aldrich), diluted to 50 μ g/ml in buffer (50 mM Tris, 0.1% Triton, 0.5 M sodium chloride [pH 8]), was added. The hydrolysis was measured by monitoring the absorbance at 405 nm for 15 min using a Tecan Safire ELISA reader (MTX Labs Systems). Results were expressed relative to the NHE activity of a positive control (HNE in diluent alone).

Statistical analysis. Data were expressed as means $\pm$ standard deviations (SD). Statistical analysis was performed with either a nonpaired Student's *t* test or one-way analysis of variance (ANOVA) using Sigma Stat 2.0.

RESULTS

Tr and E significantly contribute to anti-HIV-1 activity of CVLs from HIV-R CSWs. We first assessed levels of Tr and E in CVLs from CSWs to ascertain the amount of these proteins in the experimental samples and to confirm that elevated levels of Tr/E are associated with HIV-1 resistance, as previously demonstrated (30). ELISA measurements in Fig. 1A confirm that Tr/E protein levels in CVLs were significantly higher in the HIV-R group than those in HIV-S controls. Levels of other innate factors with documented anti-HIV-1 activity, namely, SLPI (16), LL37 (72), and lactoferrin (40), were also assessed in CVLs; however, no significant differences were found between the HIV-S and HIV-R

groups (data not shown). We then determined the anti-HIV-1 effect of CVLs from the HIV-R and HIV-S groups. Due to the constraint of sample quantity, we were unable to process individual samples for this experiment, and therefore six to eight CVLs within each HIV-S and HIV-R groups were combined into three individual samples. Three combined CVL samples per HIV-S and HIV-R group were each incubated in triplicate with cell-free HIV-1 prior to infecting TZM-bl cells for 48 h. Our results showed that CVLs from HIV-R compared to HIV-S had significantly more potent anti-HIV-1 activity against HIV-1_{ADA} (Fig. 1B). However, no protective effect was observed against HIV-1_{IIIB} from either of the groups (Fig. 1C).

Next, we determined whether the higher level of anti-HIV-1 activity of CVLs from the HIV-R group was specifically associated with the presence of a higher level of Tr/E in these samples. All remaining CVLs from HIV-R women were pooled (pCVLs) and subjected to mock or Tr/E immunodepletion. Qualitative analysis by Western blotting (WB) indicated that, compared to the mock-depleted sample, Tr/E amounts were significantly reduced in the Tr/E-immunodepleted sample (data not shown). Quantitative analysis by ELISA revealed that at least 30% of Tr/E was eliminated from the pCVL (Fig. 1D).

Figure 1E shows that pretreatment of cell-free HIV-1_{ADA} with mock-depleted pCVLs resulted in significant (almost 30%; $P < 0.001$) inhibition of HIV-1 infection of TZM-bl cells compared to virus alone. However, when HIV-1_{ADA} was pretreated with Tr/E-depleted pCVLs, the infection of TZM-bl cells was significantly ($P = 0.004$) increased compared to mock-depleted pCVLs. This increase in HIV-1 infection from pretreatment with Tr/E-depleted pCVLs represented approximately 60%, when the maximum inhibitory effect of mock-depleted pCVLs was considered 100%. In contrast to HIV-1_{ADA}, no significant anti-HIV-1 activity was observed against HIV-1_{IIIB}, in the presence or absence of Tr/E (Fig. 1F). Collectively, these findings indicate that even only 30% depletion of Tr/E in pCVLs from HIV-R CSWs accounts for over 60% of the direct anti-HIV-1 activity of CVLs, preferentially targeting HIV-1_{ADA}.

Tr and E equipotently inhibit human neutrophil elastase activity. Since one of the key properties of Tr/E is protease inhibition, this activity was evaluated as an indicator of the functional integrity of the tested proteins. Antiprotease activity was also tested to ascertain that structural (i.e., tag insertion) and manufacturing differences between the tested proteins were not dramatically affecting their functions, which would prevent comparisons of other properties of Tr/E in this study. Results of protease inhibition assay indicated that both Tr and E were equipotently active as protease inhibitors and almost completely abrogated enzymatic activity of HNE at a concentration of 10 μ g/ml (data not shown).

