

TRAPPIN-2 AND ELAFIN AGAINST HIV-1 AND HSV-2 IN THE FEMALE
GENITAL MUCOSA

CHARACTERIZATION OF ANTIVIRAL PROPERTIES OF TRAPPIN-2 AND
ELAFIN AGAINST HIV-1 AND HSV-2 IN THE FEMALE GENITAL MUCOSA

By

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment
of the Requirements for
the Degree Doctor of Philosophy

McMaster University

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DOCTOR OF PHILOSOPHY (2012)

McMaster University

(Medical Sciences)

Hamilton, Ontario

TITLE: Characterization of Antiviral Properties of Trappin-2 and Elafin
Against HIV-1 and HSV-2 in the Female Genital Mucosa

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NUMBER of PAGES: xi, 264

----- Abstract -----

Sexually transmitted infections (STIs), especially HIV/AIDS and HSV-2, continue to be a devastating burden on societies around the world. The close link between HSV-2 and HIV-1, the role of inflammation in driving these infections, and the limited success and availability of prophylactic and therapeutic measures underscore the need for continued search of alternative means of protection. Characterization of endogenous antimicrobials, especially those local to the female genital tract and actively regulating inflammatory and antiviral responses, could be beneficial for microbicidal trials. Although regulators of mucosal immunity, such as serine antiproteases, trappin-2 and elafin (Tr/E), have been associated with resistance to HIV-1, their antiviral activity remains poorly understood. Thus, the research presented in this thesis centers on characterization of antiviral properties of Tr and E individually and their potential mechanisms in defense against HSV-2 and HIV-1 in the female genital mucosa. Chapter 2 examines Tr/E contribution to antiviral host defense responses elicited by a synthetic mimic of viral dsRNA, polyI:C. Chapter 3 documents the presence and characteristics, including potential mechanisms, of antiviral activity of Tr/E against *in vitro* and *in vivo* HSV-2 infection. Chapters 4 and 5 determine the contribution of Tr/E to the natural anti-HIV-1 protection of CVL and structural characteristics, mode(s) of action, and cellular distribution/localization of antiviral Tr/E proteins. Therein, we present novel properties of each Tr/E by demonstrating their inhibitory and multiple effects against both HSV-2 and HIV-1. These effects appear to be mediated either through virus or cells and be associated with altered viral attachment/entry, transcytosis and infection, innate viral recognition, modulated inflammation and increased antiviral protection of cells. Reported antiviral activity of Tr/E was also contextual and exerted, at least in HEC-1A cells, via autocrine/paracrine mode and depended on elafin's nuclear localization and its unmodified N-terminus. Tr/E may represent viable candidates for further studies in the field of STIs.

----- Acknowledgements -----

I wish to express my deepest gratitude to my family, friends and all of the colleagues, collaborators, examiners, students, and faculty that I am indebted to for immeasurable support in my journey through graduate studies and my growth as a person and as a scientist

To my supervisor, Dr. Kenneth Rosenthal, for his endless patience, wisdom, and guidance

To members of my Supervisory Committee, Drs. Charu Kaushic, Marek Smieja, and Zhou Xing for their critical, but always constructive and positive insight, support, and invaluable contribution

To Dr. Martin Stämpfli for his great guidance and encouragement in science and life

To Dr. Susanna Goncharova, Bethany Henrick, Dr. Xiao-Dan Yao, Dr. Sumiti Jain, and Dr. Kakon Nag, who were extremely generous with their friendship, support, time, and expertise

To my colleagues, Amy Patrick and Jennifer Newton for their unconditional support and guidance

To my colleagues and friends Tanja Thurn, Tina Walker, Alba Llop, Jessica Kafka, Victor Ferreira, Nicole Barra, Amy Gillgrass, Marcia Fattouh, Tiffany Chan, Amit Thatte, as well as Drs. Aisha Nazli, Ramzi Fattouh, Mahmoud Pouladi, Amal Al-Garawi, Firoz Mian, Dusan Sajic, Amanda Kwant, Vera Tang, Navkiran Gill, David Alvarez, Katherine Arias, Beata Gajewska, Jiang-Feng Sun, Ryan Wiley, Stacey Ritz, Elizabeth Cates, Jill Johnson, Filip Swirski, Clinton Robbins, Gordon Gaschler, Caleb Zavitz, Carla Bauer, Shunsuke Takenaka, Dominik Fritz, David Smyth for all of their help, guidance, and friendship throughout my long life at McMaster University

To my family, including the Pokanai, Milovanova, and Drannik families, for their continuous support, unconditional love, and endless encouragement

To my parents, Georgiy and Lyudmila, for being true role models in life and for all of their sacrifices so I would have the opportunity to pursue my dreams and interests

To my husband, Alex, and son, Timmy, for their immeasurable and endless love, support, and understanding; for sharing with me my dreams, hopes, and passions; for always having my back and being my inspiration and a shoulder to lean on during high and low times in this journey

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----- List of Abbreviations -----

AIDS	acquired immunodeficiency syndrome
AP-1	activating protein-1
APOBEC	apolipoprotein B mRNA editing enzyme, catalytic polypeptide
APOBEC3G	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G
CSW	commercial sex workers
cTr	human recombinant trappin-2 with a C-terminal His-tag (6×His-Tr)
CVL	cervico-vaginal lavage
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (protein)
E	human elafin without a tag
ECs	epithelial cells
Eh	human elafin purified from commercial Tr/E with a C-terminal tag
eIF2 α	eukaryotic initiation factor 2 α
hE	in-house human recombinant elafin with an N-terminal HAT-tag
FGT	female genital tract
GAG	glucosaminoglycan
gp	glycoproteins
HAART	highly active antiretroviral therapy
HESN	highly HIV-exposed seronegative individuals
HIV	human immunodeficiency virus
HIV-R	HIV-exposed resistant commercial sex workers
HIV-S	HIV-exposed susceptible commercial sex workers
HNE	human neutrophil elastase
HS	heparan sulfate
HSV	herpes simplex virus
IFN	interferon

IRF	interferon regulated factor
MDA5	melanoma differentiation-associated gene-5
OAS	oligoadenylate synthetase
NF- κ B	nuclear factor-kappa B
PAMP	pathogen-associated molecular pattern
PKR	protein kinase R
polyI:C	polyinosinic-polycytidylic acid
PRRs	pattern recognition receptors
RIG-I	retinoic acid-induced protein I
RNaseL	ribonuclease L
SIV	simian immunodeficiency virus
SLPI	secretory leukocyte protease inhibitor
STIs	sexually-transmitted infection
sTr	human trappin-2 without a tag, isolated from Ad/Tr-cells supernatants
TLR	Toll-like receptor
Tr	trappin-2
RIG-I	retinoic acid inducible gene-I
WAP	whey acidic protein
2',5'-OAS	2',5'-oligoadenylate synthetase

----- Preface -----

The research documented in Chapters 2-5 of this doctoral thesis represents four independent although conceptually related bodies of work that, as of July 2012, have been previously published or are in the submission process. The work conducted in each manuscript required a collaborative effort with several colleagues, resulting in multiple authors.

CHAPTER 2

Drannik A, Nag K, Yao X-D, Henrick BM, Sallenave J-M, Rosenthal K. Trappin-2/Elafin Modulate Innate Immune Responses of Human Endometrial Epithelial Cells to PolyI:C. *PLoS One*. 2012;7(4):e35866. Epub 2012 Apr 24.

This study was conducted between 2005 and 2010. As the lead author, I designed and performed all of the experiments, analyzed all data and wrote the manuscript. Drs. X-D. Yao (research associate) and K. Nag (post-doctoral fellow) provided technical assistance and help with editing of the manuscript. B. Henrick (graduate student) contributed with experimental assistance and discussion. Dr. J-M. Sallenave (collaborator) provided unique reagent for use in the study and contributed valuable scientific input and critically appraised the manuscript. Dr. K. Rosenthal assisted in interpretation of the study and revising of the manuscript.

CHAPTER 3

Drannik A, Nag K, Sallenave J-M, Rosenthal K. Antiviral Activity of Trappin-2 and Elafin *in vitro* and *in vivo* against Genital Herpes. Submitted to *J Virol* on August 20, 2012 and is currently under revision

This study was conducted between 2005 and 2010. As the lead author, I designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Dr. K. Nag (post-doctoral fellow) provided technical assistance and discussion. Dr. J-M. Sallenave (collaborator) provided unique animals for the study and contributed valuable scientific input and critically appraised the manuscript. Dr. K. Rosenthal assisted in interpretation of the study and revising of the manuscript.

CHAPTER 4

Drannik A, Nag K, Yao X-D, Henrick BM, Jain S, Ball B, Plummer F, Wachihi C, Kimani J, Rosenthal K. Anti-HIV-1 Activity of Elafin is More Potent than Its Precursor's, Trappin-2, in Genital Epithelial Cells. *J Virol*. 2012 Apr;86(8):4599-610. Epub 2012 Feb 15.

This study was conducted between 2007 and 2010. As the principal author, I designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Dr. K. Nag (post-doctoral fellow) provided technical advice and assisted with revising of the manuscript. Dr. X-D.Yao (research associate) and B. Henrick (graduate student) provided experimental support and discussion. S. Jain (graduate student) provided experimental advice and discussion. Drs. T.B. Ball, F.A. Plummer, C. Wachihi, J. Kimani (collaborators) provided unique reagents and Dr. T.B. Ball offered scientific input and critical appraisal of the manuscript. Dr. K. Rosenthal assisted in interpretation of the study and revising of the manuscript.

CHAPTER 5

Drannik A, Nag K, Yao X-D, Henrick BM, Ball B, Plummer F, Wachihi C, Kimani J, Rosenthal K. Unrestricted N-terminus and Nuclear Localization of Elafin are Critical for Antiviral Activity against HIV-1 in Female Genital Epithelial Cells. Submitted to *PLoS One* on March 26, 2012 and is currently under revision
*Joint first authors

This study was conducted between 2008 and 2010. I designed and performed the experiments, analyzed and interpreted the data, and wrote the manuscript. Dr. K. Nag (post-doctoral fellow) provided technical support and advice and assisted with interpreting the study and writing of the manuscript. Dr. X-D. Yao (research associate) and B. Henrick (graduate student) provided experimental support and discussion. Drs. T.B. Ball, F.A. Plummer, C. Wachihi, J. Kimani (collaborators) provided unique reagents. Dr. K. Rosenthal assisted in interpretation of the study and revising of the manuscript.

----- Chapter 1 -----

Introduction

Sexually Transmitted Infections

Sexually transmitted infections (STIs) remain a major public health concern and a great cause of morbidity, financial burden, infertility, and even mortality in both industrialized and developing countries (UNAIDS, 2007; WHO, 2001). In total, over two dozen of bacterial, viral and parasitic infections are known to be transmitted *via* sexual encounter, and they account for almost 340 million STI cases annually around the globe (WHO, 2001). It is believed, though, that STIs numbers are underestimated, as social stigma prevents people from seeking medical health, and there is also no single organization gathering statistics for STIs worldwide regularly, while many countries have their own ways of reporting infection rates.

These STIs include infections, such as those caused by *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, human papilloma virus (HPV), and *Treponema pallidum* that can be successfully treated when diagnosed at the right time (CDC, 2010). However, at the other end of the spectrum are infections with limited success in treatment/cure as well as in common availability and effectiveness of preventive measures. The latter infections are human immunodeficiency virus type 1 (HIV-1), a causative agent of acquired immunodeficiency syndrome (AIDS), and herpes simplex virus 1 and 2 (HSV-1 and HSV-2) that cause human herpetic ulcerative infections.

HSV-2 and HIV-1 interplay

HSV-2 and HIV-1 often co-exist, with HSV-2 facilitating HIV-1 infection (Cohen, 2004; Sobngwi-Tambekou et al., 2009). Preexistence of HSV-2 establishes local milieu that is more conducive to the acquisition and increased transmission and progression of HIV-1 (Celum, 2004; Sheth et al., 2008). Such a milieu is likely a result of collective events, including breached integrity of mucosal barrier due to genital ulcerations (O'Farrell, 1999), microbial stimulation through innate sensing (Kurt-Jones et al., 2005; Kurt-Jones et al., 2004), increased target cell influx (Koelle et al., 2000), and altered levels of protective antimicrobial innate factors and endogenous flora (Fakioglu et al., 2008; Kaul et al., 2008). Following HSV-2 and HIV-1 acquisition, these events can

be further maintained, in part, through immune activation and inflammation due to dysregulated immune responsiveness and increased activation of cellular transcription factors activating protein (AP)-1 (Jang et al., 1991; Spandidos et al., 1989) and nuclear factor kappa B (NF- κ B) (Equils et al., 2004; Li et al., 2009a). The immune activation and inflammation may occur at any point throughout the course of each HSV-2 and HIV-1 infections. But perhaps, these events are most pivotal during the early events of virus-host interaction and surrounding the establishment of primary HSV-2 or HIV-1 infection. Hence, in our studies, we primarily focused on the early events as well as inflammatory and antiviral responses following HIV-1 and HSV-2 stimulation in the female genital epithelial cells (ECs) and mucosa.

Current prophylactic and therapeutic measures against HSV-2 and HIV-1

Considering the overall globally limited availability and accessibility to anti-HSV-2 and anti-HIV-1 drugs and their high costs, as well as their ineffectiveness in curing already established diseases, having a preventive vaccine would clearly represent a better option for controlling the spread of HSV-2 and HIV-1. However, despite our understanding now that HSV-2 and HIV-1 are predominantly mucosally transmitted pathogens (Al-Harthi and Landay, 2001; Cunningham et al., 2006), we are still far from clearly defining the characteristics of protective immune responses at mucosal surfaces, which hinders the development of effective prophylactic and therapeutic measures.

Vaccines

Indeed, despite many attempts at designing effective vaccines in the HSV-2 field, including those with glycoprotein subunits, genetically modified live virus, replication-impaired mutants, DNA-based products, and vector-based vaccines (Stanberry and Rosenthal, 2005), their overall success is inconsistent and limited (Belshe et al., 2012; Cohen, 2010). Such results could be attributed, at least in part, due to efficient manipulation of and escape from the host's immune surveillance by virus.

Similar to HSV-2, an overall success of HIV-1 vaccines remains limited, although recent findings may hold promises for having successful prophylactic and therapeutic vaccines in the near future (McMichael and Haynes, 2012; Rappuoli and Aderem, 2011). Indeed, previously, many attempts with prophylactic HIV-1 vaccines (Flynn et al., 2005; Pitisuttithum et al., 2006) failed, potentially reflecting our poor knowledge of the pathogenesis of HIV/AIDS, as well as of viral complex structure, its global diversity, and mutagenicity. Recently, however, a community-based RV144 trial in Thailand finally showed some protection (Rerks-Ngarm et al., 2009). Albeit offering only modest 30% protective effect, this trial provided an insight for future vaccine trials suggesting that the induction of both cell- and antibody-mediated responses might offer the ultimate protection. Additionally, another vaccine trial just commenced in January of 2012 in the USA; being led by a Canadian researcher Dr. Kang, this vaccine is based on a whole genetically-modified inactivated virus as an antigen and has high expectations in the HIV field. The results of this trial are not available yet, but expected to be released soon.

Excitingly, a p24-like peptide (Vacc-4x)-based HIV-1 therapeutic vaccine from Bionor Pharma (Jones, 2010) has finally shown beneficial results in a phase IIb trial, demonstrating a statistically significant 64% reduction of viral load "set point" in patients receiving Vacc-4x compared to those given placebo. Collectively, although these advances in vaccine trials are encouraging, it will take time for these measures to become widely available and accessible. Hence, to gain a better and faster control over the spread of HSV-2 and HIV-1 infections in the absence of vaccines, a different approach is needed to complement currently available therapeutic options.

Microbicides

An alternative to vaccines would be the use of substances called topical microbicides designed for vaginal or rectal application that can either inactivate virus or prevent its binding, entry, and replication mucosally (Nikolic and Piguet, 2010). Microbicides have to be effective, non-toxic, non-inflammatory at mucosal sites, and not inducing disturbances in the endogenous flora. In this respect, naturally occurring

antimicrobial factors may represent a better alternative or complimentary option to synthetic microbicides, given their nontoxicity and compatibility with the host's mucosa. Although multiple synthetic compounds have been proposed for anti-HSV-2 and anti-HIV-1 use, many did not get clearance in the clinical trials. Some were even found to have increased the risk for HIV-1, likely due to their toxicity and inflammation-inducing effects *in vivo* as was shown for nonoxynol-9 (N-9) (Fichorova et al., 2001; Lederman et al., 2006; Nikolic and Piguet, 2010; Van Damme et al., 2002).

Two major classes of microbicides have been established on the basis of their properties: (i) nonspecific compounds with broad antiviral activity; and (ii) specific molecules targeting essential viral or host cell proteins (Nikolic and Piguet, 2010; Zydowsky, 2008). Nonspecific microbicides, detergents and pH-modifying compounds, usually act in the first steps of viral contact with the mucosal barrier and can inactivate virus. Specific compounds impair viral binding/entry into cells, whereas highly specific microbicides can act at later stages, such as reverse transcriptase inhibitor-based microbicides against HIV-1. In addition, microbicides based on the delivery of genetically-modified commensal bacterial strains (Liu et al., 2007; Zydowsky, 2008) have also been proposed. For example, the probiotic *Lactobacillus reuteri* (*L. reuteri*) RC-14 that safely colonizes the human vaginal tract, has been genetically modified to produce anti-HIV proteins blocking multiple steps of HIV entry into human peripheral blood mononuclear cells. One such substance is CD4D1D2-antibody-like fusion protein that was able to bind single or dual tropic coreceptor-using HIV-1 primary isolates. The HIV entry or fusion inhibitors were fused to the native expression and secretion signals of BspA, Mlp or Sep in *L. reuteri* RC-14 and the expression cassettes were stably inserted into the chromosome. *L. reuteri* RC-14 expressed the HIV inhibitors in cell wall-associated and secreted forms. This is the first study to show that a human vaginal probiotic strain can be modified to express functional viral inhibitors and thus to potentially lower the sexual transmission of HIV.

For HSV-2 prevention, a number of microbicidal compounds, including surfactants and detergents, N-9, inhibitors of viral binding/entry into neurons or keratinocytes, PRO 2000 and cellulose sulfate, as well as an RNA-interference-based approaches have been proposed and tested in animal and clinical trials (Nikolic and Piguet, 2010). A combination of Carraguard and zinc (PC-710) was investigated by the Population Council and found to be potent in animal models (Nikolic and Piguet, 2010). Similarly, a derivative of mandelic acid, PPCM, was also recently shown to prevent HSV-2 in a murine model (Mesquita et al., 2008). Further, an RNA-interference-based microbicides against HSV-2, targeting HSV-2 UL27 and UL29 genes, have also been investigated (Nikolic and Piguet, 2010; Palliser et al., 2006). However, none of the compounds had been approved for an official use as a microbicide against HSV-2, as of 2010. Therefore, in the absence of an effective prophylactic vaccine, the two established prevention methods for HSV-2 transmission are: condoms (Wald et al., 2005) and HSV-2 antiviral drugs (Corey et al., 2004).

For HIV-1, as of 2010, there were 11 non-specific microbicides, including PRO 2000, ACIDFORM, Invisible Condom, and entry inhibitor VivaGel, that were undergoing clinical evaluation and more than 50 compounds were still in preclinical development (Nikolic and Piguet, 2010). Pertaining to specific microbicidal compounds, essentially every group of highly active antiretroviral therapy (HAART) regimen was extensively investigated in animal studies as well as in safety and efficacy clinical trials. Protease inhibitors appeared to provide more pressure to HIV-1 and led to the least of drug-resistance, whereas the use of tenofovir demonstrated exciting results (Kelly and Shattock, 2011; Nikolic and Piguet, 2010). Indeed, several proof-of-principle trials of pre-exposure prophylaxis for HIV-1 prevention have been implemented in different populations. In these trials, tenofovir was used in various combinations: either as a topical 1% gel before and after sexual exposure supplemented with oral tenofovir alone or with other antiretrovirals, or just oral combination of tenofovir, as reviewed elsewhere (Celum and Baeten, 2012). Tenofovir is a nucleotide reverse transcriptase inhibitor targeting early steps of HIV-1 replication. In the CAPRISA 004 study, vaginal tenofovir

gel application had significantly reduced the risk of HIV-1 acquisition in participants by 39%, with even higher efficacy of 54% in a high-adherence group (Abdool Karim et al., 2010). Interestingly, in this study an unexpected 51% reduction in HSV-2 incidence in the tenofovir arm was also found, concurring to the earlier data demonstrated *in vitro* (Andrei et al., 2011). Indeed, tenofovir was shown to inhibit the replication of HSV clinical isolates in multiple cell lines, including human embryonic fibroblasts, keratinocytes, and organotypic epithelial 3D rafts, to decrease HSV replication in human lymphoid and cervicovaginal tissues *ex vivo*, and to delay HSV-associated pathology and mortality in topically treated HSV-infected mice. It is believed that the active tenofovir metabolite inhibits HSV DNA-polymerase in addition to targeting HIV reverse-transcriptase (Andrei et al., 2011).

Results from other three trials, albeit with oral tenofovir regimen, were in line with results from the CAPRISA 004 study (refer to reviews (Celum and Baeten, 2012; Kelly and Shattock, 2011)); however, the results from other two studies, with vaginal and oral tenofovir, did not show any efficacy. Overall, a significant protection by vaginally applied tenofovir gel demonstrated in the CAPRISA 004 study had provided a proof of principle that microbicides could be effective against STIs.

Therapeutic approaches

The introduction of acyclovir in the early 1980s, and subsequent release of its prodrug, valacyclovir, has dramatically changed the outcome of HSV-2 infection (Conant et al., 2002; Corey et al., 2004; King, 1988). The use of these drugs is associated with significantly reduced viral shedding and HSV-2 reoccurrence frequency (Corey et al., 2004). In addition, treatment with acyclovir shows not only a consistent effect on herpes suppression, but also on reduction in HIV-1 viral load and disease progression (Lingappa et al., 2010; Ouedraogo et al., 2006). Acyclovir is a nucleoside analogue that targets the HSV-2 kinase and DNA polymerase (King, 1988). In clinic, acyclovir and its derivative, valacyclovir, have been a common anti-HSV-2 therapy (Corey et al., 2004; King, 1988; Koelle and Corey, 2008). However, these medications only reduce HSV-2 replication and

neither cure the disease, nor affect HSV-2 entry, although valacyclovir therapy was shown to significantly reduce HSV-2 acquisition by reducing viral shedding in the HSV-2 heterosexual discordant couples (Corey et al., 2004). Nonetheless, potential acyclovir-associated hepatic and renal toxicity (King, 1988), and the emergence of resistant HSV-2 strains with the long-term use of acyclovir have been a concern (Griffiths et al., 2003; Piret and Boivin, 2011) and a constant reminder of the necessity for having alternative measures to compliment common therapy.

Similar to HSV-2, the introduction of antiretroviral therapy has dramatically reduced HIV-associated health burden. Specifically, mortality, morbidity and occurrence of opportunistic infections have been significantly reduced (Palella et al., 1998; Quinn, 2008). Five major groups of drugs constituting HAART and targeting several steps/structures in HIV life cycle were introduced and used to control HIV-1 infection over the years: inhibitors of the reverse transcriptase (RTI): nucleoside/nucleotide RTI (NRTI) and non-nucleoside RTI (NNRTI); the integrase strand-transfer inhibitors (INSTIs); the antagonists of CCR5; the protease inhibitors (PI); and the entry, or fusion, inhibitors (FI), as reviewed elsewhere (Peters and Conway, 2011; Sierra et al., 2005; Wainberg et al., 2012); but new drug candidates have also been undergoing testing (Asahchop et al., 2012). However, the use of HAART does not eliminate virus reservoirs throughout the body and does not cure already established infection. Furthermore, the long-term use of HAART is also associated with the emergence of HIV resistance (Wei et al., 2002) and side effects involving metabolic dysfunction, immune reconstitution inflammatory syndrome, neurocognitive and renal disorders, and cardiovascular events (Blot and Piroth, 2012; Bonham et al., 2008; Laurence, 2004). Collectively, these findings indicate that the search for complimentary strategies and factors, capable of targeting both HSV-2 and HIV-1 and their transmission/infection steps, should be continued. Perhaps, special consideration could be given to the host's endogenous antimicrobials. Being naturally a part of the local mucosal milieu, such antimicrobials will unlikely cause the toxicity and adverse effects on mucosal barrier and endogenous flora.

The mucosal environment

HSV-2 and HIV/AIDS are largely maintained by sexual transmission through mucosa of the genital and rectal areas and the FGT accounts for about 30-40% of new HIV-1 infections in women (Hladik and McElrath, 2008). To gain access to target cells, HSV-2 and HIV-1 must successfully cross the mucosal barrier in the FGT. However, the unique morphological and functional characteristics of the FGT can influence the outcome of this host-virus interaction.

The female genital tract and epithelial cells

The FGT is a unique immunological site with its main role to protect genital mucosa from invading pathogens and to ensure the successful reproduction of the species (Hickey et al., 2011). These roles are achieved by the FGT tolerating the allogeneic sperm and allowing the successful development of semiallogeneic foetus, while inducing efficient antimicrobial immune responses protective against pathogens. All of these processes are possible due to well coordinated efforts between unique structural, morphological, and physiological characteristics, innate and adaptive immune responses of the FGT, and their tight regulation by reproductive hormones, estradiol and progesterone, as extensively reviewed in references (Hickey et al., 2011; Wira et al., 2005a).

Instead of typically organized lymphoid structures (Johansson and Lycke, 2003), like those present in the gut or the lungs (Kunisawa et al., 2005), human uterus possesses unique lymphoid aggregates that are made up predominantly by CD8+ T cells that surround a central B cell core that are further encapsulated by macrophages (Yeaman et al., 1997). Having such an organization of lymphoid tissue may largely contribute to more compartmentalized immune responses in the FGT (Johansson et al., 2001; Kozlowski et al., 2002). The predominance of IgG over IgA in the lumen (Johansson and Lycke, 2003) and the presence of TGF β -mediated inhibition of antigen presentation by estradiol in the lower FGT (Wira et al., 2002) are additional features that make the FGT a

unique mucosal site, as reviewed elsewhere (Johansson and Lycke, 2003; Wira et al., 2005a),

Additionally, while hormonal changes during the menstrual cycle allow for the successful ovulation, implantation and pregnancy resolution, they also create a window of vulnerability 7-10 days post ovulation. During this brief period around ovulation, sex hormones directly or indirectly affect the mucosal barrier and immune protection, thus increasing the susceptibility of the FGT to STIs (Wira and Fahey, 2008). Indeed, for example, progesterone has been shown to suppress proliferative capacity of lymphocytes (Lewis, 2004), which could lead to compromised innate and adaptive immune responses within the FGT and consequently inefficient microbial clearance. Further, the expression of pathogen recognition receptors (PRRs) (Lin et al., 2009) and viral binding cellular receptors, for HIV-1 (Yeaman et al., 2003) and HSV-2 (Linehan et al., 2004), has also been shown to be regulated by sex hormones. Taken together, the features presented above play a significant role in normal and pathogenic conditions of the FGT and therefore must be taken into consideration when designing the protective measures for the FGT.

In women, the mucosal barrier is represented by primary genital ECs from the upper (Fallopian tubes, uterus, and endocervix) and the lower (ectocervix and vagina) FGT that are covered by a mucus layer and bathed in the genital fluid containing immune cells and protective factors, such as complement, immunoglobulins, cytokines/chemokines, and natural antimicrobial factors (Hickey et al., 2011), including cationic proteins that will be discussed later in this chapter. The genital fluid has a low pH due to the lactic acid –produced by commensal bacteria residing in the lower FGT (Antonio et al., 1999; Mirmonsef et al., 2011); the presence of such commensals makes genital mucosa an unfavorable milieu for pathogens to thrive.

Genital epithelial cells

Genital ECs are considered as primary sentinels and defenders of the FGT (Kaushic et al., 2010; Wira et al., 2005b), since they are located at the interface between

the hostile environment and the FGT. Genital ECs are immunologically active organ, regulating the passage of microorganisms across the mucosa as well as the induction of innate and adaptive immune responses to microorganisms. Specifically, genital ECs can *sense* invading pathogens and their pathogen associated microbial patterns (PAMPs) through innate sensors or pattern-recognition receptors (PRRs); ECs can directly *respond* to pathogens through the induction of immune mediators, as well as innate and adaptive responses (Quayle, 2002); ECs can also *transmit* this information to other cells by recruiting the latter through secreted chemoattractants (Kaushic et al., 2010; Wira et al., 2011a; Wira et al., 2005b).

The first-aid antiviral responses are triggered in ECs following PRRs activation and culminate in the induction of inflammatory mediators and antiviral type I interferons (IFN), leading to the containment of viral infection and its spread (Samuel, 2001; Yoneyama and Fujita, 2010). Through the secretion of such innate factors, ECs induce the adaptive immune responses, as chemokines and cytokines would facilitate the recruitment and activation of additional immune cells, including antigen-presenting cells such as dendritic cells (DCs). These adaptive immune responses will be necessary upon re-exposure to the pathogen (Samuel, 2001) for the generation of swift and robust recall responses.

Morphological and functional characteristics of genital ECs can significantly influence the outcome of the host-HIV encounter. Indeed, the lower genital tract is covered by a thick multi-cell layer of non-keratinized stratified squamous ECs that continuously slough off over the menstrual cycle (Wira et al., 2005a), thus making it more challenging for the virus to cross, despite offering a larger surface area for the host-virus interaction. In contrast, the upper FGT appears to be more vulnerable to viral transmission due to only a thin layer of columnar ECs covering the structures. Despite the tight junctions between the cells, making the healthy monolayer impermeable to pathogens and large molecules, this single-cell layer may allow viral transmission more readily, especially in the presence of post-coital abrasions. The transitional (transformation) area between ectocervix and endocervix is another and perhaps the most

susceptible transmission site for pathogens, including HSV-2 and HIV-1, due to its propensity to being susceptible to cervical ectopy and thus having protrusions of columnar epithelium (Kaul et al., 2008) as well as being enriched with submucosal CD4+ T cells, the primary HIV- target cells (Pudney et al., 2005).

Pathogen recognition receptors (PRRs)

Viral recognition through innate sensors occurs during viral attachment and entry into a target cell and is critical to the outcome of host-virus encounters. When induced in a timely and well-controlled manner, such responses serve to control or eradicate viral infection. As primary defenders in the genital mucosa (Wira et al., 2005b), ECs are equipped with distinct types of innate sensors, including Toll-like receptors (TLRs), with endometrial ECs expressing TLRs 1-9 and cervical and vaginal ECs expressing TLRs 1, 2, 3, 5, and 6 (Kaushic et al., 2010; Nazli et al., 2009; Schaefer et al., 2005). The presence of retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs), including retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), has also been reported, but in human endometrial HEC-1A cell line (Drannik et al., 2012c).

Membrane-bound TLRs 1, 2, 4, 5, and 6 are involved in the recognition of extracellular bacterial, fungal, and viral (for TLRs 2 and 4) PAMPs. In contrast, TLRs 3, 7, 8, 9, localized inside the cell in endosomes, participate in the recognition of nucleic acids of bacterial and viral origin, namely double-stranded (ds)RNA for TLR 3, single-stranded (ss)RNA for TLR 7/8, and CpG DNA for TLR 9 (Yoneyama and Fujita, 2010). Furthermore, for those PAMPs that have not been transported into endosomes and were left in the cytoplasm, RIG-I and MDA5 primarily participate in the detection of various forms and lengths of viral dsRNA (Yoneyama and Fujita, 2010). Thus, through these PRRs, genital ECs can recognize HIV-1 and HSV-2 and their multiple PAMPs, or evolutionary-conserved motifs associated with pathogens' structural components such as glycoproteins, lipidglycans, as well as DNA and RNA nucleic acids (Finberg et al., 2005; Mogensen et al., 2010; Yoneyama and Fujita, 2010) .

Ligation of such innate sensors leads to the activation of different transcription factors, including NF- κ B, AP-1, mitogen-activated protein kinase (MAPK), and interferon-regulated factors (IRFs) (Mogensen et al., 2010). These processes culminate in the transcription of pro-inflammatory and antiviral type I IFN genes. Indeed, genital ECs have been shown to secrete chemokines such as β -chemokines macrophage inflammatory protein (MIP)-1 α , MIP-1 β , MIP-3 α , regulated upon activation, normal T-cell expressed, and secreted (RANTES), as well as cytokines IL-1 β , IL-6, IL-8, tumor necrosis factor (TNF) α , and stromal-derived factor (SDF)-1 (Andersen et al., 2006; Drannik et al., 2012c; Fahey et al., 2005; Fichorova et al., 2002; Ghosh et al., 2009; Schaefer et al., 2004). Type I IFNs are among antiviral factors secreted by genital ECs (Nazli et al., 2009). These factors contribute to the containment of viral infection through multiple mechanisms, including the recruitment and activation of specialized cells like DCs, NK and CD8+ T cells, the production of host's antiviral factors such as apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G APOBEC3G (Chen et al., 2006) and the induction of interferon-stimulated genes (Bauer et al., 2008).

Among many genes facilitating the host's antiviral protection, studies have repeatedly confirmed the beneficial role of several ISGs. For example, ISG15 and ISG56 have been shown to contribute to the regulation of virus-induced type I IFN responses (Li et al., 2009b); 2',5'-oligoadenylate synthetase (2',5'-OAS) was shown to inhibit protein synthesis through the induction of endoribonuclease (RNase) L and subsequent degradation of cellular and viral RNAs (Clemens and Williams, 1978). Protein kinase R (PKR) (Karpov, 2001), inducible nitric oxide synthase (iNOS), and myxovirus (Mx)-family proteins are other IFN-induced proteins implicated in antiviral protection (Nazli et al., 2009; Schaefer et al., 2005). Collectively, these observations confirm that genital ECs are important regulators of the cross-talk between innate and adaptive antimicrobial responses. In addition, the important attribute of genital ECs, the possession of PRRs, can be a logical target for prophylactic and/or therapeutic approaches that could improve our efforts in controlling HSV-2 and HIV-1 infections.

PolyI:C: a potent antiviral factor with a caveat

Strong efforts have been made to induce potent protective responses by stimulating innate immune receptors with various adjuvants or microbial ligands, including polyinosinic-polycytidylic acid (polyI:C) (Ashkar et al., 2004; Nazli et al., 2009). PolyI:C is a synthetic viral ligand and a mimic of viral dsRNA. Viral dsRNA is a PAMP generated during a life cycle of most, if not all, viruses (Alexopoulou et al., 2001; Yoneyama and Fujita, 2010). PolyI:C, similar to dsRNA and viral dsRNA, is recognized by TLR 3 (Nazli et al., 2009; Schaefer et al., 2005) as well as MDA5 (Yoneyama and Fujita, 2010; Yoneyama et al., 2004), and RIG-I (Kato et al., 2008). Following recognition of dsRNA, activated PRRs trigger phosphorylation, homodimerization and translocation into a nucleus of a set of transcription factors like IFN regulatory factor 3 (IRF3), IRF7, NF- κ B, and ATF2/c-jun (c-Jun) (Alexopoulou et al., 2001; Bauer et al., 2008; Wathelet et al., 1998; Yoneyama et al., 2004). Inside the nucleus, these transcription factors either work independently or interact (Maniatis et al., 1998). Specifically, IRF3 alone can directly bind to the IFN-stimulated response element (ISRE) in the promoter region of ISGs and activate a set of ISGs in the absence of type I IFN production (Mossman et al., 2001; Paladino et al., 2006). Such antiviral cascade is devoid of excessive inflammatory responses and is induced when a low viral stimulation is detected. Alternatively, in response to a high viral load, IRF3 associates with NF- κ B and c-jun, forming an enhanceosome complex on the ISRE in the promoter region of IFN β . This complex triggers the production of IFN β and induction of ISGs in an IFN-dependent mode (Lin et al., 1998; Maniatis et al., 1998; Merika et al., 1998; Wathelet et al., 1998). Although such a response may be necessary, simultaneous induction of several cascades leads to a robust inflammation, target cell recruitment, and/or tissue damage due to the production of pro-inflammatory cytokines and chemokines along with antiviral type I IFNs molecules (Nazli et al., 2009; Paladino et al., 2006).

IRF3 was shown to be important not only for polyI:C-mediated protection (Bauer et al., 2008), but also in experiments with replication-competent viruses, including HSV (Yao and Rosenthal, 2011). Further, Vero cells deficient of IRF3 were unable to mount

an IRF3-dependent and IFN β -independent responses against either incoming virus particles or a dsRNA mimetic, polyI:C (Chew et al., 2009), and IRF3-deficient animals were less protected from a viral challenge with encephalomyocarditis virus (EMCV) and showed reduced IFN β levels in serum compared to their wild-type controls (Sato et al., 2000). However, DeWitte-Orr et al. showed that fibroblasts remained 60-90% protected against viral challenge even in the absence of IRF3 and its adaptor molecule, IFN promoter stimulator-1 (IPS-1) (DeWitte-Orr et al., 2009). These observations would suggest that the involvement of IRF3 could be critical, but not essential, for antiviral protection, depending on cell and virus type as well as the length of dsRNA (DeWitte-Orr et al., 2009). Nevertheless and most importantly, viruses, like HSV (Paladino et al., 2010; Yao and Rosenthal, 2011) and HIV-1 (Doehle BP, 2009), specifically target and disrupt functional activity of IRF3 as part of their strategy in making cells more susceptible to infection, thus further confirming the importance of IRF3 in antiviral protection.

Treatment with polyI:C has been shown to induce a potent antiviral protection *in vitro* and *in vivo*, making polyI:C an attractive candidate for microbicide or vaccine adjuvant trials against STIs (Ashkar et al., 2004; Nazli et al., 2009; Schaefer et al., 2005). However, in addition to an antiviral activity, polyI:C was also shown to trigger the release of pro-inflammatory mediators (Nazli et al., 2009; Schaefer et al., 2005) and to increase the susceptibility of target cells to HIV infection (De Jong et al., 2008). Additional research is required to reconcile whether polyI:C can be used for HIV-1 prevention in the future. Taken together, the above findings support that the FGT is a dynamic mucosal site contributing to the host's protection through antiviral and inflammatory responses. These immune responses, however, may appear as a double-edged sword: while being generally beneficial to the host, they may become detrimental when not efficiently regulated or exploited by a pathogen, and therefore contribute to disease progression. Thus, it is important to have means of active regulation of immune responses in the target organ, including the FGT.

HIV: structure, life cycle, pathogenesis

HIV is a member of the *Retroviridae* family (*Lentivirus* subfamily), characterized by causing a slow and long-progressing infection (Sierra et al., 2005). It is thought that HIV has been introduced to humans as a result of multiple zoonotic infections carried over from non-human primates infected with different strains of Simian Immunodeficiency Virus (SIV) (Wolfe et al., 2004), thus giving rise to different HIV strains, in turn. The most prevalent HIV-1 strain is related to SIV infecting Chimpanzees (Gao et al., 1999), whereas the less prevalent and less pathogenic HIV-2 strain is related to SIV infecting sooty mangabeys (Korber et al., 2000).

In 2010 it was estimated that since the early 1980s, HIV-1 infections led to more than 25 million deaths and additional 33 million of infected people globally (UNAIDS, 2010). HIV-1 is primarily a mucosal pathogen, since over 80% of people living with HIV/AIDS became infected through the exposure of genital and intestinal mucosal surfaces to the virus (Royce et al., 1997). Moreover, the risk of viral transmission and infection associated with different routes of exposure varies, with the FGT contributing between 30% and 40% of transmission (Hladik and McElrath, 2008). Given that the proportion of women living with HIV remains around 52% of global estimates (UNAIDS, 2010), understanding HIV-1 transmission and defense immune responses in the female genital mucosa is critical for gaining control over HIV infection. Furthermore, despite the overall global stabilization of the HIV-1 pandemic in many previously hard-hit countries, the rise of HIV-1 in other sectors, especially in counties of Eastern Europe and Central Asia continues (UNAIDS, 2010). Of great concern is also the high rate of HIV-1 infections in some industrialized cities, as it is evident by almost 3% of infection rate in Washington DC compared to 5 to 25% in some African countries (Greenberg et al., 2009).

The pathogenesis of HIV/AIDS and HIV life cycle are closely intertwined, in that the development of the disease begins with early direct interaction between the host and HIV, setting the stage for the establishment of a primary infection. Such an interaction is

largely based on pathogen's *binding* and *recognition* when crossing the genital or intestinal mucosal barrier, as well as the *host's responsiveness* to these processes. In turn, the latter events are predetermined by the *virus* and its life cycle as well as the *environment*, female genital mucosa in our studies. These events are also true for HSV-2 and will be discussed further in the corresponding section.

HIV structure

HIV is a spherical RNA retrovirus of around 100 nm in diameter. Mature virions consist of a lipid bilayer membrane with envelope glycoproteins (gp) on its surface. These glycoproteins are arranged as heterodimers of the external gp120 non-covalently attached to the transmembrane domain gp41 and are organized in 72 triangular-shaped spikes (Gelderblom, 1991; Ozel et al., 1988). HIV lipid membrane surrounds dense truncated cone-shaped nucleocapsid (core), which contains two identical copies of the positive single stranded (ss) genomic RNA, viral protease (PR), viral reverse transcriptase (RT), integrase (IN), some viral regulatory proteins such as Vpu, Vif, Vpr and Nef, and other cellular and viral factors, including tRNAs (Knipe et al., 2007). The core is supported by a number of proteins constituting Gag p55 complex: matrix protein (MA or p17), which binds to and stabilizes the outer lipid membrane (Wu et al., 2004); capsid protein (CA or p24), constituting the actual capsid (Briggs et al., 2003); nucleocapsid protein (NC of p7/9) that is crucial for the formation and stabilization of the genomic RNA dimers and in the NC assembly (Goel et al., 2002); and p6 that has been shown to be important for viral assembly and release from the cell as well as internalization of the Vpr protein into the assembled virion (Derdowski et al., 2004; Kondo et al., 1995; Sandefur et al., 2000).

Further, the HIV viral genome contains 9 genes that transcribe 15 proteins (Knipe et al., 2007). The genes are divided into two major groups: three are structural genes, including *gag*, *pol*, and *env*; and six are regulatory/accessory genes, namely *vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*. Further, each end of the genome contains long-terminal repeat (LTR) sequences with promoters, enhancers, and other gene sites used for binding cellular

transcription factors such as NF- κ B, AP-1, and nuclear factor of activated T cells (NFAT). Normally, upon cell activation, these factors lead to the transcription of inflammatory and antiviral genes as a first-aid response to either microbial or inflammatory stimulation (Kawai and Akira, 2010). However, when activated in the case of HIV, these factors will induce not only the transcription of these protective factors, but also the generation of HIV provirus and several viral proteins through binding to viral LTR, consequentially leading to increased viral replication (Berkhout and Jeang, 1992; Chen-Park et al., 2002; Huang and Jeang, 1993; Knipe et al., 2007). HIV LTR also contains trans-activation response element (TAR) implicated in Tat-mediated *trans*-activation of viral genes transcription. As a result, tat can induce viral replication not only via tat-NF- κ B-LTR interaction, but also via binding to the TAR element, ultimately also leading to viral replication and production of viral proteins (Richter et al., 2002). Collectively, these findings indicate that HIV has evolved several mechanisms to effectively target the cell to continue the generation of virus progeny, which might be dependent on microbial and inflammatory events in the host.

HIV life cycle

HIV replication is a well coordinated and sophisticated process that involves several steps as reviewed by Sierra *et al* (Sierra et al., 2005): (i) viral binding and fusion with a target cell; (ii) reverse transcription of viral RNA; (iii) integration of proviral DNA; (iv) viral DNA transcription and (v) mRNA translation; and finally (vi) assembly, budding and maturation of virions. To better understand and compliment the material presented in Chapter 4 and 5 related to viral binding/entry, HIV-1 attachment will be discussed in greater detail than the rest of the life cycle.

HIV-1 attachment is a complex and dynamic event that could be a turning point in the HIV-cell encounter. HIV-1 entry and infection are initiated with viral attachment to a target cell surface through the engagement of canonical HIV receptors, namely the CD4 and one of the chemokine receptors CCR5 or CXCR4. Binding between HIV-1 envelope

gp120 and CD4 (Dalglish et al., 1984; Klatzmann et al., 1984) initiates this process, bringing about the conformational change in gp120 and allowing for its subsequent attachment to a secondary binding molecule, CCR5 or CXCR4 (Alkhatib et al., 1996; Feng et al., 1996). Subsequently, a gp120-CD4-CXCR4/CCR5 interaction leads to additional conformational changes resulting in the exposure and energetic insertion of the gp41 fusion domain into a permissive cell, and ultimately in virion entering the cell.

HIV-1 coreceptors CXCR4 and CCR5 are members of the chemokine receptor family and represent binding partners to naturally occurring ligands RANTES, MIP-1alpha and MIP-1beta for CCR5 and SDF-1 for CXCR4 (Alkhatib et al., 1996; Feng et al., 1996). The significance of CCR5/CXCR4 discovery as HIV-1 coreceptors comes from the fact that the use of these factors can block or reduce HIV infection in target cells (Kawamura et al., 2000). Further and perhaps even more importantly is that the homozygous mutation in the CCR5 gene almost always results in HIV-1 resistance (Liu et al., 1996). In addition, the preferential use of chemokine receptors also represented the basis in the last classification of HIV-1 strains (Grivel et al., 2010). The use of either CCR5 or CXCR4 determines the susceptibility of different cell populations to HIV-1 infection and replication, and predetermines viral tropism. That is, CCR5 is a coreceptor for monocyte/macrophage-infecting, non- syncytium-inducing R5 (M)-tropic HIV primary isolates, whereas CXCR4 is a coreceptor for T cell-infecting and syncytium-inducing X4 (T)-tropic viral isolates. There are isolates, however, that exhibit dual R5X4 tropism (Grivel et al., 2010).

The deciphered interaction between gp120/gp41 and canonical HIV-1 receptors was historically believed to be a prerequisite for a successful infection, since it was initially described for the main CD4+ HIV-1 target cell such as CD4+ T cells that become infected and largely depleted during the course of infection. However, this traditional view was challenged when cells with low or no CD4 surface expression or differential pattern of chemokine receptors expression (Bobardt et al., 2007), such as dendritic cells (DC), macrophages, or primary genital epithelial cells (ECs) (Saphire et al., 2001; Tremblay, 2010), were also found permissive to HIV-1 attachment/entry. This

posed the question of how HIV-1 gains access to these cells and whether or not it uses alternative attachment molecules or perhaps a combination of the latter and canonical receptors.

Indeed, a number of alternative, or non-canonical, HIV-1 binding receptors have been identified on cells susceptible to HIV-1 attachment/entry or infection, including DCs and genital ECs, and shown to be important in HIV-1 pathogenesis. These molecules vary vastly depending on a cell type and include C-type lectins such as langerin in LC, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (protein) DC-SIGN (Geijtenbeek et al., 2000), the type II membrane glycoprotein DCIR (dendritic cell immunoreceptor) (Lambert et al., 2008), and syndecans (Bobardt et al., 2007; Bobardt et al., 2003), to name a few. Studies show that these receptors can function separately in the absence of CD4/CCR5/CXCR4, or together and/or in the presence of canonical receptors, and the implication of the latter mechanism was recently described for integrin $\alpha 4\beta 7$ (Arthos et al., 2008), as will be discussed later.

Furthermore, alternative HIV-1 receptors have been also implicated in both passive transmission of HIV in *trans*-infection (Bobardt et al., 2007; Bobardt et al., 2003; Gallay, 2004; Geijtenbeek et al., 2000), and a productive *cis*-infection (Gringhuis et al., 2010; Mondor et al., 1998), depending on the receptor and the cell type. An example of the latter was recently identified in DCs, when gp120-DC-SIGN binding triggered a productive HIV-1 infection by augmenting the initially insufficient TLR8-ssRNA-mediated induction of NF- κ B activation (Gringhuis et al., 2010) in a Raf-1-dependent way. The role of alternative HIV-1 receptors in viral attachment/entry into the genital ECs will be discussed in the following sections. Taken together, these observations indicate that HIV-cell interaction during viral attachment is much more complex than previously thought. HIV-1 attachment has a significant bearing on the outcome of HIV-cell encounter, depending on virus and a cell type, which should be considered when designing protective antiviral measures. Alternative HIV-1 receptors may significantly alter the dynamics of the HIV-cell interaction by amplifying the stimulation that could result in a different quality of HIV-1 transmission. More research is needed to better

understand the interaction between canonical and non-canonical HIV receptors and on different cell types and its implications on HIV/AIDS pathogenesis.

Pathogenesis of HIV/AIDS: from early events to acute and chronic phases

HIV-associated immune dysfunction has long been associated with immunodeficiency, a hallmark of HIV/AIDS pathogenesis. Further, there is growing evidence supporting the idea that it is the immune activation, rather than a loss of CD4+ T cells, that is driving immune dysfunction in HIV/AIDS pathogenesis. Despite the fact that the first mention of this idea appeared in the late 1980s (Ascher and Sheppard, 1988; Grossman et al., 1993), we are still far from having a clear understanding of what is really fueling HIV/AIDS pathogenesis and in particular how chronic immune activation is sustained and caused immunodeficiency. Since anti-HIV defense involves both innate and adaptive arms of the immune system, it is likely that multiple mechanisms are underlying sustained immune activation. Whereas historically adaptive immune responses were better investigated and understood, the role of innate immune responses in HIV/AIDS was largely overlooked. However, being the first line of defense against invading pathogens, including HIV, innate responses may play a paramount role in determining the outcome of HIV-host encounter.

Innate immune activation begins with HIV attachment to cells, as well as the recognition of viral and other PAMPs and subsequent triggering of innate inflammatory and antiviral responses. These responses are generally beneficial to the host, but may become detrimental, when dysregulated, and thus contribute to disease progression partly through NF- κ B-mediated immune activation, cell recruitment and activation. These events lead to sustained immune activation and inflammation, viral replication and high cell turnover that result in functional immune exhaustion and progression to AIDS (Broliden et al., 2009; Haase, 2011; McMichael et al., 2010; Mogensen et al., 2010). Although the evidence of immune activation can be found during both acute and chronic stages of HIV/AIDS as well as locally and systemically, the events surrounding the host-

HIV initial encounter in mucosa might be the most crucial in determining the susceptibility or resistance to HIV; yet, they remain poorly understood.

