Retrocyclin, A Potent Hiv-1 Entry Inhibitor

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by

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ABSTRACT

Human immununodeficiency virus (HIV) infection is the leading cause of death due to viral infections worldwide. In the absence of an effective vaccine or consistent male condom use, there is a clear need for female-controlled preventatives such as topical vaginal microbicides. Recent attention has been focused on developing natural antimicrobial peptides, as anti-retroviral microbicides. Increasing evidence suggests that cationic antimicrobial peptides such as defensins are effective HIV-1 inhibitors. Human alpha- and beta-defensins contribute substantially to innate immune defenses against microbial and viral infections. Certain nonhuman primates also produce theta-defensins – 18 residue cyclic peptides that are potent HIV-1 entry inhibitors. Multiple human theta-defensin genes exist, but they harbor a premature termination codon that blocks translation. Consequently, the theta-defensins (retrocyclins) encoded within the human genome are not expressed as peptides. *In vivo* production of theta-defensins in rhesus macaques involves the post-translational ligation of two nonapeptides, each derived from a 12-residue "demidefensin" precursor. Neither the mechanism of this unique process nor its existence in human cells is known. To ascertain if human cells retained the ability to process demidefensins, we transfected human promyelocytic cells with plasmids containing repaired retrocyclin-like genes. The expected peptides were isolated, their sequences were verified by mass spectrometric analyses, and their anti-HIV-1 activity was confirmed *in vitro*. Our study reveals for the first time, to our knowledge, that human cells have the ability to make cyclic theta-defensins. Given this evidence that human cells could make theta-defensins, we attempted to restore endogenous expression of retrocyclin peptides. Since human theta-defensin genes are transcribed, we used aminoglycosides to read-through the premature termination codon found in the mRNA transcripts. This treatment induced the production of

intact, bioactive retrocyclin-1 peptide by human epithelial cells and cervicovaginal tissues. The ability to reawaken retrocyclins genes from their 7 million years of slumber using aminoglycosides could provide a novel way to secure enhanced resistance to HIV-1 infection.

Our studies on retrocyclin reveal that they are potential candidates to develop as topical vaginal microbicides to prevent sexual transmission of HIV-1. Mucosal surfaces of the vagina are the portals for heterosexual transmission of HIV-1 and therefore play a fundamental role in the pathogenesis of primary infection. In a search for direct biological evidence for the role of human vaginal fluid in innate host defense, we characterized the anti-HIV-1 function of cationic polypeptides within minimally manipulated vaginal fluid. In our studies, we revealed that vaginal fluid confers intrinsic anti-HIV-1 properties against both X4 and R5 strains of HIV-1, and could protect against HIV-1 infection and reduce proviral genome integration in organotypic cultures of human cervicovaginal tissue. The majority of this activity was contained in the cationic polypeptide fraction, and the depletion of cationic polypeptides using a selective cation-exchange resin ablated most of the intrinsic activity against HIV-1. By adding the cationic polypeptide fraction to depleted vaginal fluid, we were able to restore activity against HIV-1. Using a proteomic approach, we identified 18 cationic polypeptides within vaginal fluid, nearly all of which are either known antimicrobials or have other purported roles in host defense. Interestingly, physiologic concentrations of 13 of the cationic polypeptides were alone not active against HIV-1, yet in concert they partially restored the anti-HIV-1 activity of cation-depleted vaginal fluid. These results suggest that synergism between cationic polypeptides is complex and full anti-HIV-1 activity likely involves the aggregate of the cationic peptides and proteins in the acidic human vaginal fluid. Interestingly, retrocyclins retained complete anti-HIV-1 activity

in the presence of human vaginal fluid. Therefore expression of retrocyclin peptides can help activate the natural defense mechanism against HIV-1.

We next investigated the regulation of expression of retrocyclin (pseudo)gene. We identified a putative interferon response cluster upstream of the retrocyclin gene. The activity of this cluster was upregulated when treated with IFN-β although to a modest extent. Interestingly, the cluster also contained the binding site for an Interferon Consensus Sequence Binding Protein (ICSBP), a known repressor of the IFN inducible genes. Deletion of the ICSBP site or addition of a known inhibitor of ICSBP resulted in the increase in the activity of the cluster, indicating a role for ICSBP in the negative regulation of expression of retrocyclins. Collectively our data suggest that the expression of this ancestral gene is tightly regulated in both a positive and negative manner via the IFN response pathway.

To my Amma and Appa

Mrs. C.Umadevi and Mr. L.Venkataraman

&

in loving memory of my Grand uncle Dr. A. Nagarathinam.