Tr and E independently inhibit HIV-1_{ADA} infection of TZM-bl cells. Next we evaluated the ability of Tr and E to inhibit HIV-1 infection of TZM-bl cells that express canonical HIV-1 receptor components CD4, CXCR4, and CCR5 (31, 47, 54, 79) and determined if the antiviral effect of Tr/E is virus or cell dependent. Data in Fig. 2A and B show that pretreatment of HIV-1_{ADA} with Tr or E, respectively, led to a dose-dependent inhibition (up to 50%) of HIV-1 infection of TZM-bl cells. The 0.001- to 1- μ g/ml concentration range for Tr and E proteins was chosen based on our ELISA measurements showing a range of 0 to 0.6 μ g/ml of Tr/E protein detected in CVLs from CSWs in Kenya (Fig.

FIG 1 Tr and E significantly contribute to anti-HIV-1 activity of CVLs from HIV-R commercial sex workers. Tr/E protein levels were measured by ELISA in cervicovaginal lavages (CVLs) from HIV-1-susceptible (HIV-S; $n = 23$) and resistant (HIV-R; $n = 20$) Kenyan commercial sex workers (CSWs) and presented as individual data points with the mean; all values are expressed as $\mu\text{g/ml}$ (A). Three combined CVL samples within each HIV-S and HIV-R groups were diluted 1:4 and tested in triplicate for anti-HIV-1 activity by incubation with 100 TCID₅₀ of R5-tropic HIV-1_{ADA} (B) or X4-tropic HIV-1_{IIIB} (C) for 1 h at 37°C prior to addition onto TZM-bl cells for 48 h. Collectively, cells received medium alone ($n = 3$) or combined CVLs ($n = 3$), untreated HIV-1 ($n = 3$), or HIV-1 treated with combined CVLs ($n = 9$). Substrate β -Glo was added 48 h postinfection, and the level of viral infection was quantified using a luminometer. The data are shown as the mean $\pm$ SD and presented as luminescence relative light units (RLU). In panels D to F, all CVLs from HIV-R CSWs were pooled (pCVL) and either mock depleted (Mock-D) or immunodepleted of Tr/E (Tr/E-D), and the depletion efficiency was verified by ELISA (D). Depleted pCVLs were diluted 1:4 and tested as described for panels B and C for anti-HIV-1 activity against HIV-1_{ADA} (E) and HIV-1_{IIIB} (F). Overall, in panels E and F, cells received medium alone ($n = 6$) or pCVLs ($n = 6$), untreated HIV-1 ($n = 6-9$), or pCVL-treated HIV-1 ($n = 6$). The data represent values from two experiments performed in triplicate and are shown as the mean $\pm$ SD of luminescence relative light units (RLU). Statistical analysis in all panels was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test, with P values indicated on the graphs. Statistical significance was considered when P was <0.05 .

1A), as well as physiological concentrations reported elsewhere (20).

Since inhibition of HIV-1 infection in TZM-bl cells was observed after the virus was preincubated with the proteins, this suggested that a direct, or virus-mediated, interaction between Tr or E and HIV-1 likely occurred. However, when the protein-HIV mixture was added onto the target cells, we could not have excluded the possibility that the proteins prevented HIV-1 infection through alternative, or cell-mediated, mechanisms; thus, we examined this possibility next. Figure 2C confirms earlier results of a direct anti-HIV-1 effect of Tr and E when tested at a concentration of 1 $\mu\text{g/ml}$; however, it also shows that when TZM-bl cells, but not the virus, were first pretreated with Tr or E and then repeatedly washed and infected with HIV-1, no significant reduction in HIV-1 infection of TZM-bl cells was observed. These data suggest that Tr and E mainly interacted with HIV-1 rather than compo-

nents on TZM-bl cells. In contrast, no reduction in HIV-1 infection of TZM-bl cells was seen following pretreatment of HIV-1_{IIIB} (Fig. 2D to F). Collectively, our findings indicate that both Tr and E have anti-HIV-1 activity. This antiviral activity is most likely virus mediated, involving direct interaction with HIV-1 particles, rather than cell mediated, which impacts binding/entry receptors on TZM-bl cells. The observed protective effect appears to be preferential against HIV-1_{ADA}.