Early events

The natural course of HIV-1 infection includes two stages: an acute, or primary, infection and a chronic phase, with each of them having characteristic viral and immune patterns. To gain access to HIV-1 cell targets, including CD4+ T cells, subtypes of DCs, and monocytes/macrophages, HIV must successfully cross the mucosal barrier in the FGT. From the studies using human *ex vivo* tissue cultures/explants and animal models, we now know that virions can cross the female genital mucosa in both lower and upper part of the FGT (Howell et al., 1997; Joag et al., 1997; Kell et al., 1992; Miller et al., 1992; Miller et al., 2005; Zussman et al., 2003). However, specifically how HIV crosses the mucosal barrier remains unclear, since the heterogeneity of experimental models, conditions, and tissue/cell types used in those studies preclude from reaching a consensus. Indeed, several modes/routes of HIV transmission across the mucosal barrier have been proposed based on *in vitro* models and *ex vivo* explants and include: direct infection (Howell et al., 1997); HIV passage through tissue abrasions (Norvell et al., 1984); viral capture by dendrites of intraepithelial DC/Langerhans cells (Kawamura et al., 2000; Turville et al., 2002); HIV paracytosis between the cells due to disrupted barrier integrity (Nazli et al., 2010); and transcytosis (Bobardt et al., 2007; Bomsel, 1997; Saidi et al., 2007; Wu et al., 2003).

The transcytosis model, in particular, has been studied extensively over the years. This model was first proposed by Bomsel, M. in 1997 (Bomsel, 1997). Ever since, different variations of this model have been utilized to study the mechanisms involved in the transmucosal passage of HIV and the early events of cell-HIV interaction (Bobardt et al., 2007; Bomsel, 1997; Bouschbacher et al., 2008; Hocini et al., 2001; Wu et al., 2003) as well as the HIV inhibitory effect of various compounds (Bobardt et al., 2010; Hocini et al., 1997; Lorin et al., 2005; Saidi et al., 2006). In most studies utilizing this model, cell-free and cell-associated virions were endocytosed and transmitted across polarized

epithelial monolayer, albeit at low rate, without causing a productive HIV infection of genital ECs (Bobardt et al., 2007; Wu et al., 2003). However, some reports have shown a productive infection of ECs (Howell et al., 1997), while others have demonstrated that, instead of productive infection, genital ECs sequester large amounts of virus (Wu et al., 2003) and thus can be a reservoir of HIV-1 (Dezzutti et al., 2001; Saidi et al., 2007). Transcytosis as a mode of HIV transmission was shown in primary EC cultures (Bobardt et al., 2007; Bouschbacher et al., 2008) and cell lines (Saidi et al., 2007), but neither in tissue explants nor *in vivo*. Furthermore, HIV-1 transmission was shown to be augmented under pro-inflammatory conditions (Carreno et al., 2002), indicating that inflammation promotes HIV-1 transmission across ECs monolayer.

Interestingly, another study recently confirmed the detrimental role of pro-inflammatory conditions in HIV-1 passing through an epithelial monolayer and proposed a novel mechanism of viral transmission across genital ECs (Kaushic et al., 2010; Nazli et al., 2010). In this study, the researchers demonstrated that a direct interaction between HIV-1 gp120 and primary genital ECs triggered an acute (within 4h) event resulting in secretion of pro-inflammatory factors, including TNF α . The induction of TNF α , in particular, led to breaching the integrity of ECs monolayer through disruption of tight junctions between ECs and allowing for HIV and bacterial translocation across the monolayer (Nazli et al., 2010). These findings demonstrate that genital ECs can play a pivotal role in regulating the permeability of the genital mucosa for HIV and other luminal microbes. These findings also imply that despite genital ECs contributing to the natural mucosal protection through secretion of antimicrobial factors, they can also alter the permeability of this barrier by inducing a TNF α -mediated immune activation and pro-inflammatory milieu, meaning they can change the outcome of the host-HIV encounter. However, these events are likely either insignificant or infrequent in *in vivo* HIV transmission, which is supported by low rates of HIV-1 transmission via vaginal intercourse (Mastro and de Vincenzi, 1996) and that only a single or few isolates cross the barrier and establish the infection (Zhu et al., 1996).

Although the actual binding partner of HIV gp120 was not reported in this study, it might be interesting to see whether or not these events are mediated through the recognition of viral gp120 by cellular membrane-bound TLRs, for example TLR 2 and 4 or some alternative receptors. Hypothetically, given that genital ECs express a variety of TLRs and that during HIV-1 replication cycle a multitude of PAMPs can be generated (Mogensen et al., 2010), it is plausible that other sensing receptors, as was shown for TLR 7/8 and HIV ssRNA in DCs (Gringhuis et al., 2010), might also be involved in viral recognition during its passage through genital ECs.

In addition to inflammatory conditions, the role of HIV-1 interaction with genital ECs *via* binding/entry HIV-1 receptors has also been recently highlighted, suggesting a role in HIV-1 pathogenesis (Gallay, 2004). Studies demonstrate that genital ECs express a variety of HIV-1 attachment/entry receptors, including canonical CD4, CXCR4, and CCR5 receptors and a variety of non-canonical HIV-1 binding structures. The expression of both CXCR4 and CCR5 receptors has been repeatedly confirmed and shown to vary throughout the menstrual cycle (Bobardt et al., 2007; McClure et al., 2005; Yeaman et al., 2003). In contrast, the data demonstrating CD4 expression in the FGT has been inconsistent, with some studies showing its presence and dependence on hormonal regulation (Yeaman et al., 2003), and others not confirming its presence at all (Bobardt et al., 2007; McClure et al., 2005). Taken together, these results would support the role and the presence of alternative HIV receptors on genital ECs involved in promoting viral migration across genital mucosa.

Indeed, studies demonstrate that in addition to canonical HIV-1 receptors, a number of non-canonical HIV binding molecules are also present on genital ECs. Some of the better studied receptors are proteoglycans (PG) with chondroitin sulfate (CSPG) chains; syndecans, the most abundant PGs with heparan sulfate (HS) chains (Bobardt et al., 2007; Mondor et al., 1998); β 1 integrin (Maher et al., 2005); galactosylceramide (Bomsel, 1997); and a variant of salivary agglutinin gp340 (Stoddard et al., 2007). These receptors have been implicated in facilitating HIV-1 attachment, entry, and transmission across both primary cervical and vaginal ECs (Bobardt et al., 2007; Stoddard et al., 2007),

as well as immortalized cell lines (Bobardt et al., 2007; Gallay, 2004; Saidi et al., 2007). Interestingly, syndecans have been also implicated in attachment of not only HIV-1 (Gallay, 2004), but other viruses as well, including HSV-2 (Cheshenko et al., 2007), thus further highlighting their significance in promoting viral infections and perhaps even providing additional link between HSV-2 and HIV-1 infections.

Despite the demonstrated importance of these receptors in facilitating HIV-1 transmission across mucosa, precisely how syndecans work remains unclear. Recently, however, it was demonstrated that HIV-1 gp120 can recognize similar motifs on syndecans and CCR5 (de Parseval et al., 2005). This finding raises questions about the relative contribution of each of the HIV binding receptors and their potential interaction on HIV-1 transmission in different cell types. This finding also implies that syndecans may facilitate HIV-1 attachment/transmission across genital ECs in a similar way as was shown for integrin $\alpha 4\beta 7$ and CD4+ T cells (Arthos et al., 2008). In this study, Arthos et al. showed integrin $\alpha 4\beta 7$ to interact with gp120 (and possibly simultaneously with CD4 and CCR5) in CD4+CCR5+ T cells that led to the rapid activation of LFA-1 through an as yet unidentified signal, subsequently facilitating the formation of virological synapses and more efficient cell-to-cell HIV-1 spread (Arthos et al., 2008). Since among CD4+CCR5+ T cells found in mucosal tissues there are also those T cells expressing $\alpha 4\beta 7$, this mechanism may partly explain why predominantly R5, and not X4, HIV-1 isolates transmit infection. Similarly, it is plausible that gp120/syndecan/CCR5 interaction in ECs might be one of the reasons why preferentially R5-tropic HIV-1 isolates cross the mucosal barrier and transmit the infection to the immune cell targets (Keele et al., 2008; Zhu et al., 1996). It is unclear at the moment why preferentially R5 HIV isolates become the transmitter-founder viruses, but it is likely a result of collective pressure from several factors. Such factors may include mucosal innate antibacterial factors, HIV target cell availability and their appropriate receptor expression, and fitness of the virus, especially pertaining to its gp120-CCR5 interaction and subsequent signal transduction events (Arthos et al., 2008) (Grivel et al., 2010). Taken together, these findings indicate that genital ECs play a critical role in viral transmission across genital

mucosal barrier by directly recognizing and responding to HIV-1. Thus, the regulation of pro- and anti-inflammatory factors at the EC level might be crucial for controlling the passage of HIV and other pathogens across genital epithelial monolayer.

Acute and chronic phases

After direct interaction between HIV-1 and ECs and subsequent viral transmission across the mucosal barrier, more HIV target cells are recruited to submucosa and become activated. These events likely occur due to initial secretion of pro-inflammatory and antiviral cytokines and chemokines, such as MIP3- α , by genital ECs in response to viral encounter (Haase, 2011; Nazli et al., 2010; Ochiel et al., 2010) and subsequent release of MIP-1 β by CCR6+ plasmacytoid DC (pDC) that consequently attract more HIV-1 target cells, such as T cells and macrophages (Haase, 2011). Despite the induction of antiviral type I IFNs and APOBEC factors, as well as LC destroying captured HIV through langerin (McMichael et al., 2010), HIV-1 target cells become infected with predominantly R5 HIV isolates (Keele et al., 2008; Saba et al., 2010; Salazar-Gonzalez et al., 2009; Zhu et al., 1996). Based on studies in humans and non-human primates (NHPs), we know that virus establishes a few focal infections in submucosal LCs and DC-SIGN+ DCs (Hu et al., 2000), but mostly in resting CD4+ T cells (Miller et al., 2005; Zhang et al., 1999). No viral load is detected in plasma during the eclipse phase that lasts up to 10 days from the initial exposure (Haase, 2011). Virus is locally amplified in order to expand productive infection (Haase, 2010). During this window of time, virus appears to be the most vulnerable and could be an easy target for antiviral interventions (Haase, 2011). A week later, viral RNA appears in plasma. At this point, despite type I IFNs being released to contain local infection, overall immune activation and pro-inflammatory mediators facilitate the migration of virus and virus-infected cells to local lymphoid tissues and draining lymph nodes, where DC-SIGN+ DCs further amplify the infection by transmitting the virus to CD4+CCR5+ T cells, many of which also express integrin $\alpha 4\beta 7$. Subsequently, in order to combat viral replication, DCs activate and recruit NK and NKT cells and begin to induce HIV-specific T cell responses. However, these

events further augment immune activation and replication in CD4+ T cells, leading to viral systemic dissemination and distribution to the peripheral lymphoid organs, with preferential targeting of gut-associated lymphoid tissue (GALT), where activated CD4+CCR5+ memory T cells reside in abundance (Brenchley and Douek, 2008; Brenchley et al., 2004; McMichael et al., 2010). Plasma viral load continues to rise, and a massive depletion of CD4+ T cells occurs in mucosal tissue, primarily in the gut, where the majority of these cells reside. Within the first three weeks of infection, up to 80% of CD4+ memory T cells are depleted from the gut (Brenchley et al., 2004; McMichael et al., 2010), likely due to their high activation status and expression of CCR5 and also by virtue of a direct viral lytic effect or Fas-mediated apoptosis, or NK cell-mediated killing of HIV-infected cells (Haase, 2011; McMichael et al., 2010; Mogensen et al., 2010). Although subsequently the numbers of circulating in blood CD4+ T cells rebound, the mucosal pool of CD4+ T cells remains depressed. Despite DCs, NK and NKT cells activation and the formation of HIV-specific antibodies, plasma viremia continues to rise, peaking by 21-28 days and is associated with massive immune activation, which is also evident from the appearance of an acute retroviral syndrome: acute-phase inflammatory markers in blood and flu-like symptoms (McMichael et al., 2010). At this point, viral reservoirs are already established in resting cells and will not be sensitive to HAART therapy (detailed reviews are references (Haase, 2011; McMichael et al., 2010; Mogensen et al., 2010)).

Over 4-5 months, the disease becomes chronic and viremia reaches a set point, with CD8+ T cells playing a role in controlling the virus (Haase, 2011; McMichael et al., 2010; Mogensen et al., 2010). However, during this virtually asymptomatic phase, the most damage to the immune system is inflicted by sustained immune activation, established reservoirs of actively and latently infected cells (Mogensen et al., 2010; Schwenker et al., 2008), dysregulated expression and responsiveness of innate sensors, specifically TLRs 2, 3, 4, 6, 7/8 in PBMCs (Lester et al., 2008; Lester et al., 2009; Meier et al., 2007; Sachdeva et al., 2010; Sanghavi and Reinhart, 2005), and high cell (primarily CD4+ T) turn over, as evidenced by the presence of activation markers HLA-DR and

Ki67, with CD38 being considered the most predictive of disease progression (Haase, 2011; McMichael et al., 2010; Mogensen et al., 2010). Ultimately, such a sustained immune activation leads to functional immune deficiency and AIDS.

Several lines of evidence suggest that in addition to virus (Lester et al., 2008; Lester et al., 2009), per se, other factors, such as translocating pathogens and their PAMPs like LPS (Bafica et al., 2004a; Equils et al., 2001), can be directly contributing to the disease progression. Indeed, we and others have proposed a direct contribution of HIV-1 itself to immune activation, as the initiation of HAART therapy leads to almost immediate decline in correlates of immune activation, which was also associated with significantly reduced viraemia (Lester et al., 2008). Arguably, HIV-1 can activate cells, including genital EC (Nazli et al., 2010), initially during its attachment and recognition of viral PAMPs by cellular PRRs, or later through Tat- or NF- κ B-mediated induced viral replication (Mogensen et al., 2010; Schweneker et al., 2008). However, it is unlikely that immune activation is solely dependent on viral load, since naturally SIV-resistant monkeys, sooty mangabeys, have limited immunopathology and immune activation despite high levels of viraemia (Silvestri et al., 2003). Indeed, microbial translocation, which could occur early in HIV transmission when the epithelial integrity is breached upon ECs interaction with HIVgp120 (Nazli et al., 2010) or later with a “leaky gut” after massive depletion of CD4+ T cells from the gut (Brenchley et al., 2004), has also been implicated in bolstering immune activation through PAMPs recognition (Bafica et al., 2004b), since LPS was shown to activate LTR-dependent HIV replication through TLR4 (Equils et al., 2001).

Collectively, it is clear that HIV/AIDS pathogenesis is a complex and dynamic process, and we still don't have a clear understanding of all the events occurring *in vivo*. Despite the activation of inflammatory and antiviral immune responses, the immune system is “spinning its wheels” in efforts to contain the virus, yet only further fueling this activation. HIV/AIDS pathogenesis involves multiple players that, although being naturally beneficial, can be detrimental to the host in favoring the virus and the immune activation, which further limits our view of the correlates of protection against HIV-1.

Lessons learnt from HIV-resistant hosts

Perhaps, the most convincing evidence on the immune activation contributing to the establishment and disease progression comes from observations of naturally resistant SIV hosts (Silvestri et al., 2003) as well as individuals who remain HIV-1 seronegative despite repeated high-risk exposures to HIV-1 (Tomescu et al., 2011).

Indeed, sooty mangabeys, a natural SIV host, demonstrate attenuated SIV-mediated immune activation and immunopathology that protect them from developing AIDS, despite high-level viraemia (Silvestri et al., 2003). In contrast, a non-natural host and SIV-susceptible rhesus monkeys, show marked SIV-triggered type I IFN and chemokine inflammatory responses in lymph nodes that are associated with accelerated disease progression (Bosinger et al., 2009; Durudas et al., 2009). These findings indicate that SIV-induced immune activation and responsiveness are associated with disease development and progression, whereas less robust or moderated immune responses to virus are more beneficial for a better disease outcome.

Furthermore, data from human studies with highly HIV-exposed seronegative (HESN) individuals would support the results from studies with SIV-resistant sooty mangabeys. Although millions of people become infected following HIV-1 exposure, some people, albeit rarely, remain HIV-1-uninfected despite repeated exposures to the virus (Fowke et al., 1996). Several groups of such cohorts have been identified around the globe, and they include: discordant couples, where one partner remains HIV-uninfected despite being repeatedly exposed to virus through unprotected intercourse; infants born to HIV-positive mothers and subsequently exposed to HIV through breast feeding; haemophiliacs who received HIV-infected blood treatments; and individuals engaged in a high-risk sexual behavior or commercial sex workers (CSWs) (Tomescu et al., 2011). Collectively, HESN individuals are characterized as subjects without any detectable in serum anti-HIV-1 IgG and without any evidence of infection despite frequent exposure to HIV-1 and/or repeated high-risk behavior in areas with high HIV-1 prevalence (Tomescu et al., 2011). Evidence demonstrates that HIV-1 resistance in

HESN individuals likely stems from a compilation of factors including genetic predisposition (Lacap et al., 2008; Sironi et al., 2012), increased levels of certain antivirals and antiproteases in the CVL (Burgener et al., 2011; Iqbal et al., 2005; Iqbal et al., 2009; Zapata et al., 2008), potent anti-HIV-1 mucosal IgA (Broliden et al., 2001; Devito et al., 2000a; Devito et al., 2000b) and T cell responses (Kaul et al., 2000), and increased expression of genes regulating tight junction (Songok et al., 2012). Importantly, however, is that HIV-1 resistance in these subjects has also been associated with “immune quiescence” and decreased cellular activation (Begaud et al., 2006; McLaren et al., 2010; Songok et al., 2012). These findings are in line with earlier results from the monkey studies, demonstrating the lack of sustained immune activation and inflammation in naturally-resistant to SIV animals (Silvestri et al., 2003). Notably is that this immune quiescence in HESN subjects is not absolute and continuous, since robust, but transient, early activation of innate antiviral responses have been demonstrated *ex vivo* in HESN CSWs from Kenya (Su et al., 2011). Specifically, while PBMC from HIV-susceptible individuals in response to IFN- γ stimulation showed a biphasic and prolonged increase in IRF1 expression, HIV-resistant individuals demonstrated a robust, but transient IRF1 expression, suggesting that early and transitory IRF1 responsiveness is crucial for HIV-1 resistance (Su et al., 2011). This observation is in line with results from the study by Clericci et al., demonstrating increased immune responsiveness to TLR agonist by PBMC from another cohort of CSWs from Italy (Sironi et al., 2012). Interestingly, acute SIV in sooty mangabeys was also associated with early robust ISG responses and immune activation in blood and lymph nodes, but they were rapidly resolved and were not associated with disease progression (Bosinger et al., 2009). The relative significance of these systemic responses, however, remains unclear, since high viremia in sooty mangabeys persists regardless the dynamics of immune responses, and PBMC from HESM CSW remain susceptible to HIV-1 infection (Messele et al., 2001). In contrast, these findings are rather highlighting the importance of mucosal responses, especially considering the presence of broadly neutralizing mucosal IgA that are capable of inhibiting HIV-1 transcytosis across human ECs (Broliden et al., 2001; Devito et al.,

2000a) and our recent finding that cervical mononuclear cells (CMCs) from HIV-resistant (HIV-R) CSWs have altered PRRs mRNA expression compared to HIV-susceptible (HIV-S) controls (X.D. Yao, personal communication, unpublished data). It is unknown though how these CMCs from HIV-R group would respond to *in vitro* stimulation.

Collectively, these findings indicate that continuous exposure of HESN individuals to the virus induces active mucosal anti-HIV-1 responses that render these individuals resistant to mucosal HIV-1 infection. Further, since dysregulated PRRs expression/responsiveness and prolonged immune activation accompany HIV/AIDS progression (Lester et al., 2008), and since “immune quiescence” (McLaren et al., 2010; Songok et al., 2012) and robust, but transient, antiviral responses (Su et al., 2011) appear to be associated with HIV-1 resistance, it is possible that active immune regulation, rather than down-regulation, of pathogen recognition and inflammatory responses can be more beneficial in determining the fate of HIV-host encounter and disease establishment. Thus factors modulating inflammatory responses while increasing antiviral protection might be beneficial in controlling HIV and other STIs such as HSV-2 with pronounced inflammatory component driving their pathogenesis.

HSV-2

While the majority of HIV-1 encounters do not result in productive infection (Royce et al., 1997), some factors may enhance the susceptibility to HIV-1 infection. Indeed, co-infection HSV-2 is very common among HIV-positive persons (Schacker et al., 1998), and preexisting HSV-2 has been clearly implicated as a risk factor for HIV-1 (del Mar Pujades Rodriguez et al., 2002; Sobngwi-Tambekou et al., 2009).

HSV-2 belongs to a large *Herpesviridae* family (Arvin et al., 2007; Esmann, 2001); together with herpes simplex virus (HSV)-1, they are the most studied members of *alpha-herpesvirinae* subfamily and the primary causal agents of recurrent genital and facial herpetic lesions, respectively (Ahmed et al., 2003; Esmann, 2001). Nearly 60% of US adults are infected with HSV-1, and the estimated seroprevalence of HSV-2 in North

America is around 20%; it is even higher, 30-80%, in some developing countries and sub-Saharan Africa (Chen et al., 2000; Xu et al., 2006). These numbers make genital herpes one of the leading and most prevalent STI worldwide, although the role of HSV-1 in genital lesions is increasing and possibly underestimated. Even more alarming is the fact that most sexual and perinatal transmissions of HSV-2 occur during asymptomatic mucocutaneous viral shedding (Koelle and Wald, 2000), and that HSV-2 closely interplays with HIV-1 infections. Indeed, increasing epidemiological evidence indicates that HSV-2 is a risk factor for HIV-1 acquisition, transmission, and disease progression (Corey, 2007; Kapiga et al., 2007; Ouedraogo et al., 2006; Sheth et al., 2008; Todd et al., 2006; Wald and Link, 2002). Thus, it is of great importance to have efficient measures of HSV-2 prevention and treatment in order to gain control over the spread of both HSV-2 and HIV-1 infections.

HSV-2 structure, life cycle, and invasion mechanisms

The lack of preventive measures, discussed earlier in this chapter, is largely attributed to the structural and functional complexity of the virus and its pathogenesis. Virions of herpesviridae family range in size between 80 and 250 kbp and contain large double-stranded (ds) DNA genomes (Arvin et al., 2007; Mossman, 2002). The genome is surrounded by a viral envelope with multiple glycoproteins and spikes (i.e., gD, gB). The tegument contains both the envelope and the capsid. The HSV envelope contains at least 11 different membrane glycoproteins, of which five (i.e., gD, gH, gL, gC and gB) are critical for the viral entry (Novak and Peng, 2005; Spear, 2004).

In HSV virions, many among the encoded 100-200 genes are used not only to ensure proper HSV-2 replication, but also to evade the host's defense mechanisms. Examples of such evasion strategies include the ability to down-regulate the induction of protective type I IFNs and ISG-mediated responses (Mossman, 2002; Mossman et al., 2001; Yao and Rosenthal, 2011), to decrease DCs maturation and antigen presentation (Novak and Peng, 2005; Raftery et al., 2006), to suppress NK cell function (Orange et al.,

2002), and to establish latency in neuronal tissues through the expression of latency-associated transcripts (LATs) (Arvin et al., 2007; Chen et al., 2002; Corey et al., 1983).

Human ECs and neuronal cells are considered principle targets in HSV primary infection, although other cells can also be affected (Mikloska et al., 2001; Mikloska and Cunningham, 2001; Smiley et al., 1985). Primary infection of a new host is initiated after the attachment of viral gB and/or gC to the cell surface glycosaminoglycan (GAG) (Spear et al., 1992). Heparan sulfate proteoglycans, including key constituents, syndecan-1 and syndecan-2, are widely expressed among HSV target cells, such as ECs and neuronal cells. These HS moieties are predominant sites for herpes virus attachment (Lindahl et al., 1994; Tal-Singer et al., 1995), although chondroitin sulfate molecules can also be employed by the virus (Mardberg et al., 2002). For the viral entry to occur, however, further binding between viral gD and one of the cell surface entry co-receptors is required (Spear, 2004). Studies indicate that several receptors, including HVEM (herpes virus entry mediator) (Whitbeck et al., 1997) or members of the poliovirus receptor-related (PRR) immunoglobulin superfamily such as nectin-1 (Geraghty et al., 1998) and nectin-2 (Lopez et al., 2000; Warner et al., 1998) have been implicated in the binding with gD. HVEM belongs to the TNF family and activates NF- κ B and AP-1 via TNF receptor-mediated mechanisms (Harrop et al., 1998). Finally, the fusion between all four viral gB, gD, gL, and gH and the host cell membrane results in viral entry and release of tegument proteins and viral capsid into the cytosole (Jenssen, 2005). Viral attachment, entry, and infection are unique processes in that they often are governed by tissue-specific microenvironment, molecular interactions and the use of additional co-receptors as a result of activities of various tissue-specific enzymes (Spear, 2004).

Following viral attachment and entry, HSV-infection continues with the synthesis of α -proteins (i.e., ICP0, ICP4, ICP22 and ICP27), products of immediate-early genes; these proteins subsequently induce the synthesis of β -proteins (i.e., ICP6, ICP8, gB), products of early genes, and γ -proteins (i.e., UL56), products of late genes (Arvin et al., 2007; DeLuca et al., 1984; Koshizuka et al., 2002). The early genes transcribe viral

proteins necessary for the replication of HSV genome. And the late genes are responsible for providing the structural components of the virion (Novak and Peng, 2005).

Pathogenesis of HSV-2

Both innate and adaptive immune responses are activated during HSV-2 infection, with the innate immune system serving to control the initial events, and the adaptive - to execute viral clearance (detailed reviews are in references (Chan et al., 2011; Cunningham et al., 2006; Keele et al., 2008; Novak and Peng, 2005)). It is clear, however, that neither innate nor adaptive immune responses are efficient enough in dealing with HSV-2, as it is evident from the establishment of latency (Chen et al., 2002; Koelle and Corey, 2008) and the occurrence of periodic disease reactivations (Corey et al., 1983).

Following attachment and entry of HSV-2 into a target cell, virions and their multiple PAMPs (i.e., glycoproteins and nucleic acids, including dsRNA, dsDNA, and potentially ssRNA) (Finberg et al., 2005; Weber et al., 2006) are exposed to a variety of PRRs, namely TLRs 2, 3, 7/8, and 9, RNA helicases RIG-I and MDA5, and inflammasomes (Finberg et al., 2005; Rasmussen et al., 2009; Yu and Levine, 2011). Such an exposure triggers a series of signaling events, leading to activation of pathways and transcription factors, namely NF- κ B, MAPK, AP-1, and the IRF, that initiate antiviral and inflammatory responses (Kawai and Akira, 2006, 2010; Mogensen and Paludan, 2005). Among key antiviral responses mediated through these transcription factors and their corresponding pathways are the induction of Type I IFNs (Kawai and Akira, 2006; Yoneyama and Fujita, 2010; Yu and Levine, 2011) and various ISGs that were discussed earlier (Bauer et al., 2008; Li et al., 2009b).

A representative of type I IFNs, IFN β , has been considered as one of the correlates of protection against HSV-2 infection (Gill et al., 2006; Nazli et al., 2009). IFN β -mediated signaling limits viral replication and spread by activating additional antiviral pathways/factors, such as protein kinase R that phosphorylates eIF2 α and limits viral translation, or OAS/RNase L pathway leading to RNA degradation and ultimately

inhibition of viral translation and replication, as reviewed in reference (Stetson and Medzhitov, 2006). The induction of type I IFNs and ISGs following viral exposure is the host's attempt at preventing the establishment of primary viral infection and its spread and reactivation (Cunningham et al., 2006; Kawai and Akira, 2006). Viruses, however, evolved strategies to overcome host's immune surveillance. The apparent redundancy in these antiviral responses is necessary to enable the host to counteract HSV-2 evasion mechanisms, like those involving the inhibition of type I IFNs or IRF3 and IRF7-mediated responses. In the latter mechanisms, ICP0 and virus host shut-off (vhs) HSV proteins have been shown to play a role (Paladino et al., 2010; Yao and Rosenthal, 2011).

In addition to innate antiviral responses, viral recognition also triggers the induction of innate inflammatory responses that are often associated with activation of NF- κ B and the release of pro-inflammatory factors (Kawai and Akira, 2006; Yoneyama and Fujita, 2010; Yu and Levine, 2011). Interestingly, in addition to targeting and disarming antiviral responses, viruses, including HSV, have also been implicated in altering the expression and functional activity of innate viral sensors (Yao and Rosenthal, 2011), main regulators of immune responses (Kawai and Akira, 2006; Yoneyama and Fujita, 2010; Yu and Levine, 2011). Viral manipulation of PRRs expression and functioning leads to dysregulated host's immune inflammatory responses, which become detrimental to the host and further contribute to HSV infection and pathogenesis (Morrison, 2004; Mossman, 2002; Mossman et al., 2001). Studies demonstrate that HSV challenge has been associated with altered expression of TLR2 (Kurt-Jones et al., 2004) and TLR4 (Bottcher et al., 2003), as well as activation of NF- κ B, and the release of TNF α (Thapa and Carr, 2008), IL-1 β , and IL-6 (Gill et al., 2006; Li et al., 2009a). The significance of HSV innate recognition through viral PRRs and the role of dysregulated antiviral responses were highlighted by Bottcher et al. and Kurt-Jones et al. (Kurt-Jones et al., 2005; Kurt-Jones et al., 2004), demonstrating that a murine encephalitis was associated with increased levels of TLR4 mRNA (Bottcher et al., 2003), and that TLR2-/- mice had reduced HSV-1-mediated brain inflammation and mortality (Kurt-Jones et al., 2004). Kurt-Jones and colleagues also showed that cord-blood derived cells from human

neonates exhibited more vigorous responses to HSV stimulation compared to adult PBMCs, as was evident by higher levels of IL-6 and IL-8. In their study, the researchers concluded that these findings could potentially explain the sepsis syndrome seen with HSV infections in human neonates that could also be linked to TLR2 dysregulation (Kurt-Jones et al., 2005). HSV-induced immune activation can also contribute to breaching the blood-brain barrier and allowing HSV to translocate into the central nervous system (CNS) (Pasiaka et al., 2011; Thapa and Carr, 2008).

HSV-2 enters the CNS through the dorsal roots and establishes a life-long and latent infection in dorsal roots and sensory ganglia with recurrent episodes of reactivation and symptomatic or asymptomatic viral shedding. At this point, many adaptive immune responses are activated, including those involving CD4 and CD8 T cells, in an attempt to clear the virus (Chan et al., 2011; Cunningham et al., 2006; Koelle and Corey, 2008). HSV-2-specific CD8+ T cells play a role in surveillance to limit disease reactivation and viral replication in ganglia and skin (Koelle and Corey, 2008).

Most of HSV-2 infections and reactivations have minor and self-limiting symptoms, unless in immunocompromised or neonates, while viral shedding can be often even asymptomatic, thus making it difficult to screen, diagnose, treat, and prevent these events. There is no currently approved vaccine for HSV-2 (Cohen, 2010), and most antiviral treatments are primarily based on acyclovir nucleoside derivatives that target viral replication, but not the entry or multiple steps in herpes life, as was discussed earlier. Such inefficient targeting makes the control over the acquisition of primary HSV-2 infection and subsequent viral shedding and transmission difficult. A productive primary HSV-2 infection ultimately initiates a vicious cycle leading to the establishment and episodic reactivations of latent herpes infection, further resulting in continuous spread of infectious virus across the population, and also presenting a risk factor for HIV-1. In this context, naturally-occurring or synthetically-derived antiviral molecules, and especially those possessing immunomodulatory properties, could offer a promising alternative.

Antimicrobial armada in the FGT

Overview of multiple groups

In spite of the magnitude of the HIV-1 epidemic, 99.5% of unprotected sexual encounters do not result in productive HIV-1 infections (Gray et al., 2001), suggesting that viral transmission and spread are tightly controlled within the FGT through a complex and efficient interplay between physical barriers, immune responses, and mucosally secreted natural protective factors (Kaul et al., 2008; Kaushic et al., 2010; Wira et al., 2011b).

Of the estimated 800 antimicrobial peptides (AMPs) known to exist in nature, many have been shown in humans and at various mucosal sites (Izadpanah and Gallo, 2005), including the FGT (Wira et al., 2011b). Although a complete repertoire of endogenous AMPs present in the FGT is unknown, many of them have been identified and functionally characterized in the cervicovaginal lavage (CVL) fluid (Burgener et al., 2011; Cole and Cole, 2008; Hickey et al., 2011; Shaw et al., 2007; Venkataraman et al., 2005; Wira et al., 2011b); but many still remain underinvestigated. Based on structural and functional commonalities, these factors are grouped into several major families, including chemokines (Rancez et al., 2012), defensins (Ganz and Lehrer, 1994; Kagan et al., 1994; Klotman and Chang, 2006), cathelicidins (Larrick et al., 1995a; Larrick et al., 1995b; Yang et al., 2004), serpins and cystatins (Burgener et al., 2011; Fahey et al., 2011) as well as serine antiproteases (Sallenave, 2010; Schalkwijk et al., 1999).

Human genital ECs are major contributors of endogenous antimicrobials, since ECs can constitutively or in response to microbial or inflammatory stimulation secrete a variety of such protective factors that include defensins, secretory leukocyte protease inhibitor (SLPI), elafin and its precursor trappin-2 (Tr/E) (Fahey and Wira, 2002; Ghosh et al., 2010; King et al., 2002; Schaefer et al., 2005; Wira et al., 2011a), and many others (Wira et al., 2011b).

In addition to their regulation by inflammatory and microbial stimuli, the expression of these antimicrobials in the FGT can be also regulated by the sex hormones, estradiol and progesterone, and vary throughout the menstrual cycle (Fahey et al., 2008;

King et al., 2003b; King et al., 2003c). Indeed, levels of endogenous antimicrobials, such as SLPI, lactoferrin, and several defensins, were shown to transiently, yet significantly, decrease in the CVLs around ovulation, before returning to constitutive levels near the end of the secretory phase (Horne et al., 2008; Keller et al., 2007; Wira et al., 2011b). Such fluctuations in levels of endogenous antimicrobials may partly explain the window of vulnerability in the FGT in the later half of the menstrual cycle (Wira and Fahey, 2008).

Many of these peptides, such as cathelicidins, defensins, and antiproteases, are small cationic proteins with broad antibacterial, antifungal, and antiviral activities (Cole and Cole, 2008; Klotman and Chang, 2006; Sallenave, 2010), including against HSV-2 and HIV-1 (Drannik et al., 2012b; Ghosh et al., 2010; John et al., 2005; MasCasullo et al., 2005; Venkataraman et al., 2005; Wahl et al., 1997).

Cathelicidins are a family of peptides with only one member found in humans, called cathelicidin hCAP-18/LL-37 (Levinson et al., 2009; Tjabringa et al., 2005). LL-37 is a mature protein and a product of proteolytic cleavage of a C-terminus domain of its precursor, hCAP-18 (Sorensen et al., 2008). LL-37 exhibits many activities that include antimicrobial, chemotactic, and immunomodulatory (Larrick et al., 1995a; Mookherjee et al., 2006). Some of these activities have been attributed to LL-37 direct targeting of microbial cell wall (Ramanathan et al., 2002), binding to nucleic acids (Hasan et al., 2011) and LPS (Larrick et al., 1995a), and the ligation of formyl peptide receptor like (FPRL)-1 moiety, one of LL-37 cellular receptors (Brown and Hancock, 2006; De et al., 2000). Anti-HIV-1 activity of LL-37 has also been demonstrated; however, it may or may not involve FPRL-1 ligation and signaling (Bergman et al., 2007; Steinstraesser et al., 2005; Wong et al., 2011). Anti-HSV-2 activity of LL-37 has not been reported.

Among human defensins, there are two main subfamilies: α and β defensins. There are six α defensins, such as human neutrophil peptide (HNP) 1–4 and human

defensin (HD) 5 and 6, and six better studied β -defensins HBD1-6, with only four of them identified in the FGT (Cole and Cole, 2008; Klotman and Chang, 2006). Based on *in vitro* studies, defensins were shown to act either through virus, by interacting with viral envelop, or through cells, by altering cell surface expression of viral receptors or blocking cellular binding sites (Hazrati et al., 2006; Klotman and Chang, 2006; MasCasullo et al., 2005; Quinones-Mateu et al., 2003; Sun et al., 2005; Wang et al., 2004; Weinberg et al., 2006; Yasin et al., 2004). However, despite the reported anti-HSV-2 activity for human defensins 5 and 6 (Hazrati et al., 2006), they were shown to increase host's susceptibility to HIV-1 infection (Rapista et al., 2011). A similar pro-HIV-1 activity was also documented for prostate stromal protein 20 kDa (ps20) (Alvarez et al., 2008), from the whey acidic proteins (WAP) family. Interestingly, despite that ps20 appears to negatively correlate with HIV-1 infection, the other members of this WAP family, namely SLPI and Tr/E, have been shown to exhibit antiviral activities (Ghosh et al., 2010; Shugars et al., 1997).

Serine protease inhibitors SLPI, as well as elafin (E) and its precursor, trappin-2 (Tr), are among other cationic endogenous antimicrobials that have been linked to mucosal protection against sexually transmitted pathogens, such as HSV-2 and HIV-1 (Hazrati et al., 2006; MasCasullo et al., 2005; Moreau et al., 2008). SLPI is a small non-glycosylated protein with two WAP domains that was initially isolated from bronchial secretions by Thompson and Ohlsson in 1986 (Thompson and Ohlsson, 1986). SLPI is induced in different cell types and at many mucosal sites, including the FGT, as reviewed elsewhere (Drannik et al., 2011; Moreau et al., 2008; Sallenave, 2010)). In addition to its inhibitory activity against neutrophil serine proteases, cathepsin G and neutrophil elastase (Eisenberg et al., 1990), SLPI also possesses anti-inflammatory (Ding et al., 1999; Henriksen et al., 2004) and antimicrobial properties (Hiemstra et al., 1996), similar to Tr and E, as reviewed elsewhere (Drannik et al., 2011; Sallenave, 2010).

Regarding anti-inflammatory activity of SLPI, this protein was shown to reduce NF- κ B activation either through binding to bacterial PAMP, LPS (Ding et al., 1999), or

to inhibitors of NF- κ B, I κ B α or I κ B β (Lentsch et al., 1999) or through binding to DNA and competing specifically for NF- κ B DNA binding sites (Taggart et al., 2005).

Studies indicate that low HIV-1 transmission through breast milk (Wahl et al., 1997), saliva, and CVL was correlated with high levels of SLPI in the mucosal fluids (Lin et al., 2004; McNeely et al., 1995; Pillay et al., 2001), and reduced SLPI levels in CVLs were associated with increased risk of HIV-1 infection (Novak et al., 2007). SLPI was shown to exhibit antiviral activity *in vitro* against both HIV-1 and HSV-2, which appeared to be independent of antiprotease activity of SLPI and dependent on the protein's direct interaction with cells, rather than virus (MasCasullo et al., 2005; McNeely et al., 1997). Further, SLPI has been demonstrated to reduce HIV-1 infection in monocytes/macrophages (Hocini et al., 2000; Ma, 2004) and lymphocytes (Py et al., 2009), but not to interfere with transcytosis of cell-associated HIV-1 across polarized monolayer of ECs. SLPI was shown to target HIV-1 before the viral reverse transcription step in monocytes (McNeely et al., 1997) and to interact with cell surface HIV-1 binding partners scramblase 1 and 4 in T cells (Py et al., 2009), as well as annexin II in macrophages (Ma, 2004). Considering functional and structural (40% sequence identity between elafin and each SLPI domain (Moreau et al., 2008)) similarities between SLPI, Tr, and E, it could be argued that their antiviral features would also be similar. Compared to SLPI, however, antiviral activity of Tr/E, especially in the FGT, has been much less investigated, and only recently received some attention.

Trappin-2 and elafin

Antiviral activity

Indeed, increased levels of Tr/E were recently found in CVLs from highly-exposed HIV-resistant (HIV-R) commercial sex workers (CSWs) in Nairobi, Kenya (Iqbal et al., 2009). Despite repeated HIV-1 exposures, these women had no signs of infection; yet they had HIV-specific immune responses (Broliden et al., 2001; Kaul et al., 2001; Kaul et al., 2000). These findings suggest that the repeated viral exposure, at least in part, is inducing potent HIV-specific and non-specific protective responses and a local

milieu in the genital mucosa of these HESN women that would collectively prevent HIV-1 translocating across the epithelial barrier. Utilizing SELDI-TOF mass spectrometry, Iqbal and colleagues identified increased levels a 6 kDa protein elafin in CVLs of HIV-R CSWs, compared to HIV-susceptible (HIV-S) controls (Iqbal et al., 2009). Although in their patent the authors showed that recombinant elafin prevented HIV-1 infection of T cells *in vitro* (Ball, 2006), no *in vitro* HIV-inhibitory activity was reported in the original study (Iqbal et al., 2009). Interestingly, however, when HIV-S CSWs were followed prospectively, the authors reported that elevated levels of both Tr/E were associated with HIV-1 protection, thus suggesting that both proteins might be required for optimal protection against HIV-1.

Another recent study also focused on antiviral activity of Tr/E in the FGT. Ghosh et al. showed that recombinant E had an inhibitory effect against both R5 and X4 HIV-1 in TZM-bl target cells *in vitro*, but only when the virus, and not the cells, was pretreated with E. In this study, the researchers proposed a direct anti-HIV-1 activity of E as the mechanisms behind its antiviral activity (Ghosh et al., 2010).

Other studies also elucidated antiviral effects of Tr/E; these studies, however, did not focus on the genital mucosa or STIs. Nevertheless, these reports demonstrated that in a model of viral myocarditis, Tr/E-expressing animals had increased survival, reduced tissue damage and organ dysfunction, compared to control animals (Zaidi et al., 1999). And another study showed that Tr/E-expressing mice, compared to their counterparts, had increased number and activation of lung DCs that were associated with augmented Th1-polarized virus-specific lung and spleen adaptive immune responses and increased viral (Ad-LacZ) clearance from the lungs (Roghianian et al., 2006).

Despite that both Tr/E have been associated with anti-HIV-1 effect in the FGT, there was no clear understanding of how this activity was mediated and what was the contribution of Tr/E to the total anti-HIV-1 activity of CVL, considering that CVL contains a wide range of antimicrobial molecules (Hickey et al., 2011). Furthermore, equally unknown is whether or not each Tr and E individually possess anti-HIV activity

and whether they are equipotent in this antiviral activity. Moreover, although accumulated evidence highlighted the role of Tr/E in defense against HIV-1 (Ghosh et al., 2010; Iqbal et al., 2009), their contribution to defense against another sexually-transmitted and linked to HIV-1 pathogen, HSV-2, remains undefined. All of these points have led us to the research presented herein.

The following sections in the introduction will provide additional background information on trappin-2 and elafin that will facilitate the reader's understanding of the nature and activities of these proteins. Some of this information might be repeated in the introductions to each of the following chapters.

History

Tr/E are alarm serine antiproteases that, in contrast to systemic antiproteases, are secreted in tissues to offer more localized protection (Sallenave, 2010). The fact that these molecules can be isolated from sites affected by chronic inflammatory conditions, including atherosclerosis and chronic lung and skin diseases (Sallenave and Ryle, 1991; Schalkwijk et al., 1990; Sumi et al., 2002), would further support this notion. Of the two proteins, E was discovered first, when in 1990 two groups, independently, isolated E from the skin of patients with psoriasis (Schalkwijk et al., 1990; Wiedow et al., 1990) and called it skin-derived antileukoprotease (SKALP). Subsequently, in 1991, Sallenave and colleagues purified and characterized E as elastase specific inhibitor (ESI) from human lung sputum (Sallenave and Ryle, 1991). These discoveries resulted in multiple names given to the same protein. It was during the cloning of the E cDNA that was discovered that the molecule was larger than anticipated, due to the presence of the N-terminal non-inhibitory, or cementoin, domain with a binding site for transglutaminase. The whole molecule of E precursor was then given the name trappin-2, with "trappin" being an acronym for **TR**ansglutaminase substrate and **wAP** domain containing **ProteIN** (for nomenclature refer to reference (Schalkwijk et al., 1999)). In the literature, however, some do not differentiate between the proteins and use the terms Tr and E interchangeably, perhaps adding ambiguity to the field.

Structure

Tr/E are small cationic proteins that share an evolutionary-conserved four-disulfide core domain (FDC). The FDC domain is shared by other WAP family members, SLPI and ps20; but it is also common to other unrelated to WAP family proteins, as reviewed elsewhere (Bingle and Vyakarnam, 2008; Drannik et al., 2011). Better known as WAP domain, the FDC structure is rich in cysteine residues that stabilize four disulfide bonds involved in protease inhibition (Tsunemi et al., 1996). Tr is secreted as a cationic unglycosylated (95-aa) 9.9 kDa protein consisting of two parts: N- and C-terminal domains. Proteolytic cleavage of the N-terminal cementin domain liberates the C-terminal 57-residue with the inhibitory WAP domain (5.9 kDa), which becomes a soluble E (Guyot et al., 2005a). In the N-terminus of Tr, there is a transglutaminase substrate-binding domain (TSBD) with four repeated motifs of Gly-Gln-Asp-Pro-Val-Lys sequences (Nara, 1994), thus explaining the name. The N-terminus of E also contains one such a motif (Baranger et al., 2008), hence allowing both Tr/E to be covalently linked to extracellular matrix proteins like heparin by a tissue transglutaminase, most likely contributing to wound healing (Guyot et al., 2005b).

Antiprotease and antibacterial activities

Tr/E are pleiotropic molecules exhibiting antiproteolytic, antimicrobial, and immunomodulatory properties, as reviewed in references (Drannik et al., 2011; Moreau et al., 2008; Sallenave, 2010), all of which, however, could be affected by Tr/E susceptibility to oxidation and protease degradation (Guyot et al., 2008; Guyot et al., 2005a; Nobar et al., 2005). Tr/E were demonstrated to inhibit human neutrophil elastase (HNE), proteinase 3, and endogenous vascular elastase (Cowan et al., 1996; Moreau et al., 2008).

Notably, antibacterial activity of Tr/E has been studied and understood much better, compared to their antiviral effects. Tr/E were shown to directly inhibit the growth of a number of fungal, as well as Gram-positive and Gram-negative bacterial pathogens,

both *in vitro* and *in vivo* (McMichael et al., 2005a; Moreau et al., 2008; Sallenave, 2010; Simpson et al., 1999), but sparing *Lactobacillus* (Fahey et al., 2011). This direct antimicrobial effect of Tr/E is believed to be largely mediated through their cationic charge (Baranger et al., 2008; Hiemstra et al., 1996); however, binding to microbial DNA (Bellemare et al., 2010) has also been proposed. In *in vivo* experiments with *P. aeruginosa* and *S. aureus*, antibacterial activity of adenovirally-expressed Tr was associated with reduced bacterial load and inhibited neutrophilic inflammation (McMichael et al., 2005a; Simpson et al., 2001b).

Immunomodulatory activity

In addition to their antibacterial effect, Tr/E have been also shown to modulate the host's immune responses. Indeed, similar to SLPI, Tr/E were demonstrated to target binding, recognition, and mounting of inflammatory responses against microbial and inflammatory stimuli (Butler et al., 2006; Drannik et al., 2012c; Henriksen et al., 2004; McMichael et al., 2005b; Roghanian et al., 2006; Simpson et al., 2001b; Taggart et al., 2005). Specifically, in *in vitro* studies, Henriksen et al. demonstrated that Ad-mediated delivery of Tr/E reduced endothelial IL-8 release in response to oxidized low density lipoprotein, LPS, and TNF α and macrophage TNF α production in response to LPS. In both endothelial cells and macrophages, these inhibitory events were associated with reduced activation of NF- κ B, through up-regulation of its inhibitor, I κ B α (Henriksen et al., 2004). In another study, Butler and colleagues demonstrated that E-pretreated monocytes secreted reduced levels of MIP-1 in response to LPS stimulation (Butler et al., 2006). Reduced levels of MIP-1 were associated with inhibited activation of AP-1 and NF- κ B and attenuated degradation of I κ B α and I κ B β . Interestingly, it was found that reduced AP-1 activation was mediated through the inhibition of proteosomal degradation of I κ B α , but not its phosphorylation, in presence of E (Butler et al., 2006). In line with the previous study, another group has shown anti-inflammatory effect of Tr/E by demonstrating that each Tr/E were capable of binding to LPS and preventing its signaling in murine macrophages, resulting in reduced secretion of TNF α . However, in serum-free

conditions, Tr/E were shown to stimulate TNF α release (McMichael et al., 2005b), suggesting a dual activity of Tr/E on cellular immune responses under certain conditions. The latter findings are reminiscent of results from *in vivo* studies, where in mice with adenovirally expressed human Tr, local pulmonary LPS-induced immune responses were augmented, or primed, and associated with increased influx of neutrophils; whereas systemic responses were dampened and characterized by lower levels of TNF α , MIP-2, and MIP-1 compared to the lungs (Sallenave et al., 2003; Simpson et al., 2001a). In line with the previous findings, Wilkinson and colleagues demonstrated that Tr had an opsonizing effect on *P. aeruginosa* clearance through CD14-dependent activation of macrophages and recruitment of neutrophils (Wilkinson et al., 2009). Collectively, these observations demonstrate a dual activity of Tr/E with both immunomodulatory and immunostimulatory effects. It has been proposed that in the host's defense against bacteria, Tr/E might be priming local immune responses by promoting opsonisation, phagocytosis, and direct killing of bacteria through the recruitment and activation of immune cells, while regulating and dampening the magnitude and spread of systemic inflammatory responses (Sallenave, 2010). Notably, similar dual immunomodulatory properties were shown for other endogenous cationic antimicrobials, such as defensins and cathelicidins, perhaps highlighting the pleiotropic nature and a broad repertoire of their activities, through which these factors effectively contribute to the host's antimicrobial defense (Brown and Hancock, 2006).