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CHAPTER ONE: GENERAL REVIEW

1.1 Need for topical microbicides against HIV-1

The World Health Organization estimates that 15.5 million women were infected with HIV by the end of 2007, which represents about 50% of total people with HIV worldwide [1,2]. Heterosexual intercourse continues to be the predominant mode of transmission of this pandemic and women are more susceptible to HIV-1 infection than men [3-5]. Although the current HIV treatments reduce mortality and morbidity, access to these medications are not available in many developing and underdeveloped countries. In the absence of effective treatments or vaccines for HIV, many of the current efforts are aimed at developing new measures to prevent transmission of HIV. Condoms are an effective preventive measure but their use is often not negotiable by women due to socio-economic and cultural reasons. Effective female-controlled prophylactic measures that can protect against HIV-1 infection are needed. Topical microbicides, which can be used by the receptive partner, represent a new avenue for the prevention of sexual transmission of HIV.

1.2 Update on candidate microbicides

Much attention and effort has been given to developing detergents (benzalkonium chloride or octoxynol-9 or sodium lauryl sulfate or nonoxynol-9/N-9) that function as a spermicide as well as a microbicide [6-8]. The most promising amongst them nonoxynol-9/N-9, however, failed the clinical trials due to its detrimental effect on human epithelium [9-15]. Currently, there are about 29 diverse candidate microbicides in development which can be broadly categorized as i) agents that disrupt the viral envelope, ii) agents that inhibit entry or fusion of the virus, and iii) agents that enhance the normal vaginal defenses.

Several natural and synthetic polymers that can directly or indirectly disrupt the viral envelope are currently being tested. These include anionic polymers or surfactants that contain sulfated esters of polysaccharides (Example: dextran sulfate/Emmelle™ [16-18], cellulose sulfate/Ushercell™ [19,20], Carrageenan/ Carraguard™ [21-28]) or sulfonated benzene or naphthalene (Polystyrene sulfonate, Polynaphtalene sulfonate/PRO2000 [29-36]) or polycarboxylates (Carbomer974P /BufferGel™ [37-41]) and cellulose acetate phthalates [42,43]. However, some of the disadvantages of using anionic polymers as topical microbicides are that they are not broad-spectrum in their activity, they induce proinflammatory cytokines, they have a high toxicity index, they disturb the natural microflora, and they induce ulceration of the vaginal epithelium [44-48]. Natural plant extracts such as Praneem™ that exhibit anti-retroviral activity are under preclinical development. Although Phase-II efficacy trials of this herbal extract have been promising so far, the toxicological and long term effects of the repeated use of these formulations on reproductive health has yet to be evaluated [49-52].

The second category of anti-HIV-1 microbicides that are currently in the pipeline undergoing preclinical investigations include viral fusion inhibitors which block the viral proteins or host receptors (CD4, CCR5 or CXCR4) utilized by the virus for entry. Peptidic ligands for receptors CD4 (PRO 542) [53,54] or CCR5 (Maraviroc [55-59], PRO-140 [60-62], PSC-RANTES [63-65]) or CXCR4 (T22, T134, ALX40-4C, CGP 64222) [66,67] show promising anti-HIV-1 activity. An important factor to be considered here is that these chemokine receptors have other functions in the body, which if blocked may lead to undesirable side effects. Therefore, fusion inhibitors that block viral proteins are likely better compounds to develop as microbicides than those that target

the host. Many fusion inhibitors (cyanobacterial lectin Cyanovirin-N [68-73], Enfuvirtide (T20) and its derivatives [74-81]) that block gp41 and prevent entry of the virus to the host membrane have undergone clinical testing.

A more recent 'probiotic' method that acts as fortifiers of vaginal host defense is the use of capsules of *Lactobacilli*, the most common vaginal bacteria. Certain strains of *Lactobacilli sp.* release lactic acid and hydrogen peroxide which can inactivate HIV-1. Epidemiological studies have shown a correlation between increased colonization of lactobacillus and decreased HIV-1 transmission. Bio-engineered Lactobacilli that can express microbicides that target HIV-1 and serve as mucosal drug delivery systems are currently being explored [82-86]. The development of an effective microbicide against HIV-1 also depends on our emerging knowledge of the sexual transmission of the virus.

1.3 Transmission of HIV from the mucosal surface to target cells

Sexual transmission of HIV is predominantly through the mucosal surfaces, particularly the vaginal and rectal mucosa [4]. Underlying the vaginal and rectal mucosa there are dendritic cells, macrophages and T-cells that express CD4 receptor, a primary HIV-1 receptor. Apart from the CD4 receptor, the virus also utilizes secondary chemokine co-receptors CCR5 and CXCR4 to gain entry into target cells. The target cells express more of the CCR5 receptor than CXCR4 receptor on the cell surface. Consequently, R5 (M-tropic) strains of HIV-1, which utilize CCR5 as a co-receptor for entry, are the predominantly sexually transmitted strains [87-98]. Co-receptor usage plays a critical role in HIV-1 disease progression and HIV-1 transmitted *in vivo* is generally M-tropic, but later a tropism switch may occur in many indivduals to X4 (T-tropic) strains, which use