Tr and E significantly reduce attachment of HIV-1 to HEC-1A cells. To evaluate whether Tr and E could interfere with HIV-1 attachment, HEC-1A cells, which are extensively used in the HIV-1 field for their close resemblance to genital ECs (38, 58, 59), were used. Figure 3A shows that when HIV-1_{ADA} was pretreated with either Tr or E at a concentration of 1 $\mu\text{g/ml}$, significantly less HIV-1_{ADA} was captured by HEC-1A cells, as measured by p24 associated with cell lysates. Similar results were observed

FIG 2 Tr and E independently inhibit HIV-1_{ADA} infection of TZM-bl cells. Media or recombinant trappin-2 (Tr) or elafin (E) at 0.001, 0.01, 0.1, and 1 μg/ml was incubated with 100 TCID₅₀ of R5-tropic HIV-1_{ADA} (A and B) or X4-tropic HIV-1_{IIIB} (D and E) for 1 h at 37°C before adding the virus onto TZM-bl cells for 48 h. Alternatively, media or Tr and E at 1 μg/ml were incubated either with 100 TCID₅₀ of HIV-1_{ADA} or TZM-bl cells (C) and HIV-1_{IIIB} or TZM-bl cells (F) for 1 h at 37°C. TZM-bl cells were then washed 5 times with PBS. Cells not pretreated with the proteins received either medium, untreated HIV-1 (V), or HIV-1 preincubated with Tr/E (V+p). Cells pretreated with the proteins (c+p) received untreated HIV-1 alone. Substrate β-Glo was added 48 h postinfection, and the level of viral infection was quantified using a luminometer. The data are representative of one of at least three independent experiments performed in triplicate and are shown as the mean ± SD of luminescence relative light units (RLU). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test, with *P* values shown and considered significant when *P* was <0.05.

when HEC-1A cells, and not the virus, were pretreated with Tr or E and then repeatedly washed and inoculated with HIV-1, thus, indicating that Tr and E inhibited HIV-1 attachment through direct interaction with HEC-1A cells that lack canonical HIV-1 receptors. Furthermore, our results showed that attachment of only HIV-1_{ADA}, but not HIV-1_{IIIB}, was inhibited (Fig. 3B). Additionally, metabolic activity of cells pretreated with Tr or E was assessed. No significant differences in metabolic activities were observed between the Tr- and E-treated and untreated cells (data not shown). Collectively these findings indicate that Tr and E significantly impeded attachment of HIV-1_{ADA} to HEC-1A cells, which was not due to altered metabolic activity of cells but through their interaction with both viral particles and cells.

Tr and E do not alter cell surface expression of HIV-1 receptors/coreceptors on HEC-1A and TZM-bl cells. Attempts were also made to determine whether pretreatment of cells with either

Tr or E would alter cellular expression of select HIV-1 receptors/coreceptors that have been shown to contribute to viral attachment/entry in HEC-1A cells (59). We did not assess the expression of CD4 and CXCR5, since HEC-1A cells have been shown not to express these molecules (59). Instead, we chose to look at syndecan-1 (CD138), as one of the abundantly expressed alternative binding molecules that belongs to the family of HSPGs on ECs (8, 59) and is important for HIV-1 attachment to HEC-1A cells. The expression of CXCR4 was included as a negative control, since this molecule was shown not to be important for HIV-1 attachment to ECs (8, 59). The expression of both syndecan-1 and CXCR4 on TZM-bl cells was also assessed for purposes of comparison, although no changes in their expression were expected, knowing that preincubation of Tr or E with TZM-bl cells did not reduce HIV-1 infection of these cells. The results of FACS analyses of cell surface expression of syndecan-1 and CXCR4 revealed no differ-

FIG 3 Tr and E significantly reduce attachment of HIV-1 to HEC-1A cells. Media or recombinant trappin-2 (Tr) or elafin (E) at 1 μ g/ml was incubated with 100 TCID₅₀ of R5-tropic HIV-1_{ADA} (A) or X4-tropic HIV-1_{IIB} (B) or HEC-1A cells alone for 1 h at 37°C. HEC-1A cells were washed 5 times with PBS before addition of medium (–) or virus (V) for another 1.5 h at 37°C. Cells not pretreated with the proteins received either medium (–), untreated HIV-1 (V), or HIV-1 preincubated with Tr or E (V+p). Cells pretreated with the proteins (c+p) received untreated HIV-1 alone. Following 1.5 h of incubation, HEC-1A cells were lysed and the amount of p24 associated with cell lysates was determined by ELISA. The data are representative of one of three independent experiments performed in triplicate and are shown as the mean $\pm$ SD of p24 protein expressed as pg/ml. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test, with *P* values shown and considered significant when *P* < 0.05.

ence in the receptors' expression between Tr/E-treated and untreated cells that were either exposed to R5 and X4 viruses or not (data not shown). Similar results were also obtained for TZM-bl cells (data not shown), suggesting that pretreatment of either HEC-1A or TZM-bl cells with Tr or E had no effect on syndecan-1 and CXCR4 expression in the presence or absence of viral exposure.