Sources and expression

Being present in various mucosal secretions (Tjabringa et al., 2005), including CVLs (Iqbal et al., 2009), tissues, such as skin, colon, placenta, lungs, the FGT, and cells like neutrophils, macrophages, T cells, and epithelial cells (Dalgetty et al., 2008; King et al., 2003a; King et al., 2007; Marischen et al., 2009; Mihaila and Tremblay, 2001; Motta et al., 2011; Pfundt et al., 1996; Sallenave et al., 1992; Sallenave et al., 1993; Wiedow et al., 1990), Tr/E are strategically placed to regulate mucosal immune responses. *In vitro* studies demonstrate that genital ECs (Fahey and Wira, 2002), together with keratinocytes,

bronchial, and alveolar ECs (Sallenave et al., 1994), constitutively produce low amounts of Tr/E, unlike SLPI. In contrast, Tr/E secretion can be greatly induced in response to various stimuli, such as TNF α , IL-1 β , LPS, defensins, and neutrophil elastase (Pol et al., 2003; Sallenave et al., 1994; Simpson et al., 2001a; van Wetering et al., 2000), suggesting that SLPI and Tr/E play differential roles, with SLPI providing a constitutive baselines antiprotease/anti-inflammatory protection, while Tr/E - following stimulation (Sallenave, 2010). Interestingly, we and others have recently found that Tr/E secretion from the genital ECs was induced in response to a synthetic mimic of a viral dsRNA, polyI:C (Drannik et al., 2012c; Ghosh et al., 2010), hence confirming a significant role of Tr/E in antiviral immune inflammatory responses in the FGT mucosa.

Hypothesis

Based on the evidence presented above, we hypothesized that Tr/E may play a significant role in defense against HIV-1 in the FGT, and that their presence in the CVL might be critical for the natural anti-HIV-1 activity of CVLs. Given that both Tr/E were identified in the CVLs, we further hypothesized that each Tr and E individually might possess antiviral activity. Taking into account that HSV-2 represents a significant risk factor for HIV acquisition and transmission, and since no previous reports focused on anti-HSV-2 activity of Tr and E, we also considered characterizing the contribution of each Tr and E to the host's defense against HSV-2 in the female genital mucosa. Considering previously demonstrated antibacterial and immunomodulatory effects of Tr/E, we also hypothesized that Tr/E antiviral activity could be targeting viral infectivity and/or modulating host's recognition and mounting of antiviral and inflammatory immune responses. To better test both antiviral and immunomodulatory activities of each Tr and E, we utilized viral ligand, polyI:C.

Objectives

The research presented in Chapters 2, 3, 4, and 5 was designed to test our hypothesis and to elucidate and expand our knowledge on the antiviral properties of each

Tr/E individually and their potential mechanisms and targets in defense against HSV-2 and HIV-1 in the female genital mucosa.

In the first manuscript in this thesis (Chapter 2), we characterized whether or not and how each Tr/E individually contributed to antiviral host defense in the genital ECs elicited by polyI:C, a synthetic mimic of viral dsRNA, capable of inducing not only an antiviral state in target cells, but also strong inflammatory responses. To accomplish this, we used human endometrial ECs, HEC-1A line, a replication-deficient adenovirus expressing the human Tr gene, recombinant tagged and untagged Tr/E proteins, polyI:C, and a target VSV-GFP virus. We specifically determined whether Tr/E alone or in the context of polyI:C pretreatment were able to augment antiviral protection of cells in response to a challenge with VSV-GFP. We also determined whether in doing so, Tr/E acted directly on virus or indirectly through cells by blocking viral entry or modulating viral sensing, or inflammatory or antiviral responses and pathways. We focused on the expression of innate viral sensors, namely TLR 3, RIG-I, and MDA5, activation of transcription factors NF- κ B, AP-1, IRF3 and ISGs, and the induction of IFN β and pro-inflammatory factors IL-8 and IL-6.

In the second manuscript (Chapter 3), building on findings from the previous chapter, notably that Tr/E enhance antiviral protection and reduce inflammatory responses elicited by polyI:C, we sought to determine the contribution of Tr/E to antiviral defense against HSV-2 using *in vitro* and *in vivo* models. We determined whether or not each Tr/E individually acted directly on virus or indirectly through cells by increasing cellular antiviral protection mediated through IRF3 and IFN β . We also determined whether Tr/E exerted antiviral effect by altering NF- κ B-mediated inflammatory responses. In the *in vivo* model of lethal HSV-2 genital infection, we utilized Tr-transgenic mice that expressed the human Tr gene and evaluated their survival, pathology scores, as well as viral loads in the genital tract and viral translocation into the CNS.

The third manuscript (Chapter 4) builds on the work presented in Chapters 2 and 3, showing that Tr/E augment antiviral protection induced by polyI:C as well as in response to HSV-2. Here, we sought to determine the presence and characteristics and mechanisms of antiviral activity of each Tr/E against HIV-1. To this end, we examined the contribution of Tr/E to the natural anti-HIV-1 protection of CVL and also comparatively assessed antiviral activity of each of the recombinant Tr/E proteins. We also investigated whether Tr/E anti-HIV-1 activity was virus or cell-dependent and whether Tr/E had equally potent antiviral activities. In this study, we also attempted to identify whether Tr/E acted through particular HIV-binding receptors by altering their expression.

In the last manuscript in this thesis (Chapter 5), based on collective evidence from the previous chapters indicating that E was more potent compared to Tr against HSV-2 and HIV-1, we sought to investigate the specific mechanisms and prerequisites of anti-HIV-1 activity of mostly E and in genital ECs expressing non-canonical HIV receptors. Here, using tagged and untagged Tr/E proteins, we determined specific structural characteristics, mode(s) of action, and cellular distribution/localization of antiviral E. We also determined whether or not there were several potential mechanisms and levels of anti-HIV-1 activity, including the modulation of HIV-triggered innate viral recognition and pro-inflammatory responses. Importantly, we sought out to confirm our *in vitro* data with observations utilizing cervico-vaginal cells from HIV-R and HIV-S cohorts from Kenya.

Trappin-2/Elafin Modulate Innate Immune Responses of Human Endometrial Epithelial Cells to PolyI:C

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Published in PLoS ONE in April 2012, Vol 7 Issue 4: e35866 pp 1-16

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Summary and central message:

With the lack of widely available and effective preventive and therapeutic vaccines or microbicides against HSV-2 and HIV-1, strong efforts have been made to induce potent protective responses by stimulating innate immune receptors with various adjuvants or microbial ligands, including polyI:C. A synthetic viral ligand and a mimic of viral dsRNA, polyI:C was shown to induce a potent antiviral protection in the female genital mucosa. However, polyI:C was also implicated in inducing potent pro-inflammatory responses and increasing susceptibility of target cells (DC) to HIV-1 infection, all of which could limit the therapeutic potential of this molecule. Furthermore, and perhaps even more importantly, with viral dsRNA being a by-product of replication of many if not all viruses, it can be argued that dsRNA, similarly to LPS, can be present in tissues and/or circulation during viral replication and potentially contribute to immune activation driving the pathogenesis of STIs like HIV-1 and HSV-2. Thus, polyI:C was considered suitable as an innocuous and clinically relevant to the realm of STIs viral stimulant to be used to elucidate whether or not and how endogenous innate factors Tr/E contribute to antiviral host's defense mechanisms.

Using a replication-deficient adenovirus expressing the human Tr gene as well as recombinant Tr/E proteins, in Chapter 2 we demonstrated that Tr/E acted directly on a target virus, VSV-GFP, and indirectly through cells by modulating viral sensing and inflammation as well as enhancing polyI:C-induced cellular antiviral responses. Our results also demonstrated that enhanced polyI:C-driven antiviral protection of Tr/E-treated ECs was partially mediated through IRF3 activation, but not associated with higher induction of IFN β . Finally, we reported that in Ad/Tr-treated cells, not only Tr, but also E, was detected after polyI:C treatment. Collectively, these findings identified for the first time that in the female genital ECs and in the context of viral ligand, Tr/E exerted multiple antiviral mechanisms targeting several pathways and potentially involving alternative and still unidentified factors or pathways.

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Abstract

Background: Upon viral recognition, innate and adaptive antiviral immune responses are initiated by genital epithelial cells (ECs) to eradicate or contain viral infection. Such responses, however, are often accompanied by inflammation that contributes to acquisition and progression of sexually-transmitted infections (STIs). Hence, interventions/factors enhancing antiviral protection while reducing inflammation may prove beneficial in controlling the spread of STIs. Serine antiprotease trappin-2 (Tr) and its cleaved form, elafin (E), are alarm antimicrobials secreted by multiple cells, including genital epithelia.

Methodology and Principal Findings: We investigated whether and how each Tr and E (Tr/E) contribute to antiviral defenses against a synthetic mimic of viral dsRNA, polyinosine-polycytidylic acid (polyI:C) and vesicular stomatitis virus. We show that delivery of a replication-deficient adenovector expressing Tr gene (Ad/Tr) to human endometrial epithelial cells, HEC-1A, resulted in secretion of functional Tr, whereas both Tr/E were detected in response to polyI:C. Moreover, Tr/E were found to significantly reduce viral replication by either acting directly on virus or through enhancing polyI:C-driven antiviral protection. The latter was associated with reduced levels of pro-inflammatory factors IL-8, IL-6, TNF α , lowered expression of RIG-I, MDA5 and attenuated NF- κ B activation. Interestingly, enhanced polyI:C-driven antiviral protection of HEC-Ad/Tr cells was partially mediated through IRF3 activation, but not associated with higher induction of IFN β , suggesting multiple antiviral mechanisms of Tr/E and the involvement of alternative factors or pathways.

Conclusions and Significance: This is the first evidence of both Tr/E altering viral binding/entry, innate recognition and mounting of antiviral and inflammatory responses in genital ECs that could have significant implications for homeostasis of the female genital tract.

Introduction

Genital epithelial cells (ECs) provide the first line of defense against sexually-transmitted infections (STIs) (Nazli et al., 2010; Wira et al., 2005b). Upon viral sensing through pattern-recognition receptors (PRRs), ECs initiate innate and adaptive immune responses that serve to eradicate or contain viral pathogens (Samuel, 2001; Yoneyama and Fujita, 2010). ECs can directly respond to viruses and viral pathogen-associated molecular patterns (PAMPS) by secreting innate protective factors, including defensins and cathelicidins (Erhart et al., 2011) as well as members of the whey-acidic protein (WAP) family (Ghosh et al., 2010). Of the 18 human WAP proteins, only a few have been well characterized to date (Bingle and Vyakarnam, 2008), and among the better understood are serine antiproteases elafin (E) with its precursor, trappin-2 (Tr), as well as secretory leukocyte protease inhibitor (SLPI), and prostate stromal protein 20 kDa (ps20) (Bingle and Vyakarnam, 2008; Drannik et al., 2011).

The physiological role of serine antiproteases has been extensively studied over the past two decades (Schalkwijk et al., 1990), mainly due to their contribution to homeostatic equilibrium through the control of proteases, inflammation, and infections (Moreau et al., 2008; Sallenave, 2010). Together with other proteins, such as snake venom neurotoxins (Drenth et al., 1980) and whey acidic protein (Piletz et al., 1981), serine antiproteases share an evolutionary conserved canonical cysteine-rich four-disulfide core (FDC) domain, or the WAP domain, involved in protease inhibition (Tsunemi et al., 1996). Trappin-2 (9.9 kDa) (or pre-elafin) is a secreted and unglycosylated protein of 95-amino acids (aa) (Schalkwijk et al., 1999) that contains an N-terminal cementoin domain (38-aa) (Nara, 1994) and elafin (5.9 kDa), a C-terminal inhibitory WAP (57-aa) domain (Nara, 1994; Tsunemi et al., 1996). Elafin is released from the N-terminus of Tr by proteolysis, arguably most efficiently by mast cell tryptase (Guyot et al., 2010; Guyot et al., 2005a). Antiprotease activity and wound repair were the first described properties of Tr and E (Tr/E), similar to SLPI. Unlike ps20, SLPI along with Tr/E are functional neutrophil serine protease inhibitors (Bingle and Vyakarnam, 2008; Moreau et al., 2008). Inhibition of human neutrophil elastase (HNE) and proteinase

3 by the inhibitory loop on a WAP domain allows Tr/E to control tissues proteolysis associated with excessive inflammation in a neutrophil-rich environment. In turn, cross-linking between repeated hexapeptide motifs (GQDPVK) on the N-terminal portion of each Tr/E (Guyot et al., 2005b; Schalkwijk et al., 1999) and extracellular matrix proteins arguably allows Tr/E to repair compromised tissue integrity (Guyot et al., 2005b; Simpson et al., 2001b). In addition, due to their cationic nature, but not exclusively (Bellemare et al., 2010), Tr/E were shown to possess antimicrobial activity against Gram-negative and Gram-positive bacteria (Bellemare et al., 2010; Simpson et al., 1999; Wilkinson et al., 2009) and certain fungal infections (Baranger et al., 2008). Worth mentioning is that similar to SLPI, antibacterial activity of Tr/E appeared to be independent of their antiprotease function (Simpson et al., 1999). Later, anti-inflammatory features of the antiproteases were also described, showing that Tr/E and SLPI were capable of reducing activation of NF- κ B and AP-1 by altering I κ B activation (Henriksen et al., 2004) and proteosomal degradation (Butler et al., 2006), respectively, in response to inflammatory and bacterial stimulation. More recent studies, however, also reported immunomodulatory properties of Tr/E. Indeed, depending on the environment, Tr/E can either dampen inflammation (Butler et al., 2006; Simpson et al., 2001b) or promote immunostimulatory events and prime the immune system (McMichael et al., 2005b; Roghanian et al., 2006). Both Tr/E are found at mucosal surfaces (Ghosh et al., 2010; Iqbal et al., 2009), in tissues (King et al., 2003b; King et al., 2007; Sallenave et al., 1992; Schalkwijk et al., 1991; Wiedow et al., 1990) and multiple cell types, including genital ECs (Ghosh et al., 2010; King et al., 2003b) and regarded as alarm antiproteases, as they are mainly produced in response to pro-inflammatory stimuli like LPS (Simpson et al., 2001a), TNF α (Pfundt et al., 2000), and IL-1 β (King et al., 2003b; Sallenave et al., 1994). Interestingly, ECs from the female genital tract (FGT) produce Tr/E constitutively, with uterine cells capable of producing even greater amounts of Tr/E in response to a viral ligand, polyinosine-polycytidylic acid (polyI:C) (Ghosh et al., 2010), indicating the significance of these molecules in controlling the local milieu in the FGT.

Viral double-stranded RNA (dsRNA) is a PAMP generated during the life cycle of most, if not all, viruses (Alexopoulou et al., 2001; Yoneyama and Fujita, 2010). Double-stranded RNA, including viral dsRNA and its synthetic mimic polyI:C, are recognized by at least two families of PRRs: Toll-like receptors (TLRs), including TLR 3 (Nazli et al., 2009; Schaefer et al., 2005), and RNA helicases, namely retinoic acid inducible gene-I (RIG-I) (Yoneyama et al., 2005; Yoneyama et al., 2004) and melanoma differentiation associated gene 5 (MDA5) (Kato et al., 2008). Following recognition of dsRNA, activated PRRs initiate a series of signaling events, triggering phosphorylation, homodimerization and translocation into the nucleus of a set of transcription factors like interferon (IFN) regulatory factor 3 (IRF3), IRF7, NF- κ B, and ATF2/c-Jun (c-Jun) (Alexopoulou et al., 2001; Bauer et al., 2008; Wathelet et al., 1998; Yoneyama et al., 2004). Inside the nucleus, these transcription factors either work independently or interact with each other (Maniatis et al., 1998) in triggering the transcription of antiviral and inflammatory gene products, such as type I IFNs and IL-8, IL-6, and TNF α . Specifically, IRF3 alone can directly bind to the IFN-stimulated response element in the promoter region of interferon-stimulated genes (ISGs) and activate a set of ISGs and their products in the absence of type I IFN production (Mossman et al., 2001; Paladino et al., 2006). Such antiviral cascade is devoid of excessive inflammatory responses and is induced when a low viral stimulation is detected. Alternatively, in response to a high viral load, IRF3 associates with NF- κ B and c-Jun to trigger the production of IFN β and the induction of ISGs in an IFN-dependent mode (Lin et al., 1998; Wathelet et al., 1998), which also triggers robust inflammation, target cell recruitment, and/or tissue damage due to the production of pro-inflammatory cytokines and chemokines along with antiviral type I IFNs molecules (Nazli et al., 2009; Paladino et al., 2006).

Treatment with polyI:C has been shown to induce potent antiviral protection *in vitro* and *in vivo*, making polyI:C an attractive candidate for microbicide or vaccine adjuvant trials against STIs (Ashkar et al., 2004; Nazli et al., 2009; Schaefer et al., 2005). However, in addition to antiviral activity, polyI:C also triggers the release of pro-inflammatory mediators (Nazli et al., 2009; Schaefer et al., 2005), therefore potentially

negating its beneficial effects in the FGT. While pro-inflammatory factors are important for immune cell recruitment and activation, if poorly controlled, they may also be detrimental in FGT, since acquisition and pathogenesis of common STIs are associated with immune activation and inflammation (Freeman et al., 2006; Lester et al., 2008; Rebbapragada et al., 2007). Hence, interventions leading to better control of inflammatory responses may prove to be more beneficial for increased antiviral protection and overall health in the FGT. Recently, new evidence has accumulated on the role of Tr/E in protection against viruses (Ghosh et al., 2010; Iqbal et al., 2009). Indeed, Tr/E have been associated with resistance to HIV mucosal transmission in commercial sex workers (CSWs) in Kenya (Iqbal et al., 2009). Later, Ghosh *et al.* reported an anti-HIV feature of E in *in vitro* study (Ghosh et al., 2010). Furthermore, Roghanian *et al.* documented that Tr expression increased Ad/LacZ viral clearance, as well as secondary immune responses, in a murine model *in vivo* (Roghanian et al., 2006).

Collectively, these data clearly demonstrate the importance of Tr/E in antiviral protection, although specifically how Tr/E contribute to antiviral immune-inflammatory responses is still unknown. Thus, the objective of this study was to elucidate whether and how Tr/E contribute to innate antiviral and inflammatory responses in uterine ECs elicited by a viral ligand, polyI:C. Here, we describe novel antiviral and immunomodulatory properties of Tr/E. To the best of our knowledge, this is the first study to present evidence of Tr/E affecting host innate recognition and modulating antiviral and inflammatory responses in genital ECs against polyI:C.

Materials and methods

Reagents

PolyI:C and lipopolysaccharide (LPS) were reconstituted in the phosphate-buffered saline (PBS) and used at concentrations shown in figures (Sigma-Aldrich, Oakville, ON, Canada). Human recombinant (r) proteins Tr (rTr) (R&D Systems, Burlington, ON, Canada) (Schalkwijk et al., 1999) and in-house recombinant E (rE)

(described below) were used in *in vitro* experiments and as reference markers for Western blotting. The amount of Tr/E being used in this study ranges from 0.2 to 5 µg/ml to cover the physiological levels (within 1 µg/ml) reported previously (Ghosh et al., 2010; Iqbal et al., 2009) as well as concentrations achieved in supernatants of human endometrial carcinoma (HEC)-1A cells infected with a replication-deficient adenovirus (Ad) expressing human Tr gene (Ad/Tr) (over 1 µg/ml).

Cell lines

HEC-1A, Caco-2 (human colonic epithelial cells), and A549 (derived from a type II human alveolar cell carcinoma) cells (Lieber et al., 1976) were obtained from American Type Culture Collection (Rockville, MD). HEC-1A and A549 cells were cultured in McCoy's 5A Medium Modified (Invitrogen Life Technologies, Burlington, Ontario, Canada) and DMEM, respectively, supplemented with 10% fetal bovine serum (FBS), 1% HEPES, 1% l-glutamine (Invitrogen Life Technologies), and 1% penicillin-streptomycin (Sigma-Aldrich) at 37°C in 5% CO₂. Caco-2 cells were cultured in DMEM growth medium containing 5% FBS, 1% l-glutamine, 1% penicillin-streptomycin, 5mL NEAA, and 9.6mL NaHCO₃.

Adenoviral constructs and delivery in cell culture

The Ad constructs used in this study have been described in detail elsewhere (Bett et al., 1994; Sallenave et al., 1997; Sallenave et al., 1998). To express human Tr, the Ad/Tr vector, encoding gene for 95-aa human Tr, was used (Sallenave et al., 1997; Sallenave et al., 1998). This Ad construct was previously called Ad/E. E1, E3-deleted empty adenovirus Ad-dl703 (Ad/dl), coding for no transgene, was used as a control for Ad/Tr (Bett et al., 1994). Both Ad vectors were prepared at the Centre for Gene Therapeutics at McMaster University (Hamilton, ON, Canada). To generate supernatants containing Tr, HEC-1A cells were infected with MOI 0-50 plaque-forming units (PFU) of Ad/Tr (Ad/Tr-cells) or Ad/dl (Ad/dl-cells) overnight at 37°C in Opti-MEM® I Reduced Serum Medium (Invitrogen Life Technologies), washed with PBS and

incubated for 12 h in serum-containing medium. Cells were washed again and incubated for additional 24 h in serum-free cell culture medium. Cell-free supernatants were used either for protein measurement by ELISA or for antiprotease activity against HNE. Another aliquot of supernatants was further concentrated with 3 – 30 kDa MWCO centrifugal filter units (Amicon, Millipore, Billerica, MA, USA) as per supplier's instructions and used in *in vitro* studies. For routine experiments, epithelial cells were either treated with Opti-MEM medium alone (UT) or with MOI 50 PFU of Ad/dl or Ad/Tr at 37°C overnight. After PBS washes and rest for 4 h, cells were incubated in serum-containing medium alone or with polyI:C for additional 24 h.

Vesicular stomatitis virus (VSV) plaque reduction assay and antiviral assay

Vesicular stomatitis virus (VSV-GFP), a lytic IFN-sensitive virus expressing green fluorescent protein under the viral promoter (a kind gift from Dr. Brian Lichty, McMaster University), was used in a plaque reduction assay (Bauer et al., 2008) to assess the role of Tr/E in antiviral protection. This method is based on determining the ability of VSV-GFP to replicate in cell cultures in presence of biologically active antiviral factors, e.g., IFNs (Bauer et al., 2008). Briefly, HEC-1A cells were seeded in 96-well culture plates and infected with Ad (Ad-cells) as described above, followed by treatment with medium alone or polyI:C for 24 h. Induction of antiviral response was assessed by subsequently challenging cell monolayers in serum-free medium with MOI 1 PFU of VSV-GFP. GFP fluorescence intensity was visualized 24 h later on a Typhoon Trio (Amersham Bioscience, GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and quantified using Image Quant 5.2 software.

MTT viability assay

MTT assay, described elsewhere (Nazli et al., 2009), was used as per supplier's instructions to determine viability of Ad-exposed and polyI:C-treated HEC-1A cells (Biotium Inc., Hayward, CA, USA).

ELISA assays

Cell-free supernatants were stored at -70°C until assayed for human Tr/E, IL-8, TNF α , IL-6 with ELISA DuoSet kit (R&D Systems), human IFN β by ELISA kit from Antigenic America Inc. (Huntington Station, NY, USA), and IFN α subtypes by *VeriKineTM* Human Interferon-Alpha Multi-Subtype ELISA Kit (Piscataway, NJ, USA) according to the supplier's protocol. Analytes were quantified based on standard curves obtained using an ELISA reader Tecan Safire ELISA reader (MTX Labs Systems Inc.). Cut off limit for Tr/E and IL-8 was 31.25 pg/ml; for TNF α and IFN β was 15.6 pg/ml; for IFN α subtypes was 12.5 pg/ml, and levels detected below these limits were considered as undetectable.

Generation of recombinant human elafin

To prepare rE protein, cDNA fragments encoding the relevant part of Tr (amino acid residues A61–Q117) were amplified from HEC-1A cells cDNA using the following primers: 5'-ACAGGATCCGCGCAAGAGCCAGTCAAAGGTCCA-3' and: 5'-CAGGAATTCTCACTGGGGAACGAAACAGGCCATC-3'. The amplified elafin-cDNA was gel purified, restriction digested, and directionally subcloned into *Bam*H I and *Eco*R I site of bacteria expression vector pHAT10 (BD Biosciences, Rockville, MD, USA) in frame to the HAT-tag. The clone was confirmed by restriction digestion and nucleic acid sequencing of both strands. The plasmid was transformed into *Escherichia coli* BL21 strain (Codon Plus, Palo Alto, CA, USA). When the cells grew in Luria-Bertani broth containing 100 $\mu\text{g/ml}$ ampicillin to an A_{600} of 0.55–0.60 at 37°C , protein expression was induced by adding isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM for 6 h. The cells were harvested by centrifugation at 2,000 $\times g$ for 5 min, and cell pellets were washed once with ice-cold PBS (pH 7.4) and resuspended in wash buffer (50 mM Na₂PO₄, 300 mM NaCl, pH 7.0). The desired recombinant protein, rE, was found in the soluble fraction in pilot experiment. After disrupting the cells by freeze-thaw and subsequently by sonication, the lysates were centrifuged at 20,000 $\times g$ for 20 min, and the soluble fraction was collected. The soluble recombinant protein was

purified from this fraction by BD TALON metal affinity resin (BD Biosciences, Mississauga, ON, Canada) according to the manufacturer's instructions and dialyzed against PBS at 4°C. The expressed human rE was confirmed by Western blotting targeting the HAT-tag as well as by specific antibody for human E.

Preparation of cell extracts and Western blot (WB) analysis

Whole-cell extracts were prepared by using whole-cell extract buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1%NP-40, 1%SDS, 1×protease inhibitor (Roche, Mississauga, ON, Canada)) as per standard protocol. Protein amount was quantified using Bradford assay with bovine serum albumin (BSA) (Sigma-Aldrich) as a standard and Bio-Rad Dye Reagent Concentrate as a protein stain (Bio-Rad Laboratories, Mississauga, ON, Canada). WB was performed on a 10% polyacrylamide denaturing SDS-PAGE gel and PVDF membranes (Amersham, Arlington Heights, IL, USA) as per standard protocol, using the following primary antibodies: anti-human Tr/E TRAB2O (Hycult Biotech, Uden, Netherlands), tIRF3 FL-425 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), pIRF3 Ser396 (Cell Signaling, Danvers, MA, USA), RIG-I #4520 (Cell Signaling), MDA-5 R470 (Cell Signaling), c-Jun (60A8) (Cell Signaling), p-c-Jun (Ser73) (Cell Signaling), TLR3 IMG-5631-2 (Imgenex, San Diego, CA, USA), NF-κB p65 (C-20): sc-372 (Santa Cruz), GAPDH ab9485 (Abcam) antibodies at a dilution of 1:1000, except for GAPDH (1:5000) and p-NF-κB p65 (Ser 536): sc-33020 (Santa Cruz) (1:100). After incubation with corresponding horse-radish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Laboratories), blots were visualized using a SuperSignal West Femto or Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL, USA). GAPDH was used as internal loading control. Quantification of band intensities was done using MBF_ImageJ for Microscopy Software.

Transfection and luciferase assay

HEC-1A cells (5×10^5 per well) were transfected in 0.5 mL of Opti-MEM® medium (Invitrogen Life Technologies) in a 24-well plate with 20 ng of DNA of each of pgkβ-Gal and pNF-κB-Luc or pAP-1-Luc (Stratagene, La Jolla, CA, USA) plasmids

(total DNA 40 ng per well) and 50 MOI of Ad/dl or Ad/Tr overnight at 37°C utilizing Lipofectamine 2000 (Invitrogen Life Technologies) as per supplier's instructions. P_{gk}-β-Galactosidase plasmid was used as a normalization control. After transfection, cells were extensively washed with PBS and allowed to rest for 4 h followed by stimulation with polyI:C and LPS for 2-4 h. After ligand stimulation, cells were washed twice with cold PBS, lysed with 1 × reporter buffer (Enhanced Luciferase Assay Kit (Cat # 556866, BD Pharmingen) and subjected to one freeze-thaw cycle, after which cell lysates were collected and assayed in a 96-well plate for luciferase (Enhanced Luciferase Assay Kit, BD Pharmingen) and β-galactosidase (Luminescent β-Gal assay, Promega, Madison, WI, USA) activities separately as per supplier's protocol, using Opticomp I luminometer (MGM instruments).

IRF3 knockdown by RNA interference

Small interfering (siRNA) molecule (Invitrogen Life Technologies) targeting IRF3 (GenBank accession number NM_001571) within 247-1530 ORF through the following sequences: IRF3-498 (start) CAACCGCAAAGAAGGGTTGCGTTTA, or non-targeting siRNA, RNAi Negative Control (medium GC content), 12935-300, (Invitrogen Life Technologies) were used to specifically knockdown IRF3. Transfections of siRNA (8 pmol) were done using Lipofectamine RNAiMAX and Opti-MEM® I Reduced Serum Medium (Invitrogen Life Technologies) as per supplier's instructions. HEC-1A cells, 3×10^4 in a 100 μl of total volume of complete growth medium, were transfected in a 96-well BD Falcon culture plate (BD Biosciences) for 48 h before adding MOI 50 PFU of Ad/dl or Ad/Tr. Knockdown efficiency was monitored using WB.

RNA extraction and real-time quantitative PCR analysis

The protocol for RNA isolation was described elsewhere (Lester et al., 2008). Briefly, total RNA was isolated from Ad-cells cultured with medium alone or polyI:C for 6 h, using TRIzol reagent (Invitrogen Life Technologies) according to the supplier's protocol. RNA was DNase-treated with DNA-free (Ambion, Austin, TX, USA) and

complementary (cDNA) was synthesized from total RNA using SuperScript reverse transcriptase III (Invitrogen Life Technologies) as per supplier's protocol. Real-time quantitative PCR was performed in a total volume of 25 μ l using 1 \times Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 5 μ l of diluted cDNA, 500 nmol forward primer, 500 nmol reverse primer (Mobix; McMaster University, ON, Canada), and 200 nmol probe in a 96-well plate. TaqMan oligonucleotide primers and probes labeled with 6FAM at 5'-end and a non-fluorescent quencher at 3'-end were designed using Primer Express 1.5 (Applied Biosystems) and selected following the TaqMan rules of Applied Biosystems. The sequences were as follows: RIG-I: 5'-AGGGCTTTACAAATCCTGCTCTCTTCA-3' (probe), 5'-GGTGTTCAGATGCCAGAC-3' (forward), 5'-TTCCGCAAATGTGAAGTGTATAA-3'(reverse); MDA5: 5'-TTTGGCTTGCTTCGTGGCCC-3'(probe), 5'-TGATTCCCCTTCCTCAGATAC-3'(forward), 5'-TGCATCAAGATTGGCACATAGT-3'(reverse); TLR3: 5'-TGTGGATAGCTCTCC-3'(probe), 5'-CCGAAGGGTGGCCCTTA-3'(forward), 5'-AAGTTACGAAGAGGCTGGAATGG-3' (reverse); 18S rRNA: 5'-CGGAATTAACCAGACAAATCGCTCCA -3' (probe), 5'-GTGCATGGCCGTTCTTAGTT-3' (forward), 5'-TGCCAGAGTCTCGTTCGTTAT-3' (reverse). The expression of 18S ribosomal RNA (rRNA) was used as an internal control. PCR was run with the standard program: 95°C 10 min, 40 times of cycling 95°C 15 sec and 60°C 1 min in a 96-well plate with an ABI PRIZM 7900HT Sequence Detection System using the Sequence Detector Software 2.2 (Applied Biosystems). To determine the expression of ISG56, a semi-quantitative RT-PCR was performed with oligonucleotide primer sequences (Mobix): 5'-GACAGGAAGCTGAAGGAGAAA-3' (445-bp product) (forward), 5'-TcTTGCATTGTTTCTTCTACCACT-3' (reverse). PCR program was as follows: 94°C for 2.5 min, 30 cycles of 94°C for 20 sec, 55°C for 30 sec, 72°C for 1 min, 72°C for 5 min. PCR products were electrophoresed on 2% agarose gel using a 100-bp DNA ladder (Invitrogen Life Technologies) as a marker to identify PCR products. The gel was stained with a loading fluorescent dye EZ-vision N472-Q

(AMRESCO Inc., Solon, OH, USA) and visualized with UV transilluminator (Gel Doc 2000, BioRad, Mississauga, ON, Canada). Densities of DNA bands were quantified to signal volumes using ImageQuant 5.0.

Immunofluorescence staining

Immunofluorescence staining was performed as described earlier (Bauer et al., 2008; Nazli et al., 2009), but with minor modifications. Ad-cells, grown on an 8-well BD Falcon culture slides (BD Biosciences), were medium- or 25 µg/ml polyI:C-treated for 4 h, fixed with 4% paraformaldehyde, permeabilized with 0.1 % Triton X-100 in PBS for 20 min, and blocked for 1 h at ambient temperature in a blocking solution (0.1 % Triton X-100, containing 5% goat serum/BSA). IRF3 was detected using 1:100 dilution in blocking solution of IBL18781 (IBL, Gunma, Japan) antibody for 1 h. Negative control rabbit immunoglobulin fraction (DakoCytomation, Glostrup, Denmark) served as an isotype control and was diluted to match the protein content of the primary anti-IRF3 antibody. Secondary antibody, Alexa Fluor 488 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) was added to cells in a blocking solution for 1 h. The nuclei were visualized by staining with propidium iodide. Images were acquired using an inverted laser-scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany).

Protease inhibition assay (PIA)

This assay was performed by measuring inhibition of HNE activity by Tr/E as was described earlier (Simpson et al., 2001b), but with minor modifications. Elastase-inhibitory activity was measured in a 96-well plate by combining cell-free undiluted supernatant (generated as described earlier in Materials and Methods and before or after polyI:C treatment) (final volume 10 µl/well), serially diluted each rTr and rE, or medium alone, to a known quantity of purified HNE (50 ng in 10 µl/well) or diluent alone (negative control), and incubating for 30 min at 37°C. Subsequently, 50 µl of HNE substrate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma-Aldrich), diluted to 50 µg/ml in 50 mM Tris, 0.1% Triton, 0.5 M sodium chloride, pH 8 buffer were added

and the hydrolysis was recorded by monitoring the increase of absorbance at 405 for 15 min using a Tecan Safire ELISA reader (MTX Labs Systems).

Statistical analysis

Data were expressed as means \pm standard deviation (SD). Statistical analysis was performed with either unpaired Student's *t* test or a one-way analysis of variance (ANOVA) using Sigma Stat 2.03.

Results

Ad/Tr-cells secrete Tr and both Tr/E are detected in Ad/Tr-cells supernatants in response to polyI:C

Initial studies evaluated the expression of the secreted Tr from Ad/Tr-cells by ELISA that detects both Tr/E (Ghosh et al., 2010). We report that Ad/Tr-cells secreted significant amounts of Tr/E in an Ad-dose-dependent manner (Figure 1A). In contrast, untreated HEC-1A (UT) and Ad/dl-cells expressed very low to no Tr/E (Figure 1A). Since the antibodies used in the ELISA did not distinguish Tr from E in Ad/Tr-cells supernatants (Ad/Tr-sups), WB was performed to clarify the presence of both Tr/E (Vandermeeren et al., 2001). Two recombinant reference proteins, namely commercial 6×His-Tr (rTr) (Schalkwijk et al., 1999) and in-house HAT-E (rE), were used as comparative markers for Tr and E, respectively. Due to tag insertion, both reference proteins appeared 3-4 kDa higher than the appearance of untagged proteins would be expected. Thus, rE band appeared at ~ 10-11 kDa, and rTr at ~13-15 kDa. Additionally, a smaller band ~10-11 kDa was also observed in the rTr reference protein (Figure 1B, lane 8), being most likely E present in the protein preparation. This smaller band also appeared higher than would be expected for E, due to the His-tag insertion. Hence, we conclude that the commercial rTr is a mixture and contains both Tr/E that are indicated as such on WB by arrows (Figure 1B, lane 8). WB data demonstrate that supernatants of UT and Ad/dl-cells supernatants (Ad/dl-sups) did not show any detectable forms of endogenous Tr/E in the blot (Figure 1B, lane 1 – 4). In contrast, WB of Ad/Tr-sups in the absence of polyI:C stimulation revealed protein bands that appeared between Tr/E bands of the reference proteins and thus were considered as Tr (Figure 1B, lane 5). The bands in lane 5 represent secreted Tr that resulted from Ad/Tr infection of HEC-1A cells. Further, following polyI:C treatment of Ad/Tr-cells, the intensity of the Tr bands in Ad/Tr-sups was greatly increased, indicating a significantly higher amount of Tr (Figure 1B, lane 6) being produced. Interestingly, a smaller protein band also appeared (Figure 1B, lane 6) that was below the level of rE-reference marker protein, as well as the E band in the rTr

reference protein (Figure 1B, lane 7), and thus was considered as E. Taken together, these results indicate that Ad/Tr-cells secrete Tr, and both Tr/E are detected in the supernatants following Ad/Tr-cells stimulation with polyI:C.

Ad/Tr-cells secrete Tr that is functionally active against HNE

Being foremost a protease inhibitor, Tr in Ad/Tr-sups was tested in protease inhibition assay (Simpson et al., 2001b). The results of anti-protease assay showed that Ad/Tr-sups significantly inhibited HNE activity in a dose-dependent manner, compared to Ad/dl-sups (Figure 1C). Following polyI:C stimulation, Ad/Tr-sups, containing presumably both Tr/E, were also tested and found functional against HNE (data not shown), comparable to before polyI:C treatment activity.

Tr/E significantly reduce VSV-GFP infection and enhance polyI:C-driven antiviral protection in Ad/Tr-cells

Since polyI:C stimulation of ECs triggers the induction of antiviral protection (Bauer et al., 2008; Nazli et al., 2009), we next determined whether Tr/E could modulate antiviral protection. Results of standard VSV-GFP plaque reduction assays show that the delivery of Ad/Tr significantly reduced VSV-GFP replication in polyI:C-untreated cells (Figure 2A and 2B) that was beyond the anti-viral protection induced by Ad delivery alone (i.e., in Ad/dl group). Ad delivery is known to activate innate immune responses (Sakurai et al., 2008), and the fact that susceptibility of HEC-1A cells to VSV-GFP infection (Figure 2B) was already reduced by treating the cells with Ad/dl alone confirms the Ad-induced innate activation. Since polyI:C-untreated Ad/Tr-cells secrete only Tr (Figure 1B), we conclude that the presence of Tr was associated with significantly increased antiviral protection of Ad/Tr-cells in the absence of polyI:C. Following polyI:C treatment, VSV-GFP replication was further reduced across the groups in Ad-cells (Figure 2A and 2B); viral replication in Ad/Tr-cells also remained significantly reduced (with up to 50% reduction, $p < 0.05$) and antiviral protection increased, compared to

polyI:C-treated Ad/dl-cells (Figure 2A and 2B). Based on these results and those shown in Figure 1B, we deduce that the presence of both Tr/E was associated with significantly increased cellular antiviral protection in Ad/Tr-cells after polyI:C treatment. Altogether, these observations clearly indicate that exogenous Tr expression in Ad/Tr-cells before VSV-GFP challenge has a separate antiviral protective mechanism in addition to the Ad- and polyI:C-induced responses; furthermore, both Tr/E appear to mediate enhanced polyI:C-induced cellular antiviral protection in Ad/Tr-cells.

We next attempted to elucidate specific mode(s) of Tr/E antiviral activity. Given the possibility of additional Tr/E being released from Ad/Tr-cells after polyI:C stimulation and upon VSV-GFP challenge and thus potentially acting on both virus and cells, we tested if both or each Tr/E individually were acting through two separate mechanisms: (i) direct antiviral activity exerted during the virus/cell encounter and viral binding/entry, targeting either virus or cells; and (ii) indirect cell-associated immunomodulatory activity that targets polyI:C-triggered cellular antiviral responses and protection. To minimize the Ad-associated effects, we used secreted/soluble proteins delivered to Ad-uninfected HEC-1A cells. Because we could not use Ad/Tr-sups after polyI:C stimulation as a source of both Tr/E, since they would contain residual polyI:C and thus mask the effect of Tr/E alone, a commercial rTr (with a C-terminus His-tag) was used as a source of both Tr/E, based on results shown in Figure 1B. For comparative assessment of each Tr/E individually, we used Ad/Tr-sups before polyI:C stimulation as a source of secreted Tr (no tag), and rE (with an N-terminus HAT-tag) was used as a source of E. All the proteins were initially compared for their antiprotease activity and found equally potent against HNE at around 10 $\mu\text{g/ml}$ (data not shown). Figure 2C shows that when virus, but not cells, was pre-treated with recombinant rTr for 1 h before addition onto cells, viral replication was significantly reduced by about 20% ($p < 0.05$), compared to media alone. Of note, no further reduction in viral replication was noticed when a higher dose of rTr was used. These results clearly indicate that rTr (a mixture of Tr/E) has a statistically significant, albeit modest, antiviral effect, which appears to be due to direct, or virus-mediated, activity of the proteins. We further show that

pretreatment of HEC-1A cells with rTr, followed by co-culturing with polyI:C for 24 h before VSV-GFP challenge, significantly reduced viral replication (up to 30%, $p < 0.05$) and enhanced polyI:C-induced antiviral protection, compared to polyI:C alone (Figure 2D). Interestingly, when cells were pretreated with rTr, then washed and challenged with VSV-GFP 24 h later without prior polyI:C stimulation, no significant decrease in viral replication was observed (data not shown), suggesting that rTr does not induce potent antiviral cellular responses without polyI:C stimulation and reduces VSV-GFP replication only following direct contact with virus. Collectively, these results suggest that secreted/soluble rTr, as a mixture of both Tr/ E, demonstrated two distinct properties: a virus-mediated antiviral activity and the modulation/enhancement of polyI:C-induced cellular antiviral responses, suggesting that the presence of both Tr/E is required for both of these activities.

Next we assessed antiviral properties of individual secreted/soluble Tr/E preparations. We show that VSV-GFP replication was reduced (up to 40%, $p < 0.05$) in Ad/Tr-sups-treated cells (Figure 2E), compared to controls, but only after polyI:C treatment (Figure 2E, main graph and insert), suggesting that Tr from Ad/Tr-sups does not exhibit potent direct antiviral activity, but is capable of enhancing polyI:C-induced cellular responses. Insert in Figure 2E demonstrates that, although a trend toward a decreased viral replication was noted when Ad/Tr-sups were pre-incubated with the virus, there was no significant inhibitory effect observed from Ad/Tr-sups, compared to their controls. Furthermore, surprisingly, in contrast to rTr or Ad/Tr-sups, rE did not exhibit any antiviral properties (data not shown), indicating differential antiviral properties of the tested proteins that could be potentially related to their structural differences. Collectively, these results clearly indicate that both Tr/E appear to be required for antiviral protection mediated via both mechanisms.

Exogenous expression of Tr and polyI:C stimulation do not lead to impaired cell viability or metabolic activity of Ad/Tr-cells

Considering that polyI:C can induce apoptosis (Dogusan et al., 2008), we determined whether Ad/Tr-cells had impaired cell viability and metabolic activity, using a standard MTT Cell Viability Assay (Nazli et al., 2009). Our results determined no significant changes in viability and metabolic activity among the groups following either Ad, with recovery period of 4 h versus 24 h (data not shown), or polyI:C treatment (Figure 2F and 2G). Therefore, we can exclude the impairment in the treated cells as the cause of differences between Ad/Tr- and Ad/dl-cells.

Ad/Tr-cells respond to polyI:C treatment with modulated production of IFN β

We next elucidated the immunomodulatory effect of Tr/E in Ad/Tr-cells in context of polyI:C stimulation. Type I IFNs are considered a hallmark of antiviral response, with IFN β being a key correlate of polyI:C-induced antiviral protection (Bauer et al., 2008; Nazli et al., 2009; Schaefer et al., 2005). We determined whether enhanced antiviral protection in presence of Tr/E was associated with higher levels of IFN β , using quantitative real-time RT-PCR and commercial ELISA. Figure 3 demonstrates that polyI:C treatment of Ad-cells triggered a significant induction of IFN β expression/secretion in contrast to untreated Ad-cells. Interestingly, Ad/Tr-cells responded to polyI:C treatment with significantly dampened, but not completely abrogated, levels of IFN β , compared to Ad/dl-cells. Since IFN β levels were reduced at both mRNA (Figure 3A) and protein (Figure 3B) levels, it is likely that IFN β expression was affected primarily at the transcription level. A similar dampening effect was observed when IFN β protein was assessed at earlier time point (6 h) or in response to a lower dose of polyI:C (0.1 μ g/ml) (data not shown). We have also attempted to measure other members of type I IFNs as well. Namely, multi-subtypes of IFN α were measured by commercial ELISA (Hobbs and Pestka, 1982); however, levels of proteins detected in

all supernatants were below the sensitivity of the ELISA and thus considered undetectable (data not shown).

Enhanced polyI:C-driven antiviral protection is associated with hyperactivation of IRF3 in Ad/Tr-cells

Phosphorylation and nuclear translocation of IRF3 are key events in the transcriptional activation of inducible ISG cellular genes (Bauer et al., 2008; Paladino et al., 2006), and polyI:C was previously shown to induce activation of IRF3 *in vitro* (Bauer et al., 2008; Nazli et al., 2009). Qualitative and quantitative analyses of phosphorylated IRF3 (pIRF3) showed that, compared to control cells, IRF3 phosphorylation in Ad/Tr-polyI:C-treated cells was initially modestly reduced at 1 h, but then subsequently increased after 2 h (Figure 4A, pIRF3 WB panel and quantifying histogram) and remained increased for up to 24 h (data not shown). In contrast, no significant changes were evident in the total IRF3 (tIRF3) protein amount for Ad/Tr-cells, compared to controls (Figure 4A, tIRF3 WB panel). Confocal imaging at 4 h post polyI:C stimulation (Figure 4B) corroborated WB findings of increased IRF3 phosphorylation in Ad/Tr-cells, since significantly more Ad/Tr-cells appeared with IRF3 translocated into the nucleus, compared to Ad/dl-cells (Figure 4B, polyI:C panel). Taken together, these results suggest that exogenous expression of Tr/E promotes hyperactivation of IRF3 that is associated with increased antiviral protection of Ad/Tr-cells against VSV-GFP challenge.

Tr/E significantly decrease mRNA expression of ISG56 in Ad/Tr-cells following polyI:C stimulation

ISG15 and ISG56 have been implicated in antiviral protection and linked to activation of IRF3 in response to polyI:C (Bauer et al., 2008; Noyce et al., 2006). Results of RT-PCR demonstrated no difference in mRNA levels of ISG15 between Ad/Tr and Ad/dl groups after polyI:C stimulation (data not shown). In contrast, mRNA expression

of ISG56 (Figure 5A and 5B) appeared significantly reduced in Ad/Tr-cells in response to polyI:C.

Tr/E alter phosphorylation and transcriptional activity of NF- κ B in Ad/Tr-cells in response to polyI:C

We next elucidated phosphorylation and activation of other transcription factors, including NF- κ B and c-Jun, that have also been shown to contribute to antiviral responses (Alexopoulou et al., 2001; Wathélet et al., 1998; Yoneyama et al., 2004). Qualitative and quantitative results show that NF- κ B p65 phosphorylation in Ad/Tr-cells was attenuated after 1 h post-polyI:C exposure and remained decreased until 8 h, compared to the control group (Figure 6A, NF- κ B WB panel and Figure 6B, NF- κ B quantifying histogram). Our results further revealed that overall c-Jun phosphorylation was only transiently reduced in Ad/Tr-cells between 1 h and 4 h and returned to levels comparable to such of controls at 8 h post polyI:C treatment (Figure 6A, pc-Jun WB panel and Figure 6B, pc-Jun quantifying histogram). These results prompted us to next evaluate the effect of Tr expression on transcriptional activity of NF- κ B and c-Jun, which was assessed by luciferase reporter gene assay. Data shown in Figure 6C, NF- κ B/Luc panel, demonstrate that transcriptional activity of NF- κ B in Ad/dl-polyI:C-treated cells was significantly induced at 4 h, compared to untreated and LPS-treated Ad/dl-cells. However, polyI:C-induced NF- κ B transcriptional activity in Ad/Tr-cells was significantly reduced, compared to Ad/dl-cells. In contrast, transcriptional activity of AP-1 was not different between the groups following stimulation with either of the ligands (Figure 6C, AP-1/Luc panel). Collectively, our data show that Tr/E expression attenuated NF- κ B activation not only at the phosphorylation level, but also at the level of its transcriptional activity, which was more pronounced compared to changes observed for c-Jun/AP-1.

Tr/E significantly reduce levels of dsRNA sensors RIG-I and MDA5 in polyI:C-treated Ad/Tr-cells

PolyI:C-induced antiviral protection of primary genital ECs was previously associated with heightened expression of TLR3 (Schaefer et al., 2005). Thus, we evaluated the expression levels of dsRNA sensors in Ad/Tr-cells following polyI:C treatment. Figures 6D-F show that in contrast to TLR3, polyI:C stimulation induced a significant (over 40 times) increase in mRNA expression of RIG-I and MDA5 (Figure 6D-E, left panels) in all UT and Ad-cells. Further, compared to UT and Ad/dl-cells, Ad/Tr-cells surprisingly had significantly attenuated expression of RIG-I and MDA5 at both mRNA and protein levels (Figure 6D-E, left and right panels). RIG-I expression in Ad/Tr-cells appeared to be affected at earlier time point, around 1 h, compared to MDA5 expression that was attenuated at 8 h after polyI:C stimulation. Moreover, reduced expression levels of RIG-I and MDA5 were sustained for up to 24 h following polyI:C treatment (data not shown). The expression of TLR3, however, was not different between the groups (Figure 6F). Collectively, these data indicate that mRNA and protein expression of RIG-I and MDA5 are increased in response to polyI:C stimulation, but the magnitude of expression is reduced in Ad/Tr-cells. Further, that we observed differential pattern of expression among RIG-I, MDA5, and TLR3 could indicate either a different kinetics of responses of these sensors, or that each RIG-I, MDA5, and TLR3 respond differentially to polyI:C, known to be a mixture of various lengths of dsRNA (Kato et al., 2008).