CXCR4 as a co-receptor (Reviewed in [99]). There are several strains of HIV due to the error prone nature of reverse transcriptase. Based on the genetic similarities of these strains they are classified into 3 major groups: Major group (M), Outlier group (O) and New group (N) (Reviewed in [100]). More than 90% of HIV-1 infections belong to HIV-1 group M, while Group O appears to be restricted to west-central Africa and group N, isolated in 1998 in Cameroon, is extremely rare. Within group M there are atleast 9 different genetically distinct subtypes or clades of HIV-1 (subtypes A, B, C, D, F, G, H, J and K) [100-102]. Subtype B has been the most common subtype that has been isolated from many parts of the world [101]. An individual can be infected with more than one subtype of HIV-1 and occasionally, the two viruses of different subtypes can hybridize to create a more virulent recombinant virus [102-104]. Moreover, it has been observed that certain HIV-1 subtypes are predominantly associated with a specific mode of transmission. In particular, subtype B is spread mostly by homosexual contact and intravenous drug use (essentially via blood), while subtype C was associated with heterosexual epidemics (via a mucosal route) [105]. Whether there are biological causes for the observed differences in transmission routes remains a subject of debate.

The mechanism by which the virus shuttles across the mucosal epithelium and infects the underlying target cells is not clear. One of the proposed mechanisms involves M cells, specialized cells present in the gastrointestinal and rectal epithelium that aid in delivering the virus across the tight epithelial layer. The dendritic cells (DCs) can directly bind the HIV-1 envelope protein gp120 via a C-type lectin DC-SIGN and shuttle the virus across the epithelial layer in order to deliver it to the underlying CD4+ T-cells [106,107].

The fusion of HIV into target cells involves the binding of the host CD4 receptor and appropriate secondary receptor to the viral envelope glycoproteins gp120 and gp41 subunits. The mechanism of viral fusion involves two helical regions of gp41, an N-terminal heptad repeat (HR1) and a C-terminal heptad repeat (HR2) [108-110]. The HR1 and HR2 helical regions form a six-helix-bundle, in which three α-helices formed by HR2 peptides pack in an antiparallel manner against a central HR1 homotrimer. This six-helix bundle aids and stabilizes the formation of a pore on the host cell membrane, which allows the transfer of viral genome and proteins into the host cells [109,110]. Despite a wealth of knowledge on HIV propagation / infection of immune cells, early events from the breach of vaginal mucosa to the establishment of infection are poorly understood [4]. Moreover, the vaginal mucosa is a complex environment consisting of numerous endogenous antimicrobial components that contribute to innate host defense.

1.4 Endogenous antimicrobial components of vaginal mucosa

Cationic antimicrobial proteins are integral components of the vaginal mucosa that contribute to the innate antiviral activity. Many antimicrobial peptides including lysozyme, lactoferrin, calprotectin, secretory leukocyte protease inhibitor (SLPI), human neutrophil peptides (HNP-1, -2 & -3) and β-defensins [111,112], are released into the vaginal fluid by the epithelial cells and neutrophils, and likely contribute to the innate defense of this surface.

Although reported evidence is available for the activity of vaginal fluid against pathogens, attention to its role in protecting against HIV-1 virus has been given only recently. Several studies have analyzed the antiviral properties of the three most abundant proteins on mucosal surfaces: lysozyme, lactoferrin and SLPI. Lysozyme and lactoferrin have been shown to reduce the

absorption and penetration of HIV-1 virus *in vitro* [113,114] although the activity was only modest. Recent data suggests that SLPI, secreted by the mucosal epithelial and acinar cells, blocks HIV-1 infection of monocytes and T cells by preventing internalization of the virus [114-117]. Several reports have shown that the level of SLPI is reduced in vaginal fluid of HIV-infected persons. However, other reports suggest that SLPI has no effect on HIV replication [118], and this debate persists.

Other known effectors of natural host defense present in vaginal mucosal secretions are defensins, a large family of cationic antimicrobial peptides. Defensins are the most widely studied family of antimicrobial peptides. They contain six cysteines that form three disulphide- bridges and are predominantly composed of β-sheets. Based on the position of the cysteines and the intramolecular disulphide bonds, defensins are grouped in to three subfamilies: α-defensins, β-defensins and θ-defensins [119-127]. Alpha-defensins (HNP-1 to -3) inhibit HIV-1 replication *in vitro* by two mechanisms: in the absence of serum, they inhibit HIV-1 replication before viral integration in $CD4^+$ T cells, and in the presence of serum, they interfere with the signaling pathways on target cells and block the nuclear import and transcription of the HIV-1 genome [128-130]. Human β-defensins have been shown to inhibit HIV-1 replication by modulating the CXCR4 co-receptor and also by interacting directly with the HIV-1 virions [131]. It is to be noted that many of the above mentioned studies analyzed the effect of only one or two antimicrobial peptides, and thus the synergistic activity of these peptides in a biologically relevant system was not explored. Using an integrated proteomic approach we identified the cationic antimicrobial peptides of vaginal fluid. Further we demonstrated that these peptides contribute to the innate anti-retroviral activity of vaginal fluid [132] (**Chapter 3**).