Knockdown of endogenous Tr/E by siRNA significantly increased shedding of infectious HIV-1 from HEC-1A cells. We next assessed the role of endogenous Tr/E in postentry events of HIV-1's life cycle and the effect of Tr/E on the ability of HEC-1A cells to release infectious virus. Endogenous expression of Tr/E in HEC-1A cells was knocked down by utilizing Tr/E-specific small interfering RNA (siRNA). Data in Fig. 4A show that following specific Tr/E siRNA silencing in HEC-1A cells, Tr/E protein levels were reduced at least 50% in supernatants of HEC-1A cells stimulated with poly(I:C). When HEC-1A cells were exposed to HIV-1 and the amount of infectious particles was detected 24 h later using TZM-bl target cells, we observed that HEC-1A cells treated with Tr/E siRNA released significantly more infectious particles than cells treated with control siRNA (Fig. 4B and C). TZM-bl data were also in line with significantly higher p24 levels measured in Tr/E siRNA-treated compared to control siRNA-treated cells for R5 virus (54 ± 4 and 38 ± 2 pg/ml, respectively; *P* = 0.004) and for X4 virus (87 ± 13 and 34 ± 9 pg/ml, respectively; *P* =

FIG 4 Knockdown of endogenous Tr/E by siRNA significantly increased shedding of infectious HIV-1 from HEC-1A cells. HEC-1A cells were left untreated (UT) or were transfected with a nontargeting control (ctrl) or Tr/E siRNA and subsequently received poly(I:C) treatment for 24 h. Tr/E protein levels were determined in HEC-1A supernatants by ELISA and expressed as ng/ml (A). In panel B, cells were siRNA transfected as described above and exposed to 10 ng p24 of R5-tropic HIV-1_{ADA} or X4-tropic HIV-1_{IIB} for 2 h. Following virus exposure, HEC-1A cells were extensively washed and medium was replaced for 24 h. To detect viral shedding, supernatants from HEC-1A cells were used to infect TZM-bl cells that were monitored for 48 h. Substrate β -Glo was added 48 h postinfection, and the level of viral infection was quantified using a luminometer (B and C). The data are representative of one of three independent experiments performed in triplicate and are shown as the mean $\pm$ SD of luminescence relative light units (RLU). Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's post hoc test, with *P* values shown and considered significant when *P* was < 0.05.

0.01). These results indicate that endogenously produced Tr/E significantly impacts the ability of HEC-1A cells to process and/or release infectious viral particles following HIV-1 exposure (Fig. 4B and C). It is noteworthy that the release of both HIV-1_{ADA} and HIV-1_{IIB} was increased from cells with reduced endogenous Tr/E expression.

Tr and E significantly reduce transcytosis of HIV-1 through a monolayer of polarized HEC-1A cells. Antiviral activities of Tr and E were also evaluated in a transcytosis assay (31, 38, 59). This assay was used to assess transfer of cell-free HIV-1 across a tight epithelial monolayer of polarized HEC-1A cells (with TER measuring above 350 Ω /cm²), grown on a semipermeable membrane. The results show that HEC-1A cells, apically pretreated with var-

FIG 5 Tr and E significantly reduce transcytosis of HIV-1 through a monolayer of polarized HEC-1A cells. HEC-1A cells were grown as a polarized monolayer in a Transwell system and incubated with medium or a range of recombinant Tr and E for 1 h before the addition of 10 ng p24 of R5-tropic HIV-1_{ADA} (A and B) or X4-tropic HIV-1_{IIB} (C and D) for 8 h at 37°C to the apical chamber. Data are representative of one of three independent experiments performed in triplicate and are shown as the mean $\pm$ SD. Amounts of infectious HIV-1 particles in the basolateral chamber were determined using TZM-bl cells and are expressed as the percentage of infectious particles recovered in the presence of Tr/E compared to the percentage of virus recovered in the virus control alone, taken as 100%. Statistical analysis was performed using one-way analysis of variance (ANOVA) with *P* values shown and considered significant when *P* was <0.05 .

ious doses of Tr or E before addition of HIV-1, passed significantly fewer infectious HIV-1 particles across the monolayer into the basolateral compartment (Fig. 5A to D). The inhibitory effect of Tr and E against HIV-1 transfer was dose dependent. In addition, pretreatment with E appeared to reduce transfer of not only HIV-1_{ADA}, but also HIV-1_{IIB}, although its effect on HIV-1_{IIB} was modest yet statistically significant (Fig. 5). Similar resistance (i.e., TERs) between apical and basolateral chambers before and after HIV-1 exposure ensured that the observed differences in virus transfer were not due to altered integrity of the epithelial monolayer. Altogether, these findings indicate that both Tr and E have the ability to reduce the amount of infectious HIV-1 particles crossing a tight epithelial barrier that could be attributed to either direct or indirect anti-HIV-1 activities of the proteins.