IRF3 is required for polyI:C-driven antiviral protection in Ad/Tr-cells

We next determined whether IRF3 was required for enhanced polyI:C-driven antiviral state in Ad/Tr-cells. IRF3 was knocked down by utilizing IRF3-specific small interfering RNA and cells were subsequently treated, or not, with polyI:C before challenging with VSV-GFP. The greatest knockdown efficiency was observed between 72 h and 96 h post transfection (data not shown). Results of WB demonstrated no

apparent expression of tIRF3 after siRNA treatment (Figure 7A). Further, VSV-GFP replication in polyI:C-untreated Ad/dl- and Ad/Tr-cells was not significantly altered when IRF3 was knocked down (Figure 7B). This observation suggests that IRF3 is dispensable for antiviral defense against VSV-GFP infection in both Ad/dl and Ad/Tr groups in the absence of polyI:C treatment and that markedly reduced viral replication in Ad/Tr-cells was attributed to other, yet unidentified, factor(s). In contrast, when polyI:C was added, VSV-GFP replication was initially markedly reduced in both Ad/dl and Ad/Tr groups in the presence of IRF3, but then significantly increased in the absence of IRF3; yet, Ad/Tr-cells still remained more protected than Ad/dl group. This observation implies that enhancement of polyI:C-triggered antiviral protection in Ad/Tr-cells was only partially dependent on IRF3. Figure 7B also showed that VSV-GFP replication was restored about 50% of the original viral load detected in each of the groups in absence of IRF3 and polyI:C stimulation, suggesting that additional mechanisms/factors were contributing to polyI:C-induced antiviral protection. Collectively, these findings indicate that IRF3 plays an equally important role in antiviral protection in both Ad/dl and Ad/Tr groups, and that the presence of IRF3 is important, but not essential, for enhanced polyI:C-induced antiviral protection in Ad/Tr-cell, as other factors, perhaps upstream of IRF3, may also be contributing to this protection.

Tr/E significantly reduce pro-inflammatory cytokines in ECs following polyI:C and LPS stimulation

PolyI:C stimulation induces not only antiviral, but also pro-inflammatory factors (Nazli et al., 2009; Schaefer et al., 2005) that are regulated by viral sensors and transcription factors, including NF- κ B and c-Jun. We therefore assessed levels of pro-inflammatory mediators IL-8, TNF α , and IL-6 in Ad/Tr-cells treated with polyI:C. Figures 8A-C demonstrate that Ad/Tr-cells, regardless of their origin (i.e., genital HEC-1A or gut Caco-2, etc.), secreted significantly lower levels of IL-8 24 h after polyI:C and LPS treatment, compared to controls; TNF α and IL-6 were similarly reduced in Ad-cells

in response to polyI:C (data not shown). Interestingly, stimulation of HEC-1A with LPS did not produce a significant increase in IL-8, compared to untreated cells, possibly due to a low baseline expression of TLR4 in the genital EC (Nazli et al., 2009). These results clearly indicate that Tr/E controlled the release of pro-inflammatory mediators in ligand-treated cells. Interestingly, Tr/E secreted by Ad/Tr-cells appeared to significantly contribute to reduced IL-8 levels. Indeed, Tr/E neutralization with specific anti-Tr/E TRAB20 (HyCult Biotech) antibodies (under the control of antiprotease assay) that were added to Ad/Tr-cells 1 h prior to 0.1 $\mu\text{g/ml}$ of polyI:C treatment and subsequently co-cultured with polyI:C for additional 24 h to neutralize any secreted Tr/E, led to significantly higher polyI:C-induced IL-8 secretion (up to 40 %, $p=0.03$) in Ad/Tr-cells (data not shown). Figures 8D and 8E further show that HEC-1A cells pre-treated with either Ad/Tr-sups, rTr or rE before subsequent co-culture with polyI:C also released lower levels of IL-8 in response to polyI:C. Of note, anti-inflammatory effects of secreted/soluble proteins, compared to Ad/Tr-cells, appeared to be less potent and context-dependent, which forced us to use a lower dose of 0.1 $\mu\text{g/ml}$ of polyI:C. Additionally, either Ad/Tr-sups or rTr/rE alone did not trigger any significant IL-8 production in absence of polyI:C. Collectively, our findings indicate that Tr/E are capable of inhibiting inflammatory responses in ECs from various sources and against both viral and bacterial PAMPs, and that secreted/soluble Tr/E significantly contribute to reduction in polyI:C-induced IL-8 secretion. However, stimulation of immune responses can also be observed depending on experimental conditions.

Discussion

WAP proteins, including antiproteases Tr/E, SLPI, and ps20, are pleiotropic molecules known to play multiple and significant roles in health and disease (Drannik et al., 2011; Moreau et al., 2008; Sallenave, 2010). Indeed, ps20 has been reported as a potential diagnostic marker in prostate cancer (McAlhany et al., 2004) and as a novel negative signature protein in HIV infection (Alvarez et al., 2008). In contrast, SLPI and Tr/E show significant therapeutic potential in atherosclerosis as well as cardiovascular (Alam et al., 2012; Henriksen et al., 2004; Shaw and Wiedow, 2011; Vergnolle N, 2011), lung (McElvaney et al., 1993),(Zani et al., 2011) and gut disorders (Motta et al., 2011; Vergnolle N, 2011). Additionally, higher levels of Tr/E in CVLs of HIV-resistant CSWs (Iqbal et al., 2009) and the testing of the Lactobacilli-based elafin delivery system for combating STIs in the FGT (Fahey et al., 2011) would further support this notion. Our data showed that delivery of Ad/Tr to HEC-1A cells resulted in secretion of functional Tr, while both Tr/E were detected following treatment of these cells with polyI:C. Moreover, polyI:C treatment further resulted in Tr/E-enhanced antiviral protection and significantly reduced pro-inflammatory IL-8, IL-6, TNF α that were associated with lower expression of viral innate sensors RIG-I and MDA5 and altered NF- κ B activation in Ad/Tr-cells. Notably, increased antiviral protection was due in part to Tr/E ability to act directly on virus or by modulating polyI:C-driven cellular antiviral responses. Interestingly, such Tr/E-augmented cellular responses triggered by polyI:C were partially mediated through IRF3 activation, but not higher induction of IFN β , thus suggesting multiple antiviral mechanisms of Tr/E and the involvement of alternative and still unidentified factors or pathways.

This is the first study that comparatively assessed the presence and potential mechanisms of antiviral activity of each Tr/E. Here, we presented evidence showing two distinct, but likely complimentary antiviral properties of Tr/E: (i) direct antiviral activity exerted during the virus/cell interaction and targeting virus, but not cells; and (ii) indirect and cell-associated immunomodulatory activity, targeting polyI:C-triggered cellular antiviral responses. The virus-mediated activity was observed in the absence of polyI:C

stimulation and in the presence of Tr in Ad/Tr-cells (presumably present in both Ad/Tr-sups and Ad/Tr-cells). Because only Tr was detected in polyI:C-untreated Ad/Tr-cells, and because the expression of IFN β was low and not different between untreated Ad/dl and Ad/Tr groups, we conclude that in the absence of polyI:C, Tr alone was mediating antiviral activity in Ad/Tr-cells by acting either directly on virus or indirectly through cells. However, we failed to transfer this direct antiviral effect of Tr via Ad/Tr-sups, possibly due to the absence of an additive protective effect from augmented intracellular Tr (as would be expected in Ad/Tr-cells), or due to an inefficient delivery of Tr in supernatants and a “diluting” effect from other antiviral factors released in response to Ad/dl delivery (Fig. 2E, insert).

Additionally, the presence of both Tr/E, as in rTr preparation, was also protective against VSV-GFP challenge in the absence of polyI:C treatment. Although no reports describing direct antiviral activity of Tr are published to date and no precise mechanisms of Tr/E direct antiviral effect have been identified, our observation with rTr is in line with Ghosh *et al.* findings, describing a direct antiviral effect of E as a mode of action against HIV (Ghosh et al., 2010). Interestingly, rTr also appeared to have only virus- and not cell-mediated protective effects, similar to E mentioned earlier (Ghosh et al., 2010). In contrast, a close WAP member SLPI, was shown to have only cell-mediated antiviral effects, at least against HIV (Ma, 2004) and herpes simplex virus (HSV) (MasCasullo et al., 2005). Examples of same-family members having differential antiviral mechanisms have also been described for other innate molecules, such as human defensins against HIV (Quinones-Mateu et al., 2003; Rapista et al., 2011) and HSV (Hazrati et al., 2006). These reports suggest that molecules even from the same group, may possess their own, potentially different in potency and targets, exquisite antiviral activities and yet still uniquely contribute to overall mucosal protection against STIs.

Our results further suggest that the presence of both Tr/E might be required for each virus- (direct) and cell-associated (indirect) antiviral effect, as was evident from our data using Ad/Tr-cells, Ad/Tr-sups, and rTr, indicating that perhaps the most efficient antiviral protection depends on collaborative work of both Tr/E. Interestingly, when HIV-

susceptible, but uninfected, CSWs were followed prospectively, those who remained HIV-negative had elevated levels of both Tr/E detected in CVLs (Iqbal et al., 2009). Additionally, when characterizing the specificity of proteins secreted after Ad/Tr infection, our ELISA and WB results also showed that Ad/Tr-cells secreted both Tr/E independently in response to polyI:C, while only Tr was detected without polyI:C stimulation. Although Ghosh *et al.* also reported that primary uterine EC produced Tr/E in response to polyI:C (Ghosh et al., 2010), the independent production of E was never demonstrated. Further, only Tr (13-16 kDa) was previously identified in supernatants from LPS-stimulated alveolar ECs (Sallenave et al., 1994). On conjuncture, these results suggest that expression of each Tr/E could be a tissue/cell- or ligand-specific defense mechanism against an unknown protease that was potentially activated in response to a viral ligand. It might be important in the future to clarify whether primary genital EC from the FGT produce each Tr/E independently in response to polyI:C, similarly to Ad/Tr-cells.

It is unclear why tested rE failed to show antiviral activity against VSV-GFP; it could be attributed, however, to a HAT-tag insertion at the N-terminus of rE. Indeed, all rTr, rE, and secreted Tr were equally functional against HNE (data not shown) and capable of inhibiting IL-8 production in response to polyI:C (Figure 8). Yet, while rTr with a His-C-terminus tag exhibited antiviral activity, rE with a HAT-N-terminus tag did not. This observation suggests that blocking N-terminus, but not C-terminus, appears to be critical for antiviral activity of rE. An earlier study by McMichael *et al.* supports this argument, since they showed that the N-terminus of Tr had a better affinity for LPS than its C-terminus end (McMichael et al., 2005b). Additionally, it is unclear why we observed increased levels of IL-8 with higher concentrations of rTr and rE. But one possible explanation could be that the proteins were initially delivered and left on cells in serum-free conditions, thus promoting the activation of pro-inflammatory events as was previously shown for Tr/E in response to LPS (McMichael et al., 2005b).

The second antiviral property of Tr/E observed in our study was an indirect cell-mediated immunomodulatory activity of Tr/E, targeting polyI:C-induced antiviral cellular

responses. In contrast to responses to bacterial or pro-inflammatory stimuli (Butler et al., 2006; Henriksen et al., 2004), the scope and specific mechanism(s) of viral ligand-triggered immunomodulatory activity of Tr/E have never been fully investigated. Our data demonstrate that this indirect cell-associated activity is targeting viral recognition through modulation of RNA helicase expression as well as the induction of key inflammatory and antiviral innate signaling pathways and mediators.

Our results showed that polyI:C-triggered antiviral cellular protection was significantly enhanced in the presence of Tr (in Ad/Tr-sups) and Tr/E (in Ad/Tr-cells and in rTr). We also showed that polyI:C-mediated activation of IRF3 was further induced in Ad/Tr-cells, compared to controls, whereas IFN β expression was dampened. Interestingly, human β defensin 3 (Semple et al., 2011) and cathelicidin LL37 (Hasan et al., 2011) that were previously shown to have antiviral, including anti-HIV, activity (Steinstraesser et al., 2005a; Zapata et al., 2008), were also reported to inhibit IFN β production *in vitro* in response to LPS and polyI:C, respectively. These observations further support our results and strengthen the earlier argument of Tr/E acting either directly against VSV-GFP or through cells and additional factors/pathways in Ad/Tr-cells. Furthermore, moderation of immune-inflammatory responses and thus curbing undesirable immune activation might be one of the protective mechanisms of innate antimicrobials at mucosal sites. Additionally, in searching for ISGs typically associated with antiviral protection and IRF3 activation (Noyce et al., 2006), we found that expression of ISG15 was not significantly changed, unlike ISG56 being reduced and in agreement with IFN β data. It is not entirely understood why such discordance was observed; however, it could be due to the fact that ISG15 was shown to be regulated by either IRF3 or IFN β (Bauer et al., 2008; Harty et al., 2009), unlike ISG56 that was shown to be under the regulation of IFN β or viruses (Guo et al., 2000; Terenzi et al., 2006) and thus following IFN β pattern of induction as shown in our study. The alternative explanation could be that these two genes follow a different temporal pattern of activation that was overlooked here.

This is the first report on the involvement of serine antiproteases, Tr/E in particular, in antiviral signaling pathways. As no prior data are available on the role of Tr/E in IFN β and IRF3 induction, further and more detailed investigations might be required to explain why in Ad/Tr-cells IFN β and ISG56 levels were reduced while IRF3 activation was increased. We hypothesize, however, that this phenomenon could be an attempt of Tr/E to control antiviral inflammatory events through RIG-I/MDA5 and NF- κ B downregulation while increasing cellular protection through activation of IRF3 and/or alternative factors or pathways. Although most of the studies show ISG56 to be associated with upregulated IRF3 [62], our finding is in line with data from Li *et al.* showing that a knockdown of ISG56 was associated with increased IRF3 activation and inhibition of VSV-GFP replication (Li et al., 2009b) as a result of ISG56 mediating MITA-TBK1 interaction and subsequent downstream activation of IRF3. It is also possible that alternative factors/pathways, in addition to IRF3, regulate IFN β and ISG56 expression and contribute to Tr/E-enhanced antiviral protection, which is also supported by our IRF3 siRNA data. Collectively, these data indicate that in the presence of Tr/E, antiviral protection is increased and that direct or indirect antiviral effect(s) of Tr/E depend, but not exclusively, on IRF3 and other factors, perhaps upstream of IRF3.

Inflammation is one of the leading factors predisposing to acquisition and disease progression of STIs in the FGT (Lester et al., 2008; Lester et al., 2009; Rebbapragada et al., 2007). This notion is supported by the fact that “immune quiescence” and reduced immune activation are crucial for resistance against STIs (McLaren et al., 2010), while dysregulated TLR expression and immune-inflammatory responses are detrimental (Lester et al., 2008; Lester et al., 2009; Rebbapragada et al., 2007). Here, we showed that Tr/E individually or as a mixture, as well as in Ad/Tr-cells and as secreted/soluble proteins in Ad/Tr-sups, were capable of reducing IL-8, IL-6, and TNF α expression in response to polyI:C. Moreover, in Ad/Tr-cells we also observed significantly reduced activation and transcriptional activity of NF- κ B. The IL-8 inhibitory effect was not specific to human endometrial ECs, or to polyI:C, indicating that similar effects could be observed at other mucosal surfaces and in response to different microbial ligands. We

further showed that, compared to controls, mRNA and protein levels of RIG-I and MDA5 (mainly at a later time point), but not TLR3, were significantly diminished in response to polyI:C and in presence of Tr/E. Immunomodulatory properties of both Tr/E demonstrated in models of pro-inflammatory and bacterial (LPS) stimulations were shown to depend on inhibition of NF- κ B and AP-1 activation (Butler et al., 2006; Henriksen et al., 2004), thus further supporting our NF- κ B data. However, Tr/E inhibitory effect targeting antiviral immune responses, including viral sensing, has not been previously reported. Hence, modulation of expression of RIG-I, MDA5, and pro-inflammatory mediators shown here could represent novel antiviral functions of Tr/E, possibly even executed at different levels, namely receptors and transcription factors. That we observed differential pattern of RIG-I, MDA5, and TLR3 expression could indicate either different temporal kinetics of responses of these sensors, or that each RIG-I, MDA5, and TLR3 respond differentially to polyI:C, being a mixture of variable lengths of dsRNA (Kato et al., 2008). Further, the lack of polyI:C-triggered TLR3 induction in HEC-1A compared to primary genital ECs, also likely reflects tissue or structure-dependent differences between the cells, suggesting that primary genital ECs may exhibit distinct results. Collectively, these observations suggest that Tr/E can alter innate viral recognition and mounting of antiviral immune-inflammatory responses.

The precise mechanism(s) of immunomodulatory effects of Tr/E, as both secreted and/or intracellularly expressed proteins, on viral sensors, cytokines, and IFN β in response to polyI:C treatment is still largely unknown. This is partly because the existence of the cognate receptor for Tr/E remains elusive, and it is equally unknown whether Tr/E require a receptor to function. We propose that reduced levels of IL-8, IL-6, TNF α and IFN β in Ad/Tr cells in response to polyI:C are likely a result of overall attenuation of RIG-I and MDA5 levels, as they are known to regulate the expression of pro-inflammatory and antiviral mediators (Kato et al., 2006; Yoneyama and Fujita, 2010; Yoneyama et al., 2004) through activation of main signaling pathways, such as NF- κ B that is downregulated in our study (Yoneyama et al., 2004). It remains to be elucidated, however, how Tr/E specifically inhibit RIG-I and MDA5 expression. It is plausible that

Tr/E directly bind to polyI:C, as was shown for binding of LL37 to polyI:C (Hasan et al., 2011), as well as Tr/E binding to LPS (McMichael et al., 2005b). Such an interaction may alter binding/recognition of polyI:C by its cognate receptors, including cell-surface scavenger receptor A or intracellular sensors RIG-I, MDA5, and TLR3, which in turn could explain our reduced expression levels of RIG-I and MDA5. Another possible site of inhibition by Tr/E could be downstream of receptors/viral sensors and involve Tr/E binding to DNA and competing for specific DNA binding sites with transcription factors including NF- κ B, as was shown for SLPI as one of its anti-inflammatory mechanisms in response to LPS (Taggart et al., 2005).

A noteworthy observation of this study is that while Tr from Ad/Tr-sups and rE were found functional against HNE and able to inhibit polyI:C-induced IL-8 production, they did not show any antiviral activity, suggesting that antiprotease, anti-inflammatory, and antiviral activities of the tested proteins may not necessarily be co-dependent or predictive of each other; nonetheless, they can be complimentary. This observation is supported by earlier reports, showing both a protease non-inhibitory N-terminus and an inhibitory C-terminus of Tr exhibiting comparable antibacterial and antifungal functions (Baranger et al., 2008; Simpson et al., 1999). In contrast, Mulligan *et al.* showed that SLPI Gly(72) mutant, unlike other mutants tested in that study, lost its *in vivo* immunosuppressive activity against NF- κ B activation and neutrophil recruitment in the lungs that appeared to be most closely related to SLPI's trypsin-inhibiting activity (Mulligan et al., 2000). Although being an important property of both Tr/E, the inhibition of HNE activity is not considered a critical function for our studies, since epithelial cells do not make neutrophil elastase (Pham, 2006) and thus, the earlier discussed Tr/E-mediated changes are most unlikely attributed to antielastase activity of the proteins. The above observations indicate that perhaps additional structure-function studies might be warranted in the future to specifically address the cross-talk between antiprotease, anti-inflammatory, and antiviral properties of Tr/E and their specific roles in defense against viruses.

Overall, our data support and further extend earlier observations on immunomodulatory effects of Tr/E (Butler et al., 2006; Henriksen et al., 2004). This work demonstrates that in genital ECs and in response to polyI:C, Tr/E antiviral effects are mediated through direct or virus targeting activity and indirect or cell-associated immunomodulatory function(s) that target host innate recognition and mounting of antiviral and inflammatory responses. While dampening of IFN β , a key antiviral mediator, may seem counterintuitive and detrimental to antiviral defenses, our findings suggest that directly or indirectly increased antiviral protection and moderated, or finely-tuned, inflammation, might be more advantageous to a host in the context of viral exposure. In conclusion, this study clearly demonstrates the importance of Tr/E in antiviral protection. Our findings also propose the existence of multiple targets and potentially several and unique modes of action for each of the proteins, which warrant additional research in the future.

Acknowledgments

The authors appreciate the expert technical support of J. Newton and A. Patrick and thank Drs. M. Stämpfli and B. Lichty (McMaster University) for supplying reagents.

Figure legends

Figure 1. Ad/Tr-cells secrete Tr that is functionally active against HNE and both Tr/E are detected in Ad/Tr-cells supernatants in response to polyI:C. HEC-1A cells were either treated with medium alone or with MOI 10-50 of Ad/dl or Ad/Tr. Supernatants were collected 36 h after Ad removal and tested for Tr/E expression by ELISA (A) or for antiprotease activity (C), where results are expressed as percent reduction over a positive control (media alone plus HNE and a substrate). The data are representative of two independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's *t* test with * representing significant difference between the groups, $p < 0.05$. (B) Immunoblotting analysis of supernatants from HEC-1A cells developed using TRAB20 antibodies. Cells were either left untreated (lanes 1,2) or treated with MOI 50 of Ad/dl (lanes 3,4) or Ad/Tr (lanes 5,6) and then incubated for 24 h in presence of medium alone (-) or 25 μ g/ml polyI:C (+). Two recombinant reference proteins, namely in-house HAT-E (rE) (lane 7) and commercial 6 \times His-Tr (rTr) (lane 8), were used as comparative markers for E and Tr. Bands corresponding to the forms of Ad-induced Tr and E are indicated on the blot by arrows.

Figure 2. Tr/E significantly reduce VSV-GFP infection and enhance polyI:C-driven antiviral protection in Ad/Tr-cells. Untreated (UT) or treated with MOI 50 of Ad/dl or Ad/Tr HEC-1A cells were medium- or polyI:C 0.1 – 25 μ g/ml-treated for 24 h, followed by MOI 1 VSV-GFP. GFP fluorescence intensity was visualized 24 h later using a Typhoon scanner (A), and relative fluorescence intensity to virus positive control was determined and presented as % of UT control (B). (C) VSV-GFP load after pre-incubation of 5 μ g/ml of rTr either with MOI 1 of VSV-GFP (rTr+v) or cells (rTr+c) for 1 h, followed by their washing and addition of VSV-GFP. Medium-treated VSV-GFP (v) served as a positive control. (D) HEC-1A cells were treated with 5 μ g/ml of rTr for 1 h in serum-free medium, after which medium or 0.1 μ g/ml polyI:C was added for 24 h followed by MOI 1 of VSV-GFP. This polyI:C dose was chosen because anti-inflammatory effects of secreted/soluble proteins, compared to Ad/Tr-cells, were less

potent. (E) HEC-1A cells were pretreated with equal volumes of supernatants from Ad/dl-cells (Ad/dl-sups) and Ad/Tr-cells (Ad/Tr-sups, 5 µg/ml final concentration) for 1 h before medium or 0.1 or 5 µg/ml polyI:C was added for 24 h followed by MOI 1 VSV-GFP. Lower polyI:C doses were used to better visualize VSV-GFP viral replication 24 h later, using a Typhoon scanner. Insert in panel (E) depicts VSV-GFP load after pre-incubation of same volumes and concentrations of Ad/dl- and Ad/Tr-sups with either MOI 1 VSV-GFP (dl+v) or (Tr+v), respectively, or cells (dl+c) or (Tr+c) for 1 h, followed by washing and addition of MOI 1 VSV-GFP. Medium-treated VSV-GFP (v) served as an untreated control. The data are representative of at least two independent experiments performed in triplicate and shown as mean ± SD. Statistical analysis was performed using ANOVA or Student's *t* test in (C), and * representing significant difference, when $p < 0.05$. (F and G) Cell growth and metabolic activity of HEC-1A cells were determined by standard MTT Cell Viability Assay Kit following infection with MOI 10-50 of Ad/dl or Ad/Tr (F) or after stimulation with increasing doses of polyI:C (G). Cell viability was expressed as % of untreated cells, which served as a negative control group, and was designated 100%; the results are expressed as % of negative control.

Figure 3. Ad/Tr-cells respond to polyI:C treatment with reduced production of IFNβ. HEC-1A cells were either treated with medium alone (UT) or with MOI 50 of Ad/dl or Ad/Tr and incubated for 6-24 h in presence of media or 25 µg/ml of polyI:C. (A) At 6 h post treatment, IFNβ mRNA from total RNA was determined by real-time quantitative RT-PCR. Values are normalized to a housekeeping gene 18S in the same sample and presented as fold induction over UT cells in absence of polyI:C treatment. (B) At 24 h of polyI:C stimulation, supernatants were tested for IFNβ expression by ELISA. Data are representative of at least two independent experiments performed in triplicate and expressed as the mean ± SD, shown in pg/ml. Statistical analysis was performed using Student's *t* test with * representing significant difference between the groups, $p < 0.05$.

Figure 4. Enhanced polyI:C-driven antiviral protection in Ad/Tr-cells is associated with hyperactivation of IRF3. (A) Western blots and their analyses of phosphorylated IRF3, total IRF3, and GAPDH proteins were performed from whole-cell extracts of HEC-Ad cells that were either left untreated or treated with 25 µg/ml polyI:C during indicated time points. (B) Immunofluorescence analysis of IRF3 nuclear translocation following either medium alone or polyI:C 25 µg/ml treatment for 4 h. Representative staining is shown for IRF3 (green), nuclear stain (PI) (red), and composite (yellow) at magnification 2520×. The data are representative of three independent experiments with similar results.

Figure 5. Exogenous expression of Tr/E significantly decreases mRNA expression of ISG56 in Ad/Tr-cells following polyI:C stimulation. HEC-1A cells were either treated with medium alone or with MOI 50 of Ad/dl or Ad/Tr and incubated in presence of media or 25 µg/ml of polyI:C. (A) At 6 h post treatment, total RNA was harvested and mRNA levels of ISG56 were assessed by conventional RT-PCR. (B) Quantification of ISG56 expression using ImageQuant software. Values are normalized to a housekeeping gene 18S in the same sample and presented as relative fold induction over untreated cells, shown in arbitrary units. The data are representative of at least two independent experiments performed in triplicate and are shown as the mean ± SD. Statistical analysis was performed using Student's *t* test with * representing significant difference between the groups, $p < 0.05$.

Figure 6. Altered NF-κB phosphorylation and transcriptional activity and reduced RIG-I and MDA5 expression in polyI:C-treated Ad/Tr-cells. Western blots (A) and their quantification (B) of phosphorylated p65 subunit of NF-κB (pNF-κB) as well as c-Jun (pc-Jun) and GAPDH proteins were performed from whole-cell extracts of Ad-cells that were either left untreated or treated with 25 µg/ml polyI:C during indicated time points. Transcription activity (C) of NF-κB (NF-κB/Luc panel) and (AP-1/Luc panel) was assessed by luciferase reporter assay by transfecting HEC-1A cells with pgkβ-Gal and pNF-κB-Luc or pAP-1-Luc plasmids (total DNA 40 ng per well) (p) alone or together with 50 MOI of Ad/dl or Ad/Tr overnight, washing,

allowing to rest for 4 h and stimulating with 25 $\mu\text{g/ml}$ polyI:C and 1 $\mu\text{g/ml}$ LPS for 4 h. Then luciferase and β -galactosidase activities were determined in cell lysates and expressed as relative luciferase units using galactosidase plasmid as normalization control. Data are presented as mean \pm SD and are representative of three experiments for NF- κ B and two - for AP-1. (*D-F, left panels*) Total RNA was harvested and mRNA expression of RIG-I, MDA5, and TLR3 was determined by real-time quantitative RT-PCR at 6 h post treatment with polyI:C 25 $\mu\text{g/ml}$. Values are normalized to a housekeeping gene 18S in the same sample and presented as fold induction over untreated cells. (*D-F, right panels*) Western blot analysis of RIG-I, MDA5, TLR3, and GAPDH protein was performed from whole-cell extracts of Ad-cells that were either treated or not with 25 $\mu\text{g/ml}$ polyI:C during indicated time points. The data are representative of at least two independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's *t* test with * representing significant difference between the groups, $p < 0.05$.

Figure 7. IRF3 is required for polyI:C-driven antiviral protection in Ad/Tr-cells. (*A*) HEC-1A cells were left untreated or transfected with a non-targeting control siRNA (ctrl siRNA), or IRF3 siRNA (IRF3 siRNA) for 48 h. Two days after siRNA delivery, HEC-1A received MOI 50 of Ad/dl or Ad/Tr followed by 25 $\mu\text{g/ml}$ of polyI:C treatment for 24 h and Western blot analyses of total IRF3 (tIRF3) and GAPDH proteins were performed from whole-cell extracts 96 h post-transfection. (*B*) Twenty four hours following polyI:C treatment, cells were infected with MOI 1 of VSV-GFP for another 24 h. Levels of GFP fluorescence were visualized and quantified using a Typhoon scanner. The fluorescence reading of treated cultures was normalized to untreated (control) cultures and presented as percentage relative fluorescence. The data are representative of two independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's *t* test with * representing significant difference between the groups, $p < 0.05$.

Figure 8. Tr/E significantly reduce protein secretion of IL-8 in ECs following polyI:C and LPS stimulation. Secretion of IL-8 in supernatants from

HEC-1A (A), A549 (B), and Caco-2 (C) cells initially infected with MOI 50 of Ad/dl or Ad/Tr and subsequently stimulated with either 25 µg/ml of polyI:C or 1 µg/ml of LPS for 24 h. Following stimulation, supernatants were tested for IL-8 secretion by ELISA. Data are representative of at least two independent experiments performed in triplicate and expressed as the mean ± SD, shown in pg/ml. Statistical analysis was performed using Student's *t* test with * representing significant difference between the groups, $p < 0.05$. (D) Secretion of IL-8 in supernatants from HEC-1A cells that were pre-treated with HEC-Ad/dl and HEC-Ad/Tr supernatants before polyI:C stimulation. HEC-1A received 50 µl of concentrated supernatants containing around 10 µg/ml of Tr/E in Ad/Tr supernatants and 0.004 µg/ml of Tr/E in Ad/dl sups for 1 h, to which additional 50 µl of medium alone or polyI:C to the final concentration of 0.1 µg/ml were added for 24 h. (E) Secretion of IL-8 in supernatants from HEC-1A cells treated with commercial 6×His-Tr (rTr) or in-house HAT-E (rE) for 1 h and then stimulated with medium alone or 0.1 µg/ml of polyI:C for 24 h. A lower dose of polyI:C was used in (D) and (E), since anti-inflammatory effects of secreted/soluble proteins, compared to Ad/Tr-cells, were less potent. Statistical analysis was performed using ANOVA, and * representing significant difference between the polyI:C group and rTr groups; † representing significant difference between the polyI:C and rE groups, $p < 0.05$.

Figure 1.

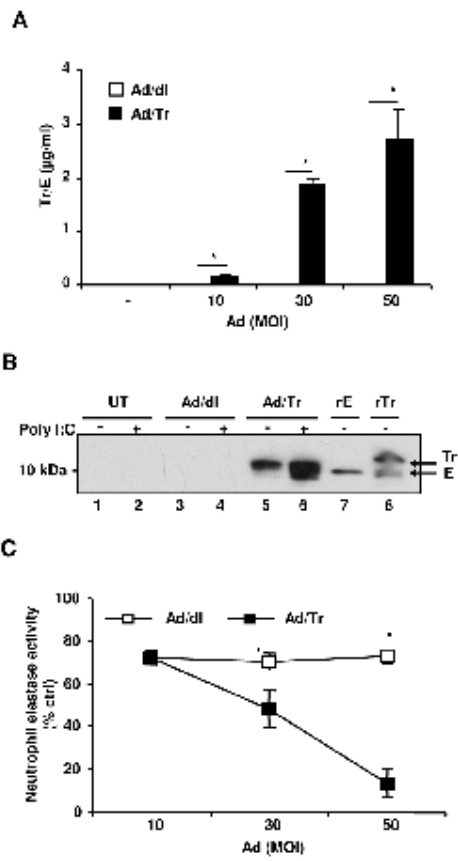


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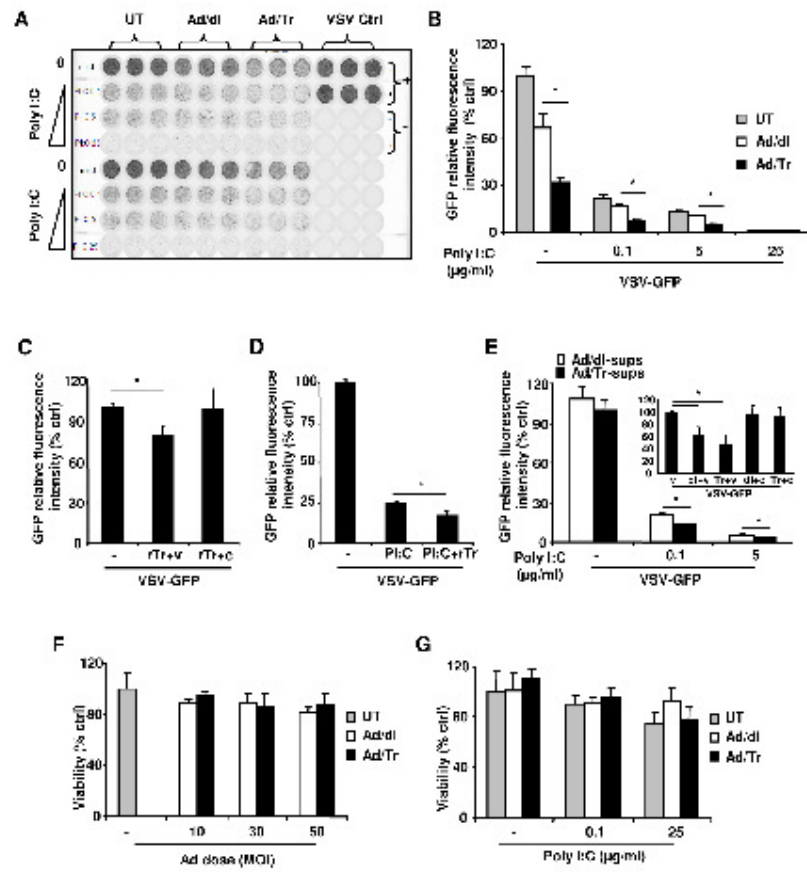


Figure 3.

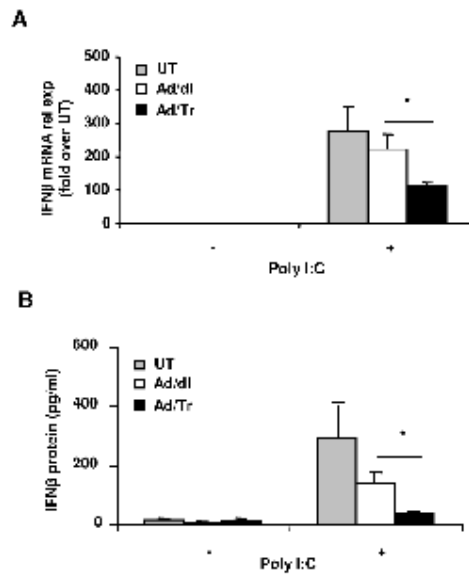
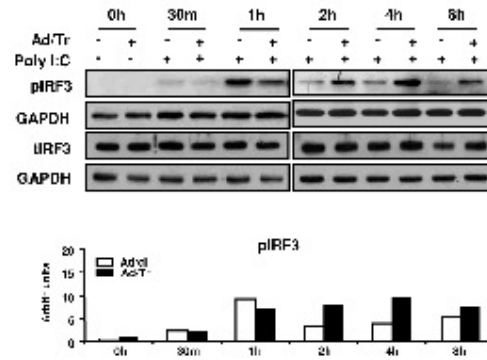


Figure 4.

A



B

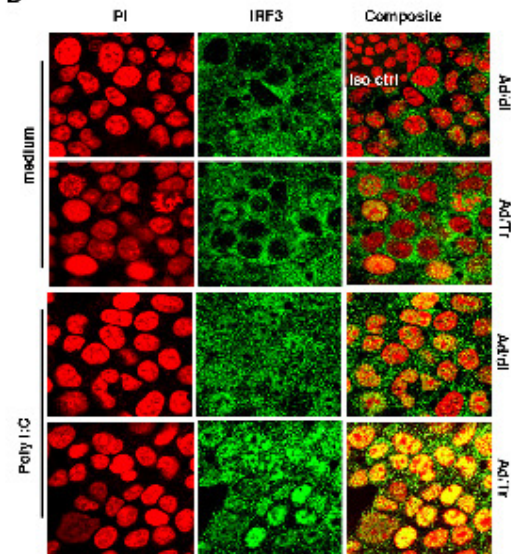


Figure 5.

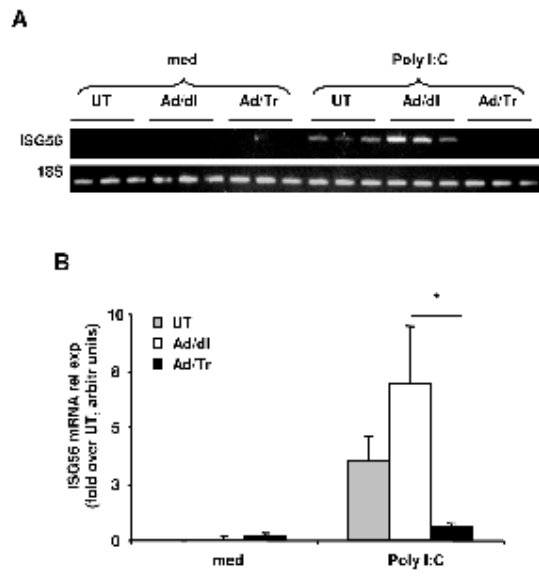


Figure 6.

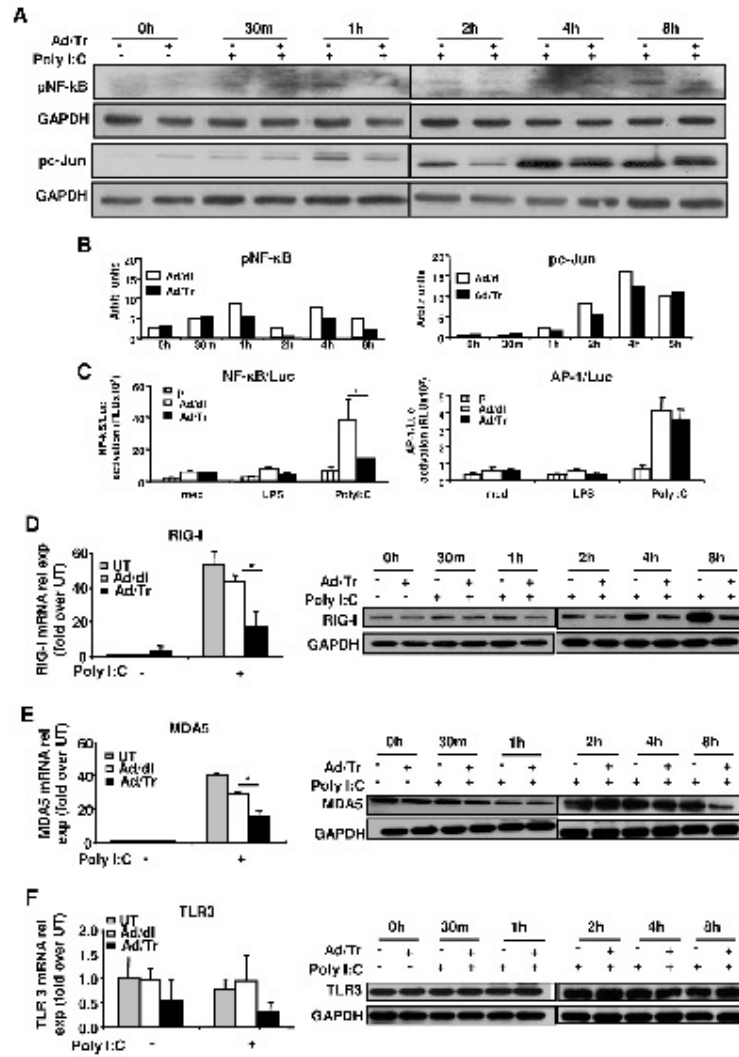


Figure 7.

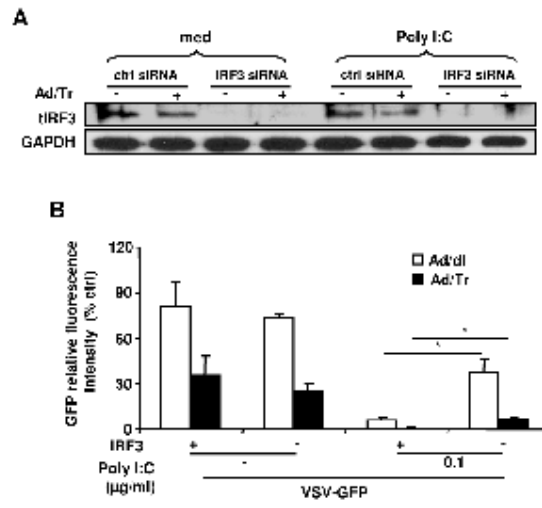
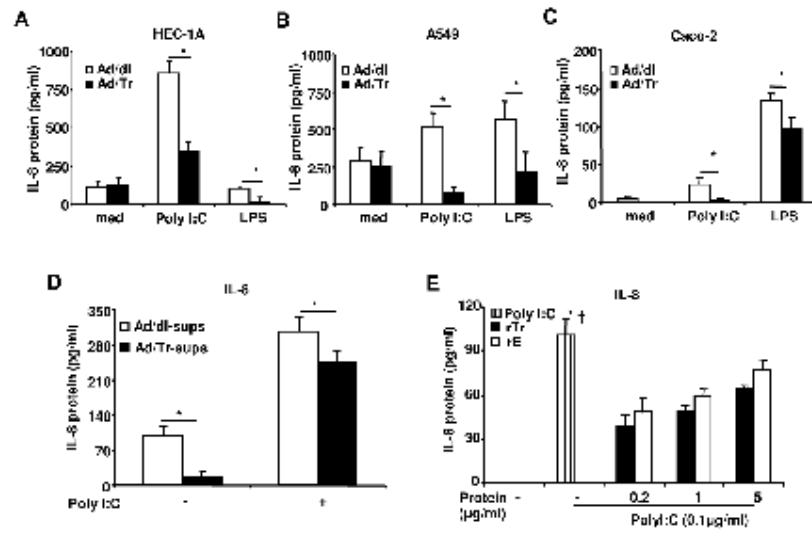


Figure 8.



Antiviral Activity of Trappin-2 and Elafin *in vitro* and *in vivo* against Genital Herpes

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This manuscript has been prepared for submission to Journal of Virology

Summary and central message:

Building on findings from the previous chapter, notably that Tr/E enhance antiviral protection and reduce inflammatory responses elicited by polyI:C, in Chapter 3 we sought to determine the contribution of Tr/E to antiviral defense against a sexually-transmitted pathogen. HSV-2 is a primary cause of genital herpes, a life-long chronic infection with episodic reactivations of mucocutaneous viral shedding that are often asymptomatic. HSV-2 infection is also one of the leading co-infections and predisposing factor for HIV/AIDS acquisition and transmission and no effective preventative measures are available to date to control HSV-2 infection. Thus, the identification of novel antiviral factors at mucosal sites, especially the FGT as one of the main portals of viral entry, can be a good strategy for targeting both HSV-2 and HIV-1 infections. Our *in vitro* studies demonstrated that Tr/E increased antiviral protection and reduced viral load in human endometrial ECs, HEC-1A, following HSV-2 challenge. Furthermore, we found that Tr/E reduced viral attachment to ECs by acting on both virus and cells and that E appeared to be more potent in its antiviral activity in comparison to Tr. Our results also demonstrated that reduced viral replication was associated with lower secretion of pro-inflammatory factors IL-8 and TNF α and decreased NF- κ B nuclear translocation. Interestingly, the more protected cells also demonstrated enhanced IRF3 nuclear translocation and increased levels of secreted IFN β in response to HSV-2 challenge compared to controls.

Finally, our *in vivo* studies in Tr transgenic mice (Etg), intravaginally inoculated with lethal HSV-2 dose, showed that Etg mice had reduced viral translocation from the genital tract into the CNS and reduced viral replication in the CNS, compared to Wt controls, that was accompanied by significantly reduced levels of TNF α in the target organs after HSV-2 challenge. These Etg animals also exhibited a trend of increased survival and lower pathology scores. Altogether, this study provided first and important experimental evidence highlighting the presence and potential targets of anti-HSV-2 activity of Tr/E in the female genital mucosa.

Antiviral Activity of Trappin-2 and Elafin *in vitro* and *in vivo* against Genital Herpes

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Running title: Trappin-2/Elafin in HSV-2 infection in Genital Mucosa

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Word count for abstract: 245

Word count for text (excluding the references, table footnotes, and figure legends): 7,115

ABSTRACT

Herpes simplex virus type 2 (HSV-2) is a primary cause of recurrent genital mucocutaneous lesions. Most sexual and perinatal transmissions occur asymptotically, and HSV-2 increases acquisition and transmission of HIV-1. We recently demonstrated that antiprotease elafin and its precursor trappin-2 (Tr/E) inhibited HIV-1 attachment/entry and transcytosis across human genital epithelial cells (ECs) and enhanced polyI:C-induced antiviral protection while modulating inflammatory responses in ECs. Here, we assessed Tr/E contribution to the defense against HSV-2. Our *in vitro* studies demonstrate that endometrial (HEC-1A) and endocervical (End1/E6E7) ECs pretreated with human Tr-expressing adenovirus (Ad/Tr) or recombinant (r) Tr/E proteins were more protected against HSV-2 infection, and that rTr/E reduced viral attachment/entry to ECs by either acting on virus or cells. Furthermore, lower viral replication was associated with reduced secretion of pro-inflammatory IL-8 and TNF α and decreased NF- κ B nuclear translocation. Additionally, the more protected Ad/Tr-treated ECs also demonstrated enhanced IRF3 nuclear translocation and higher secretion of antiviral IFN β in response to HSV-2. Interestingly, E was found ~7 times more potent against HSV-2 than Tr. Finally, our *in vivo* studies of intravaginal lethal HSV-2 infection in Tr-transgenic mice (Etg) showed that, despite similar viral replication in the genital tract (GT), Etg appeared to have delayed viral translocation from the GT, significantly reduced viral burden and TNF α levels in the central nervous system, and a trend to increased survival compared to controls. Collectively, this is the first experimental evidence highlighting the anti-HSV-2 activity of Tr/E in the female genital mucosa.

INTRODUCTION

The estimated seroprevalence of herpes simplex virus 2 (HSV-2) in North America is nearly 20% and is even higher around 30-80% in some developing countries and sub-Saharan Africa (Chen et al., 2000; Xu et al., 2006). These numbers make genital herpes one of the leading and most prevalent sexually transmitted infections (STI) worldwide. Most sexual and perinatal transmission of HSV-2 occur during asymptomatic, or “mute”, mucocutaneous viral shedding (Koelle and Wald, 2000), when a person is unaware of transmitting the pathogen to others. Even more alarming is the fact that HSV-2 is closely linked to human immunodeficiency virus-1 (HIV-1) infections, by being a risk factor for HIV-1 acquisition (Freeman et al., 2006) and transmission (Corey, 2007b; Ouedraogo et al., 2006).

As a natural consequence of attachment, entry, and infection, viruses, including HSV-2, become exposed to a variety of innate sensors, or pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs), RNA helicases, and inflammasomes (Rasmussen et al., 2009; Yu and Levine, 2011). Subsequently, viral recognition triggers a series of intracellular signal transduction events that activate key transcription factors involved in antiviral and immune-inflammatory responses. Specifically, upon activation, mitogen-activated protein kinase (MAPK), nuclear factor (NF)- κ B (Li et al., 2009a) and the IFN regulatory factors (IRF) (Mogensen and Paludan, 2005) coordinate the expression of genes with antiviral and inflammatory activity. Type I interferons (IFNs) (Kawai and Akira, 2006), with IFN β leading the way in defense against HSV-2 (Gill et al., 2006; Nazli et al., 2009), as well as interferon-stimulated genes (ISGs) (Bauer et al., 2008; Li et al., 2009b) are only a few examples of factors contributing to antiviral defense.

Exposure to HSV-2 also triggers the release of pro-inflammatory mediators, including TNF α (Thapa and Carr, 2008b), IL-1 β , and IL-6 (Gill et al., 2006; Li et al., 2009a). Such factors contribute not only to the induction of protective innate and adaptive immune responses (Gill et al., 2006; Thapa and Carr, 2008a), but also, if poorly controlled, to the development of systemic inflammatory reactions, as seen in neonatal

sepsis (Kurt-Jones et al., 2005), or in breaching blood-brain barrier and HSV translocating into the central nervous system (CNS) (Kurt-Jones et al., 2004; Thapa and Carr, 2008b). Upon entry into the CNS, HSV-2 establishes a life-long and latent infection in dorsal roots and sensory ganglia with recurrent episodes of re-activation and symptomatic or asymptomatic viral shedding. While in humans this process is not usually life-threatening, unless generalized, murine models of HSV infections demonstrate high morbidity and mortality from viral CNS dissemination (Thapa and Carr, 2008b). Regardless of the severity and presentation of herpetic lesions, the “mute” transmission of HSV-2, its life-long latency and the interplay between genital herpes and HIV-1 infections (Rebbapragada et al., 2007) places HSV-2 among high priority infections, requiring the development of novel and efficient therapeutic and preventative measures, especially those protecting the genital mucosal.