Elafin has greater anti-HIV-1 activity than trappin-2. To identify the comparative potency of Tr and elafin E, we utilized the functional HIV-1 infection and transcytosis experimental data for HIV-1_{ADA} virus. The data sets were plotted in a scattered diagram to determine the 50% inhibitory concentrations (IC₅₀) of Tr and E pertaining to the relevant anti-HIV-1 activity. The IC₅₀ for Tr and E regarding infectivity of TZM-bl cells were estimated to be 0.04 ± 0.013 μ g/ml and 0.0003 ± 0.0002 μ g/ml, respectively (Fig. 6A). The relevant IC₅₀ for Tr and E regarding transcytosis in HEC-1A cells were determined as 0.05 ± 0.015 μ g/ml and 0.0006 ± 0.0001 μ g/ml, respectively (Fig. 6B). Comparison of IC₅₀ for Tr and E revealed that E is approximately 130 times more potent than Tr in its ability to inhibit HIV-1 infectivity of TZM-bl cells. Additionally, E is approximately 80 times more effective in attenuating transcytosis of HIV-1_{ADA} virus particle through HEC-1A cells. Collectively, these results revealed that E is superior to its precursor, Tr, regarding anti-HIV-1 activities, and this superiority trend is equally evident for TZM-bl cells that express canonical HIV-1 receptors or for noncanonical HIV-1 receptor-bearing cells.

However, the overall efficacy of anti-HIV-1 activity of E is approximately 1.5 times higher for direct treatment of HIV-1 than the treatment of noncanonical receptor-bearing cells, namely, HEC-1A.

DISCUSSION

To date, our knowledge of correlates of HIV-1 protection, especially in mucosal fluids, remains limited. Despite the fact that numerous endogenous factors were shown to have anti-HIV-1 effects, their precise mechanism of action often remains elusive. Furthermore, their *in vitro* anti-HIV-1 activity and the *in vivo* contributions to HIV-1 resistance may not always support or predict each other. The presented study provides evidence on *in vitro* anti-HIV-1 effects of serine antiproteases Tr and E and their *in*

FIG 6 Determination of IC₅₀ of trappin-2 and elafin mediating HIV-1 infectivity and transcytosis. Readouts from dose-dependent functional studies were used for determining efficiency parameters of relevant experimental points. The maximum inhibitory effect was considered 100%, and the other values were computed accordingly and plotted against relevant doses of trappin-2 (Tr; asterisks) and elafin (E; solid circles) as percentages of maximal inhibitory effect. The relevant IC₅₀ for Tr and E are indicated by open arrowheads and solid arrowheads, respectively, regarding inhibitory effects of Tr and E on HIV-1 infectivity on TZM-bl cells (A) and HIV-1 transcytosis on HEC-1A cells (B). Error bars represent SD.

vivo role in HIV-1 resistance. Here, we elucidated antiviral properties of Tr/E by assessing their contribution to anti-HIV-1 activity of CVLs and by testing recombinant Tr and E individually against HIV-1 *in vitro*. Our results showed that elevated Tr/E levels were associated with clinical HIV-1 resistance of CSWs and anti-HIV-1 activity of CVLs *in vitro*; immunodepletion of Tr/E from CVLs confirmed that Tr and E significantly contributed to this inhibitory effect. Further, recombinant Tr and E each demonstrated both virus- and cell-mediated antiviral activities that led to inhibition of HIV-1 infection, attachment, and transcytosis through genital ECs. Interestingly, cell-mediated effects of Tr and E were only evident on ECs that did not express all canonical cell-surface HIV-1 receptors and were not associated with altered expression of syndecan-1 or CXCR4. Furthermore, knockdown of endogenous Tr/E increased HIV-1 shedding of both R5 and X4 HIV-1 from ECs. Finally, our data indicate that despite being equally active antiproteases, E is significantly more potent in its anti-HIV-1 activity than Tr, and antiviral properties of both Tr and E are context dependent. We believe this is the first report to describe anti-HIV-1 activity of Tr and to link mucosal HIV-1 resistance with novel anti-HIV-1 activities of Tr and E shown *in vitro*.