Human genital epithelial cells (ECs), being the principle target in HSV-2 infection, respond to viral encounter by releasing innate antimicrobial factors in efforts to eradicate or contain viral pathogens (Ghosh et al., 2010; Iqbal et al., 2009). Serine protease inhibitors elafin (E) and its precursor, trappin-2 (Tr), along with secretory leukocyte protease inhibitor (SLPI), and defensins belong to a large family of cationic antimicrobials that have been linked to endogenous mucosal protection against sexually transmitted pathogens, including HSV-2 and HIV-1 (Hazrati et al., 2006; Iqbal et al., 2009; MasCasullo et al., 2005; Venkataraman et al., 2005). Trappin-2 and elafin (Tr/E), as well as SLPI, all share the cystein-rich fold with four disulfide bonds called the whey acidic protein (WAP) domain, involved in protease inhibition (Tsunemi et al., 1996). Secreted as unglycosylated protein, Tr (9.9 kDa) (Schalkwijk et al., 1999) (or pre- or full-length elafin) contains an N-terminal cementin domain (38-aa) (Nara, 1994), and elafin (5.9 kDa), a C-terminal 57-residue domain with a WAP structure (13-14). Inhibiting human neutrophil elastase and proteinase 3 through the WAP domain allows Tr/E to control excessive inflammation and tissue damage. Additionally, cross-linking between the transglutaminase-binding motifs located on the N-terminus of each Tr/E (Baranger et al., 2008; Nara, 1994) and extracellular matrix proteins like heparin and fibronectin

makes Tr/E indispensable for repairing compromised tissue integrity (Guyot et al., 2005b).

Tr/E possess broad-spectrum antibacterial (Simpson et al., 1999) and antifungal properties (Baranger et al., 2008). Recently, we and others have shown that Tr/E exert immunomodulatory activity, where depending on the environment, they can either dampen inflammation or promote immunostimulatory events and prime the immune system (Drannik et al., 2012c; McMichael et al., 2005b; Roghanian et al., 2006). Both Tr/E are found at mucosal surfaces and secretions, including cervicovaginal lavage fluid (CVL) (Ghosh et al., 2010; Iqbal et al., 2009; King et al., 2003b); in tissues, like skin, placenta, genital and gastrointestinal tract (King et al., 2003b; King et al., 2007; Pfundt et al., 1996; Pfundt et al., 2000; Sallenave et al., 1992; Schalkwijk et al., 1991; Wiedow et al., 1990); and multiple cell types, such as neutrophils, macrophages, keratinocytes, and epithelial cells (23, 25-26), including genital ECs (Ghosh et al., 2010; King et al., 2003b). Tr/E are regarded as alarm antiproteases, since they are mainly produced in response to pro-inflammatory stimuli such as LPS (Simpson et al., 2001a), TNF α (21), and IL-1 β (King et al., 2003b; Sallenave et al., 1994). Additionally, Tr/E were recently shown to be induced and secreted by genital ECs in response to polyinosinic/polycytidylic acid (polyI:C), a surrogate of a viral dsRNA (Drannik et al., 2012b; Drannik et al., 2012c; Ghosh et al., 2010), thus further supporting their importance in the inflammatory environment.

We and others recently demonstrated the importance of Tr/E in increased antiviral protection against HIV-1 as well as enhanced polyI:C-induced antiviral immune responses (Drannik et al., 2011; Drannik et al., 2012b; Drannik et al., 2012c; Ghosh et al., 2010; Iqbal et al., 2009). Specifically, elevated Tr/E in cervicovaginal lavage fluid (CVLs) of HIV-exposed seronegative commercial sex workers (CSWs) were found to be associated with mucosal HIV-1 resistance (Iqbal et al., 2009) and to significantly contribute to CVL natural anti-HIV-1 activity *in vitro* (Drannik et al., 2012b). However, the contribution of Tr/E to defense against HSV-2 genital infections remains undefined.

The objective of this study was to elucidate whether and how each Tr/E individually contributes to anti-HSV-2 defense mechanisms in female genital mucosa.

MATERIALS AND METHODS

Reagents. Two commercial Tr and E proteins were tested: (i) human recombinant 6×His-trappin-2 (Tr) (with a C terminus His-tag) (R&D Systems, Burlington, ON, Canada) (Drannik et al., 2012b; Drannik et al., 2012c); (ii) commercial human recombinant elafin (E) (with no tag) HC4011 (Hycult Biotech, Uden, Netherlands) (Drannik et al., 2012b). PolyI:C (Sigma-Aldrich, Oakville, ON, Canada) was reconstituted in the phosphate-buffered saline (PBS) .

Cell lines and viruses. Human endometrial carcinoma-(HEC)-1A (ATCC HTB-112, Rockville, MD, USA) and Vero African green monkey kidney cells (ATCC CCL81) were maintained in McCoy's 5A Medium Modified (Invitrogen Life Technologies, Burlington, Ontario, Canada) and in α -MEM medium (Invitrogen Life Technologies), correspondingly, supplemented with 10% fetal bovine serum, 1% HEPES, 1% l-glutamine (Invitrogen Life Technologies), and 1% penicillin-streptomycin (Sigma-Aldrich, Oakville, Ontario, Canada). Endocervical End1/E6E7 cell line (kind gift from Dr. R. Fichorova, Brigham & Women's Hospital, Boston , MA, USA) was generated as described before (Fichorova et al., 1997) and maintained in keratinocyte serum-free medium (GIBCO/BRL, Life Technologies) supplemented with 50 μ g/ml bovine pituitary extract, 0.1 ng/ml epidermal growth factor, 100 units/ml penicillin, 100 μ g/ml streptomycin, and CaCl₂ to a final calcium concentration of 0.4 mM. All cells were cultured at 37°C in 5% CO₂. Stocks of HSV-2 (strain 333) were generated using Vero cells and stored at -70°C until used.

HSV-2 *in vitro* model. For viral infections, ECs were first pretreated with Tr/E for 1 h in serum-free medium followed by HSV-2 inoculum in same serum-free medium for additional 2 h, after which virus was removed, cells repeatedly washed with PBS, and complete growth medium added for 24 or 48 h. Subsequently, supernatants were collected and stored cell-free at -70°C until further use. Viral titers were determined by plaque assay using Vero cells grown on 12-well plates to 70-80% confluence. Virus-

containing samples were serially diluted in serum-free medium and added to Vero cell monolayers. Infected monolayers were incubated at 37°C for 2 h, rocked every 15 min for viral absorption, and subsequently overlaid with complete α -MEM culture medium further supplemented with 0.05% human immune serum globulin (Canadian Blood Services). Infection was allowed to occur for 48 h at 37°C. Monolayers were then fixed and stained with crystal violet, and viral plaques were counted under a light microscope and expressed as plaque forming units (PFU) per milliliter of HSV-2.

Adenoviral constructs and delivery in cell culture. The replication-deficient adenoviral constructs (Ad) used in this study have been described in detail elsewhere (Bett et al., 1994; Drannik et al., 2012c; Sallenave et al., 1997; Sallenave et al., 1998). To express human Tr, the Ad/Tr vector, encoding gene for 95-aa human Tr, was used (Sallenave et al., 1997; Sallenave et al., 1998). This adenoviral construct was previously called Ad/E. E1,E3-deleted empty adenovirus Ad-dl703 (Ad/dl), coding for no transgene, was used as a control for Ad/Tr (Bett et al., 1994). Both Ad vectors were prepared at the Centre for Gene Therapeutics at McMaster University (Hamilton, ON, Canada). ECs were either treated with Opti-MEM® I Reduced Serum Medium (Invitrogen Life Technologies) alone (UT) or with MOI 50 PFU of Ad/dl or Ad/Tr at 37°C overnight. After repeated PBS washes and rest for 4 h, cells were exposed to various MOIs of HSV-2 in a serum-free medium for 2 h, then repeatedly washed with PBS and complete growth medium was added for additional 24-48 h. Viral titers were determined in cell-free supernatants as described within Materials and Methods section above, pertaining to cell lines and viruses.

Viral attachment. Viral attachment was determined as described previously (Hazrati et al., 2006), but with slight modifications. Briefly, HEC-1A cells and virus were first pretreated with either medium or Tr/E for 1 h at 37°C. Cells pretreated with medium, received either virus alone or virus pretreated with proteins. Cells that were pretreated with proteins first, were either repeatedly washed or not and exposed to HSV-2 virus of

multiplicity of infection (MOI) 1 (1 PFU per 1 cell) for 5 hours at 4°C to allow for viral attachment. After repeated washes with PBS to remove unbound virus, cell-associated virus was detected by probing Western blots of whole cell lysates with anti-gD primary antibody (P1103; Virusys, Atlanta, GA, USA) and anti-GAPDH (ab9485, Abcam) to control for protein loading.

MTT viability assay. MTT assay (Biotium Inc., Hayward, CA, USA) was used as per manufacturer's instructions to determine viability of HEC-1A cells after Ad and HSV-2 exposure and was described elsewhere (Drannik et al., 2012c; Nazli et al., 2009).

Tr/E knockdown by RNA interference. Small interfering (siRNA) molecule (Invitrogen Life Technologies) (GenBank accession number NM_002638) targeting human Tr/E within 67-420 ORF through the following sequences, starting from position 202: CCCGUUAAAGGACAAGUUU and non-targeting siRNA, RNAi Negative Control (medium GC content), 12935-300, (Invitrogen Life Technologies) were used to specifically knockdown Tr/E. Transfections of siRNA (8 pmol) were done using Lipofectamine RNAiMAX and Opti-MEM medium (Invitrogen Life Technologies) as per supplier's instructions. HEC-1A cells, 3×10^4 in a 100 μ l of total volume complete growth medium, were transfected in a 96-well BD Falcon culture plate (BD Biosciences) for 48-72 h before challenging with HSV-2 MOI 0.1 and 1. Knockdown efficiency was monitored using Tr/E ELISA 24 h after viral challenge.

ELISA assays. Cell-free samples were stored at -70°C until assayed for human Tr/E, IL-8, IL-6, and TNF α with ELISA DuoSet kit (R&D Systems) and for human IFN β by ELISA kit from Antigenic America Inc. (Huntington Station, NY, USA), according to the supplier's protocol. For animal experiments, murine TNF α , IL-6, MIP-2, and IFN γ were measured using ELISA DuoSet kits (R&D Systems) and for IFN β detection ELISA was conducted using PBL biomedical kit from PBL (Piscataway, NJ, USA). Analytes were quantified based on standard curves obtained using an ELISA reader Tecan Safire ELISA reader (MTX Labs Systems Inc.) as per supplier's protocol.

Preparation of cell extracts and Western blot (WB) analysis. Whole-cell extracts were prepared by using whole-cell extract buffer (50mM Tris-HCl, pH 8.0, 150mM NaCl, 1%NP-40, 1%SDS, 1 × protease inhibitor (Roche, Mississauga, ON, Canada) as per standard protocol. Protein amount was quantified using Bradford assay with bovine serum albumin (Sigma-Aldrich) as a standard and Bio-Rad Dye Reagent Concentrate as a protein stain (Bio-Rad Laboratories, Mississauga, ON, Canada). WB was performed on a 10% polyacrylamide denaturing SDS-PAGE gel and PVDF membranes (Amersham, Arlington Heights, IL, USA) as per standard protocol, using the following primary antibodies: anti-gD primary antibody (P1103; Virusys, Atlanta, GA, USA) and anti-GAPDH (ab9485, Abcam) to control for protein loading. After incubation with corresponding horse-radish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad Laboratories), blots were visualized using a SuperSignal West Femto or Pico Chemiluminescent Substrate kit (Thermo Scientific, Rockford, IL, USA). Quantification of band intensities was done using MBF_ImageJ for Microscopy Software.

Immunofluorescence staining. Immunofluorescence staining was performed as described before, but with minor modifications. HEC-1A cells grown on an 8-well BD Falcon culture slides (BD Biosciences) were pretreated with Tr and E proteins for 1 h before receiving either medium, or HSV-1 MOI 1 per well for 4 h. Following treatment, cells were fixed and blocked as described elsewhere (Bauer et al., 2008; Nazli et al., 2009). IRF3 was detected using 1:100 dilution (in blocking solution) of IBL18781 (IBL, Gunma, Japan) for 1 h. NF- κ B p65 sc-372 (SantaCruz Biotechnologies, Santa Cruz, CA, USA) (1:500) was used to detect nuclear translocation of NF- κ B p65. Negative control rabbit immunoglobulin fraction (DakoCytomation, Glostrup, Denmark) served as an isotype control and was diluted to match the protein content of the primary anti-IRF3 antibody. Secondary antibody, Alexa Fluor 488 conjugated goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) was added to cells in a blocking solution for 1 h.

Nuclei were visualized by staining with propidium iodide. Images were acquired using an inverted laser-scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany).

RNA extraction and real-time quantitative PCR analysis. The protocol for RNA isolation was described elsewhere (Drannik et al., 2012c; Lester et al., 2008). Briefly, total RNA was isolated from HEC-1A-Ad-infected cells cultured with medium alone or HSV-2 for 6 h, using TRIzol reagent (Invitrogen Life Technologies) and DNase-treated (Ambion, Austin, TX, USA), as per supplier's recommendations. RNA was quantified using the Agilent 2100 Bio-Analyzer (Agilent, Santa Clara, CA, USA). Total RNA was reverse transcribed into complementary (cDNA) with SuperScript reverse transcriptase III (Invitrogen Life Technologies). Real-time quantitative PCR was performed in a total volume of 25 μ l using 1 \times Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 5 μ l of diluted cDNA, 500 nmol forward primer, 500 nmol reverse primer (Mobix; McMaster University, ON, Canada), and 200 nmol probe in a 96-well plate. TaqMan oligonucleotide primers and probes labeled with 6FAM at 5'-end and a non-fluorescent quencher at 3'-end were designed using Primer Express 1.5 and purchased from Applied Biosystems: IFN β : 5'-CGCCGCAGTGACCATCTA-3' (forward), 5'-CCAGGAGGTTCTCAACAATAGTC-3' (reverse); 18S rRNA: 5'-CGGAATTAACCAGACAAATCGCTCCA -3' (probe), 5'-GTGCATGGCCGTTCTTAGTT-3' (forward), 5'-TGCCAGAGTCTCGTTCGTTAT-3' (reverse). The expression of 18S ribosomal (r) RNA was used as an internal control. PCR was run with the standard program: 95°C 10 min, 40 times of cycling 95°C 15 sec and 60°C 1 min in a 96-well plate with a ABI PRIZM 7900HT Sequence Detection System using the Sequence Detector Software 2.2 (Applied Biosystems).

Animals. Female mice used in this study included wild type (WT) C57Bl/6 mice (Charles River Canada, ST. Constant, Quebec, Canada) and EcDNA mice, also on a C57Bl/6 background, that were generated to express the gene for human full-length elafin (pre-elafin), or trappin-2, under MCMV promoter as described elsewhere (Sallenave et

al., 2003). Presence of Tr gene was routinely confirmed with genotypic analysis as per standard protocol. All mice were 8-16 weeks old and maintained in Level B housing conditions in a 12-hour light-dark cycle. All experimental protocols involving mice were approved by the Animal Research Ethics Board of McMaster University.

HSV-2 *in vivo* model. Five days before viral challenge, mice were injected subcutaneously with 2 mg of Depo-Provera (Depo) (Upjohn, Don Mills, Ont., Canada) to facilitate vaginal infection, since Depo is a long-acting progesterone formulation that induces a diestrus-like state in the genital tract. Mice were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg), injected intraperitoneally (IP), and their tails were lifted and 1×10^4 PFU/mouse was administered intravaginally (i.vag.) in a total volume of 10 μ l in PBS. Mice were kept on their backs under the influence of anesthesia between 45 min to 1 h to allow the inoculum to be retained. Mice were monitored daily for survival and disease progression using the following five-point scale: 0, no apparent inflammation and infection; 1, slight redness of external vaginal os; 2, swelling and redness of external vaginal os; 3, severe swelling and redness of both vaginal os and surrounding tissue, hair loss in genital area; 4, genital ulceration with severe redness, swelling, and hair loss of genital and surrounding tissue; 5, severe genital ulceration extending to surrounding tissue or signs of paralysis. Animals were euthanized at stage 5. Vaginal washes were collected daily over 8 days by pipeting twice consecutively with 30 μ l of PBS in and out of the vagina several times. On days 2, 6, and 8 post-inoculation, whole genital tracts as well as spinal cords and brainstems were removed, placed in 1 ml of PBS, and processed individually via homogenization as described earlier (Drannik et al., 2004). Viral titers were determined in supernatants using plaque assay as described earlier in this section.

Statistical analysis. Statistical analysis was performed with either non-paired Student's *t* test for differences between two groups or a one-way analysis of variance (ANOVA) for more than two groups, using Sigma Stat 2.0. Survival curves were compared with log

rank test using GraphPad Prism Version 4.0. In all cases, data were expressed as means \pm standard deviation (SD) and values of $p < 0.05$ were considered significant.

Results

Tr/E reduce HSV-2 replication in genital ECs. To elucidate if and how Tr/E contribute to defense against HSV-2 challenge in genital ECs, the role of exogenous Tr/E proteins was assessed. A replication-deficient adenovirus construct expressing the human Tr gene (Ad/Tr) (Drannik et al., 2012c; Sallenave et al., 1997; Sallenave et al., 1998) was used to exogenously express human Tr in endometrial HEC-1A and endocervical End1/E6E7 EC lines. To elucidate whether each Tr/E individually exhibit anti-HSV-2 activity, as well as to counteract detrimental side effects of adenovirus-expressed Tr, we also utilized recombinant Tr and E with HEC-1A cells. Fig. 1A shows that supernatants from HEC-1A cells infected with Ad/Tr (Ad/Tr-cells) had significantly ($p < 0.05$) lower HSV-2 titers, compared to HEC-1A cells infected with a control Ad/dl vector (Bett et al., 1994), (Ad/dl-cells). Although viral load in Ad/dl-cells alone appeared to be reduced compared to untreated (UT) cells, the reduction in HSV-2 shedding in Ad/Tr-cells was significantly greater, indicating that exogenous Tr further reduced viral replication and augmented anti-HSV-2 protection in ECs. Further, similar inhibition of Tr on HSV-2 replication was also demonstrated in endocervical End/E6E7 ECs (Fig. 1B). To determine if each Tr/E exhibit anti-HSV-2 activity, recombinant (r) Tr/E proteins were used to pretreat HEC-1A cells. Data in Fig. 1C and D demonstrate that each rTr/E independently significantly ($p < 0.05$) reduced viral titers in HEC-1A cells, compared to medium alone. The inhibition of HSV-2 replication was dose-dependent and with physiologically relevant (0.04-1 $\mu\text{g/ml}$) protein concentrations for female genital tract (Ghosh et al., 2010). Additionally, we ruled out impaired cell viability as a potential cause of reduced susceptibility to viral infection by performing an MTT viability assay (data not shown). Taken together, our results demonstrate that Tr/E each individually, in concentrations not toxic to cells, have the capacity to inhibit HSV-2 replication and enhance antiviral protection.

E has greater anti-HSV-2 activity than Tr. We next determined the comparative potency of each Tr/E against HSV-2 by identifying the 50% inhibitory concentration (IC_{50}) values of Tr/E, given that tested recombinant Tr/E proteins possessed similar antiprotease activity (Drannik et al., 2012b). The IC_{50} values for Tr/E with respect to

infectivity of HEC-1A cells were estimated to be $0.07 \pm 0.01 \mu\text{g/ml}$ and $0.01 \pm 0.008 \mu\text{g/ml}$ (Fig. 1E). Comparison of IC_{50} values for each Tr/E revealed that anti-HSV-2 efficacy of E was about 7 times greater than for Tr. Collectively, these results revealed that E is superior to its precursor, Tr, with respect to anti-HSV-2 activity.

Tr/E are required for anti-HSV-2 protection of HEC-1A cells. To corroborate results shown in Figure 1, Tr/E were knocked down in HEC-1A cells by Tr/E-specific small interfering (si) RNA and cells were subsequently challenged with HSV-2. Figure 2A shows that levels of Tr/E detected by ELISA in HEC-1A cells supernatants were significantly ($p < 0.05$) reduced after Tr/E siRNA knockdown and HSV-2 treatment, compared to control siRNA- and HSV-2-treated cells. Further, cells that maintained their endogenous Tr/E production were significantly ($p < 0.05$) protected against HSV-2 challenge with MOI 0.1 (Fig. 2B); however, this protection was overcome when a higher viral dose was used. Additionally, all siRNA-untreated cells had Tr/E levels within 50 pg/ml and viral loads similar to control siRNA-treated cells (data not shown). Overall, these results suggest that endogenous Tr/E play an important role in anti-HSV-2 defense in genital ECs, but their protective effect might be limited to lower viral doses.

Tr/E reduce binding/attachment of HSV-2 to HEC-1A cells. Binding between HSV-2 glycoprotein D (gD) and cognate cell surface receptor(s) is important for viral entry into cells (Spear, 2004). Thus, we determined if each Tr/E interfered with viral attachment to cell surface at the entry level. To this end, Western blotting (WB) was performed to assess the amount of HSV-2 gD present in cell lysates as a representative of HSV-2 attached to HEC-1A cells. Fig. 3A and B demonstrate that undetectable amount of gD was present in cells pretreated with medium alone (Fig. 3A, lanes 1&2) or with Tr/E before virus addition (Fig. 3A, lanes 5, 6, 7, 8), compared to cells exposed to HSV-2 alone (Fig. 3A, lanes 3&4). We next elucidated whether Tr/E act on cells or the virus. Fig. 3C and D show that no gD was detected in media-treated cells, in contrast to cells treated with virus alone (Fig. 3C, lanes 1 & 2, respectively). Further, when cells were first pretreated with Tr or E (Fig. 3C, lane 3&5, respectively), repeatedly washed and exposed to HSV-2 inoculum, reduced amounts of gD were detected, compared to the virus alone

group (Fig. 3C, lane 2). Interestingly, when the virus, and not the cells, was first pretreated with Tr or E (Fig. 3C, lane 4&6, respectively) and then added onto untreated cells, similarly reduced amounts of gD were detected. Fig. 3B and D show quantifying histograms of corresponding bands depicted in Fig. 3A and C. Notably, the inhibitory effect of E (Fig. 3A, lanes 7&8 and Fig. 3C, lanes 5&6) was more dramatic compared to Tr (Fig. 3A, lanes 5&6 and Fig. 3C, lanes 3&4). Collectively, these results clearly indicate that Tr/E each individually can inhibit HSV-2 at the entry level by reducing viral attachment to genital ECs, which was mediated through direct interaction with HSV-2 and as well as indirect interaction with the cells.

Ad/Tr-cells respond to HSV-2 challenge with increased IFN β secretion. The protective role of IFN β against HSV-2 infection is well established (Bauer et al., 2008; Gill et al., 2006; Murphy et al., 2003; Nazli et al., 2009). We elucidated whether Tr/E pretreatment of genital ECs could increase IFN β expression in response to HSV-2 challenge. Fig. 4A shows that mRNA levels of IFN β in Ad/Tr-cells were significantly ($p=0.007$) increased at 6 h after low dose HSV-2 challenge of HEC-1A cells, compared to Ad/dl-cells. Further, the stimulatory effect was also evident from ELISA data, showing significantly ($p=0.039$) increased levels of IFN β protein in supernatants of Ad/Tr-cells, compared to Ad/dl-controls, at 24 h post infection (Fig. 4B). Overall, these data indicate that genital ECs exposed to Ad-expressed Tr before HSV-2 challenge secrete higher levels of IFN β upon HSV-2 exposure.

Tr/E-treated ECs exhibit increased HSV-2-induced nuclear translocation of IRF3. Interferon regulatory factor (IRF) 3 is one of the key factors contributing to polyI:C induced antiviral protection *via* activation of ISGs either with or without the induction of IFN β (Bauer et al., 2008; Drannik et al., 2012c; Guo et al., 2000; Mossman et al., 2001). Here, we determined whether Ad/Tr-cells, with reduced viral load and induced IFN β secretion, would exhibit increased nuclear translocation of IRF3 following HSV-2 challenge. Immunofluorescence staining for IRF3 nuclear translocation demonstrates that in the presence of medium alone, adenovirus-untreated HEC-1A cells (UT) or Ad/dl- and Ad/Tr-cells did not have significant translocation of IRF3 (green) into the nuclear

area (red), as no merged (yellow) color was present within the nuclear area (Fig. 4C and D). In contrast, in HSV-2-treated rows, Ad/Tr-cells showed markedly increased number of cells with IRF3 nuclear translocation compared to control cells (Fig. 4C). Additionally, HEC-1A cells pretreated with recombinant Tr/E proteins also showed enhanced IRF3 nuclear translocation (Fig. 4D), with more notable effect observed for E-treated cells. However, the stimulatory effect of the tested Tr/E proteins on IRF3 nuclear translocation was less prominent, compared to Ad/Tr-cells. Taken together, these results indicate that pre-exposure of HEC-1A cells to either Ad-expressed Tr or recombinant Tr/E proteins enhanced nuclear translocation of IRF3 that was also associated with increased anti-HSV-2 protection of cells.

Tr/E-treated ECs secrete significantly reduced HSV-2-induced pro-inflammatory cytokines. A physiologically relevant outcome of the attachment, entry, and infection of cells with HSV-2 is the release of immune-inflammatory factors (Gill et al., 2006; Li et al., 2009a; Thapa and Carr, 2008b), which may contribute to the pathogenesis and inflammation associated with genital herpes (Cook et al., 2004; Corey et al., 1983). Given that Tr/E were previously shown to exert anti-inflammatory effects against bacterial (Butler et al., 2006; McMichael et al., 2005a) and viral (Drannik et al., 2011; Drannik et al., 2012b; Drannik et al., 2012c) ligands, we next determined whether pretreatment of genital ECs with Tr/E affected HSV-2-induced levels of pro-inflammatory IL-8 and TNF α . Figure 5 shows that either Ad/Tr-cells (Fig. 5A) or HEC-1A cells first pretreated with 1 μ g/ml of each recombinant Tr/E before viral challenge (Fig. 5B and C) responded to HSV-2 infection with significantly ($p < 0.05$) reduced levels of IL-8 and TNF α at 24 h, compared to Ad/dl- or media alone groups, respectively. The secreted TNF α in Ad-treated cells supernatants, however, was below the ELISA detection level (data not shown). Altogether, our data demonstrate that exogenous Tr/E modulated the secretion of inflammatory factors by ECs in response to HSV-2 challenge, which was also associated with reduced viral replication and attachment to cells.

Tr/E-treated ECs exhibit reduced HSV-2-triggered NF- κ B nuclear translocation.

NF- κ B is one of the key transcription factors that regulate production of antiviral and pro-

inflammatory factors, like IL-8 and TNF α , upon encounter with viral ligands (Li et al., 2009a; Yoneyama et al., 2004), and Tr/E were previously shown to reduce NF- κ B activation in response to bacterial (Henriksen et al., 2004) and viral ligand stimulation (Drannik et al., 2012c). Thus, given reduced secretion of IL-8 and TNF α observed in this study, we next determined if Tr/E pretreatment of HEC-1A cells inhibits NF- κ B activation after HSV-2 exposure. Immunofluorescence staining was performed assessing the localization of p65 subunit of NF- κ B (green) in HEC-1A cells, with nuclei visualized with propidium iodide (PI) (red) and nuclear translocation of p65 visualized as yellow. Qualitative analysis of representative confocal images (Fig. 5D and E) shows that treatment with medium alone did not trigger significant activation and nuclear translocation of p65 in either HEC-1A cells untreated with Ad vector (UT), Ad/Tr- or their controls, Ad/dl-cells, as there was no yellow visualized in the nuclear area of the cells across the groups (Fig. 5D, medium panels). Conversely, HSV-2 treatment alone triggered significant p65 nuclear translocation, which was reduced in Ad/Tr-, but not Ad/dl-cells (Fig. 5D, HSV-2 panels). Although some nuclear localization of p65 was noted in Ad/Tr- and Tr-treated groups, the intensity of the observed signal was substantially reduced, compared to UT- or Ad/dl-HSV-2-treated groups. Additionally, HEC-1A cells pretreated with each recombinant Tr/E (Fig. 5E), also showed reduced HSV-2-induced NF- κ B nuclear translocation, with more notable effect observed for E-treated cells. Collectively, these data indicate that pretreatment with each Tr/E before viral challenge reduces HSV-2-triggered nuclear translocation of p65 subunit of NF- κ B, which was also associated with reduced secretion of pro-inflammatory IL-8 and TNF α .

Tr transgenic (Etg) mice have significantly lower viral load and reduced TNF α protein levels in the CNS following intravaginal HSV-2 challenge. Considering our *in vitro* findings in human genital ECs, we tested antiviral properties of Tr in a murine model of genital HSV-2 infection. Since mice do not produce Tr/E, consequently we used Tr transgenic mice (earlier referred to as the full-length elafin transgenic mice, thus the name (Etg)) (Sallenave et al., 2003), but on a C57Bl/6 background, along with their corresponding wild type C57Bl/6 counterparts (Wt). Mice were intravaginally inoculated

with a lethal 1×10^4 pfu/mouse dose of HSV-2. Our data show that Etg and Wt control mice had no significant differences in viral replication in the genital tract (GT), including vaginal washes, throughout the time course of the genital HSV-2 infection (Fig. 6A and B). Interestingly, despite no differences in viral load in the GT between the groups, Etg appeared to have reduced viral load in the CNS at day 2 (Fig. 6C), possibly due to delayed or less efficient viral translocation from the GT of Etg into the CNS. Indeed, despite earlier viral clearance from the GT, virus appeared in Wt CNS 4 days earlier than in Etg mice and remained at significantly higher levels on Days 6 and 8 (Fig. 6C). Moreover, a pattern of reduced viral load in the CNS of Etg mice was continuously observed throughout the infection, reaching statistical significance by day 8 (Fig. 6C). Furthermore, reduced viral replication in the CNS in Etg compared to control mice was also associated with significantly reduced levels of TNF α in the GT and CNS at day 8 (Fig. 6D), as well as significantly attenuated pathology scores at day 5 (Fig. 6E), and a trend toward a better survival starting at day 8 (Fig. 6F). Taken together, these findings indicate that even though viral load in the GT of Etg animals was not more efficiently cleared compared to controls, viral spread and replication in the CNS was less efficient and better controlled in Etg mice, likely being related to decreased released levels of TNF α in the GT and CNS. The latter would suggest a link between reduced inflammation and less efficient spread of HSV-2. Levels of other pro-inflammatory factors, namely MIP-2, IL-6, IFN β , and IFN γ were also measured, but only MIP-2 was consistently elevated in Etg GT at day 0 before viral inoculation and later reduced at day 8 in the CNS similarly to TNF α , albeit not reaching statistical significance (data not shown). Overall, these data indicate that in the presence of human Tr, mice had a better viral containment and control of its spread, resulting in lower levels of pro-inflammatory factors locally and systemically.

Discussion

High prevalence of HSV-2 infection (Fisman et al., 2002; Nahmias et al., 1990), its serious complications in neonates and immunocompromised (Koelle and Corey, 2008), and the close interplay with HIV-1 (Corey, 2007) are worrisome and continue to remind us of the lack of efficient control of this STI. Identification of novel molecules capable of controlling primary HSV-2 infection and inflammatory responses at mucosal sites may advance our efforts in designing effective measures to curb both HSV-2 and HIV-1 infections.

We evaluated the contribution of each Tr/E to the host's defense against HSV-2 by utilizing human genital ECs *in vitro* and a model of genital HSV-2 infection in Tr transgenic mice *in vivo*. Our results demonstrated that Tr/E likely targeted HSV-2 infection at entry and post-entry events by acting directly on virus and indirectly on cells by targeting viral attachment and cellular antiviral and inflammatory responses. Cells pretreated with Tr/E secreted lower levels of pro-inflammatory factors IL-8 and TNF α and had attenuated nuclear translocation of NF- κ B following HSV-2 challenge. Moreover, reduced viral replication was also associated with increased IFN β secretions and nuclear translocation of IRF3. Interestingly, we found that tested recombinant E was more potent against HSV-2 than its precursor, Tr. Finally, our results from *in vivo* experiments showed that Tr transgenic animals, compared to Wt controls, showed a consistent trend of increased survival; better disease outcome was also associated with lower viral translocation from the GT into the CNS, as well as reduced levels of TNF α at the target organs. Collectively, these observations describe antiviral activities of Tr/E in defense against HSV-2 infection in the female genital mucosa.

It was not surprising to observe such complex and multifaceted antiviral activity for Tr/E, since other innate factors, including SLPI, defensins, and lactoferrin have also been ascribed anti-HSV-2 activity mediated through multiple mechanisms (Jenssen, 2005; Jenssen et al., 2008; MasCasullo et al., 2005; Wang et al., 2004; Yasin et al., 2004). Although it is unclear at the moment how specifically Tr/E inhibited viral

attachment to HEC-1A cells, our results would propose that Tr/E may act through both virus and target cells.

Despite the fact that no published evidence is available to date on direct interaction between Tr/E and HSV-2, this activity of Tr/E is still plausible. Indeed, α -defensins such as human neutrophil peptides (HNP)1-3 and several synthetic defensins were shown to bind to carbohydrate moieties, likely O- or N-linked glycans on HSV-2 glycoprotein B-2 (gB-2) and HIV-1 glycoprotein (gp)120, and thus directly interfere with viral attachment, entry, and infectivity (Wang et al., 2004; Yasin et al., 2004). Structural homology between Tr/E and defensins in sharing intramolecular disulfide motifs (Ganz, 1994; Grutter et al., 1988), as well as our recent observations on direct antiviral activities of Tr/E against vesicular stomatitis virus (VSV) (Drannik et al., 2012c) and HIV-1 (Drannik et al., 2012b), (A.G. Drannik, K. Nag, X.D. Yao, B.M. Henrick, W.B. Ball, F.A. Plummer, C. Wachihi, J. Kimani, and K.L. Rosenthal, submitted for publication) would also strengthen this argument. We propose that binding between Tr/E and viral carbohydrate moieties could be a potential mechanism of direct anti-HSV-2 activity of Tr/E. Future in-depth elucidation of this inhibitory effect of Tr/E might be worth pursuing, given that HSV-2 and HIV-1 share O- or N-linked glycans on their glycoproteins.

Our findings also indicate that Tr/E acts indirectly through cells. Considering that Tr/E can bind to heparin and fibronectin (Guyot et al., 2005b), the interaction between Tr/E and cell-surface HSV-2 binding/entry receptors, including heparan sulfate (HS) chains and nectin-1 and nectin-2 members of immunoglobulin superfamily (Spear, 2004), can be an additional mode of Tr/E antiviral activity. This suggested mechanism could explain why we observed decreased viral titers and attachment of HSV-2 to HEC-1A cells, knowing that these cells, in resemblance to primary genital ECs, highly express HS moieties (Saidi et al., 2007). Indirect inhibitory anti-HSV-2 effect of SLPI would support our data, since SLPI was found to exert its protective antiviral effect by interacting through ECs and not virus (Ma, 2004). However, the involvement of annexin II in anti-HSV-2 defense, which was important for anti-HIV-1 activity of SLPI, was ruled out

(MasCasullo et al., 2005). The observed inhibitory effect of Tr/E on HSV-2 attachment may have significant implications, since viral attachment to ECs is one of the critical steps required for subsequent fusion and entry of HSV-2 into ECs. Thus, our results indicate that Tr/E may affect the pathogenesis of HSV-2 by interfering with viral entry and potentially the establishment of primary HSV-2 infection mucosally as well as by hindering entry/access and spread of virus in the nervous system.

The fact that Tr/E demonstrated both cell- and virus-associated protective anti-HSV-2 effects, whereas SLPI was shown to interact only with cells, could be a result of only a remote (40%) homology between SLPI and Tr/E. Notably, a divergent effect in anti-HSV-2 activity among members of the same family was also shown for defensins. For example, while α -defensins HNP1-3 and HD5 were preferentially binding to HSV-2 gB molecules, they did not bind to cellular HS moieties; in contrast, while HBD3 bound both gB and HS, human β -defensin (HBD) 1 and HBD2 bound neither (Hazrati et al., 2006).

Silencing of endogenous Tr/E with siRNA further corroborated the importance of Tr/E in anti-HSV-2 defense in the genital ECs. Our results also showed that the inhibitory effect of endogenous Tr/E was overcome with higher viral inoculum, suggesting a context-dependent nature of anti-HSV-2 activity of endogenous Tr/E that can be limited by HSV-2 evasion mechanisms. One such mechanism may involve the regulation of Tr/E production, since we found significantly reduced mRNA and protein level of Tr/E in cells challenged with a high dose (MOI 5) of HSV-2 (data not shown). Interestingly, a similar evasion strategy of HSV-2 was reported earlier for SLPI due to viral early-gene expression (Fakioglu et al., 2008) as well as for type I IFNs and the expression of HSV-2 virion host shutoff (vhs) protein (Duerst and Morrison, 2004; Murphy et al., 2003). The latter studies support our earlier findings showing that vhs, in a dose-dependent way, inhibited innate immune sensing, IRF3 activation, and IFN β expression in human vaginal ECs (Yao and Rosenthal, 2011). Altogether, these data indicate that although Tr/E appear to be important in the host's defense against HSV-2, these molecules could represent a

vulnerable target of the viral evasion mechanisms that should be taken into consideration when designing protective measures against STIs.

In this study, E appeared ~ 7 times more potent against HSV-2 than its precursor, Tr. Interestingly, we reported a similar observation on E being over a 100 times more potent than Tr against HIV-1 (Drannik et al., 2012b). These findings are also in line with the earlier mentioned report on a divergent anti-HSV-2 effect of defensins (Hazrati et al., 2006). It is unclear at the moment why E was more potent than Tr against HSV-2 infection. However, our data likely reflect the end result of elafin's greater ability to inhibit viral binding/entry and replication, to enter the nucleus, as well as to attenuate NF- κ B phosphorylation/nuclear translocation, and to modulate gene and protein expression of pro-inflammatory mediators.

Here we found that IL-8 and TNF α , as well as NF- κ B nuclear translocation, were significantly diminished following exposure to each Ad/Tr and rTr/E, indicating that Tr/E can moderate HSV-2-induced antiviral immune-inflammatory responses. We argue that the observed moderation of inflammatory responses could be a result of Tr/E acting not only at the entry level, by reducing the number of attached virions, but also at the post-entry level, or intracellularly, perhaps directly (DNA binding) or indirectly (up-stream) targeting NF- κ B transcriptional activity. The intracellular mechanism would be supported by earlier reports showing Tr/E-mediated reduction in LPS-induced AP-1 and NF- κ B by targeting the ubiquitin-proteasome pathway (Butler et al., 2006) and up-regulating I κ B α (Henriksen et al., 2004). Moreover, the study demonstrating that SLPI can bind to DNA and compete for NF- κ B binding sites, thus preventing NF- κ B activation (Taggart et al., 2005), would also support the proposed intracellular mode of anti-HSV-2 action of Tr/E, as well as our recent finding of the dependence of anti-HIV-1 activity of Tr/E on its intranuclear localization (A.G. Drannik, K. Nag, X.D. Yao, B.M. Henrick, W.B. Ball, F.A. Plummer, C. Wachihi, J. Kimani, and K.L. Rosenthal, submitted for publication). Collectively, these observations may support DNA binding by Tr/E as one of plausible mechanisms of Tr/E antiviral activity that would explain both our reduced viral titers as well as anti-inflammatory mediators.

In support of the argument that antimicrobial factors can act at multiple levels, α -defensin 1 was shown, in the absence of serum, to act directly on HIV-1, but, in the presence of serum, to inhibit HIV-1 replication by interfering with PKC phosphorylation in primary CD4+ T cells (Chang et al., 2005). Interestingly, the latter report is similar to an earlier study by McMichael *et al.* showing that in serum-containing conditions, Tr/E had an inhibitory effect on LPS-induced release of TNF α (McMichael et al., 2005b). However, the same study reported that in serum-free conditions and at higher concentrations, Tr/E had a stimulatory activity on TNF α release.

IRF3 has been shown to mediate antiviral protection through the activation of ISGs that may or not depend on the induction of IFN β (Samuel, 2001; Yoneyama and Fujita, 2010). Considering the above, it is likely that increased antiviral protection observed in Tr/E-treated and HSV-2-exposed cells was due in part to increased IRF3 nuclear translocation and consequentially augmented IFN β induction. However, other IRFs, including IRF7 and IRF9, could also contribute, since IRF3 was shown to act in homo- and heterodimers (Hiscott, 2007). Overall, this is the first report of Tr/E-mediated increased IFN β secretion and IRF3 nuclear translocation in context of a viral infection that are also linked with increased antiviral protection of genital ECs against HSV-2 challenge. These observations clearly indicate a potential interaction between Tr/E and IRF3 pathway, perhaps by acting through the upstream regulators of IRF3 induction. Taking into account that viruses, including HSV-2 and HIV-1, specifically target and disrupt functional activity of IRF3 as part of their evasion strategy (Doehle BP, 2009; Yao and Rosenthal, 2011), such interaction might be of importance in future applications of antiviral properties of Tr/E.

A protective role of innate and adaptive immune responses mediated by NK cells, CD4+ and CD8+ cells, IL-15 and IFN β (Chan et al., 2011; Gill et al., 2006) has been well characterized and established in murine models of HSV-2 genital infection. However, a beneficial role of restricted antiviral and inflammatory responses was also recently highlighted (Duerst and Morrison, 2007; Thapa and Carr, 2008b). *In vivo* experiments using a murine transgenic model of lethal HSV-2 intravaginal infection showed that Etg

mice, expressing human Tr, had reduced viral replication in the CNS, which could be attributed to a better containment and less efficient viral translocation/spread from the local target organ, GT, into the CNS. It is unclear at the moment why viral clearance was not affected in the GT of Etg mice as would be expected based on our *in vitro* data. However, this lack of antiviral protection in the GT could be related to the experimental conditions of high HSV-2 inoculum and a progesterone-driven system. Arguably, these conditions could be dampening specifically antiviral activity of Tr, but sparing its immunomodulatory effects as would be supported by decreased levels of TNF α in GT and CNS in Etg group. These findings could imply that Tr-mediated protection is perhaps conditional and can be overwhelmed by a higher viral load or other parameters of the infection, which was also seen in our siRNA experiments.

It is also unclear why viral translocation into the CNS was initially delayed or reduced in Etg animals at day 2, despite similar viral load in the GT. However, the sustained lower HSV-2 replication in the CNS of Etg mice is likely related to lower TNF α levels in the GT and CNS, thus suggesting that immunomodulatory effects might be less conducive to viral systemic dissemination and might be better for a disease outcome. Our results are in line with studies demonstrating that mice lacking RNAase L or with neutralized TNF α had a better disease outcome and reduced mortality in the genital HSV-2 infection (Duerst and Morrison, 2007; Thapa and Carr, 2008b), hence confirming the beneficial role of Tr-mediated lower inflammation and viral translocation in genital HSV-2 infection. However, that only a trend of increased survival and a modest improvement in disease presentation were observed of Etg mice is a limiting factor in interpreting the significance of our observations and their extrapolations on human studies.

Although this is not the first study using Etg animals against viral challenge (Roghianian et al., 2006; Zaidi et al., 1999), it is the first report pertaining to HSV-2 infection. Nevertheless, our study shares key observations with earlier publications. Despite the differential experimental designs and suggested antiviral mechanisms of Tr, our findings of increased antiviral protection and reduced mortality are in agreement with

Zaidi *et al.* with acute model of viral myocarditis and Roghanian *et al.* with a model of adenoviral lung challenge assessing adaptive immunity (Roghanian et al., 2006; Zaidi et al., 1999). That beneficial effects of Tr/E were observed in such distinct models attests to the existence of differential mechanisms of Tr/E antiviral protection that could be attributed to the pleiotropic nature of the molecules as well as the target organ specificity. Taken together, our results from *in vivo* studies provide evidence indicating the existence of a protective and contextual anti-HSV-2 effect of Tr/E.

In conclusion, this is the first study showing Tr/E anti-HSV-2 effect *in vitro* and *in vivo*. This study presented novel evidence of multifactorial and complex activities of Tr/E that targeted virus/cell interaction and attachment/entry as well as mounting of antiviral and inflammatory cellular responses to HSV-2. These observations importantly compliment and expand previously shown antibacterial and anti-HIV-1 activities of Tr/E, and may translate into a broader use of microbicidal potentials of Tr/E in the female genital mucosa.

Acknowledgments

We would like to thank Dr. Ficherova for kindly providing End1/E6E7 cells for our experiments. We appreciate the expert and valuable technical support of J. Newton, A. Patrick, and R. Persuad. Finally, we acknowledge the secretarial assistance of M. Allan.

This work was supported by a grant as part of the Comprehensive T Cell Vaccine Immune Monitoring Consortium (CTC-VIMC), a key component of the Collaboration for AIDS Vaccine Development (CAVD) funded by the Bill & Melinda Gates Foundation. A.G.D. was supported by a Studentship Award from the Ontario HIV Treatment Network (OHTN) and K.L.R. by a Career Scientist Award from the OHTN.

Figure legends

FIG 1 Tr and E reduce HSV-2 replication in genital ECs, and E has greater anti-HSV-2 activity than Tr. Genital ECs cells were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr and inoculated with HSV-2 MOI 0.1-5. Viral titers in supernatants were determined 24 h post infection by standard plaque assay and presented as log-transformed plaque-forming units per ml (pfu/ml) for HEC-1A cells (*A*) and for End1/E6E7 (*B*). In *C* and *D*, HEC-1A cells were pretreated with Tr (*C*) and E (*D*) for 1 h and subsequently exposed to HSV-2 MOI 1 for 2 h and, after repeated washes, cultured for additional 24 h. Viral titers determined in supernatants by plaque assay. For all, the data are shown as the mean \pm SD and are representative of three independent experiments performed in triplicate. Statistical analysis was performed using Student's *t*-test (*A*, *B*) or ANOVA with Tukey post-hoc test (*C*, *D*), with significance indicated in graphs. For determining IC₅₀ values of Tr and E anti-HSV-2 activity, readouts from dose-dependent functional studies were used for determining efficiency parameters of relevant experimental points. The maximum inhibitory effect was considered as 100% effect, and the other values were computed accordingly and plotted against relevant doses of Tr (asterisk, *) and E (filled circle, ●) as percentage of maximal inhibitory effect. The relevant IC₅₀ values for Tr and E are indicated with filled arrowhead and open arrowhead, respectively, pertaining to inhibitory effects of Tr and E on HSV-2 infectivity of HEC-1A cells (*E*). Error bars, SD.

FIG 2 Tr/E are required for anti-HSV-2 protection of HEC-1A cells. HEC-1A cells were either left untreated (UT) or transfected with a non-targeting control siRNA (ctrl siRNA), or Tr/E siRNA for 48-72 h with subsequent challenge with HSV-2 MOI 0.1 and 1 for 2 h. After repeated washes to remove viral inoculum, cells were cultured for additional 24 h. Levels of Tr/E in supernatants were measured by ELISA (*A*) and viral titers were determined by plaque assay (*B*). The data are representative of at least two independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's *t*-test with significance indicated in graphs.

FIG 3 Tr and E reduce binding/attachment of HSV-2 to HEC-1A cells. In *A*, HEC-1A cells and HSV-2 were either treated with medium alone or with 1 μ g/ml of Tr or E for 1 h

at 37°C before HSV-2 MOI 1 was added to cells and allowed to attach at 4°C for 5 h. After viral challenge, cells were repeatedly washed and whole cell lysates prepared and assessed for attached virus by immunoblotting using anti-gD antibodies and GAPDH as a loading control. Relative intensities of WB bands were quantified using MBF_ImageJ for Microscopy Software (*B*). In *C*, cells (*C*) were either pretreated with medium (med) or Tr and E as above, repeatedly washed and received HSV-2 MOI 1 for 5 h at 4°C as above. Alternatively, virus was either left untreated (*V*) or pretreated with Tr or E before being added to media-treated cells for 5 h at 4°C. WB in *C* and its quantification in *D* were performed as described above. The data shown are representative of two experiments.

FIG 4 Increased IFN β secretion and enhanced IRF3 nuclear translocation in Tr/E-pretreated HEC-1A cells after HSV-2 challenge. IFN β release from HEC-Ad/Tr cells following exposure to MOI 1 of HSV-2 (*A*) HEC-1A cells were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr and incubated for 6-24 h in presence of media or MOI 1 of HSV-2. At 6 h post treatment, total RNA was harvested and relative expression of IFN β mRNA by real-time quantitative RT-PCR was assessed. Values were normalized to internal control 18S (*A*). At 6 and 24 h post treatment with HSV-2 MOI 1, secreted IFN β was measured by ELISA in cell-free supernatants and presented as pg/ml (*B*). The data are representative of at three independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's *t*-test with significance indicated in graphs. In *C, D*, immunofluorescence analysis of IRF3 nuclear translocation is demonstrated for HEC-1A cells that were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr (*C*) or pretreated with Tr or E for 1 h (*D*) and subsequently exposed to either medium alone, or HSV-1 MOI 1 for 4 h. Representative staining is shown for IRF3 (green), nuclear stain propidium iodide (red), and composite (yellow) at magnification 2520 \times . The data are representative of two independent experiments with similar results.

FIG 5 Tr/E-treated HEC-1A cells secrete significantly reduced pro-inflammatory cytokines and have reduced nuclear translocation of NF- κ B in response to HSV-2 challenge. HEC-1A cells were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr

(A,D) or 1 $\mu\text{g/ml}$ of Tr or E (B,C,E) and incubated for 4-24 h in presence of media or HSV-2 MOI 1. At 24 h post viral challenge, secreted IL-8 and TNF α were measured by ELISA in cell-free supernatants and presented as pg/ml (A,B,C). The data are representative of at three independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's *t*-test with significance indicated in graphs. In D,E, immunofluorescence analysis of NF- κ B p65 subunit nuclear translocation is demonstrated for HEC-1A cells that were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr (D) or pretreated with Tr or E for 1 h (E) and subsequently exposed to either medium alone, or HSV-1 MOI 1 for 4 h. Representative staining is shown for NF- κ B p65 (green), nuclear stain propidium iodide (red), and composite (yellow) at magnification 2520 \times . The data are representative of two independent experiments with similar results.

FIG 6 Tr transgenic (Etg) mice have significantly lower viral load and reduced TNF α protein levels in the CNS.. Wild type (Wt) control mice or transgenic mice expressing the gene for human Tr (Etg) were Depo-treated and five days later intravaginally inoculated with 1×10^4 pfu per mouse of wild type HSV-2 strain 333. Viral titers determined by plaque assay in vaginal washes (A), in supernatants of homogenized genital tract (GT) (B), and spinal cord with brainstem (SC+BS) (C), as well as protein levels of TNF α determined by ELISA in supernatants of GT and SC+BS (D), pathology score (E), and survival (F) are shown. For survival and disease score, cumulative data (n=30-34) are presented. For viral titers and TNF α levels, the representative data of three independent experiments (n=3-4 per group, per time point) and are shown as the mean \pm SD. Statistical analyses of survival curves were performed with log rank test using GraphPad Prism. Statistical analyses of disease scores, viral titers, and TNF α levels were performed using Student's *t*-test with significance indicated in graphs.