Higher levels of Tr/E have been previously associated with HIV-1 resistance in HIV-R CSWs (30). However, the direct contribution of Tr/E to mucosal protection against HIV-1 in HIV-R CSWs has not been investigated. We elucidated whether elevated levels of Tr/E in CVLs from HIV-R CSWs were associated with increased anti-HIV-1 activity of the CVLs and whether Tr and E were significantly contributing to this activity. We first ascertained levels of Tr/E in CVLs from HIV-R group. Our results confirmed that Tr/E levels were significantly higher in HIV-R compared to HIV-S CSWs, which was in line with an earlier report (30). Worth mentioning is that although our study was also conducted within the Pumwani cohort from Nairobi, Kenya, this is an open and ongoing cohort with continuous recruitment of new participants, and thus no significant overlap in samples was found between our study and that by Iqbal et al. (30). Furthermore, levels of SLPI, LL37, and lactoferrin were also determined in our study to explore the possibility that other antimicrobial factors with documented *in vitro* anti-HIV-1 activity (5, 39, 58, 66, 72) were contributing to HIV-1 resistance in CSWs. However, we found no differences in CVL levels of SLPI, LL37, and lactoferrin between the HIV-R and HIV-S groups of CSWs. Elevated levels of Tr/E observed in CVLs from HIV-R CSWs clearly emphasize the role of Tr and E in mucosal protection against HIV-1, although these findings do not negate the significance of other innate effectors found in CVL. In fact, SLPI, LL37, and lactoferrin represent only a fraction of many cationic anti-HIV-1 molecules of cervicovaginal fluid, whose synergistic effect as a compilation of factors, and not individually, is responsible for the natural antiviral protection within the FGT (77). Taken together, these findings indicate that the presence of elevated Tr/E and other factors, but not SLPI, LL37 or lactoferrin, might be more critical for mediating mucosal HIV-1 resistance in the genital tract of Kenyan CSWs, although this might be different for other cohorts.

The protective effect of natural Tr/E against HIV-1 was further demonstrated when CVLs from HIV-R, but not HIV-S CSWs, showed significantly greater inhibition of HIV-1 infection of TZM-bl cells and when immunodepletion of Tr/E from HIV-R CVLs resulted in significant ablation of total antiviral activity of

CVLs. Even though only a third of the Tr/E amount was removed from pooled CVLs, it accounted for up to 60% of the total anti-HIV-1 activity of CVLs, clearly indicating direct and significant contribution of Tr/E to natural anti-HIV-1 activity of CVLs. Interestingly, an earlier study showing antibody depletion of Tr/E from secretions of primary genital ECs cultured *in vitro* supports our findings by showing a reversal of anti-HIV-1 activity of antibody-treated secretions (20) and further confirms the contribution of Tr/E to anti-HIV-1 activity of mucosal fluids. On the contrary, Ghosh et al. found no correlation between Tr/E levels and anti-HIV-1 protection of CVLs from HIV-negative or HIV-positive women, and similar results were also found for SLPI. It is unclear why no correlation was found for Tr/E, but one explanation proposed by the authors was the low number of samples. Alternatively, the observed discrepancies may also stem from differences between cohorts and their levels of Tr/E measured in CVLs, CVL samples collected, or even functionality of Tr/E in collected samples. The above factors would advocate for future studies to reconcile the observed disparities. However, the determination of IC₅₀ from our study revealed that CVLs from the tested cohorts contained a sufficient level of Tr/E to provide significant protection against local HIV-1 challenge in the FGT.

In this study, we not only tested anti-HIV-1 activity of Tr, but also comparatively assessed the antiviral capacity of Tr and E by determining their inhibitory activities against both R5 and X4 HIV-1 strains and utilizing multiple assays and two human genital EC lines. *In vitro* testing demonstrated several anti-HIV-1 activities of Tr and E preparations and showed that each independently inhibited infection of TZM-bl cells, as well as reduced HIV-1 attachment and transcytosis through a monolayer of HEC-1A genital ECs, with preferential effect against R5 HIV-1. Interestingly, though, our findings demonstrated that anti-HIV-1 effects of recombinant Tr and E appeared to be contextual, since they were dependent either on the virus and its tropism or on cells and their specific components. Precisely, our studies with ECs that expressed canonical HIV-1 binding/entry molecules or not showed distinct virus-mediated anti-HIV-1 activity of Tr and E. Furthermore, we also presented evidence of alternative, or cell-mediated, antiviral effects of each of the Tr and E preparations that were never reported before. The latter antiviral activity of Tr/E, however, was only evident when HEC-1A cells, lacking canonical but expressing alternative HIV-1 binding/entry receptors, were independently pretreated with Tr or E before viral challenge. Our results are supported by an earlier study demonstrating E inhibiting HIV-1 infection of TZM-bl cells (20). In the latter study, however, antiviral activity of only E, but not Tr and E individually, were reported. Furthermore, their tested E preparation showed inhibitory effect against both R5 and X4 HIV-1 strains, unlike in our study, and it was only protective when viruses were pretreated with the protein. Since only TZM-bl cells were utilized in the above study, the authors failed to demonstrate the cell-mediated effect of E observed here. It was not unexpected to see multiple anti-HIV-1 activities of Tr and E, since other innate cationic antimicrobials, such as SLPI, lactoferrin, and defensins, have also been ascribed various antiviral properties (11, 26, 39, 44, 58), through which they all contribute to unique and potent antiviral activity of CVL. Worth mentioning is that SLPI's anti-HIV-1 effect was found to be primarily cell mediated and protective through interaction with scramblase 1 in T cells (56) and annexin II in macrophages (39), but not protective at all in the genital ECs

(29). These functional disparities perhaps show the consequences of only 40% of structural homology between Tr/E and SLPI and also indicate that the roles of SLPI and Tr/E in anti-HIV-1 defense in the FGT are not redundant, but rather unique and perhaps predetermined by their specific receptors' availability or affinity. Taken together, these observations underscore the important and complex anti-HIV-1 activities of Tr and E in inhibition of infection, attachment, and transcytosis of HIV-1 in the genital ECs.

It remains to be determined, however, how specifically Tr and E mediated their anti-HIV-1 activity. We speculated that it could be dependent on the cationic nature of Tr/E proteins potentially affecting the integrity of the virion. Alternatively, the HIV-1 inhibitory effect of Tr and E could involve their ability to bind to either viral glycoproteins or cellular structures (i.e., receptors), thus altering the attachment/entry and postentry events in HIV-1 life cycle. Since Tr and E were shown to bind to heparin and other matrix proteins (4, 22), and since HEC-1A cells express alternative HIV-1-interacting molecules like HSPG/syndecans, CSPG, GalCer, and mannose receptors (67, 84), in addition to canonical CXCR4 (59), we hypothesized that cell-mediated anti-HIV-1 activity of Tr and E might be related to preferential masking of alternative HIV-1 receptors on HEC-1A cells or changing their cell surface expression. Our data, however, showed no changes in syndecan-1 or CXCR4 expression after pretreatment of HEC-1A cells with Tr or E before viral exposure, which would not support our hypothesis. It is possible, however, that the changes in expression of these molecules occurred at a different time from the assessed time point, and hence these results must be interpreted with caution. Alternatively, it is possible that Tr and E had a masking effect on HIV-1 receptors/coreceptors, as opposed to triggering changes in their expression, which may warrant future investigations. Previous reports suggested a role of HSPG and GalCer in HIV-1 infection of TZM-bl cells, when pretreatment with GalCer mimetics (3, 18) and a syndecan-Fc hybrid (7) inhibited HIV-1 infection in these cells. From those studies, however, it was not clear whether those mimetics were exclusively blocking only alternative receptors or canonical receptors as well, or if high expression of canonical receptors would change the affinity to alternative ones located on TZM-bl. All of these arguments, on conjecture, could explain why we did not see any inhibition of HIV-1 infection when TZM-bl cells were pretreated with Tr or E preparations.

We also demonstrated that siRNA knockdown of endogenous Tr/E increased the release of both HIV-1_{ADA} and HIV-1_{IIB}. To date, there are no published reports that utilized the knockdown approach to assess the role of Tr/E in antimicrobial and especially antiviral defense mechanisms. However, the fact that rodents, who are naturally devoid of Tr/E, show increased susceptibility to *Staphylococcus aureus* infection and higher microbial load in lungs compared to their Tr/E-expressing counterparts (42) could relate to our findings (4, 70, 80). Furthermore, elevated levels of Tr/E found in CVLs of HIV-R CSWs would also imply a negative relationship between Tr/E and viral susceptibility/clearance. It is unclear why increased viral shedding of both HIV-1_{ADA} and HIV-1_{IIB} strains was observed following knockdown of endogenous Tr/E, unlike the preferentially anti-R5 HIV-1 effect from experiments with recombinant Tr and E preparations. However, these findings would indicate that endogenous Tr/E, compared to the exogenous Tr and E preparations used in this study, may exhibit differential anti-HIV-1 activity that is hypothetically more intracellular and even intranuclear, as would be suggested by our recent

studies investigating the possible link between anti-HIV-1 activity of Tr and E and their cellular distribution (A. G. Drannik et al., unpublished data).