Figure 1.

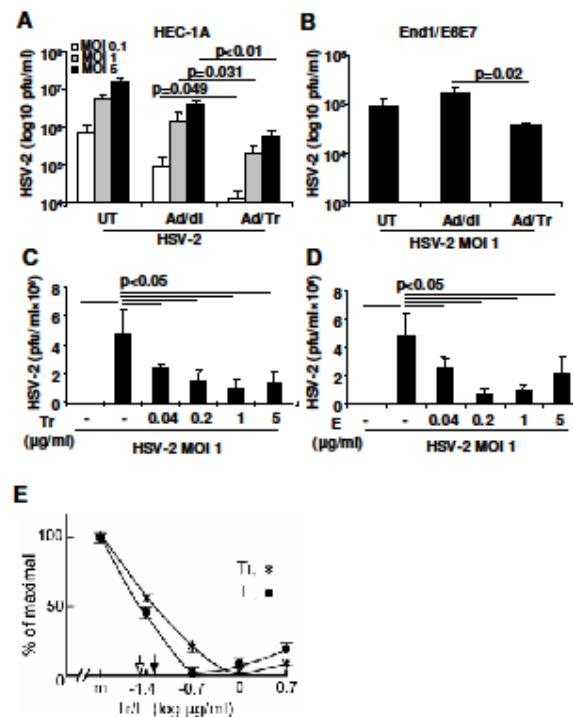
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FIG 1 Tr and E reduce HSV-2 replication in genital ECs, and E has greater anti-HSV-2 activity than Tr. Genital ECs were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr and inoculated with HSV-2 MOI 0.1-5. Viral titers in supernatants were determined 24 h post infection by standard plaque assay and presented as log-transformed plaque-forming units per ml (pfu/ml) for HEC-1A cells (A) and for End1/E6E7 (B). In C and D, HEC-1A cells were pretreated with Tr (C) and E (D) for 1 h and subsequently exposed to HSV-2 MOI 1 for 2 h and, after repeated washes, cultured for additional 24 h. Viral titers determined in supernatants by plaque assay. For all, the data are shown as the mean \pm SD and are representative of three independent experiments performed in triplicate. Statistical analysis was performed using Student's t-test (A, B) or ANOVA with Tukey post-hoc test (C, D), with significance indicated in graphs. For determining IC₅₀ values of Tr and E anti-HSV-2 activity, readouts from dose-dependent functional studies were used for determining efficiency parameters of relevant experimental points. The maximum inhibitory effect was considered as 100% effect, and the other values were computed accordingly and plotted against relevant doses of Tr (asterisk, *) and E (filled circle, ●) as percentage of maximal inhibitory effect. The relevant IC₅₀ values for Tr (asterisk, *) and E (filled circle, ●) are indicated with filled arrowhead and open arrowhead, respectively, pertaining to inhibitory effects of Tr and E on HSV-2 infectivity of HEC-1A cells (E). Error bars, SD.

Figure 2.

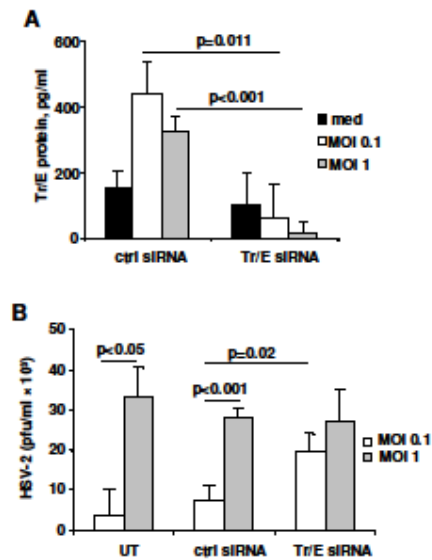
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FIG 2 Tr/E are required for anti-HSV-2 protection of HEC-1A cells. HEC-1A cells were either left untreated (UT) or transfected with a non-targeting control siRNA (ctrl siRNA), or Tr/E siRNA for 48-72 h with subsequent challenge with HSV-2 MOI 0.1 and 1 for 2 h. After repeated washes to remove viral inoculum, cells were cultured for additional 24 h. Levels of Tr/E in supernatants were measured by ELISA (A) and viral titers were determined by plaque assay (B). The data are representative of at least two independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's *t*-test with significance indicated in graphs.

Figure 3.

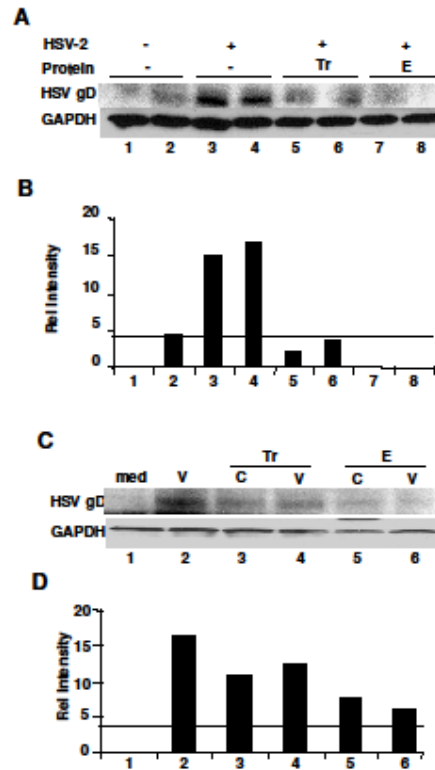
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FIG 3 Tr and E reduce binding/attachment of HSV-2 to HEC-1A cells. In **A**, HEC-1A cells and HSV-2 were either treated with medium alone or with 1 μ g/ml of Tr or E for 1 h at 37°C before HSV-2 MOI 1 was added to cells and allowed to attach at 4°C for 5 h. After viral challenge, cells were repeatedly washed and whole cell lysates prepared and assessed for attached virus by immunoblotting using anti-gD antibodies and GAPDH as a loading control. Relative intensities of WB bands were quantified using MBF_ImageJ for Microscopy Software (**B**). In **C**, cells (**C**) were either pretreated with medium or Tr and E as above, repeatedly washed and received HSV-2 MOI 1 for 5 h at 4°C as above. Alternatively, virus was either left untreated (**V**) or pretreated with Tr or E before being added to media-treated cells for 5 h at 4°C. WB in **C** and its quantification in **D** were performed as described above. The data shown are representative of two experiments.

Figure 4.

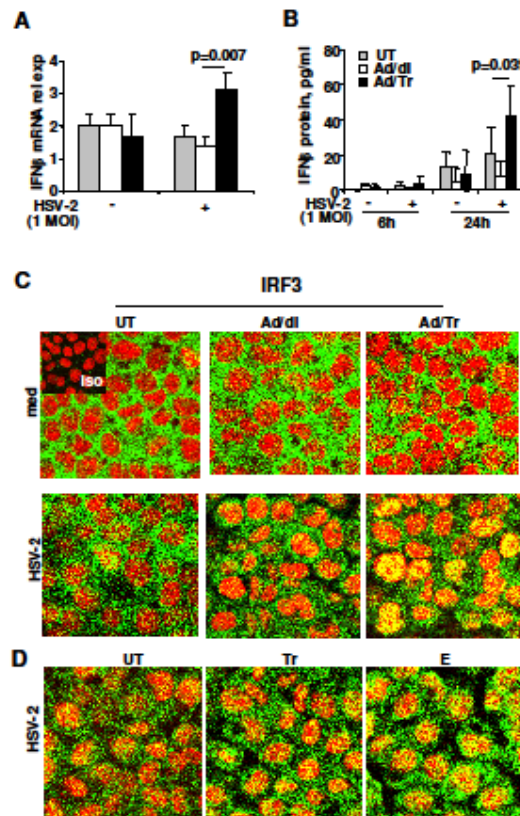
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FIG 4 Increased IFN β secretion and enhanced IRF3 nuclear translocation in Tr/E-pretreated HEC-1A cells after HSV-2 challenge. IFN β release from HEC-Ad/Tr cells following exposure to MOI 1 of HSV-2 (A) HEC-1A cells were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr and incubated for 6-24 h in presence of media or MOI 1 of HSV-2. At 6 h post treatment, total RNA was harvested and relative expression of IFN β mRNA by real-time quantitative RT-PCR was assessed. Values were normalized to internal control 18S (A). At 6 and 24 h post treatment with HSV-2 MOI 1, secreted IFN β was measured by ELISA in cell-free supernatants and presented as pg/ml (B). The data are representative of at three independent experiments performed in triplicate and are shown as the mean \pm SD. Statistical analysis was performed using Student's t-test with significance indicated in graphs. In C,D, immunofluorescence analysis of IRF3 nuclear translocation is demonstrated for HEC-1A cells that were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr (C) or pretreated with Tr or E for 1 h (D) and subsequently exposed to either medium alone, or HSV-1 MOI 1 for 4 h. Representative staining is shown for IRF3 (green), nuclear stain propidium iodide (red), and composite (yellow) at magnification 2520 \times . The data are representative of two independent experiments with similar results.

Figure 5.

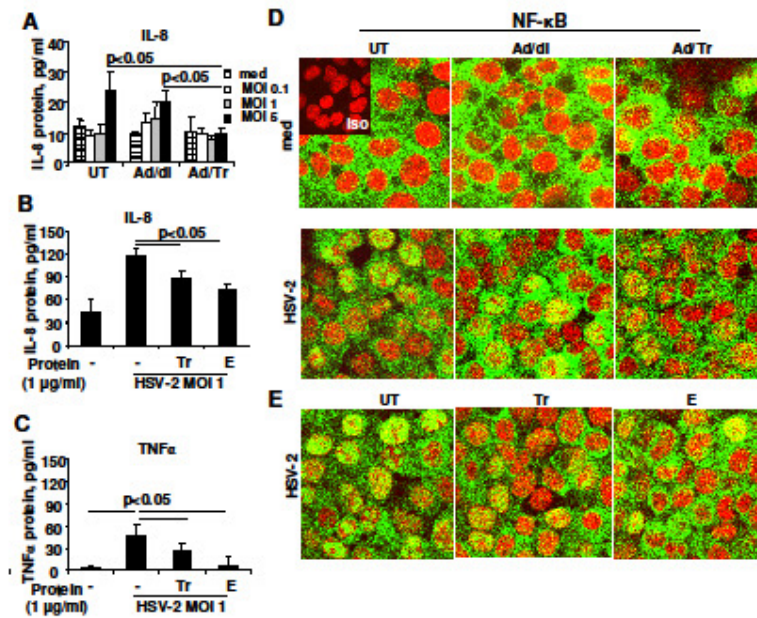
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FIG 5 Tr/E-treated HEC-1A cells secrete significantly reduced pro-inflammatory cytokines and have reduced nuclear translocation of NF-κB in response to HSV-2 challenge. HEC-1A cells were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr (A, D) or 1 μg/ml of Tr or E (B, C, E) and incubated for 4-24 h in presence of media or HSV-2 MOI 1. At 24 h post viral challenge, secreted IL-8 and TNFα were measured by ELISA in cell-free supernatants and presented as pg/ml (A, B, C). The data are representative of at three independent experiments performed in triplicate and are shown as the mean ± SD. Statistical analysis was performed using Student's *t*-test with significance indicated in graphs. In D, E, immunofluorescence analysis of NF-κB p65 subunit nuclear translocation is demonstrated for HEC-1A cells that were left untreated or treated with MOI 0-50 of Ad/dl or Ad/Tr (D) or pretreated with Tr or E for 1 h (E) and subsequently exposed to either medium alone, or HSV-1 MOI 1 for 4 h. Representative staining is shown for NF-κB p65 (green), nuclear stain propidium iodide (red), and composite (yellow) at magnification 2520x. The data are representative of two independent experiments with similar results.

Figure 6.

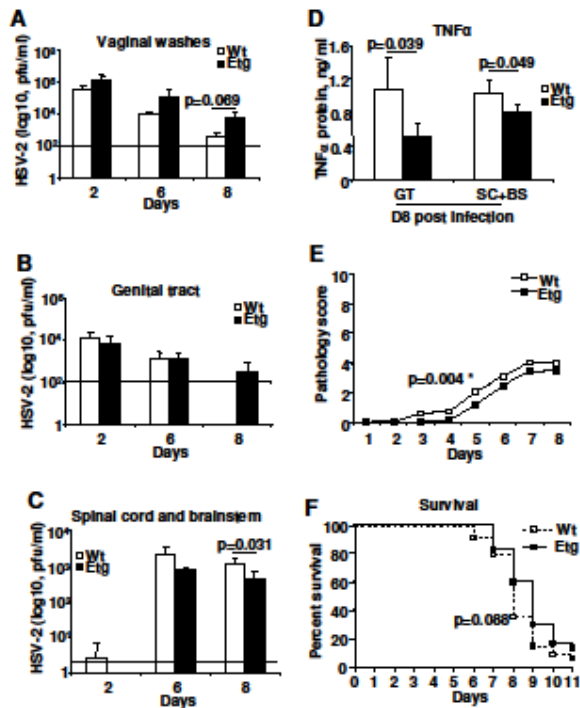
Drannik *et al.*

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----- Chapter 4 -----

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

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Published in Journal of Virology in February 2012 Vol 86 no 8 pp 4599-4610
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Summary and central message:

It is becoming increasingly clear that the early events, the local pro-inflammatory environment, and dysregulated innate immune responses at mucosal sites, the portal of HIV-1 entry, are critical factors in predetermining the outcome of viral encounter and thus can significantly contribute to one's resistance or susceptibility to HIV-1. Knowing that elevated Tr/E were identified in CVLs from HIV-resistant CSWs and having established (Chapters 2 and 3) that Tr/E increased antiviral protection and modulated innate viral recognition and inflammatory responses following polyI:C and HSV-2 stimulation, we next (Chapters 4 and 5) elucidated the role of Tr/E in the host's defense against HIV-1. To this end, we determined the contribution of Tr/E to the natural anti-HIV-1 protection of CVL and also comparatively assessed antiviral activity of each of the recombinant Tr/E proteins (Chapter 4). Here, we confirmed that CVLs from HIV-1-resistant (HIV-R) compared to HIV-1-susceptible (HIV-S) commercial sex workers (CSWs) contained significantly higher amounts of Tr/E. We also demonstrated that immunodepletion of 30% of Tr/E from CVL accounted for up to 60% of total anti-HIV-1 activity of CVLs of HIV-R CSWs. Further, pretreatment of HIV-1, but not cells, with each recombinant Tr/E proteins inhibited HIV-1 infection of TZM-bl ECs that expressed

canonical HIV-1 receptors. Conversely, in ECs that lacked the canonical receptors, Tr/E pretreatment of either HIV-1 or cells resulted in significantly reduced HIV-1 attachment and transcytosis, and reduced attachment was not associated with altered expression of syndecan-1 or CXCR4. Interestingly, E was found to be more potent against HIV-1 than Tr, despite their equipotent antiprotease activity. This study provided the first experimental evidence showing that Tr/E significantly contributed to the natural anti-HIV-1 activity of CVL; that Tr, and even more profoundly E, affected cell attachment and transcytosis of HIV-1 in genital ECs and that these effects were contextual, notably dependent on receptor type and virus strain.



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-

Received 14 October 2011 Accepted 6 February 2012
Published ahead of print 15 February 2012

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doi:10.1128/JVI.06561-11

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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 omwaptin from snake venom (49). Tr is secreted as a cationic glycosylated (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 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 systematically 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 (JCS3-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 × 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 medium. 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 × 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 × 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 IIIIB (X4-tropic) HIV-1 strain HIV-1_{IIIIB} was prepared in peripheral blood mononuclear cells (PBMCs), and the ADA (RS-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 × 10³ for HIV-1_{IIIIB} and 2.77 × 10⁴ 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 × 10⁶ 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_{IIIIB} 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_{IIIIB} 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_{IIIIB} 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 × 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 × 10⁵ 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 Dnuset 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

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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 1 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_{IIIIB} 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_{IIIIB} 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 BiotLegend (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_{IIIIB} 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_{IIIIB} 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.

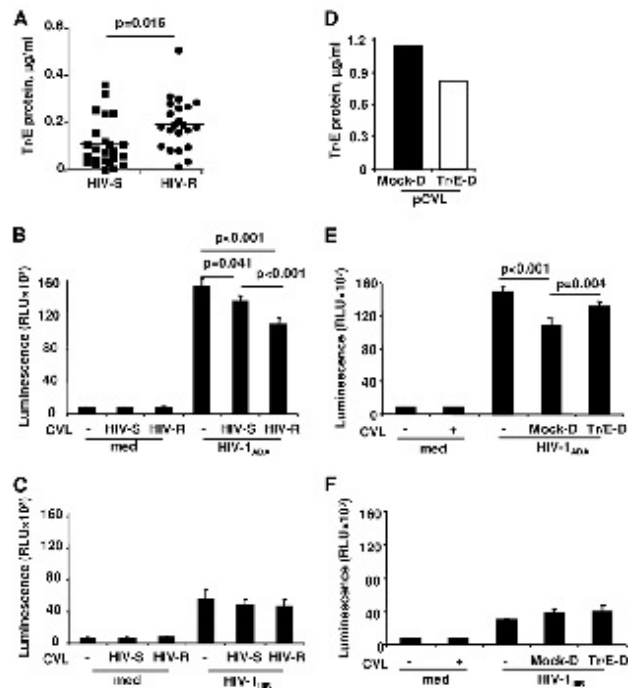


FIG 1 Tr and E significantly contribute to anti-HIV-1 activity of CVLs from HIV-R commercial sex workers. Tr/E protein levels were measured by ELISA in cervicovaginal lavages (CVLs) from HIV-1-susceptible (HIV-S; $n = 25$) and resistant (HIV-R; $n = 20$) Kenyan commercial sex workers (CSWs) and presented 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 RS-tropic HIV-1_{ADA} (B) or X4-tropic HIV-1_{III} (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_{III} (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-

ments on TZM-bl cells. In contrast, no reduction in HIV-1 infection of TZM-bl cells was seen following pretreatment of HIV-1_{III} (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

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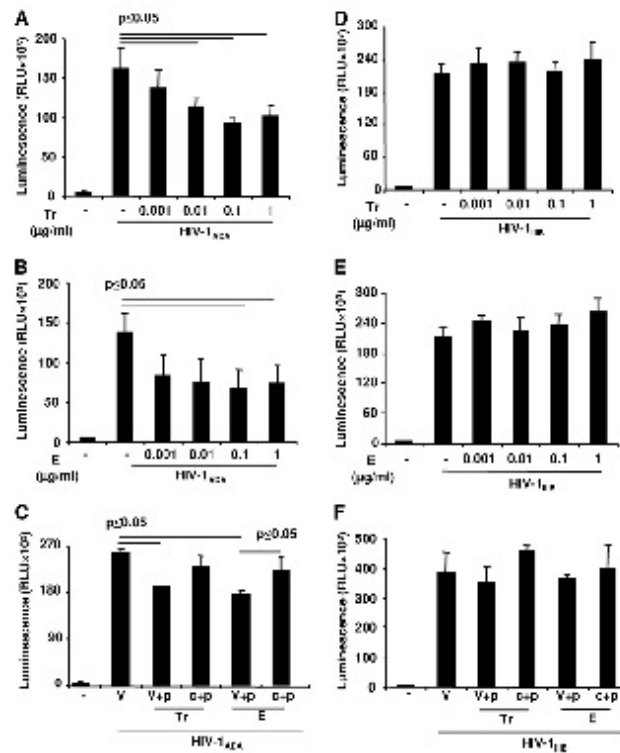


FIG 2 Tr and E independently inhibit HIV-1_{ADA} infection of T2M-bl cells. Media or recombinant trappin-2 (Tr) or elafin (E) at 0.001, 0.01, 0.1, and 1 μg/ml was incubated with 100 TCID₅₀ of R5-tropic HIV-1_{ADA} (A and B) or X4-tropic HIV-1_{IIIB} (D and E) for 1 h at 37°C before adding the virus onto T2M-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 T2M-bl cells (C) and HIV-1_{IIIB} or T2M-bl cells (F) for 1 h at 37°C. T2M-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 T2M-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 T2M-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 T2M-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-

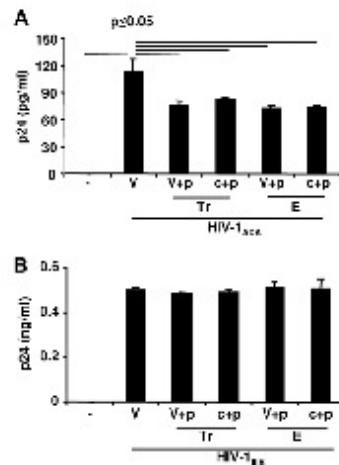


FIG 3 Tr and E significantly reduce attachment of HIV-1 to HEC-1A cells. Media or recombinant trappin-2 (Tr) or elafin (E) at 1 μ g/ml was incubated with 100 TCID₅₀ of R5-tropic HIV-1_{ADA} (A) or X4-tropic HIV-1_{IIIIB} (B) or HEC-1A cells alone for 1 h at 37°C. HEC-1A cells were washed 5 times with 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* =

Effects of Trappin-2/Elafin on HIV-1 in Genital ECs

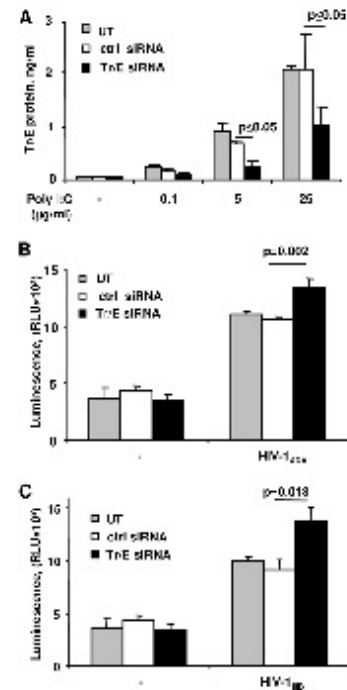


FIG 4 Knockdown of endogenous Tr/E by siRNA significantly increased shedding of infectious HIV-1 from HEC-1A cells. HEC-1A cells were left untreated (UT) or were transfected with a nontargeting control (ctrl) or Tr/E siRNA and subsequently received poly(I-C) treatment for 24 h. Tr/E protein levels were 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_{IIIIB} 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_{IIIIB} 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-

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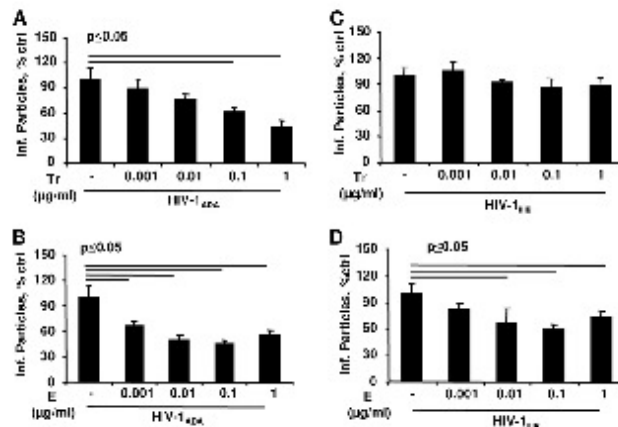


FIG 5 Tr and E significantly reduce transcytosis of HIV-1 through a monolayer of polarized HEC-1A cells. HEC-1A cells were grown as a polarized monolayer in a Transwell system and incubated with medium or a range of recombinant Tr and E for 1 h before the addition of 10 ng p24 of R5-tropic HIV-1_{ADA} (A and B) or X4-tropic HIV-1_{XB} (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.

lous 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_{XB}, although its effect on HIV-1_{XB} 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 \pm 0.013 μ g/ml and 0.0003 \pm 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 \pm 0.015 μ g/ml and 0.0006 \pm 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*

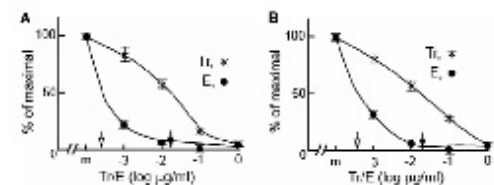


FIG 6 Determination of IC₅₀ of trappin-2 and elafin mediating HIV-1 infectivity and transcytosis. Readouts from dose-dependent functional studies were used for determining efficiency parameters of relevant experimental points. The maximum inhibitory effect was considered 100%, and the other values were 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.

in 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

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(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_{IBB}. 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_{IBB} 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_{IBB}, 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. Sallanave, 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.

ACKNOWLEDGMENTS

We would like to sincerely thank all of the women of the Pumwani cohort for their generous participation in this study. We also appreciate expert technical support of J. Newton and A. Patrick as well as all of the dedicated and hard work of the clinical staff at the Pumwani clinic with resarveys and sample collection.

This work was supported by a grant as part of the Comprehensive T Cell Vaccine Immune Monitoring Consortium (CTC-VIMC), a key component of the Collaboration for AIDS Vaccine Development (CAVD) funded by the Bill & Melinda Gates Foundation. A.G.D. was supported by a Studentship Award from the Ontario HIV Treatment Network (OHTN) and K.L.R. by a Career Scientist Award from the OHTN.

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----- Chapter 5 -----

**Unrestricted N-terminus and Nuclear Localization of Elafin
are Critical for Antiviral Activity against HIV-1 in Female
Genital Epithelial Cells**

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This manuscript has been submitted to PLoS ONE on March 26, 2012 and is currently under revision

Summary and central message:

Based on collective evidence from the previous chapters, we inferred that it was likely E, and not Tr, that was exhibiting antiviral activity in our tested Tr/E protein preparations. Thus, in Chapter 5 we investigated the mechanisms, by which E was mediating anti-HIV-1 effects on human endometrial HEC-1A cells lacking canonical HIV-1 receptors. We used tagged and untagged Tr/E proteins to demonstrate that exogenous proteins can be internalized and function on same ECs as autocrine/paracrine factors, and that anti-HIV-1 activity of E depends on its unrestricted, or unmodified, N-terminus and nuclear localization, but not its antiprotease activity. Furthermore, our results demonstrated that N-unrestricted E also attenuated NF- κ B/p65 nuclear translocation in response to R5 HIV-1 that was also associated with decreased secretion of IL-8 and expression of viral innate sensors, including TLR3 and RIG-I following viral challenge. Most importantly, we found that previously demonstrated elevated levels of Tr/E in CVL of HIV-R CSWs were associated with lower mRNA levels of TLR2, TLR3, TLR4, and RIG-I in the genital ECs from the resistant cohort, suggesting a link between Tr/E, HIV-1 resistance and modulated innate viral recognition in the female genital tract. Collectively, in this study we presented evidence that E can function in

autocrine/paracrine mode and that unmodified N-terminus is critical for elafin's intranuclear localization and its anti-HIV-1 activities.

Unrestricted N-terminus and Nuclear Localization of Elafin are Critical for Antiviral Activity against HIV-1 in Female Genital Epithelial Cells

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Running title: Anti-HIV-1 activity of intranuclear elafin in ECs

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Keywords: HIV-1 resistance, trappin-2/elafin, genital epithelial cells, cervicovaginal lavage (CVLs), NF- κ B, pattern recognition receptors (PRRs), autocrine/paracrine

Abstract

Serine antiprotease elafin (E) and its precursor, trappin-2 (Tr), were identified as biomarkers of resistance to HIV-1. We recently showed that Tr and E (Tr/E) contribute to critical anti-HIV activity of cervicovaginal lavage fluid (CVL) of HIV-resistant (HIV-R) commercial sex workers (CSW). Additionally, E was found to be more potent than Tr in modifying susceptibility to HIV-1 in genital epithelial cells, HEC-1A, lacking canonical HIV-1 receptors, but not in TZM-bl cells, bearing canonical receptors. However, the mechanisms by which E is mediating anti-HIV-1 effects on HEC-1A cells remain elusive. In this study, using tagged and untagged Tr/E proteins, we demonstrated that E can function as an autocrine/paracrine factor in HEC-1A cells, and that anti-HIV-1 activity of E depends on its unrestricted N-terminus, but not its antiprotease activity. Specifically, our results showed that exogenously added E with unrestricted N-terminus was able to enter cells, particularly the nucleus and to reduce viral attachment and transcytosis. Further, unrestricted E attenuated NF- κ B/p65 nuclear translocation in HEC-1A cells in response to R5-HIV-1_{ADA}, but not X4-HIV-1_{IIB}, as well as significantly decreased levels of IL-8 secretion and expression of pattern recognition receptors (PRRs), including TLR3 and RIG-I in HEC-1A cells. Most importantly, we found that elevated Tr/E in CVL of HIV-R CSWs were associated with lower mRNA levels of TLR2, TLR3, TLR4, and RIG-I in the genital ECs of this cohort, suggesting a link between Tr/E, HIV-1 resistance and modulated innate viral recognition in the female genital tract (FGT). Collectively, our data indicate that unrestricted N-terminus is critical for elafin's intranuclear localization and its anti-HIV-1 activities.

Introduction

Since human immunodeficiency virus-1 (HIV-1) is primarily a mucosal pathogen (Brenchley and Douek, 2008; Shattock et al., 2008) and nearly half of new HIV-1 infections are due to heterosexual acquisition (Hladik and McElrath, 2008; UNAIDS, 2010), our comprehensive assessment and efforts must be concentrated on understanding the mechanisms of mucosal protection, especially in the female genital tract (FGT). Indeed, despite low probability (1:122 to 1:1000) of HIV-1 transmission per sexual intercourse (Mastro and de Vincenzi, 1996) and overall global stabilization of HIV-1 incidence since 1999, women still represent more than 50% of all people living with HIV-1/AIDS (UNAIDS, 2010). This further underscores the need to focus on the susceptibility and defense mechanisms within the female genital mucosa, in order to address HIV-1 prevention.

To gain access and establish HIV-1 infection in submucosal primary target cells, HIV-1 has to interact with and traverse genital epithelial cells (ECs). Depending on the stage of viral life cycle, HIV-1 can be recognized by a number of pattern-recognition receptors (PRRs), including Toll-like receptors (TLR) 2, 3, 4, 7, 8 and 9, as well as RNA helicases, retinoic acid inducible gene (RIG)-I and melanoma-differentiation-associated gene 5 (MDA5) (Mogensen et al., 2010). Viral sensing through PRRs on ECs triggers a series of signal transduction events and activation of key transcription factors, including nuclear factor-kappa B (NF- κ B) (Yoneyama and Fujita, 2010). Despite that recognition and signaling events undoubtedly contribute to the induction of potent innate (Ghosh et al., 2010a; Ghosh et al., 2010b; Shust et al., 2010; Venkataraman et al., 2005) and adaptive (Kaul et al., 2000; Kaul et al., 1999) immune responses against HIV-1, they have also been implicated in disease pathogenesis and progression, perhaps through triggering robust, yet inefficient, immune responses that lead to immune activation, progressive cell loss, and ultimately inability to clear the pathogen (Grossman et al., 2006; McMichael et al., 2010). While microbicides and vaccines still hold promise to prevent acquisition, establishment, and spread of HIV-1, their success clearly depends on understanding of factors and early events in host/virus interaction that allow or divert

establishment of the infection at the portal of entry. Hence, studies conducted in highly HIV-exposed but seronegative individuals, that remain free of infection and disease, can shed light on the correlates and events determining resistance against HIV-1 in the female genital mucosa.

Cervicovaginal lavage (CVL) fluid can significantly influence infectivity at the genital epithelium. Recent studies demonstrated that treatment with CVL, which contains multiple secreted proteins, including trappin-2 (Tr) and elafin (E), attenuated HIV-1 infectivity in genital ECs (Drannik et al., 2012a; Ghosh et al., 2010a; Venkataraman et al., 2005). Tr/E are serine antiproteases and members of the whey acidic protein (WAP) family that contain a characteristic and evolutionary conserved four-disulfide core (FDC), or WAP, domain involved in protease inhibition (Moreau et al., 2008). Trappin-2 (Tr) (9.9 kDa) is a secreted precursor molecule that has two domains. In its N-terminal domain, Tr contains a transglutaminase substrate-binding domain (TSBD) with four repeated motifs with the consensus sequence Gly-Gln-Asp-Pro-Val-Lys (Nara, 1994). Proteolytic cleavage of this domain produces elafin (E) (5.9 kDa) that contains the WAP inhibitory domain (Guyot et al., 2005a) and also one TSBD [12]. Tr/E can interact with extracellular matrix proteins, including heparin, laminin, fibronectin, collagen IV, elastin and fibrinogen through crosslinking of TSBD, thus most likely contributing to wound healing (Guyot et al., 2005b). Tr/E have been demonstrated to have antimicrobial activity similar to their structural homolog secretory leucocyte protease inhibitor (SLPI) (Moreau et al., 2008). The antimicrobial property of Tr/E has specifically been shown against Gram-negative and Gram-positive bacteria, fungi, and HIV-1 (Baranger et al., 2008; Drannik et al., 2011; Drannik et al., 2012a; Ghosh et al., 2010b). The predicted mechanism for antimicrobial activity is mainly attributed to the cationic nature of these molecules (Baranger et al., 2008), presumably by disrupting the negative charges of viral envelope or bacterial cell wall; but it has never been experimentally proven. Interestingly, many of the WAP-containing proteins, such as eppin (Yenugu et al., 2004), mouse SWAM1 and SWAM2 (Hagiwara et al., 2003), and omwaprin from snake venom (Nair et al., 2007), do not exhibit antiprotease activity and still demonstrate antimicrobial activity.

Tr/E have also been linked to lung (Wang et al., 2008; Zani et al., 2011), gut (Flach et al., 2006) and skin disorders (Schalkwijk et al., 1990; Wiedow et al., 1990). Together with SLPI, Tr/E are constitutively expressed in mucosal secretions (Ghosh et al., 2010a; Ghosh et al., 2010b) and can be elevated in the presence of inflammatory stimuli such as IL-1 β , TNF α , and polyI:C (Ghosh et al., 2010b; King et al., 2003b). Tr/E are produced by multiple cell types, including genital mucosa (King et al., 2003b). Together with SLPI and a plethora of other innate effector molecules, namely defensins, cathelicidins, lysozyme, and lactoferrin, Tr/E are believed to play a significant role in protecting the FGT against STIs, like HIV-1. In contrast to SLPI and Tr/E, however, another WAP member, namely prostate stromal protein 20 kDa (ps20), was recently shown to make target cells more permissive to HIV-1 infection (Alvarez et al., 2008).

Recently, we and others have shown that both Tr and E possess anti-HIV-1 activities (Drannik et al., 2011; Drannik et al., 2012a; Ghosh et al., 2010b). Interestingly, we showed that anti-HIV-1 activity was dependent on cellular background and virus strains. Specifically, Tr/E demonstrated direct anti-HIV-1 activity against R5-HIV-1_{ADA}, but not against X4-HIV-1_{IIIB}. Moreover, we demonstrated that pretreatment of HIV-1 canonical receptor-bearing cells (TZM-bl) with exogenous Tr/E inhibited HIV-1 infection, whereas attachment and transcytosis of R5-HIV-1_{ADA} and viral shedding of R5-HIV-1_{ADA} and X4-HIV-1_{IIIB} strains were affected in HIV-1 canonical receptor-lacking cells (HEC-1A). We further found that E is approximately 130 times more effective than Tr with respect to anti-HIV-1 activity. Importantly, we confirmed that higher levels of Tr/E in CVL of HIV-1 resistance (HIV-R) commercial sex workers (CSW) and that it accounts for approximately 60% of the natural anti-HIV-1 activity of CVL in *in vitro* assays (Drannik et al., 2012a). All of these results conclusively establish the significance of E as an important anti-HIV-1 molecule and warrant revealing the relevant mechanisms of its anti-HIV-1 action. In this study, using biochemical methodologies, we show that anti-HIV-1 activity of E on HEC-1A genital ECs that lack canonical HIV-1 receptors is dependent on its intracellular localization and autocrine/paracrine activities.

Materials and Methods

Reagents and cell lines

Polyinosinic/polycytidylic acid (polyI:C) (Sigma-Aldrich, Oakville, ON, Canada) was reconstituted in PBS at indicated concentration. Human endometrial carcinoma (HEC-1A) and TZM-bl (JC53-BL) cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA) and maintained in McCoy's 5A Medium Modified (Invitrogen Life Technologies, Burlington, Ontario, Canada) and 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.

Protease inhibition assay

Elastase-inhibitory activity was measured as described previously (Drannik et al., 2012a; Simpson et al., 2001b). Briefly, Tr/E protein (final volume 10 µl/well), or diluent alone, was combined in a 96-well plate with 50 ng in 10 µl/well of purified human neutrophil elastase (HNE) (Sigma-Aldrich) or diluent alone (negative control), and incubated for 30 min at 37°C. Subsequently, 50 µl of HNE substrate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma-Aldrich), diluted to 50 µg/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).

Study participants

The study participants were described in more detail elsewhere (Drannik et al., 2012a). Briefly, women within a cohort of Pumwani CSWs from Nairobi, Kenya, were enrolled during scheduled biannual resurveys in two study groups: HIV-R, HIV-S. This is an ongoing, open cohort with participants enrolled between years 1989 and 2009. Within the cohort, women who remained HIV negative for 7 years of follow-up, as assessed by both serology and 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 relatively HIV-resistant HIV-R (Fowke et al., 1996). Participants, who were HIV-uninfected CSWs but had been followed up for less than 7 years, were defined as HIV-S. All the participants in the cohort had similar socio-economic and genetic backgrounds. No CSWs enrolled in this study were found to have co-existing 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

Cervical EC samples were collected using scraper and endocervical cytobrush following standard protocol. The EC cells were purified from the scraped materials using a standard Ficoll gravitational protocol, which were dissolved in 0.8 ml of TRIzol and stored at -70°C. Cervical ECs were available for each of the groups as follows: (HIV-S, N=10 and HIV-R, N=10).

Trappin-2 (Tr) and elafin (E) proteins for in vitro experiments

The following Tr and E protein preparations were used in this study: human commercial recombinant Tr (cTr) (with a C terminus His-tag) (R&D Systems, Burlington, ON, Canada) (Drannik et al., 2012a), which is a mixture of Tr and E (Drannik et al., manuscript in revision); secreted trappin-2 (sTr) (no tag) that was generated following infection of HEC-1A cells with replication-deficient adenovirus vector encoding gene for human Tr, as described elsewhere (Drannik et al., manuscript in revision); human commercial recombinant elafin (E) (no tag) HC4011 (Hycult Biotech, Uden, Netherlands); in-house human recombinant HAT-E (hE) (with N terminus tag) that was generated as described earlier (Drannik et al., 2012a); the C-terminally 6×His-tagged E (Eh) was purified from cTr by immunodepletion of Tr using cyanogen bromide (CNBr) beads, as per supplier's protocol (Sigma-Aldrich), that were chemically conjugated to goat-anti-Tr/E antibodies, raised against the N-terminus of Tr (AF1747, R&D Systems). Antibody-conjugated beads were prepared as described previously (Drannik et al.,

2012a), and loaded into a column. The cTr was reconstituted in PBS and slowly passed through the column for 10 times at 4°C. Flow through (Eh) was collected and concentrated by sequentially using 30 kDa and 3 kDa molecular cut-off (Millipore). Samples were aliquoted and stored at -80°C, and used as necessary. Due to expensive preparation and thus sample limitation, Eh was used only in limited comparison experiments with cTr as well as in confocal microscopy. Concentrations of Tr and E used in this study were based on a dose-dependent anti-HIV-1 activity of the proteins that was reported earlier (Drannik et al., 2012a).

HIV-1 stock preparation and related assays

The lab-adapted HIV-1_{III_B} strain (X4-tropic) was prepared in PBMCs and HIV-1_{ADA} virus (R5-tropic) was prepared in adherent macrophages purified from human PBMC as was described elsewhere (Jain and Rosenthal, 2011). A dose of 10 ng/ml of p24 was used for transcytosis and immunofluorescence experiments. Median tissue culture infectious dose (TCID₅₀) for each final stock was determined using the Reed-Muench method in TZM-bl cells (5.00×10^3 for X4-HIV-1_{III_B} and 2.77×10^4 for the R5-HIV-1_{ADA} stock); 100 TCID₅₀ of each HIV-1 stocks were used in HIV-1 attachment assays. HIV-1 attachment assays and transcytosis assays were performed as described previously (Drannik et al., 2012a), and are also briefly described here in figure legends.

MTT viability assay

MTT assay (Biotium Inc., Hayward, CA, USA) was used as per manufacturer's instructions to determine viability of HEC-1A cells and was described elsewhere (Drannik et al., 2012a; Nazli et al., 2009).

ELISA assays

CVLs and cell-free supernatants were stored at -70°C until assayed for human Tr/E, IL-8, and TNF- α with ELISA DuoSet kit (R&D Systems) according to the supplier's protocol. Cut off limit for Tr/E and IL-8 was 31.25 pg/ml; TNF- α 15.6 pg/ml. p24 was detected by

HIV-p24 ELISA as per supplier's protocol (HIV-1 p24 Antigen Capture Assay, Advanced BioScience Laboratories, Inc., Kensington, MD, USA) with a cut off limit of 3.1 pg/ml. Analytes were quantified based on standard curves obtained using an ELISA reader Tecan Safire ELISA reader (MTX Labs Systems Inc.).

Immunofluorescence staining

Immunofluorescence staining was performed as described elsewhere [6,34], but with minor modifications. HEC-1A cells grown on an 8-well BD Falcon culture slides (BD Biosciences) were pretreated with various Tr and E protein preparations, for 1 h or with polyI:C (25 µg/ml per well), or 10 ng HIV-1 p24 for 4 h. Following the above treatments, cells were fixed and blocked as described previously. NF-κB p65 sc-372 (SantaCruz Biotechnologies, Santa Cruz, CA, USA) (1:500) were used to detect nuclear translocation of NF-κB p65. Negative control rabbit immunoglobulin fraction (DakoCytomation, Glostrup, Denmark) was used as an isotype control. TRAB2O (HM2062, Hycult Biotech, Uden, Netherlands) primary antibodies were used to detect Tr/E. HAT and 6×His tags were detected using anti-HAT (LS-C51508, LifeSpan Biosciences, Inc., Seattle, WA, USA), and anti-His (#2365, Cell Signaling, Denvers, MA, USA) antibodies, respectively. Corresponding Alexa Fluor 488 conjugated IgG (Molecular Probes, Eugene, OR, USA) were used as secondary antibody. Nuclei were visualized by staining with propidium iodide. Images were acquired using an inverted laser-scanning confocal microscope (LSM 510, Zeiss, Oberkochen, Germany).

RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

Total RNA was isolated from cervical ECs and HEC-1A cells using TRIzol (Invitrogen Life Technologies). DNase-treated (Ambion, Austin, TX, USA), total RNA was reverse transcribed with SuperScript reverse transcriptase III (Invitrogen Life Technologies). RT-qPCR was performed in a total volume of 25 µl using Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and taqman protocol. The following primers were used: RIG-I: 5'-AGGGCTTTACAAATCCTGCTCTCTTCA-3' (probe),

5'-GGTGTTCAGATGCCAGAC-3' (forward), 5'-TTCCGCAAATGTGAAGTGTATAA-3'(reverse); MDA5: 5'-TTTGGCTTGCTTCGTGGCCC-3'(probe), 5'-TGATTCCCCTTCCTCAGATAC-3'(forward), 5'-TGCATCAAGATTGGCACATAGT-3'(reverse); TLR2: 5'-TCCTGCTGATCCTGC-3' (probe), 5'- TGGCATGTGCTGTGCTCTG-3' (forward), 5'-GGAAACGGTGGCACAGGAC-3' (reverse); TLR3: 5'-TGTGGATAGCTCTCC-3'(probe), 5'-CCGAAGGGTGGCCCTTA-3'(forward), 5'-AAGTTACGAAGAGGCTGGAATGG-3' (reverse); TLR4: 5'-TGTGCAACACCTTCAG-3' (probe), 5'- GGTGGAAGTTGAACGAATGGA-3' (forward), 5'- AACTCAGCACAGGCATGCC-3' (reverse); TLR7: 5'-ATTCTCCCCTACAGATC-3' (probe), 5'-TTCCTTGTGCGCCGTGTAA-3' (forward), 5'-TCAGCGCATCAAAGCATTTA-3' (reverse); TLR8: 5'-ATGAGCTGCGCTACC-3' (probe), 5'-GCCTCTGTTACTGACTGGGTGAT-3' (forward), 5'-TTGTCTCGGCTCTCTTCAAGG-3' (reverse); 18S rRNA: 5'-CGGAATTAACCAGACAAATCGCTCCA -3' (probe), 5'-GTGCATGGCCGTTCTTAGTT-3' (forward), 5'-TGCCAGAGTCTCGTTCGTTAT-3' (reverse). The expression of 18S ribosomal RNA was used as an internal control. PCRs were performed with an ABI PRIZM 7900HT Sequence Detection System using the Sequence Detector Software 2.2 (Applied Biosystems).

Statistical analysis

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

Results

Unrestricted N-terminus of elafin is critical for anti-HIV-1, but not antiprotease, activity.

Figure 1A provides schematics of various Trappin-2 (Tr) and elafin (E) proteins, including human commercial Tr - a mixture of both Tr and E with a C-terminal His-tag (cTr); human secreted Tr with no tag (sTr); human commercial E without a tag (E); human E purified from cTr with a C-terminal tag (Eh); and human in-house E with an N-terminal tag (hE) that were studied here for comparative analysis and to ascertain whether both Tr and E exert anti-HIV-1 activity. Since antiprotease activity is a major function of Tr/E, this activity of the proteins was evaluated as an indicator of their functional integrity. Results of neutrophil-elastase assay indicated that all the tested proteins were equipotently active as protease inhibitors, except sTr that was 10 times more potent compared to the other proteins (Fig. 1B). Eh preparation was not evaluated for antiprotease function due to sample limitation. However, when we tested anti-HIV-1 activity of these Tr/E protein preparations, surprisingly we observed that, in contrast to cTr and E, hE and sTr did not exhibit anti-HIV-1 activities, as assessed in attachment and transcytosis assays (Fig. 2A-D). Specifically, the attachment of R5-HIV-1_{ADA} strain was significantly reduced, when either cells or the virus were pretreated with cTr and E, but not with sTr or hE (Fig. 2A). This observation was also in line with results of transcytosis assay (Fig. 2C and D). Unlike the attachment results, however, E also inhibited transcytosis of X4-HIV-1_{IIIB} (Fig. 2D). Notably, Eh had similar HIV-1 inhibitory activity (data not shown). Additionally, metabolic activity of cells was assessed to rule out their impaired viability and metabolic activity as causes of reduced attachment and transcytosis and no differences among the groups were found (data not shown). Importantly, these results also indicate that unrestricted N-terminus of E is critical for anti-HIV-1 activities of the protein, but not for its antiprotease activity. Further, sTr which had significantly elevated antiprotease activity lacked anti-HIV-1 activity.

Cellular localization of exogenous elafin in genital epithelial cells

Since E treatment modifies anti-HIV-1 susceptibility of cells, including attachment and transcytosis of HIV-1 in HEC-1A cells, we hypothesized that E may not only be interacting with the plasma membrane components related to HIV-1 binding and entry, but also acting at post-entry or at the intracellular level. Therefore, we next determined the cellular distribution of exogenously added Tr/E to HEC-1A cells using immunofluorescence confocal microscopy. Control HEC-1 cells treated with PBS and stained with anti-Tr/E (TRAB20) antibodies and merged with propidium iodide-stained nuclei showed a low level of endogenously expressed E in the nuclei of HEC-1A cells (Fig. 3A). Interestingly, exogenous E, added as cTr or E, was rapidly taken up by cells (within 1 h) and accumulated to high levels in the nucleus (Fig. 3B & 3C). In order to clearly distinguish exogenous and endogenous E, we next used and tracked tagged-E with anti-His or anti-Hat antibodies. Control HEC-1 cells treated with PBS and stained with anti-His (Fig. 3D) or anti-Hat (Fig. 3G) antibodies were negative. HEC-1 cells treated with exogenous cTr and Eh and stained with anti-His were distributed largely to the perinuclear and nuclear region (Fig. 3E and F). Unexpectedly, hE that has an N-terminal Hat tag was localized near the periphery of the plasma membrane and not detected in the nucleus (Fig. 3H). Together these data indicate that low levels of endogenous E and exogenously added E bearing unrestricted N-terminus, rapidly enters the nucleus of HEC-1A cells. Further, the nuclear localization of E was associated with anti-HIV-1 responsiveness of HEC-1A cells. Moreover, since endogenous E in HEC-1A cells can be present intracellularly (Fig. 3A) and be secreted in response to bacterial (data not shown) and viral (Drannik et al., 2012a) ligands, and since extracellular E can also enter HEC-1A cells, we propose that anti-HIV-1 activity of E (Fig. 2) can be a result of its autocrine/paracrine function in HEC-1A cells.

Elafin attenuates HIV-1-induced nuclear translocation of NF- κ B

Since extracellularly added E can enter into HEC-1A cells that is associated with reduced HIV-1 attachment and transcytosis in HEC-1A cells, we hypothesized that E might be influencing HIV-1-induced intracellular signaling pathways. To evaluate this, HEC-1A

cells were pretreated with medium/buffer (UT) alone or with cTr, E, or hE proteins and then challenged with polyI:C or HIV-1, and NF- κ B/p65 nuclear translocation was assessed. Confocal immunofluorescence microscopy revealed that compared with UT-cells, E or cTr treatment significantly inhibited polyI:C- or R5-HIV-1_{ADA}-induced nuclear translocation of NF- κ B (Fig. 4). Notably, polyI:C challenge produced slightly greater visible effect than R5-HIV-1_{ADA} challenge and was used as a positive control for the experiment. Further, X4-HIV-1_{IIB} did not cause any significant NF- κ B /p65 nuclear translocation (data not shown). Interestingly, unlike E or cTr, exogenous hE treatment failed to block NF- κ B nuclear translocation and produced imagery similar to UT cells (data not shown).

Elafin inhibits IL-8 secretion and expression of several PRR genes in response to R5-HIV-1_{ADA}

Considering previous data demonstrating the anti-inflammatory properties of Tr/E against bacterial ligands (Butler et al., 2006), we hypothesized that E may affect HIV-1-induced secretion of pro-inflammatory factors IL-8 and TNF α from HEC-1A cells, implicated in STI/HIV-1 dissemination and pathogenesis (Nazli et al., 2010). ELISA measurements demonstrated significantly lower levels of IL-8 in the basolateral compartment of HEC-1A cells after pretreatment with E or cTr, but not with hE (data not shown), following R5-HIV-1_{ADA} (Fig. 5A), but not X4-HIV-1_{IIB} exposure (Fig. 5B). Levels of TNF α , however, were negligible and around the level of detection across the groups for both R5- and X4-HIV-1 virus-treated samples (data not shown). These results suggest that E treatment alters HIV-1-induced inflammatory responsiveness of HEC-1A cells.

Next we examined the expression of innate viral sensors implicated in HIV-1 recognition/pathogenesis (Mogensen et al., 2010) in the presence of either E, cTr, or hE and following HIV-1 challenge. Results showed that cells pretreated with E and cTr, but not hE (data not shown), had lower mRNA expression of TLR3 and RIG-I following R5-HIV-1_{ADA}, compared to virus alone group (Fig. 5C and D). In contrast to R5, no differences in TLR3 or RIG-I expression were observed for X4-HIV-1_{IIB} (data not

shown). Overall, the data indicate that HEC-1A cells, pretreated with E, showed significantly lower level of induction of innate sensors in response to viral stimulation.

Genital ECs from HIV-resistant (HIV-R) commercial sex workers (CSWs) have significantly lower expression of several PRRs

Given our *in vitro* data (Fig. 5), we investigated whether HIV-R CSWs, who remain HIV-free and have higher Tr and E in their CVLs (Drannik et al., 2012a; Iqbal et al., 2009), also had reduced expression of innate viral sensors in genital ECs. Quantitative RT-PCR of cervical ECs revealed that HIV-R CSWs indeed had significantly reduced mRNA levels of TLR2, 3, 4 and RIG-I compared to susceptible (HIV-S) CSWs (Fig. 6). Collectively, our data indicate that HIV-1 resistance may be associated with increased levels of E-mediated decreased expression of mucosal innate PRRs on genital ECs at the portal of viral entry.

Discussion

Here, we comparatively evaluated different Tr and E protein preparations with respect to their HIV-1 inhibitory and antiprotease activities. To determine how E is mediating anti-HIV-1 effects in genital HEC-1A cells, we assessed its intracellular localization and effect on viral recognition and mounting HIV-1-driven inflammatory responses using HIV-1 non-canonical receptor-bearing genital epithelial HEC-1A cells, as well as tagged and non-tagged proteins. Our results revealed that both E and Tr can function as autocrine/paracrine factors in HEC-1A cells, but that it is E, and not Tr, that likely acts against HIV-1. We further observed that anti-HIV-1 activity of E is highly associated with autocrine/paracrine, but not antiproteolytic, property of E and also depends on its unrestricted N-terminus. Specifically, we showed that E attenuated R5-HIV-1_{ADA}-induced NF- κ B/p65 nuclear translocation, as well as levels of IL-8 secretion and expression of pattern recognition receptors TLR3 and RIG-I that were associated with reduced HIV-1 attachment and transcytosis in HEC-1A cells. Importantly, our results showed that previously reported elevated Tr/E in CVL of HIV-R CSW are associated with significantly lower mRNA levels of TLR2, 3, 4, and RIG-I in the genital ECs of this cohort, suggesting a link between Tr/E, HIV resistance and modulated viral recognition in the FGT. Collectively, we propose that E-mediated altered intracellular innate activation is most likely contributing to the HIV-R property of these subjects.

The antiprotease activity of Tr/E depends on the four-disulfide core structure, or the WAP motif, that is localized to the C-terminal inhibitory domain of Tr/E (Schalkwijk et al., 1999; Tsunemi et al., 1996) and is also present in SLPI (Eisenberg et al., 1990; Grutter et al., 1988). Our finding that all Tr and E preparations used in this study were nearly equally active against HNE allowed for comparative assessment of anti-HIV-1 and immunomodulatory properties of Tr/E, despite the structural and manufacturing differences present among the tested proteins. Since not all the tested Tr and E protein preparations exhibited anti-HIV-1 activity, this provided the first experimental evidence that antiprotease and anti-HIV-1 activities of E do not correlate with each other. Our data were supported by earlier reports demonstrating that A62D/M63L, a Tr variant lacking

antiprotease properties, had normal antibacterial and antifungal activities (Baranger et al., 2008), and also by the fact that SLPI's mutant with no antiprotease activity demonstrated intact anti-HIV-1 activity (McNeely et al., 1997). Collectively, these observations highlight that E exerts antiviral effects through mechanisms and structures distinct from those mediating its antiprotease function.

Our results demonstrated that in HEC-1A cells endogenous Tr/E were present either intracellularly, including the nuclear area, or secreted extracellularly in response to bacterial and viral ligands (data not shown) (Drannik et al., 2012a). We further showed that extracellular Tr/E could enter HEC-1A cells within 1 h of exposure and localize within nuclear/perinuclear areas, similar to endogenous proteins. Since anti-HIV-1 inhibitory activity of Tr/E was highly associated with the proteins' entry into the cells, and not their antiprotease activity, we conclude that Tr/E in their defense against HIV-1 were likely acting through autocrine/paracrine function; we believe the latter function is separate and possibly complimentary to other virus- and cell surface receptor-mediated anti-HIV-1 activities of the tested Tr/E proteins.

Our results showed that E and cTr/Eh, but not sTr or hE, exhibited anti-HIV-1 activity. We propose that most likely it is the presence of E, but not Tr, that is important for antiviral activity of the tested Tr and E proteins. Based on the collective findings that cTr is a mixture of both Tr and E (Drannik et al., manuscript in revision), that sTr did not show any HIV-1 inhibition, and that anti-HIV-1 activity of E was 130 times more potent than Tr (Drannik et al., 2012a), we suggest that it is the presence of E in cTr that highly likely is attributing to anti-HIV-1 activity of cTr. Moreover, our recent observations on the limited antiviral activity of sTr against vesicular stomatitis virus (VSV-GFP) (Drannik et al., manuscript in revision) would also support this notion.

Furthermore, that E and Eh, but not hE, exerted HIV-1 inhibitory activity strongly suggested that the N-terminus tagging most likely blocked the antiviral property of hE, as well as its ability to act in autocrine/paracrine manner and to enter nuclear/perinuclear area following cell pretreatment. These findings indicated that the N-terminus of E is critical for anti-HIV-1 activity, and proposed an alternative to the general conception that

cationic property of Tr/E might be critical for antiviral activities. Indeed, our bioinformatics analysis of isoelectric points (GENETYX, version 8.2.0) revealed that E (isoelectric point 8.07) is less cationic than its precursor Tr (isoelectric point 8.97), yet E is 130 times more potent than Tr with respect to anti-HIV-1 activity, clearly indicating that cationic property is not predetermining anti-HIV-1 activity of E (Drannik et al., 2012a). The antimicrobial property of several other cationic antimicrobials has also been attributed to the N-terminal domain, as was shown for lactoferrin (Hiemstra et al., 2004; Mann et al., 1994). Additionally, our earlier studies showing the lack of antiviral effect of hE against VSV-GFP challenge would also support our current findings (Drannik et al., manuscript in revision).

NF- κ B is one of the transcription factors regulating production of pro-inflammatory cytokines, and nuclear translocation of NF- κ B is directly associated with secretion of TNF α and IL-8 (Zenhom et al., 2011) as well as HIV-1 pathogenesis and replication (Broliden et al., 2009; Hayashi et al., 2010; Pereira et al., 2000). It was reported that SLPI can affect NF- κ B nuclear translocation and influence gene regulation (Taggart et al., 2005); on conjecture, we proposed that, being a functional homolog of SLPI, most likely E performs its anti-HIV-1 effect through modulation of NF- κ B nuclear translocation and regulation of pro-inflammatory and innate sensing genes. However, the mechanism of direct virus- or cell surface receptor-mediated, anti-HIV-1 activity of E might be different. Although Tr/E secretion can be elevated in the presence of inflammatory stimuli, our earlier study (Drannik et al., manuscript in revision) indicated that, in response to polyI:C treatment, exogenous E treatment can also enhance inflammatory stimuli secretion, albeit at higher protein concentrations, which indicates a close feedback loop between these two circuitries.

Our findings that apical treatment with E to the HEC-1A cells under challenge of R5-HIV-1_{ADA} resulted in reduced secretion of IL-8 into the basolateral compartment might have important implications for activation of additional HIV-1 target cells located in the genital submucosa. This could limit local inflammation and the attraction of HIV susceptible cells. This notion is also supported by the fact that elevated levels of Tr/E in

CVL of HIV-R CSWs are associated with higher anti-HIV-1 activity *in vitro* (Drannik et al., 2012a) and mucosal resistance *in vivo*.

The acquisition and disease progression to HIV-1/AIDS is associated with immune activation (Mogensen et al., 2010; Schwenker et al., 2008) and dysregulation of expression and responsiveness of viral sensors (Lester et al., 2008; Lester et al., 2009; Meier et al., 2007; Sachdeva et al., 2010; Sanghavi and Reinhart, 2005). Specifically, we recently showed that chronic untreated HIV-1 infection was associated with aberrant expression and responsiveness of TLR2, 3, 4, 6, 7/8 in PBMCs (Lester et al., 2008; Lester et al., 2009). Further, Sooty Mangabeys, a natural host of simian immunodeficiency virus (SIV), show no signs of immune activation/inflammation, despite high systemic viraemia (Silvestri et al., 2003). In contrast, a non-natural and SIV-susceptible reservoir, African Green Monkeys, show marked inflammatory responses and disease progression. These observations suggest that HIV-induced alterations in innate immune responsiveness may initiate and perpetuate immune activation, dysfunction, and inflammation that manifest in HIV-1/AIDS pathogenesis and disease acceleration. Since resistance to HIV-1 has been associated with “immune quiescence” and decreased cellular activation (McLaren et al., 2010) on the one hand, and robust, but transient IRF1 antiviral responses (Su et al., 2011), in peripheral blood- derived monocytes on the other hand, it appears that modulation of pathogen recognition and controlling untimely or unnecessary inflammatory responses can be beneficial and pivotal in determining the fate of HIV-host encounter and disease establishment. Considering that Tr/E were shown to down-modulate antibacterial (Butler et al., 2006; McMichael et al., 2005b) and antiviral innate immune responses (Drannik et al., 2011), without negatively affecting antimicrobial protection (Drannik et al., manuscript in revision), one can argue that it might be beneficial in the case of HIV-1 to have Tr/E at mucosal surfaces to modulate innate immune responsiveness while increasing antiviral protection.

Importantly, we found that E not only influenced HIV-1 infection, but also affected the relevant intracellular molecular circuitry as demonstrated by reduced IL-8 secretion, nuclear translocation of NF- κ B, and decreased mRNA expression of several

innate viral sensing receptors, for which specific mechanisms remain to be determined. These data are reminiscent of our earlier findings in response to viral dsRNA mimic, polyI:C, showing that Tr/E mediated increased polyI:C-driven antiviral protection that was associated with reduced pro-inflammatory factors as well as lower expression levels of RIG-I and MDA5, and NF- κ B activation (Drannik et al., manuscript in revision). Our data from cervical ECs from CSWs further supports the argument that moderated, or lower, expression of local innate PRRs in context of repeated viral exposures might be a predetermining factor in resistance to HIV-1 mucosal transmission, and that E may play a direct role in these events. What remains to be seen is the functional phenotype and responsiveness of ECs, and additional cell subtypes, isolated from the genital tract of HIV-R CSWs and cultured *ex vivo*.

In conclusion, although we realize that our *in vitro* experiments only partially recreated the conditions present in the genital tract of CSWs, our study highlighted potential relationships/mechanisms and targets of E's antiviral activities in genital ECs that could be critical in reducing susceptibility to HIV-1 mucosal infection and potentially used in microbicide trials. While presented evidence may point toward a promising anti-HIV-1 activity of E alone, its ultimate protective anti-HIV-1 effect might be more evident *in vivo* at mucosal sites, acting in synergy with many other innate effector proteins, including defensins, cathelicidins, lactoferrin, and SLPI.

Acknowledgements

We would like to sincerely thank all the women of Pumwani cohort and the clinical staff at the Pumwani clinic for their generous participation and dedicated and hard work. We also express our appreciation of expert technical support of J. Newton and A. Patrick. This work was supported by a grant as part of the Comprehensive T Cell Vaccine Immune Monitoring Consortium (CTC-VIMC), a key component of the Collaboration for AIDS Vaccine Development (CAVD) funded by the Bill & Melinda Gates Foundation. A.G.D. was supported by a Studentship Award from the Ontario HIV Treatment Network (OHTN) and K.L.R. by a Career Scientist Award from the OHTN.

Disclosure Statement: The authors declare no conflict of interests.

Footnotes:

Abbreviations: CSW, commercial sex workers; CVLs, cervico-vaginal lavage fluid samples; ECs, epithelial cells; E, human elafin without a tag; Eh, human elafin purified from commercial Tr/E with a C-terminal tag; hE, in-house human recombinant elafin with an N-terminal tag; FGT, female genital tract; HNE, human neutrophil elastase; NF- κ B, nuclear factor-kappa B; PAMP, pathogen-associated molecular pattern; polyI:C, polyinosinic/polycytidylic acid; PRRs, pattern recognition receptors; SLPI, secretory leukocyte protease inhibitor; STIs, sexually-transmitted infections; TLR, Toll-like receptor; Tr, trappin-2; cTr, human commercial recombinant trappin-2 with a C-terminal His-tag (6 \times His-Tr); human secreted trappin-2 with no tag (sTr); HIV-R, HIV-exposed but resistant commercial sex workers; RIG-I, retinoic acid inducible gene-I; HIV-S, HIV-exposed and susceptible commercial sex workers; WAP, whey acidic protein.

Figure legends

FIGURE 1. Schematics and antiprotease properties of trappin-2 and elafin protein preparations. (A) Schematic diagrams of tested human trappin-2 (Tr) and elafin (E) (Tr/E) protein preparations. sTr, human secreted Tr (no tag); TG, transglutaminase domain; WAP, whey acidic protein domain; cTr, human commercial recombinant 6×His-trappin-2, a mixture of both Tr and E (with a C terminus His-tag); Eh, human recombinant E purified from cTr (with a C terminus His-tag); E, human commercial recombinant elafin (no tag); hE, in-house human recombinant HAT-E (with an N terminus HAT-tag). (B) Antiprotease activity of different Tr/E proteins was tested using purified human neutrophil elastase (HNE) to the various concentrations of Tr or E (final volume 10 µl/well), measuring a residual activity of enzyme using a chromogenic HNE specific substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide, by monitoring the change in absorbance at 405 nm for 15 min. Results are expressed relative to the NHE activity of a positive control (HNE in diluent alone); cTr (○); sTr, (◇); E, (Δ); hE, (□).

FIGURE 2. Unrestricted N-terminus of elafin is critical for anti-HIV-1, but not antiprotease, activity. For attachment assay (A,B), media or cTr, E, hE, or sTr at 1 µg/ml were incubated with 100 TCID₅₀ of R5-HIV-1_{ADA} (A) or X4-HIV-1_{IIIB} (B) or HEC-1A cells, grown in a 96-well plate, alone for 1 h at 37°C. Before addition of medium (-) or virus (V) for another 1.5 h at 37°C, the cells were washed 5 times with PBS. Subsequently, cells not pretreated with the proteins received either medium (-), untreated HIV-1 (V), or HIV-1 preincubated with Tr or E (V+p). The cells pretreated with the proteins (c+p) received untreated HIV-1 alone. Following 1.5 h of incubation, viral inoculum 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 × *g* for 5 min. The amount of total cell lysate-associated p24 was determined by ELISA. The data are representative of one of three independent experiments performed in triplicate and shown as the mean ± SD of p24 protein expressed as pg/ml. Statistical analysis was performed using one-way analysis of variance (ANOVA), with *p* values considered

significant when $p < 0.05$. For transcytosis (*C,D*), 1×10^5 HEC-1A cells were seeded 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) over minimum of 3 days. Cells that reached transepithelial resistance (TER) 350 – 470 Ω/cm^2 (EVOM; World Precision Instruments, Sarasota, FL, USA) were considered confluent and were pretreated apically with media or 1 $\mu\text{g}/\text{ml}$ of Tr or E proteins as above for 1 h before the addition of 10 ng p24 of R5-HIV-1_{ADA} (*C*) or X4-HIV-1_{IIIB} (*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. Amount of infectious HIV-1 particles in the basolateral chamber were determined using TZM-bl cells. Basolateral supernatants were 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 (Drannik et al., 2012a). Data are expressed as percentage of infectious particles recovered in the presence of Tr or 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) or Student's *t* with *p* values considered significant when $p < 0.05$. Concentrations of Tr and E used in these experiments were based on a dose-dependent anti-HIV-1 activity of the proteins that was reported earlier (Drannik et al., 2012a).

FIGURE 3. Cellular localization of exogenous elafin in genital epithelial cells.

Immunofluorescence staining of cellular distribution of 1 $\mu\text{g}/\text{ml}$ of human Tr or E (green) proteins, detected using TRAB20 (*A,B,C*), HAT-tag- (*D,E,F*), and His-tag-specific antibodies (*G,H*). Nuclei were visualized with propidium iodide (PI) as red.

Representative staining of three different experiments is shown for Tr or E proteins at 1 h post exposure and merged image shows human Tr or E visualized as yellow in composite panels. Scale bar, 20 μm .

FIGURE 4. Elafin attenuates HIV-1-induced nuclear translocation of NF- κ B.

Immunofluorescence staining of NF- κ B/p65 and its nuclear translocation following either medium alone or polyI:C 25 μ g/ml, or 10 ng of R5-HIV-1_{ADA} p24 treatment for 4 h in presence or absence of pre-treatment with 1 μ g/ml of cTr or E proteins is demonstrated. Representative staining is shown for p65 (green) visualized by Alexa488, nuclear stain (red) visualized by propidium iodide (PI), and composite (yellow). Representative data set from three independent experiments is shown. Scale bar, 20 μ m.

FIGURE 5. Elafin inhibits IL-8 secretion and mRNA expression of TLR3 and RIG-I

genes in response to R5-HIV-1_{ADA}. HEC-1A cells were grown as a polarized monolayer in a transwell system and incubated with media or 1 μ g/ml of cTr or E for 1 h before the addition of 10 ng p24 of R5-HIV-1_{ADA} (A) or X4-HIV-1_{IIB} strains (B) for 8 h at 37° C to the apical chamber. Levels of IL-8 protein were determined in basolateral compartment by ELISA and shown as the mean \pm SD of pg/ml. Statistical analysis was performed using one-way analysis of variance (ANOVA), with p values indicated on a graph. Statistical significance was considered when $p < 0.05$. TLR3 (C) and RIG-I (D) mRNA expression was assessed by harvesting total RNA and performing RT-qPCR at 8 h post treatment with 10 ng of R5-HIV-1_{ADA} p24. Values are normalized against 18S and presented as relative expression. Representative data set from three independent experiments is shown as the mean \pm SD. Statistical analysis was performed using one-way analysis of variance (ANOVA), with p considered significant when $p < 0.05$.

FIGURE 6. Genital ECs from HIV-resistant (HIV-R) commercial sex workers (CSWs) have significantly lower mRNA expression of TLR2, TLR4, and RIG-I. Relative mRNA expression levels of TLR 2, TLR4, and RIG-I in cervical ECs of HIV-S (■, N=10) and HIV-R (●, N=10) were assessed by RT-qPCR. The relative quantity of expression of these genes was normalized against 18S. The data are shown as the mean \pm SD. Statistical analysis was performed using one-way analysis of variance (ANOVA), with p considered significant when $p < 0.05$.

Figure 1.

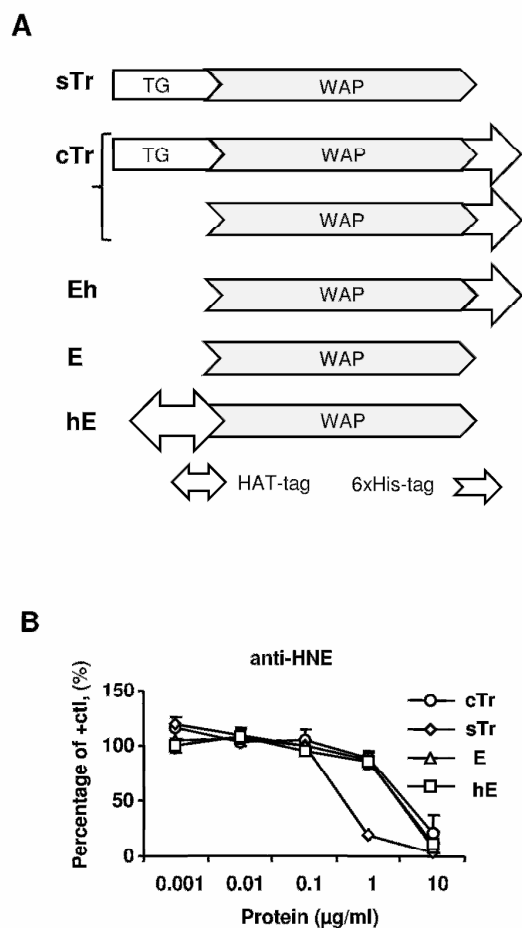
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Figure 2.

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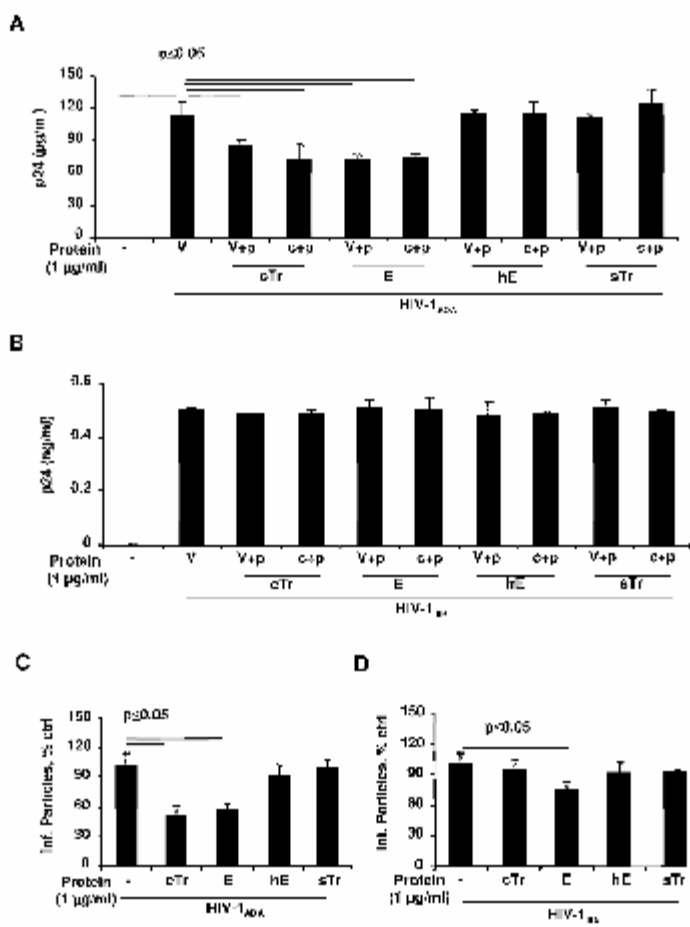


Figure 3.

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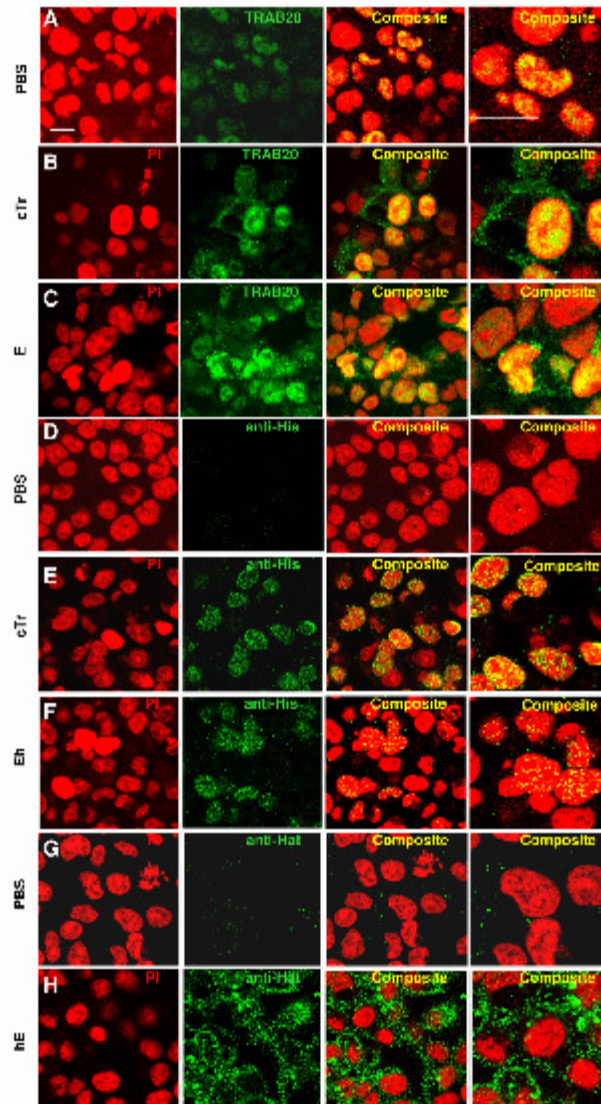


Figure 4.

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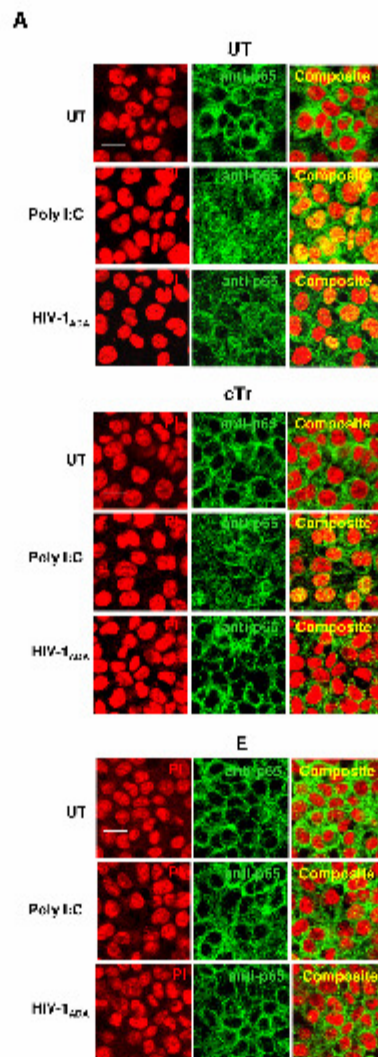


Figure 5.

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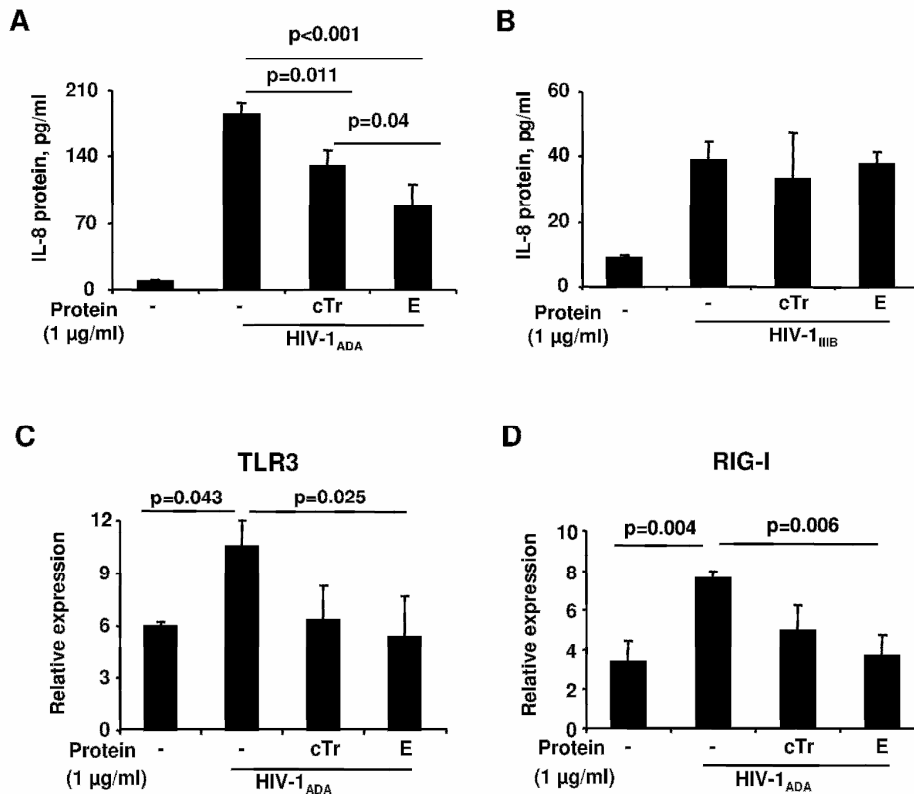
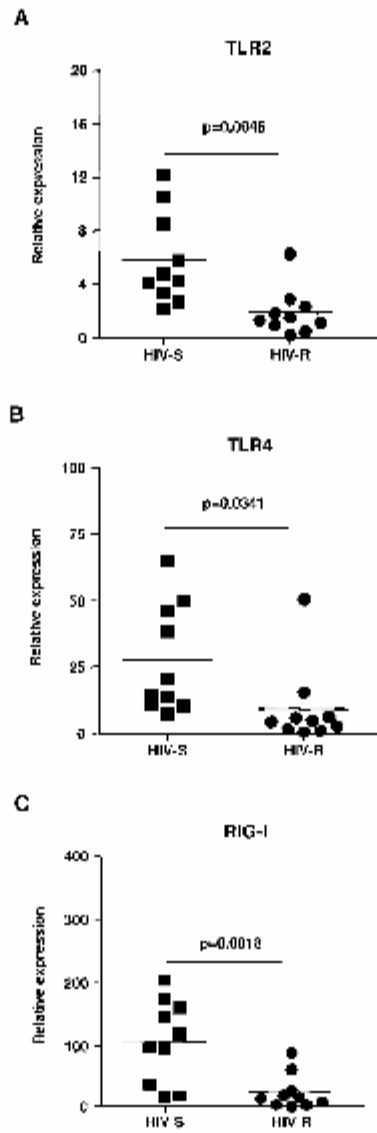


Figure 6.

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----- Chapter 6 -----

Discussion

Sexually transmitted infections, especially HIV/AIDS, remain a devastating problem with a heavy burden on economic, financial, and health aspects of societies globally (UNAIDS, 2007). Even more worrisome is the close link between HIV-1 and other STIs such as genital herpes (Sobngwi-Tambekou et al., 2009), where HSV-2 is a major risk factor increasing HIV-1 acquisition, viral shedding, and HIV-1 transmission (Celum, 2004). The persistently high numbers of HSV-2 and HIV-1 infections (Chen et al., 2000; Xu et al., 2006) indicate that the overall success of currently available/accessible prophylactic and therapeutic measures against HSV-2 and HIV-1 are not sufficient for gaining optimal control over these infections. Thus, the search for additional factors/means capable of altering key steps in HSV-2 and HIV-1 infections with the special focus on the FGT should be continued as they will advance our current protective strategies.

Despite that HIV-1 is largely transmitted heterosexually, and that women represent at least 50% of people newly infected with HIV-1 globally (UNAIDS, 2010) the estimated probability of viral transmission during sexual intercourse is low and around 1 infection in 122 exposures (Mastro and de Vincenzi, 1996). We now know that such a protective effect is due in part to natural antimicrobial activity of mucosal fluid from the FGT that has been linked to the presence of endogenous protective factors (John et al., 2005; Shust et al., 2010). Indeed, several innate factors including RANTES, MIP-1 α , MIP-1 β , and lactoferrin have been recognized for their contribution to the host's defense against HIV-1, as reviewed elsewhere (Hickey et al., 2011; Kaushic et al., 2010; Wira et al., 2011b).

Recently, the serine antiproteases Tr and E were also associated with mucosal HIV-1 resistance in HESN women in Africa (Iqbal et al., 2009); however, specific mechanisms of their antiviral activity in the FGT remain poorly understood. Given that the overall success in developing protective measures against HSV-2 and HIV-1 remains marginal (Cohen, 2010; McMichael and Haynes, 2012; Rappuoli and Aderem, 2011), continued characterization of CVL composition and identification/characterization of

novel endogenous factors with natural antiviral activities against the infections may significantly improve our efforts in controlling the spread of these STIs.

Thus, the objective of this research was to individually characterize the antiviral activity of each Tr and E separately against HSV-2 and HIV-1 in the female genital mucosa. Specifically, we assessed whether or not and how each Tr/E contributed to antiviral host defense responses elicited by polyI:C, a synthetic mimic of viral dsRNA (Chapter 2). Further, we elucidated the presence and characteristics of antiviral activity of each Tr/E, including potential mechanisms and targets, against replication competent sexually-transmitted pathogens, namely HSV-2 (Chapter 3) and HIV-1 (Chapters 4-5).

Tr/E: key findings and proposed antiviral actions

Serine antiproteases Tr/E are pleiotropic molecules, with a number of significant roles ascribed to them in infection, inflammation, and immunity (Drannik et al., 2011; Sallenave, 2010). Previously, the contribution of Tr/E to health was mostly credited to their inhibition of proteases and tissue repair, direct antibacterial and antifungal activity, bacterial opsonization, as well as the induction and modulation of innate and adaptive immune responses (Drannik et al., 2011; Moreau et al., 2008; Sallenave, 2010). For the first time, here Tr/E were individually compared and assessed for the presence and the characteristics of their antiviral activity using *in vitro* and *in vivo* models with polyI:C, HSV-2 and HIV-1. To accomplish this goal, we used multiple approaches of exogenous expression of Tr/E, including through a replication-deficient adenovirus expressing the human Tr gene, the delivery of soluble tagged and untagged recombinant Tr/E protein preparations, and the utilization of Tr transgenic mice.

Our key findings clearly established the beneficial contribution of each Tr/E individually to the host's defense against both HSV-2 and HIV-1, which has never been previously reported. Specifically, our results demonstrated that Tr/E targeted both HSV-2 and HIV-1 by acting *directly* on *virus* or *indirectly* on *cells*, or through *both*, depending on virus and cell types. Furthermore, we demonstrated that Tr/E-mediated increased antiviral resistance and reduced inflammation were associated with alterations in one or

more areas, including: (i) viral attachment/entry (VSV-GFP, HSV-2, and HIV-1), transcytosis (HIV-1), infection of target cells (HSV-2, HIV-1), viral replication and translocation (HSV-2); (ii) viral innate recognition (polyI:C, HIV-1); (iii) mounting of cellular inflammatory responses (polyI:C, HSV-2, and HIV-1); and (iiii) mounting of cellular antiviral responses (polyI:C, HSV-2, and HIV-1). Our findings also demonstrated that antiviral activity was *independent of antiprotease* activity of Tr/E; *contextual* and exerted, at least in HEC-1A cells, via *autocrine/paracrine* mode; *dependent* on elafin's *unmodified N-terminus* and its *nuclear localization*, and *E* exhibiting *more potent* antiviral activity than Tr.

The discussion below elaborates on points presented above. Since many similar characteristics of antiviral activity of Tr/E were observed against both HSV-2 and HIV-1, antiviral activity of Tr/E is discussed in the following sections, not on the basis of individual virus, but rather based on common antiviral characteristics.

Antimicrobial mechanisms of Tr/E

For many years, the antimicrobial activities of Tr/E against *bacteria* were better understood than against *viruses*. Indeed, some studies have shown that exogenous Tr/E can directly interact with bacterial ligands, such as LPS (McMichael et al., 2005b). In contrast, other studies demonstrated that Tr was able to opsonize bacteria, as was shown for *P. aeruginosa*, thus facilitating a more efficient, CD14-dependent, clearance by macrophages (Wilkinson et al., 2009) or directly interact with pathogens (Baranger et al., 2008; Bellemare et al., 2010; Simpson et al., 1999), or to alter host's innate immune responsiveness triggered by microbial stimulation (McMichael et al., 2005a; Sallenave et al., 2003; Simpson et al., 2001b).

Earlier studies on antiviral effects of Tr/E also revealed a similar heterogeneity in antiviral mechanisms. For example, Zaidi et al. demonstrated a protective effect of Tr on disease outcome and target organ function in an acute model of murine viral myocarditis (Zaidi et al., 2000). Whereas Roghanian et al. showed a Tr-mediated increase in viral clearance that was attributed to the induction of potent Th1 adaptive immune responses in

lungs and spleens in a murine model of adenoviral lung challenge (Roghalian et al., 2006). Finally, Ghosh *et al.* demonstrated that reduced viral infection of target cells was likely attributed to the direct anti-HIV-1 effect of E, which is also in line with our findings (Ghosh et al., 2010). That the beneficial effects of Tr/E were observed in such distinct models attests to the plethora of antimicrobial mechanisms that have likely evolved in an arms race between pathogens and innate antimicrobials over the years of vertebrate evolution. In turn, the existence of multiple antimicrobial mechanisms confirms Tr/E pleiotropic nature and underscores their significance in being versatile and able to tailor their protective effects to better suit the target organ's needs and specificity in defense against invading pathogen and at different sites.

Regardless of these observations, whether or not and how Tr/E work against sexually-transmitted viruses in the human genital ECs remained unresolved and became the purpose of this research.

Coexistence, but no co-dependence of antiprotease and antiviral activities of Tr/E

Tr/E inhibitory activity against serine proteases was one of the first discovered and believed to be the only function for these proteins (Wiedow et al., 1990). The later discovered antibacterial activity of Tr/E and SLPI, however, was shown to be independent from their ability to inhibit proteases (Baranger et al., 2008). In contrast, such a relationship between antiviral activity of Tr/E and their inhibition of proteases has not been elucidated. With this in mind, we tested all of our Tr/E protein preparations to ascertain their protease inhibition activity and also used this function as a control, at least in part, of Tr/E functional integrity. The antiprotease activity was also utilized as a “common denominator” in comparative assessment of Tr/E antiviral activity, in light of varied manufacturing sources of the protein preparations included in our studies. Interestingly, we found that not all of the tested Tr/E proteins exhibited antiviral activity, yet all were almost equipotent in their antiprotease activity. This led us to conclude that antiprotease and antiviral (observed against VSV-GFP, HSV-2, and HIV-1) activities of

both Tr/E did not correlate with each other and that these antiproteases acted against viruses through mechanisms and structures distinct from those mediating their antiprotease function. This finding may have significant implications for the WAP and STIs fields, since it may focus future research on the identification of novel functions as well as structural determinants of Tr/E that are specifically responsible for antiviral, and not antiprotease, activity of the proteins.

Prerequisites of Tr/E antiviral activity in HEC-1A cells

Our studies revealed that endogenous Tr/E in HEC-1 A cells were localized intracellularly, in both perinuclear and nuclear compartments. Tr/E were also secreted in response to bacterial (FimH and flagellin, unpublished data) and viral ligands, notably polyI:C (Chapter 2 and 4), which was in accordance with Ghosh et al. showing that primary genital ECs also responded to polyI:C treatment by secreting Tr/E (Ghosh et al., 2010). More importantly, we demonstrated that exogenous Tr/E entered HEC-1A cells within 1 hour of exposure and localized within nuclear/perinuclear areas, similarly to the endogenous proteins. Exogenous Tr/E proteins were able to enter the nucleus, but E only when its N-terminus was not modified or blocked by a tag. Thus, we concluded that Tr/E were able to enter the cell and the nucleus in an autocrine/paracrine mode, and the presence of unblocked N-terminus of E was crucial for its nuclear localization.

Another exciting finding that emanated from our experiments was that anti-HIV-1 activity of Tr/E, namely reduced HIV-1 attachment/entry, transcytosis, and immune activation (i.e., sensing and secretion of pro-inflammatory mediators), was highly associated with the proteins' entry into the nuclear compartment of the cell and thus elafin's unblocked N-terminus, but not with its antiprotease activity. Thus, the unmodified N-terminus of E appears to be a prerequisite for the protein's anti-HIV-1 activity, since a HAT-tagged hE did not exert any virus- or cell-associated antiviral activity against HIV-1. Hence, we collectively concluded that in the defense against HIV-1 in HEC-1A cells, Tr/E likely functioned in an autocrine/paracrine mode and most importantly by acting directly on virus, as well as indirectly on cells through cellular

uptake and nuclear localization of the proteins, and especially E with an unmodified N-terminus.

Although we did not test a HAT-tagged E against HSV-2, considering that similar direct and indirect antiviral activities of Tr/E were observed against HSV-2 and HIV-1, we propose that Tr/E are likely to act in a similar mode against both viruses. Yet, the details of these mechanisms remain to be fully elucidated in future research.

Interestingly, while HAT-tagged hE did not demonstrate any antiviral activity, it still inhibited polyI:C-induced IL-8 secretion. These results are supported by similar observations from secreted sTr, and collectively they would suggest that antiviral and anti-inflammatory activities of Tr/E are two separate entities that are likely independent, yet complimentary to each other. Another conclusion is that the presence of unblocked N-terminus and the nuclear localization of the proteins may not be as crucial for anti-inflammatory activity of the proteins, compared to their antiviral effect, because the former can be executed at different levels, including extra- and intracellularly. It might be interesting to determine in future studies if Tr/E can directly interact either with dsRNA or its cellular binding receptors (e.g. scavenger receptors (Limmon et al., 2008)), as a mode of Tr/E anti-inflammatory activity against viruses.

Our observations on Tr/E autocrine/paracrine mode of action and intranuclear entry of the proteins with an unmodified N-terminus are novel and exciting. Yet, the physiological significance of these findings might be unclear, since they were generated using HEC-1A cells and not primary human genital ECs. As was discussed earlier in Chapter 2, primary ECs, albeit unlikely, may react significantly differently from HEC-1A cells in response to viral stimulation or protein treatments. Nevertheless, considering the similarity between our polyI:C-mediated induction of Tr/E in HEC-1A cells (Chapter 2) and polyI:C-driven release of Tr/E shown by Ghosh et al. in primary genital ECs (Ghosh et al., 2010), and given that HEC-1A share a common origin and HIV-1 receptors with primary cells (Bobardt et al., 2007; Saidi et al., 2007), it could be hypothesized that primary genital ECs may also use Tr/E in autocrine/paracrine mode in their antiviral defense, similarly to HEC-1A cells. In this scenario, primary genital ECs could secrete

Tr/E as an extracellular shield against viruses and their ligands and then reuptake the proteins to also protect the cells from internalized virions, which could be potentially tested by preventing the reuptake of secreted Tr/E via Tr/E-specific antibody depletion. Altogether, these observations indicate that Tr/E can likely exert their antiviral activity through different forms of Tr/E, namely *secreted* and *intracellularly* stored proteins and through structures/entities located at different levels (i.e., extra- and intracellularly).

The protective role of secreted Tr/E proteins in antiviral defense has been clearly demonstrated. It can be supported by our data demonstrating that Ab-neutralization of Tr/E in Ad/Tr-cells significantly reversed immunomodulatory effect of Tr/E on polyI:C-induced IL-8 secretion (Chapter 2). Additionally, Ghosh et al. reported that Ab-depletion of Tr/E from secretions of primary genital ECs, grown *in vitro*, reversed anti-HIV-1 activity of the secretions (Ghosh et al., 2010). Further, we and others (Iqbal et al., 2009) showed that elevated levels of secreted Tr/E were associated with clinical HIV-1 resistance in HIV-R CSWs *in vivo* as well as direct contribution to anti-HIV-1 activity of CVLs *in vitro*, where 30% of Tr/E Ab-depletion significantly reduced HIV-1 suppressive activity of CVLs from HIV-R CSWs (Chapter 4) would also attest to the notion of the importance of secreted Tr/E in the FGT. Inferring from these observations, we can conclude that Tr/E may act through different mechanisms and at multiple levels/entities, which are discussed below.

Targeting both: virus and cells

Our data demonstrated that when either virus or cells (with some exceptions discussed later) were pretreated separately with Tr/E proteins, viral adherence, translocation and infection of target cells were reduced in both instances. These findings proposed the existence of two independent levels/pathways of Tr/E-mediated antiviral activity, separately targeting *virus* and *cells*.

Targeting virus

In this research, the direct antiviral effect of Tr/E was observed against all three viruses, namely VSV-GFP (Chapter 2), HSV-2 (Chapter 3), and HIV-1 (Chapter 4 and 5), when viruses and not cells were pre-treated with the recombinant proteins. The outcome of this effect was a reduced viral attachment/binding (HSV-2 and HIV-1) and infection of target cells (VSV-GFP, HSV-2, and HIV-1). But perhaps the most convincing evidence of Tr/E antiviral activity comes from our data linking elevated levels of the proteins with clinical HIV-1 resistance in CSWs as well as increased protection against HIV-1 in *in vitro* assays. In the Ab-depletion experiments using anti-Tr/E antibodies, we demonstrated that Tr/E account for up to 60% of natural anti-HIV-1 activity of CVL from the resistant cohort (Chapter 4). In the latter assays, inhibition of HIV-1 infection of TZM-bl target cells was also observed when virus was pretreated with CVLs, similarly to the recombinant Tr/E used in our study.

Interestingly, though, we showed that Tr/E, both recombinant and naturally occurring from CVLs of HIV-R CSWs, preferentially inhibited R5- and not X4-tropic virus, although with a few exceptions. Specifically, in the experiments using siRNA knockdown of endogenous Tr/E and in HIV-1 transcytosis assays with recombinant E pretreatment, we showed that both R5 and X4 were affected, which was in line with Ghosh et al., as discussed in Chapter 4 (Ghosh et al., 2010). It is unclear at the moment why preferentially R5 and not X4 HIV-1 strain was affected in our studies. It is possible, however, that our results highlight the presence of structural and/or functional distinctions between recombinant and naturally occurring Tr/E proteins, since siRNA inhibition of endogenous Tr/E increased the release of both R5 and X4 viral strains. Such differences could also exist between secreted and intracellularly present Tr/E, which may warrant additional studies in the future. Additionally, since X4 HIV-1 strains were shown to bind more efficiently to HEC-1A cells due to their gp120 ability to bind HSPG with higher affinity than R5 strains (Moulard et al., 2000) (Bobardt et al., 2003; Moulard et al., 2000), the observed discrepancies might also be explained by the fact that Tr/E cannot compete with a high affinity binding of X4 HIV-1 or that the proteins get displaced after initial binding to HSPG, which remains to be shown.

Nevertheless, that particularly the infectivity of R5 HIV-1 was inhibited is an important finding that may have implications for reducing the risk of HIV-1 acquisition, given that most of the founder-transmitter HIV-1 strains are R5-tropic (Keele et al., 2008). Given a broad spectrum of antimicrobial activity of Tr/E against bacterial, and fungal pathogens, as discussed in Chapter 1 and our review (Drannik et al., 2011), it would be important in the future to determine the scope of antiviral activity, in particular against HIV, given the immense diversity and variability in fitness and infectiousness of this pathogen globally. Also, it would be advisable in future investigations to expand the panel of both R5 and X4 HIV-1, considering our siRNA data that naturally-occurring Tr/E targeted both viral strains. It would also be of significance to determine Tr/E antiviral potential against cell-associated HIV-1 stocks, given that the most efficient HIV-1 transfer occurs during cell-cell interaction (Bobardt et al., 2007; Bomsel, 1997).

Additionally, that the observed inhibitory activity of Tr/E was directed against both HSV-2 and HIV-1 suggests that Tr/E employed either similar mechanism(s) (i.e., destabilization of viral capsid, etc.) or targeted similar structures/receptors shared between the viruses. Although neither we nor others have presented any specific mechanisms of direct interaction between Tr/E and the viruses, several α -defensins were reported to bind to carbohydrate moieties, likely O- or N-linked glycans, on HSV-2 gB-2 and on HIV-1 gp120, and thus interfere with viral attachment, entry, and infectivity (Wang et al., 2004) (Yasin et al., 2004). However, there could be alternative mechanisms that need not be mutually exclusive. Thus, it might be worth focusing future research on identifying specific targets of each Tr/E on HSV-2 and HIV-1 and the first plausible target tested could be O- or N-linked glycans on viral glycoproteins. Overall, these observations may have a significant bearing on the control of these STIs as the identification of common viral targets of Tr/E may assist in customizing the design of protective vaccines or microbicides against these pathogens.

Targeting cells

Tr/E exhibited antiviral activity by acting on cells, since cells pretreated separately from virus with Tr/E proteins showed reduced virus attachment and infection. Additionally, we found that viral inhibition was associated with modulated viral innate recognition and inflammatory responses as well as enhanced cellular antiviral responses. We hypothesized that this cell-associated antiviral effect could be a result of a direct interaction between Tr/E and cellular structures or as an indirect consequence of upstream events. Specifically, we proposed that Tr/E antiviral effect could be executed at multiple levels (i.e., cell surface or intracellularly) and potentially even altering more than one, such as: (i) virus binding receptors (cell surface); (ii) viral sensors or PRRs (plasma membrane or intracellularly); (iii) inflammatory pathways (intracellularly); and (iv) antiviral pathways (intracellularly).