Here, the anti-HIV-1 effect, with either CVLs or recombinant proteins, was preferentially observed against HIV-1_{ADA} and not HIV-1_{IIB}, although the E preparation showed reduced transcytosis of both R5 and X4 HIV-1 strains. Nevertheless, given that mucosally transmitted HIV-1 is primarily R5 in nature, it is promising to see the contribution of Tr/E to the inhibitory effect of CVLs primarily against HIV-1_{ADA}. Additional investigations may elucidate this preferential effect, but we hypothesize it can be attributed to potential binding preferences and affinities between Tr and E and the tested viruses, which needs to be confirmed by testing against a panel of X4 and R5 HIV-1 strains.

Our comparative analysis of anti-HIV-1 activities demonstrated that E is significantly more potent than Tr in its activity to inhibit infection and transcytosis of HIV-1_{ADA}. One of the limitations of this study was that tested Tr and E proteins were obtained from different manufacturing sources, arguably posing a question of the validity of the comparative analysis of the proteins' functions. However, the fact that tested Tr and E were equipotent in their antiprotease activity served as a common ground for the comparison of their anti-HIV-1 functions, which brings us to another question of why E appeared more potent in its anti-HIV-1 activity than its precursor Tr. The answer to this question is largely unknown as we are still far from a complete understanding of the basic structure/function relationships and the stemming antimicrobial and immunomodulatory activities of Tr and E. In fact, the field is still full of relevant questions posed over the years of the Tr/E antibacterial era, such as why Tr and its two components, namely, the N-terminus cementin and the C-terminus E, exhibited differential LPS binding with the N terminus binding both smooth and rough forms of LPS more avidly than Tr and E (43). In speculating why E was found superior to Tr in its anti-HIV-1 activity, we initially thought of net charge playing a role. However, this would appear counterintuitive since Tr is more cationic in nature than E (4). That Tr has four transglutaminase-binding sites on its N terminus, whereas E has only one, could also hypothetically have an impact on the protein-virus or protein-cell interaction, potentially altering electrostatic or other forces in place or the proteins' binding affinity. Another possibility is the smaller molecular size of E compared to Tr, which could allow for better spatial accessibility and congruency between matching sites on the virus and the proteins. The more potent antiviral activity of lactoferrin than its precursor, lactoferrin, may confirm this supposition (2). Alternatively, since E is approximately 130 times more potent than Tr regarding inhibition of HIV-1 infection and approximately 80% more effective in blocking HIV-1 transcytosis through genital ECs, we propose that it is the presence of E that is essential for anti-HIV-1 activity of CVL. This assessment is further reinforced by our recent observations of limited antiviral activity of Tr alone against green fluorescent protein-expressing vesicular stomatitis virus (VSV-GFP) challenge (A. G. Drannik, K. Nag, X.-D. Yao, B.M. Henrick, J.-M. Sallenave, and K. L. Rosenthal, submitted for publication). Therefore, we conclude that even though the presence of both Tr and E might be important for resistance against HIV-1, as would be suggested by elevated levels of both Tr/E found in CVLs of HIV-R CSWs from Kenya (30), only E may possess the essential anti-HIV-1 activity, with Tr serv-

ing as a source for E or playing another auxiliary role or roles potentially important in HIV-1–host encounter.

Although we realize that Tr and E are not the only anti-HIV-1 molecules providing antiviral defense in the FGT and that their anti-HIV-1 effects are not absolute, but contextual, we believe these results still provide valuable insights into additional correlates of protection against HIV-1. In conclusion, this study emphasizes the direct and significant involvement of Tr/E on anti-HIV-1 mucosal defense of CVLs from CSWs, and our *in vitro* results elaborate on anti-HIV-1 properties of Tr and E, highlighting potential mechanisms and targets of their antiviral activities that could be further explored and used in microbicide trials.

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