Virus binding receptors

We hypothesized that the cell-surface activity, which was observed as reduced binding/entry of both HSV-2 and HIV-1, was dependent partly on the interaction between Tr/E and virus-binding receptors, thus potentially preventing efficient attachment and consequently viral entry into cells. Our data supported this conjuncture, at least for HIV-1, showing that viral attachment to HEC-1A cells expressing non-canonical HIV-1 receptors, such as heparan sulfate moieties with syndecans, was significantly reduced, whereas TZM-bl cells expressing canonical receptors were not protected from HIV-1 infection. However, since viral entry per se was not assessed in our studies, we cannot differentiate between the effects of Tr/E on viral attachment/binding versus entry. Nevertheless, considering that Tr/E were shown to bind to extracellular matrix proteins as discussed earlier (Guyot et al., 2005b), this led us to believe that Tr/E could be acting, at least in part, through non-canonical HIV-1 receptors like syndecan-1, a key molecule implicated in HIV-1 attachment to genital ECs (Bobardt et al., 2010; Bobardt et al., 2007; Saidi et al., 2007). Our results, however, revealed no association between reduced HIV-1 binding to HEC-1A cells and the expression of syndecan-1, and it is still unclear why HEC-1A cells were more protected by Tr/E than their TZM-bl controls. The alternative

mechanism of this protection, however, could be potentially hidden in the cellular ability to uptake Tr/E. The latter could be dependent on the expression of yet elusive Tr/E receptors, the existence of which remains to be discovered; this is in contrast to SLPI that has been shown to bind to annexin II (Ma, 2004) as well as phospholipid scramblase 1 and 4 (Py et al., 2009).

Contrary to HIV-1, the involvement of syndecan-1, or any other receptor, in anti-HSV-2 activity of Tr/E was never investigated here. Further, that VSV-GFP attachment was not affected, compared to other viruses used in this study, implies that this virus has little in common with HSV-2 and HIV-1 in binding/entry mechanisms, at least in HEC-1A cells, that can be affected by Tr/E.

Altogether, these findings indicate that antiviral activity of Tr/E is not absolute, but rather *contextual*, which can be supported by its dependence on virus and cell types as well as by its variable in potency antiviral protection depending on the viral inoculum (as was observed in Chapter 3 with HSV-2). Nevertheless, given the fact that HSV-2 and HIV-1 share many proteoglycan molecules allowing viral binding/entry (Hazrati et al., 2006), and that Tr/E protected cells from both viruses, it might be of significance to identify target(s) of Tr/E cell-surface mediated antiviral activity.

Viral sensors and inflammatory responses

Innate sensing is critical to the protection against viral infections. Through the activation of signaling pathways, viral recognition stimulates the induction of protective innate and adaptive antiviral and inflammatory immune responses (Kawai and Akira, 2006). While generally being beneficial, these processes may become detrimental to the host when not efficiently controlled or exploited by pathogens (Kurt-Jones et al., 2005; Kurt-Jones et al., 2004; Mogensen et al., 2010). Following HSV-2 recognition, the induction of Type I IFNs, and IFN β in particular, is initiated and correlates with antiviral protection (Gill et al., 2006; Nazli et al., 2009). Type I IFN signaling, in turn, limits viral translation, replication, and spread by activating additional antiviral pathways/factors, such as PKR and OAS/RNase L (Kawai and Akira, 2006). However, in addition to

antiviral responses, potent inflammatory responses have also been associated with HSV challenge (Chan et al., 2011; Cunningham et al., 2006; Koelle and Corey, 2008; Thapa and Carr, 2008). Moreover, HSV-2 has been shown to manipulate PRRs expression, including in the genital ECs, where it masks its presence and disarms cells of protective antiviral IRF3- and IFN β -mediated responses in human vaginal epithelial cell line (Yao and Rosenthal, 2011) and also upregulates TLR4 expression with subsequent induction of NF- κ B activation and IL-6 and IFN β production in human cervical cells (Li et al., 2009a). Additionally, studies demonstrated that murine encephalitis was associated with increased levels of TLR4 mRNA (Bottcher et al., 2003) and robust TNF α responses (Thapa and Carr, 2008). Kurt-Jones with colleagues also showed that TLR2-/- mice had reduced HSV-1-mediated brain inflammation and mortality (Kurt-Jones et al., 2004). Collectively, these observations highlight the detrimental contribution of immune activation to HSV-induced host's responses and disease pathogenesis.

In turn, increased HIV-1/SIV acquisition and disease progression were associated with higher and prolonged immune activation, increased levels of IL-8, TNF α and type I IFNs, as well as high cell turnover and dysregulated expression and functioning of innate viral sensors (Bosinger et al., 2009; Haase, 2010, 2011; Lester et al., 2008; McMichael et al., 2010; Mogensen et al., 2010; Silvestri et al., 2003). In contrast, HIV-1 resistance was linked to initially robust but transient innate responses, as well as immune quiescence and reduced immune activation (McLaren et al., 2010; Silvestri et al., 2003; Songok et al., 2012), as was discussed earlier in the introduction. These observations indicate that local pro-inflammatory milieu following virus/host interaction and recognition is by far the main contributor to this vicious cycle perpetuating sustained immune activation, yet with inadequate viral control and culminating disease progression, as reviewed in references (Haase, 2011; McMichael et al., 2010; Mogensen et al., 2010). Since altered innate recognition and sustained pro-inflammatory responses are so critical in promoting HSV-2 and HIV-1 establishment and progression, having local factors with the ability to control the inflammatory arm of these responses, yet without compromising antiviral protection, might be indispensable for controlling STIs in the genital mucosa.

We demonstrated here that Tr/E-mediated increased antiviral protection was associated with modulated expression of RIG-I and MDA-5 with polyI:C treatment (Chapter 2) and RIG-I, MDA-5, and TLR3 following HIV-1 challenge (Chapter 5), and secretion of pro-inflammatory factors IL-8, IL-6, and TNF α was reduced after all of the treatments. Interestingly, we observed no differences in HSV-2-induced PRRs expression in contrast to our observations for polyI:C and HIV-1 treatments (Chapter 2 & 5). These results may suggest the existence of different mechanisms of Tr/E-mediated regulation of inflammatory responses that could be virus- or ligand- dependent. Furthermore, these results could be different in primary genital ECs, which remains to be determined.

Additionally, the HIV-1 *in vitro* data were also supported by our novel and clinically relevant findings showing that higher Tr/E vaginal levels in HIV-R cohort of CSWs were associated with reduced mRNA levels of TLR2, TLR3, TLR4, and RIG-I in the genital ECs of these women. Together, these findings clearly link Tr/E with modulated viral sensing, reduced immune activation and HIV-1 mucosal resistance. Although this is the first report demonstrating that Tr/E may play a role in anti-HIV-1 protection by exerting immunomodulatory effects, other studies showing that immunoquiescence and reduced immune activation were associated with HIV-1 resistance (McLaren et al., 2010; Songok et al., 2012) would support our findings. However, alternative observations have also been reported, showing a connection between HIV-1 resistance and higher, as opposed to lower, functional activity of TLR ligand-activated PBMC from HIV-R CSWs in Italy (Biasin et al., 2010; Sironi et al., 2012). The authors proposed that activated innate immune responses in HIV-R CSWs result in stronger adaptive antiviral events and ultimately better viral clearance. Or alternatively, it could be argued that early robust, but transient, and well regulated immune responses might be more beneficial for early control of HIV-1 infection and prevention of the diseases progression (Bosinger et al., 2009; Su et al., 2011). Despite recent advances in understanding HIV-1 pathogenesis, it is still unclear whether it is immune modulation of sustained immune activation or rather strong but transient activation of early antiviral responses that should be the ultimate strategy in preventing and treating viral infections

like HIV-1. What is clear, however, is that active, timely and efficient immune regulation should be in place to control any of the above events. Additional research might be required in the future to better characterize early events and key players governing the susceptibility or protection against HIV-1 in the FGT, where Tr/E appear to be viable candidates for continued investigations in light of their immunomodulatory and antiviral activities.

With respect to HSV-2 infection, our *in vivo* data in an intravaginal model of genital herpes (Chapter 3) would also support the beneficial role of Tr/E in antiviral defense in the FGT. This model was the first evidence of Tr/E anti-HSV-2 effects, which was also somewhat supportive of our *in vitro* findings. Our data showed that Tr transgenic mice overexpressing human Tr had a reduced viral burden in the CNS and delayed viral translocation from the genital tract into the CNS that were also accompanied by reduced expression levels of TNF α in the target organs later in the infection. These animals also demonstrated a trend of increased survival and milder disease presentation compared to their Wt controls, natural knockouts for Tr. Although this is the first study for each Tr/E and HSV-2 and only showed a trend, another study on antiviral activity of Tr/E is in line with our findings by showing a more favorable disease outcome and a better target organ function in Tr transgenic mice (Zaidi et al., 1999). Taken together, our findings have established the first link between Tr/E presence in the female genital mucosa, reduced inflammatory responses and milder genital herpes.

Although earlier studies documented a Tr/E-mediated inhibition of secreted MIP-1, IL-8, and TNF α , as well as NF- κ B and AP-1 activation in response to LPS (Butler et al., 2006) and oxidized LDL and LPS (Henriksen et al., 2004), our observations on Tr/E modulating viral PRRs and pro-inflammatory responses in the context of viral exposure are novel. These findings are also of importance for identifying a particular pathway affected by Tr/E, namely NF- κ B, whose less robust activation was associated with increased antiviral protection (in context of all three stimuli). Moreover, as one of the key transcription factors at the cross-roads of many pro-inflammatory and antiviral responses, NF- κ B activity has been implicated in viral infections, including for HSV-2 (Li et al.,

2009a) and HIV-1 (Equils et al., 2001). Thus, the significance of showing that Tr/E exert immunomodulatory effect on NF- κ B pathways is apparent, although our studies did not reveal any specific mechanisms of how this inhibition may be occurring. However, in light of earlier data showing that Tr/E attenuated LPS-induced NF- κ B activation (Henriksen et al., 2004) by targeting I κ B, this sparing effect on the NF- κ B inhibitors could be a plausible mechanism of Tr/E antiviral activity that can be tested in the future studies. These inhibitory events could also be a downstream consequence of “tickling” cells with Tr/E-reduced viral inoculum or Tr/E-sequestered dsRNA, leading to altered recognition by PRRs and secretion of pro-inflammatory mediators, as was shown for LL-37 and binding to polyI:C (Hasan et al., 2011). Alternatively and considering our data on Tr/E intracellular/intranuclear localization, these inhibitory events could result from direct DNA binding of Tr/E and competing for NF- κ B sites, as was shown for SLPI (Taggart et al., 2005).

Potential implications of these inhibitory effects of Tr/E could be seen in different preventive or therapeutic applications of Tr/E to control both HSV-2 and HIV-1 infections. In fact, testing of one such application is already underway and results are expected to be released soon. The commensal Lactobacilli were engineered to express Tr/E following intravaginal delivery and during peaking estrogen levels to provide antiprotease and antimicrobial protection within the FGT during the period of vulnerability around and shortly after ovulation (Fahey et al., 2011; Wira and Fahey, 2008).

Cellular antiviral responses

IRF3 was shown to be important not only for polyI:C-mediated protection (Chew et al., 2009), but also in defense against viruses (Menachery et al., 2010). Viruses, like HSV (Paladino et al., 2010) and HIV-1 (Doehle et al., 2009), often target and disrupt functional activity of IRF3, which further confirms the importance of IRF3 in antiviral protection. Alone or in collaboration with other transcription factors, IRF3 induces antiviral protection through activation of ISGs. This process can be IFN β -dependent and

also accompanied by inflammatory mediators. Alternatively, IRF3 can directly activate ISGs without inducing IFN β and the inflammatory component (Mossman et al., 2001; Paladino et al., 2006). Despite recent efforts in understanding the role of IRF3 and IFN β in pathogenesis of HIV-1 and HSV-2, this area remains underinvestigated, and so is the role of WAP proteins in antiviral defense mechanisms.

Our research presents the first evidence linking Tr/E with increased antiviral protection and IRF3 involvement. We found that in the presence of Tr/E (either via Ad/Tr or recombinant Tr and E) and following polyI:C, HSV-2, and HIV-1 stimulation, IRF3 activation/nuclear translocation was increased in protected cells compared to their controls. These data would suggest that Tr/E likely mediated enhanced antiviral protection partly through IRF3. However, other factors or pathways could also be involved, since IRF3 siRNA knockdown did not significantly attenuate polyI:C-induced antiviral protection of Ad/Tr-infected cells (Chapter 2). These findings confirm that, at least in context of polyI:C, Tr/E might be acting not only through IRF3, but other factors/pathways as well. Findings showing that fibroblasts remained 60-90% protected against viral challenge even in the absence of IPS-1 and IRF3 (DeWitte-Orr et al., 2009) would support this notion.

Interestingly, however, while IRF3 activation was increased in both polyI:C- and HSV-2 treated cells (IFN responses were not assessed with HIV-1), IFN β secretion was found to be downregulated with polyI:C treatment, but increased after HSV-2 challenge. It is not clear why we observed such differences, but it could be related to the original effect of the stimulus on antiviral responses. Since HSV-2 was shown to down regulate both IRF3- and IFN β -mediated responses in human vaginal ECs as part of its immune evasion (Yao and Rosenthal, 2011), it appeared as if Tr/E counteracted these processes and increased expression of IFN β , the main correlate of protection against HSV-2 (Chan et al., 2011; Koelle and Corey, 2008; Nazli et al., 2009). Alternatively, polyI:C treatment induced potent pro-inflammatory, as well as IFN β responses and antiviral protection in control cells. Yet, modulated IL-8, TNF α and IFN β levels were associated with Tr/E-enhanced antiviral cellular protection. Thus, it may appear that in the context of viral

exposure, Tr/E were able to stimulate IRF3 and to modulate or customize the secretion of IFN β and other pro-inflammatory factors, thus providing active immune regulation and optimal antiviral protection. The latter could be a desirable feature for innate protective factors like Tr/E, especially at mucosal sites.

Although our studies have identified IRF3 as a potential target of Tr/E in responses to dsRNA and HSV-2, many questions remain unanswered. For example, it would be important to determine which ISGs or IRFs (alternative to those already tested here) might be mediating Tr/E antiviral activity, or whether IRF3 activation would be beneficial in HIV-1 context, given that HIV-1 was shown to target IRF3 pathways in CD4+ T cells (Doehle et al., 2009) and that robust but transient IRF1 responses were suggested as correlates of HIV-1 protection (Su et al., 2011).

Potency of antiviral activity: elafin versus trappin-2

One of the goals for this research was to identify antiviral properties of each Tr/E. Thus, we individually assessed and compared each Tr and E for their antiviral potential by using different protein preparations and approaches, as discussed earlier. Although it would be advisable for future comparative analyses to use proteins derived from a common expression/manufacturing approach, our studies, along with the assessment of antiprotease activity to mitigate the manufacturing differences, revealed interesting observations. Specifically, E was found to be more potent in its antiviral activity than Tr, and this trend was observed for both HSV-2 and HIV-1. The reason for this discrepancy is unknown at the moment, but we propose it might be due to the differences in structure-function relationship for each of the proteins that would predetermine the range and efficiency of their functions. Indeed, it was shown for a bovine lactoferricin to more potently interfere with HSV-1 intracellular trafficking compared to its larger precursor, lactoferrin (Marr et al., 2009). Thus, we propose that perhaps due to a smaller size of E this molecule was able to better exert both virus- and cell-associated antiviral effects than its precursor, Tr. Alternatively, it could be argued that these E-mediated superior antiviral effects were attributed to E's better functioning as an autocrine/paracrine factor and

entering the nucleus more efficiently as well as reacting to the treatment with both dsRNA and viruses (HSV-2 and R5 HIV-1) more quickly. Alternatively, it could be argued that each Tr and E exert their own specific function that depends on the targeting entity (i.e., inhibitory activity against virus versus modulation of dsRNA-induced expression of pro-inflammatory factors). This notion can be supported by the fact that secreted sTr from Ad/Tr-supernatants was not antiviral, yet it was still inhibiting polyI:C-induced secretion of IL-8 (Chapter 2).

These lines of reasoning led us to propose that most likely it was E, but not Tr, that was contributing mostly to antiviral activity of Tr/E proteins in our experimental system, at least against HIV-1. Indeed, we determined that commercial recombinant cTr was a mixture of both Tr and E, and that secreted sTr was not antiviral (VSV-GFP and HIV-1; not tested against HSV-2), while Eh fraction of cTr was (only tested against HIV-1). Given the latter findings and that antiviral activity of E was more potent than cTr against each of the viruses, we concluded that it was the presence of E in cTr that was most likely attributing to antiviral activity of cTr. Furthermore, considering the above, it is highly likely that it was E and not Tr that was antiviral in our studies, at least against HIV-1, which remains to be determined for HSV-2.

Collectively, these observations confirm virus- and cell-associated activities of each Tr and E, depending on virus and cell type. That the abovementioned events were associated with increased antiviral protection and reduced viral load indicates that Tr/E may significantly change the course of HSV-2/HIV-1 encounters in favor of the host by altering virus-induced sensing, as well as antiviral and inflammatory responses. Although many findings presented here are novel and exciting, they are also largely circumstantial and not direct evidence of Tr/E targeting each of the indicated areas involved in the host/virus interaction. This argument underscores the need for additional and more in-depth research in the future, using key findings identified in this research.

Model

Based on the collective evidence available to date, as well as our *in vitro* and *in vivo* studies, our current understanding of how Tr/E may work against HSV-2 and HIV-1 can be proposed in the following model. Following viral recognition (i.e., whole virion, dsRNA, etc.), the production/secretion of endogenous Tr/E that are naturally present in the cytoplasm and the nucleus is increased. At this point, Tr/E may be secreted and subsequently re-internalized by the same or other cell populations, provided cellular uptake of Tr/E is possible, and therefore work in an autocrine/paracrine fashion. Alternatively, Tr/E may stay within the cell and hypothetically shuttle between the cytoplasm and the nucleus. Once secreted, Tr/E can first target virus/viral dsRNA, plausibly through direct viral killing or sequestration of viral dsRNA, or blocking cell binding sites, which remains to be seen. This targeting can occur either outside the cell or at its surface, or upon re-entry into the Tr/E-accepting cell.

The interaction with viral particles/dsRNA can also happen intracellularly with the endogenously present Tr/E proteins. Regardless of the mechanism is, such Tr/E targeting of the viral material will likely reduce the inoculum size and its presentation to PRRs, consequently leading to attenuated activation of downstream events, such as viral recognition, signaling, and mounting of inflammatory and antiviral cellular responses. Collectively, all of these events can result in moderated immune activation, release of pro-inflammatory and antiviral mediators, and reduced viral shedding or translocation.

Additionally, this direct targeting of the *viral* side can be enriched by Tr/E affecting the *cellular* part through the interference with viral attachment, entry and/or any of the steps such as viral recognition, signaling events and the secretion of inflammatory and antiviral mediators. The latter events can stem from either direct interaction between Tr/E and cellular components (i.e., PRRs, transcription factors, DNA, etc.) and take place anywhere between the plasma membrane and the nucleus, or as a downstream consequence of reduced viral attachment. At this point, transcriptional activity of NF- κ B and the secretion of pro-inflammatory mediators will likely be dampened, possibly due to direct intranuclear competition with NF- κ B for DNA binding or any other upstream

event. Conversely, functional activity of IRF3 as a representative of cellular antiviral responses will likely be enhanced and partly responsible for increased antiviral resistance, with or without the contribution from the secreted antiviral IFN β .

Given the prompt nuclear entrance of exogenous E and considering the nuclear localization of naturally present Tr/E, it can be argued that antiviral activity of Tr/E, at least against HIV-1, may not necessarily have to start at the virus level, but rather in the nucleus. This idea is based on the evidence that HIV-1 gp120 interaction with primary genital ECs triggered a swift and early pro-inflammatory event that was associated with TNF α -dependent alteration in intercellular tight junctions, allowing for microbial translocation across ECs monolayer (Kaushic et al., 2010; Nazli et al., 2010). Thus, having Tr/E strategically located in the nucleus and being capable of inhibiting NF- κ B, as one of the key factors responsible for pro-inflammatory mediators, may prove to be beneficial in controlling the early events of virus/host interaction that are crucial in predetermining the “infection” or “resistance” outcome.

That Tr/E attenuated attachment/entry, recognition, and pro-inflammatory events associated with exposure to either the viruses or a mimic of viral dsRNA may significantly affect the pathogenesis of each of the infections and at several stages. Indeed, Tr/E can intervene with the dynamics of viral acquisition and transmission through ECs and potentially the establishment of primary infection in a subsequent target cell. This interference can occur through reduced entry and transcytosis/shedding of virus through the ECs, as well as modulated viral recognition and decreased secretion of pro-inflammatory responses, but increased antiviral protection. Additionally, Tr/E may also affect the spread/translocation of virus to distant tissues, or systemically, by reducing virus-induced immune activation, which ultimately may reduce the progression of already established disease.

The proposed model is based on the results obtained using physiologically relevant concentrations of the proteins in the FGT, but utilizing human genital ECs line, and not the primary ECs. Thus, alternative events/outcomes could be observed under different conditions, which should be taken into consideration when drawing final

conclusions from our research and especially when translating these observations into clinically relevant applications of Tr/E. Based on our data, it is clear that the intracellular circuitry regulating the events of viral recognition, as well as mounting of cellular pro-inflammatory and antiviral responses, is affected by Tr and E individually and is associated with increased antiviral resistance. However, our knowledge and understanding of Tr/E antiviral mechanisms remain limited, making it difficult to specifically pinpoint all the direct Tr/E targets during host/virus encounter. Hence, more research is warranted in the future to decipher all the specific mechanisms of Tr/E antiviral activity against HSV-2 and HIV-1.

Future directions

Overall, it would be important for the advancement of WAP and STIs fields if specific targets of direct and indirect antiviral activity of Tr/E are identified in the future. To this end, it would be recommended to test against a large number of plausible binding partners of Tr/E from each of the sides: viral (for both HSV-2 and HIV-1 and including viral nucleic acids and glycoproteins) and cellular (for both canonical and non-canonical binding receptors for each of the viruses). It would be important to preferentially use human primary genital ECs or tissue and Tr/E proteins of similar manufacturing source. It would be critical to demonstrate a direct interaction, or co-localization, between Tr/E and these potential binding molecules proposed above.

Additionally, it might be prudent to elucidate whether or not Tr/E alters conformation, rather than expression levels, of virus binding cellular receptors. Finally, it would be important to know whether the cell-associated antiviral activity of Tr/E also depends of the ability of cells to internalize Tr/E, in addition to what our studies already have shown for unblocked N-terminus and nuclear localization of E. In this regard, identifying specific receptor(s) for Tr/E will be of critical importance for many other potential Tr/E properties, such as binding of Tr/E to dsRNA. This interaction can be just as possible as Tr/E binding to LPS (McMichael et al., 2005b) or as was also shown for LL-37 to bind to dsRNA (polyI:C) (Hasan et al., 2011).

Furthermore, since our data showed that cell-associated anti-HIV-1 effect of Tr/E was cell type specific, additional studies to determine if such protection also depends on cellular ability to internalize Tr/E might be warranted. In this respect, it would be important to determine how such an uptake is mediated, either *via* any specific Tr/E receptor (that remains to be elusive) or an alternative mechanism.

Additionally, it might be worthwhile to investigate whether Tr/E can be used as a pre or post-exposure prophylactic measure for STIs (as a gel or suppositories). Tr/E can also be offered in the acute or chronic phases of infections (in IV bolus), especially HIV-1, to alter viral load and immune activation, as well as the development/progression of atherosclerosis and cardiovascular events as complications of antiretroviral therapy (Blot and Piroth, 2012), in light of earlier shown beneficial effects of Tr/E in atherosclerosis and good tolerability of intravenous administrations of E (Proteo Inc) (Shaw and Wiedow, 2011).

Conclusion and implications

Taken together, this research has identified several novel immunobiological properties of Tr/E that advance our understanding of these molecules as modulators of mucosal immunity against HSV-2 and HIV-1 in the female genital mucosa. Our findings have also established the presence of multiple levels at which antiviral activities of Tr/E against STIs in the female genital ECs can be executed.

The significance and implications of these studies can be largely determined by the fact that our observations confirm and vastly expand previously demonstrated beneficial properties of Tr/E against HIV-1. Additionally, the antiviral effect of Tr/E against HSV-2 is novel and has never been reported before. Overall, our data may have important clinical implications, considering our *in vitro* evidence that Tr/E can interfere with several steps of the pathogenesis of each HSV-2 and HIV-1 discussed earlier as well as HIV-1 clinical resistance. Due to the multifaceted mode of action and strategic presence of Tr/E at the interface between the hostile environment and the tissue,

including the FGT, our results, and the growing list of clinical trials with Tr/E application is further attesting to this notion.

It is clear that Tr/E are not a panacea against pathogens, including HSV-2 and HSV-1, as the antiviral activity of Tr/E is contextual and can be limited by unfavorable conditions. Moreover, Tr/E just as many other factors, likely do not act in solitude but rather in synergy with the rest of the protective armada in CVL and likely all mucosal fluids. Nevertheless, it is our hope that the demonstrated contribution of Tr/E to the host's antiviral activity, and especially to the natural anti-HIV-1 activity of the CVL, will be translated into more and broader prophylactic and therapeutic applications, or can be considered as inducible targets of such measures, to combat the spread of STIs.

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(For Chapters 1-6)

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Appendix I

War and Peace between WAP and HIV: Role of SLPI, Trappin-2, Elafin and ps20 in Susceptibility to HIV Infection



War and peace between WAP and HIV: role of SLPI, trappin-2, elafin and ps20 in susceptibility to HIV infection

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Abstract

Despite tremendous advances in our understanding of HIV/AIDS since the first cases were reported 30 years ago, we are still a long way from understanding critical steps of HIV acquisition, pathogenesis and correlates of protection. Our new understanding of the importance of the mucosa as a target for HIV infection, as well as our recent observations showing that altered expression and responses of innate pattern recognition receptors are significantly associated with pathogenesis and resistance to HIV infection, indicate that correlates of immunity to HIV are more likely to be associated with mucosal and innate responses. Most of the heterosexual encounters do not result in productive HIV infection, suggesting that the female genital tract is protected against HIV by innate defence molecules, such as antiproteases, secreted mucosally. The present review highlights the role and significance of the serine protease inhibitors SLPI (secretory leucocyte protease inhibitor), trappin-2, elafin and ps20 (prostate stromal protein 20 kDa) in HIV susceptibility and infection. Interestingly, in contrast with SLPI, trappin-2 and elafin, ps20 has been shown to enhance HIV infectivity. Thus understanding the balance and interaction of these factors in mucosal fluids may significantly influence HIV infection.

HIV

Since the first case report of HIV/AIDS 30 years ago, tremendous advances have been made in changing the course of this pandemic, particularly with antiretroviral drug treatments. However, we are still a long way from having effective HIV preventive measures in place. Indeed, our understanding of critical steps in HIV/AIDS acquisition and pathogenesis remains poor, and climbing rates of HIV cases further confirm this notion. Having multiple cell targets of HIV infection as well as routes of viral transmission with their physiological/structural differences also adds complexity to designing effective preventive measures. The lack of clear immunological correlates of protection against HIV, partly attributable to the enormous global diversity of the virus, is another challenge in HIV prevention.

We are now aware that HIV transmission occurs primarily at mucosal sites; acute HIV infection is followed by explosive virus replication in T and Langerhan's cells as well as massive depletion of CD4⁺ T-cells in the mucosa, especially in

the gut [1]. It is also clear now that HIV pathogenesis is driven by immune activation and high cell turnover [2-4]. Previously, the search for immune correlates in HIV was mainly focused on adaptive immune responses; however, in light of our new understanding of HIV pathogenesis, these correlates may be associated with mucosal and innate responses. Currently, the microbial translocation theory posits that immune activation in HIV is driven by a 'leaky' gut [5]. But our recent studies on CSWs (commercial sex workers) in Kenya indicated that untreated HIV infection is associated with significantly enhanced expression and responsiveness of innate PRRs (pattern-recognition receptors) that may be the penultimate driver of immune activation in HIV [6,7]. We also found that expression of selected PRRs was significantly altered in CMCs (cervical mononuclear cells) and CECs (cervical epithelial cells) of HIV-R (HIV-resistant) compared with HIV-S (HIV-susceptible) and HIV-P (HIV-positive) CSWs (X.D. Yao, R.T. Lester, R.W. O'Keefe, B.M. Henrick, W. Jaoko, C. Wachibi, T.B. Ball, F.A. Plummer and K.L. Rosenthal, unpublished work). These observations further support our hypothesis that altered innate viral recognition and responsiveness at mucosal sites might be critical events, perpetuating immune activation and disease progression or resistance to HIV/AIDS.

It is still debatable whether ECs (epithelial cells) represent a direct target for HIV infection, regardless of their anatomical location. What is clear, however, is the significant role of genital ECs in early steps of HIV transmission. Indeed, the ability of genital ECs to directly recognize and respond

Keywords: AIDS, elafin, HIV, secretory leucocyte protease inhibitor (SLPI), trappin-2, whey acidic protein (WAP), whey acidic protein four-distal core domain (WFDC domain).

Abbreviations used: CSW, commercial sex worker; CVI, cervico-vaginal lavage sample; dsDNA, double-stranded DNA; E, elafin; EC, epithelial cell; FDC, four-distal core; FG, female genital tract; HIV-R, HIV-resistant; HIV-S, HIV-susceptible; ICAM-1, intercellular adhesion molecule 1; IFN α , interferon α ; IL, interleukin; IFA-1, lymphocyte function associated antigen 1; MDA-5, methylcytosine diarsine 5; poly(I:C), poly inosine-polycytidylic acid; PRR, pattern recognition receptor; ps20, prostate stromal protein 20 kDa; RIG-1, retinoic acid-inducible gene 1; rtr/rf, recombinant trappin-2 and elafin; SLPI, secretory leucocyte protease inhibitor; TNF α , tumour necrosis factor α ; T, trappin-2; WAP, whey acidic protein; WFDC, WAP FDC.

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to HIV may largely predetermine the outcome of the host–pathogen interaction [8,9]. Recent data indicate that women are disproportionately affected by this infection; however, most of the heterosexual encounters do not result in productive HIV infection [10]. This finding suggests that the FGT (female genital tract) is protected from HIV by endogenous defense molecules secreted mucosally and by multiple cells types, including ECs. In fact, we now know that CVLs (cervico-vaginal lavage samples) from healthy women show a pronounced activity against HIV and HSV (herpes simplex virus) that is attributed to the presence of cationic polypeptides [11–13]. These protective innate factors belong to different groups, encompassing human defensins [14], cathelicidins [15] and antiproteases [16–19]. Although members of these groups share limited homology in structure, they are closely related in their broad antimicrobial and immunomodulatory properties.

General characteristics of antiproteases SLPI (secretory leucocyte protease inhibitor), Tr (trappin-2) and E (elafin)

The group of neutrophil serine protease inhibitors includes nearly 20 members, but the nature and properties of SLPI, Tr and E are characterized the most. All three, SLPI, Tr and E, belong to the WAP (whey acidic protein) family of polypeptides that share a domain with eight characteristic cysteine residues that form four disulfide bonds, thus the name ‘four-disulfide core’ domain [20]. The FDC (four-disulfide core) domain is also called a WAP domain, as it is similar to a whey acidic protein, initially identified from rodent milk [21]. Hence FDC-containing proteins from a WAP group are often referred to as WFDC (WAP FDC) proteins. However, note that it is not only WAP proteins that have an FDC domain [20,22], hence calling all proteins with an FDC domain WFDC proteins is inaccurate.

SLPI is a non-glycosylated cationic 11.7 kDa protein of 107 amino acids and two WAP domains, as reviewed in [23]. In the early 1970s, SLPI was originally isolated from bronchial secretions [24] and was later found to have antiprotease, antimicrobial and anti-inflammatory properties and to be present in different cell types and locations, including FGT [22,23]. SLPI shows inhibitory activity against neutrophil serine proteases cathepsin G and neutrophil elastase that appears to be linked to the second WAP domain. Tr is another unglycosylated protein of 95 amino acids (9.9 kDa) that has E, as a C-terminal WAP domain of 57 amino acids (5.9 kDa) [25] and an N-terminal cementoin domain of 38 amino acids. Tr and E exhibit inhibitory properties against neutrophil serine proteases elastase and proteinase 3 [23]. E is generated by proteolytic cleavage of the N-terminus cementoin domain of Tr by mast cell tryptase [26], and appears to share 40% homology with SLPI [23].

Serine antiproteases SLPI, Tr and E are detectable in both serum and mucosal secretions [27], and are present in many cell types and tissues [28], including genital ECs, where they

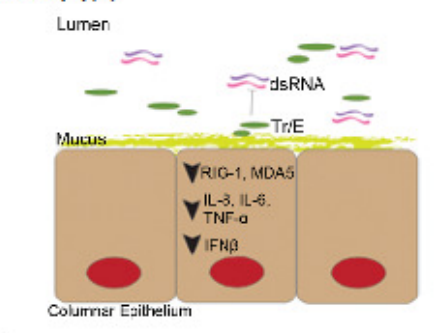
are secreted in response to pro-inflammatory stimuli [16]. Interestingly, we recently confirmed and extended a previous observation [16] by showing that human endometrial cells HEC-1A secreted both Tr and E in response to a synthetic mimic of viral dsRNA (double-stranded RNA), poly(I:C) (polyinosine-polycytidylic acid) (A.G. Drannik, K. Nag, X.-D. Yao, B.M. Henrick, J.-M. Sallenave and K.L. Rosenthal, unpublished work). Since dsRNA is considered to be a by-product of many, if not all, virus infections [29], our finding may indicate that in the presence of viral antigens, both Tr and E are present in CVL and act against viruses in the FGT.

Similar to SLPI, Tr and E primarily function to protect tissues from excessive proteolysis due to neutrophil activation [30,31] and multiple pathogens via direct or indirect mechanisms [16,32–35]. Anti-inflammatory features of these serine antiproteases were also extensively characterized, but primarily in response to bacterial antigens. Specifically, SLPI, Tr and E were shown to reduce the expression of pro-inflammatory factors as a result of their inhibitory effect on NF- κ B (nuclear factor κ B) and AP-1 (activating protein 1) by altering I κ B (inhibitor of NF- κ B) activation [36] and proteasomal degradation [37] respectively. Worth mentioning is that we also showed recently that both Tr and E were capable of reducing pro-inflammatory factors IL-8 (interleukin-8), IL-6 and TNF α (tumour necrosis factor α) as well as antiviral IFN β (interferon β) in response to poly(I:C) (Figure 1). Our results also demonstrated that Tr and E significantly inhibited VSV (vesicular stomatitis virus)-GFP (green fluorescent protein) replication, directly or indirectly, and enhanced poly(I:C)-induced antiviral protection (A.G. Drannik, K. Nag, X.-D. Yao, B.M. Henrick, J.-M. Sallenave and K.L. Rosenthal, unpublished work). Interestingly, enhanced antiviral protection was not associated with higher induction of IFN β in the present study. We showed further that reduced inflammatory factors in the presence of Tr and E were associated with lower expression of innate viral sensors RIG-I (retinoic acid-inducible gene I) and MDA-5 (methylene dianiline 5; Figure 1). We believe this was the first evidence of Tr and E affecting host innate recognition and modulation of antiviral and inflammatory responses in genital ECs that is significant for health and disease of the female reproductive tract (A.G. Drannik, K. Nag, X.-D. Yao, B.M. Henrick, J.-M. Sallenave and K.L. Rosenthal, unpublished work). More recently, however, dual immunomodulatory properties of Tr and E were also reported; that is, depending on the environment, Tr and E can either dampen inflammation [37,38] or promote immunostimulatory events and prime the immune system [39,40], suggesting that we still do not understand all the conditions and factors regulating Tr and E functions.

Anti-HIV activity of SLPI, Tr and E

Interest in SLPI, Tr and E continues to grow as more evidence is accumulated on their anti-HIV properties [16,19,41]. Initially, it was discovered that low HIV transmission through

Figure 1 | Tr and E inhibit the expression of innate viral sensors RIG-I and MDA-5 as well as pro-inflammatory and anti-viral mediators in genital epithelial cells in response to a mimic of viral dsRNA, poly(I:C).



saliva and CVL was correlated with high levels of SLPI in the mucosal fluids. Subsequently, the potent anti-HIV activity of SLPI was shown to be independent of its antiprotease activity and mediated through its direct effect on cells, but not HIV [41]. Specifically, two potential modes of anti-HIV action of SLPI have been described to date: (i) SLPI interferes with HIV fusion with the T-cell plasma membrane through binding to scramblase 1, a membrane protein that interacts with CD4 and controls the movement of the phospholipid bilayer of the plasma membrane [42]; (ii) SLPI blocks viral entry/fusion with a myeloid cell as a result of binding to annexin II, a macrophage receptor for a phosphatidylserine moiety that HIV carries on its outer coat on exiting from an infected cell [34]. Interestingly, SLPI appears to be protective against HIV only in models using monocytes/macrophages and T-cells, but not genital ECs, despite the fact that SLPI is also secreted by genital ECs [43]. The lack of protection on ECs could suggest that anti-HIV activity of SLPI might be limited or predetermined by the cell or a receptor type, on which HIV is acting. In other words, SLPI might have differential ability to block HIV receptors, depending on their types, cell distribution and SLPI affinity to them.

Considering the previously demonstrated resemblance between SLPI, Tr and E in structure and function, one would anticipate that anti-HIV features of the antiproteases would also be similar. Indeed, E was recently identified as a biomarker of HIV resistance in CSWs in Kenya [16,19,32,41]. Iqbal et al. have demonstrated using SELDI-TOF (surface-enhanced laser-desorption ionization-time-of-flight) MS that expression of a 6 kDa protein E was significantly higher in CVLs of HIV-R CSWs, compared with HIV-S controls [19]. Although no specific mechanism of action was proposed, in their patent the investigators showed that recombinant E prevented the infection of T-cells *in vitro* [44]. Interestingly, however, when HIV-S CSWs were followed prospectively, the authors reported that elevated levels of both Tr and E were associated with HIV protection. This finding indicates

Table 1 | Functions of SLPI, Tr, E and ps20 in addition to their antiprotease activity

Action	Reference(s)
SLPI	
Antibacterial, antifungal, antiviral (HIV), anti-inflammatory, tissue repair	[22-24,32,34,36,49]
Tr and E	
Antibacterial, antifungal	[22,23,33,35-42,44,49]
Anti-inflammatory, tissue repair, priming innate system	
Antiviral (HIV)	[16,19]
Antiviral (VSV, HIV), inhibition of viral innate sensors	(A.G. Drannik, X.-D. Yao, B.M. Henrick, K. Nag, S. Jain and K.L. Rosenthal, unpublished work)
ps20	
Growth inhibition, pro-angiogenic, pro-viral (HIV), cell recruitment, migration, adhesion, cancer suppressor, regulation of extracellular matrix	[22,45-49]

that both proteins might be required for optimal protection against HIV [19], which warrants additional investigation in the future. Recently, another group has also shown a protective effect of E against HIV and suggested that it was the result of a direct effect of E on HIV [16].

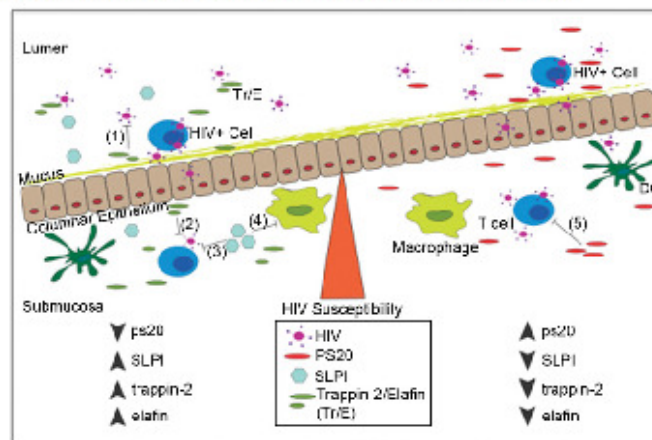
In agreement with the above data, we also showed recently that rTr/rE (recombinant Tr and E) each independently inhibited HIV attachment and transcytosis across human genital ECs *in vitro*. Treatment of HIV or cells with rTr/rE significantly decreased virus attachment to ECs, suggesting direct and indirect mechanisms of anti-HIV activity of Tr and E (A.G. Drannik, X.-D. Yao, B.M. Henrick, K. Nag, S. Jain and K.L. Rosenthal, unpublished work). Furthermore, we also confirmed significantly higher expression of Tr and E in CVLs of HIV-R, and this was associated with reduced mRNA of TLR-2, TLR-4 and RIG-I in ECs from HIV-R compared with HIV-S, as determined by quantitative reverse transcription-PCR. Collectively, these findings highlight important and multifaceted role(s) of Tr and E in innate viral recognition and ultimately protection against HIV in FGT.

ps20: an HIV-enhancing factor

Recently, another serine antiprotease was implicated in HIV [45,46]. Similar to SLPI, Tr and E, ps20 (prostate stromal protein 20 kDa) belongs to the WAP family of proteins that share FDC domain [46] and participates in wound repair and cell migration. However, in contrast with SLPI, Tr and E, ps20 does not possess antiprotease activity and has also been identified as a pro- or enhancing HIV factor, or marker of HIV permissiveness of CD4⁺ memory T-cells. Human ps20 was initially purified from the prostate gland stroma

Figure 2 | The HIV-modulating effect of WFDC family proteins Tr, E, SLPI and ps20

(1) Tr and E inhibit HIV transcytosis through a monolayer of genital ECs by reducing attachment of virus to ECs due to direct anti-HIV and indirect effects of Tr and E (2) E inhibits HIV infection of CD4⁺ T-cells by potentially binding to HIV binding sites on T-cells (3) SLPI inhibits HIV infection of macrophages by blocking binding between PS (phosphatidylserine) and annexin II (4) SLPI inhibits HIV infection of CD4⁺ T-cells by preventing binding between HIV and CD4 through the formation of SLPI-scramblase complexes (5) ps20 enhances HIV infection of CD4⁺ T-cells by promoting HIV entry via fusion as a result of increased expression of cell-surface CD54 and thus increases cell-free and cell-cell spread of HIV.



as a growth inhibitor and a correlate of stromal-epithelial cell differentiation and interaction [47,48]. Primary biological functions of ps20 relate to cancer inhibition, angiogenesis, regulation of extracellular matrix, cell migration and wound repair [47–49]. Interestingly, HIV appears to make ps20 a Trojan horse. Specifically, HIV utilizes the ability of ps20 to affect cell–cell adhesion and migration through the activation of the LFA-1 (lymphocyte function-associated antigen 1)/CD54 integrin pathway by up-regulating expression of CD54 [ICAM-1 (intercellular adhesion molecule 1)], thus making CD4⁺ memory T-cells more susceptible to infection. This integrin pathway has been shown to be important for normal cell conjugation. Hence, its activation promotes cell–cell adhesion and more efficient intercellular HIV transfer and entry into CD4⁺ T-cells [46].

A WAP protein axis in HIV?

From the evidence presented in the present review, it is clear that, despite having common familial roots, SLPI, Tr, E and ps20 exhibit differential functions in the context of HIV (Table 1). With protective anti-HIV effects of SLPI, Tr and E on the one hand and pro-HIV action of ps20 on the other, one might wonder whether HIV susceptibility and infection could be the result of imbalanced ratios of WAP proteins at routes of viral entry. Could there be an axis, or a yin yang, in WAP proteins and susceptibility to HIV infection

(Figure 2)? The answer to this question is unclear at the moment, since we still have limited understanding of how these serine antiproteases interact and whether their targets and mechanisms of actions overlap. In other words, we do not really know whether SLPI, Tr and E can regulate the expression of adhesion molecules, such as ICAM-1 and LFA-1, and whether SLPI, Tr and E can negate the enhancing effect of ps20 on HIV by simply changing the expression of the integrins. Clearly, the ubiquitous expression of SLPI, Tr, E and ps20, their contribution to host homeostasis and the emerging evidence of their role in HIV infection underscore an urgent need for more research in future on the role of these molecules in HIV. It might be important to know the patterns and ratios of expression between SLPI, Tr, E and ps20 in mucosal fluids or the same HIV target cells, and whether there is a relationship between altered, or imbalanced, homeostatic expression of SLPI, Tr, E and ps20 and HIV susceptibility. In addition, it might be of significance to know whether ps20 is secreted mucosally and whether this enhancing effect of ps20 on HIV can be relevant to ECs, given the primarily mucosal/sexual nature of HIV transmission.

Concluding remarks and future directions

The present review highlights the significance of innate mucosal factors SLPI, Tr, E and ps20 in mediating HIV susceptibility and infection. Building on our current but

limited understanding of the inhibitory effects of Tr, E and SLPI versus the enhancing effects of ps20 on HIV, we propose a potential model of how these innate factors may influence HIV transmission in the FGT through their modulation of viral recognition and mounting of antiviral immune-inflammatory responses (Figure 2).

Although recent efforts in HIV vaccine trials provide a glimmer of hope that an effective prophylactic vaccine can be developed, it may take a decade before it becomes widely available [50]. Therefore efforts to continuously advance our understanding of the role of innate mucosal factors or biomarkers of resistance to HIV, such as the WAP proteins, will inform mucosal HIV vaccine development.

Acknowledgements

We thank K. Nag, X.-D. Yao, S. Jain (all at McMaster University) and J.-M. Sallenave (Institut Pasteur, Paris, France) for research assistance and the expert technical support of J. Newton and A. Patrick (both at McMaster University).

Funding

This work was supported by a grant as part of the Comprehensive T Cell Vaccine Immune Monitoring Consortium (CTC-VIMC), a key component of the Collaboration for AIDS Vaccine Development (CAVD) funded by the Bill and Melinda Gates Foundation. A.G.D. was supported by a studentship from the Ontario HIV Treatment Network (OHTN) and K.L.R. was supported by a Career Scientist Award from the OHTN.

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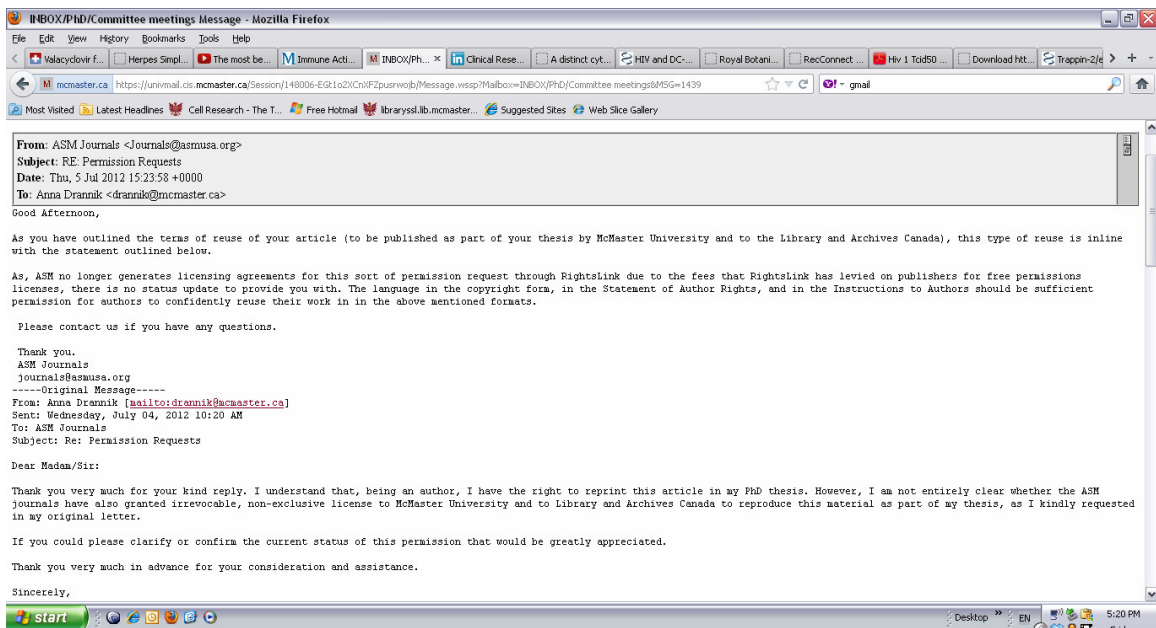
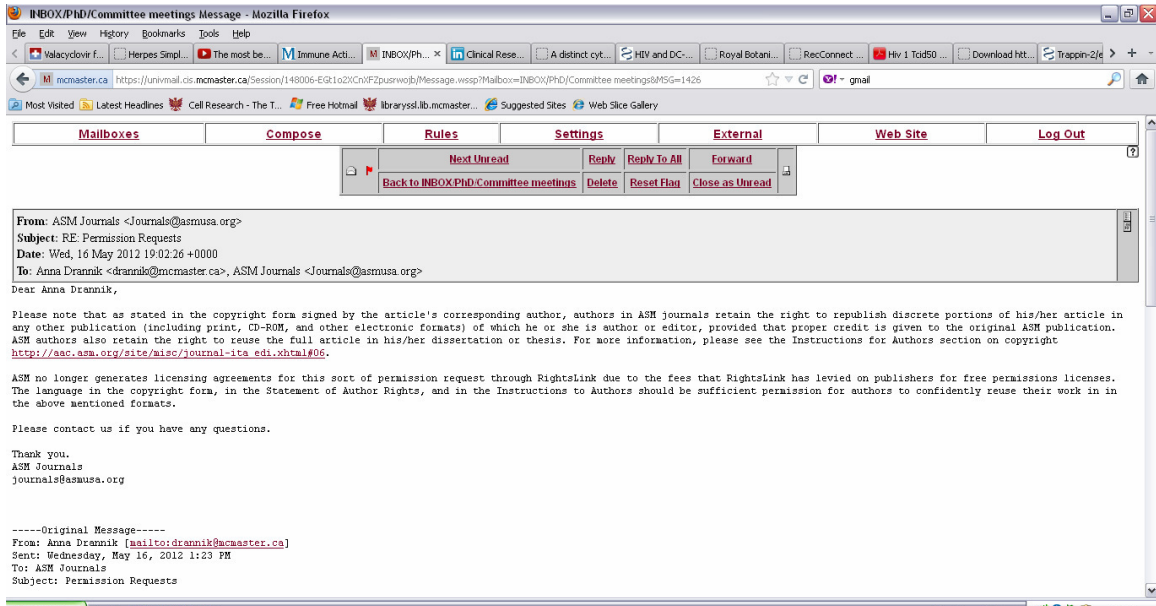
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Received 17 June 2011
doi:10.1042/BS10391427

Appendix II

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Anna Drannik
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16 May 2012

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