Despite the presence of these numerous antiviral peptides, HIV-1 transmission is on the rise. In our efforts to develop effective therapeutics against this virus, we have focused our attention on the third subclass of defensins, known as theta-defensins. Synthetic human theta-defensins (retrocyclins) were found to be potent inhibitors of HIV-1 entry [120,128,133-135], and are the subject of **Chapter 2**.

1.5 Retrocyclins: a potent anti-retroviral peptide

Theta-defensins are the most recently identified defensin subfamily, isolated initially from leukocytes and bone marrow of the rhesus monkey *Macacca mulatta* [136]. Theta-defensins are expressed in Old World monkeys and orangutans; however θ-defensin genes (DEFT) in humans are pseudogenes [134]. A premature termination codon mutation in the signal peptide region prevents translation of human DEFT mRNA [134]. The structure of a human DEFT pseudogene and predicted cDNA is shown in **Figure 1.1A**. Multiple copies of the human DEFT pseudogenes, likely derived by gene duplication, are located in the chromosome 8p23 region which is consistent with the location of many α-defensin and β–defensin genes [134,137,138]. Phylogenetic analysis reveals that DEFT genes may have been derived by mutation of α-defensin genes (DEFA) in Old World monkeys and must have arisen during evolution after the orangutan and human lineages diverged. Interestingly, orangutans have both the intact DEFT gene and the DEFT pseudogenes in their genome [134]. Sequence homology between orangutan alpha defensin (DEFA) and human theta-defensin gene (DEFT) is shown in the **Figure 1.1B**.

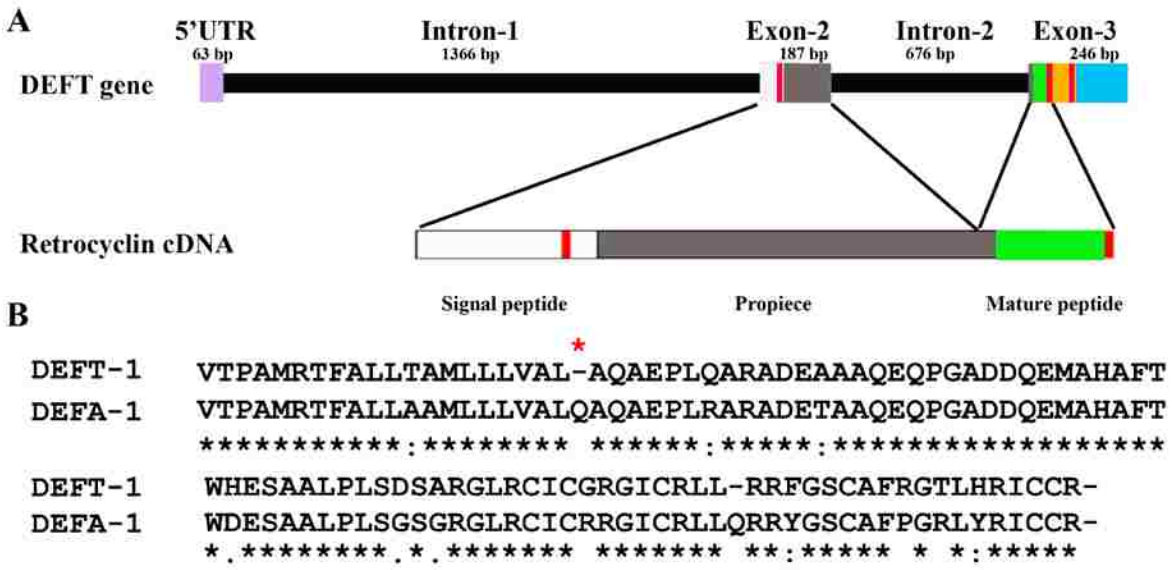

Figure 1.1: Structure of Human DEFT-pseudogene. A) Structure of Human DEFT-1 pseudogene showing the relative length and position of the 3 exons (5'UTR, Exon-2 and Exon-3) and 2 introns. The red lines represents termination codon, orange block represents truncated C-terminal domain of alpha-defensin and the blue block represents the 3' region of the pseudogene. B) An alignment of translated human DEFT-1 pseudogene and alpha-defensin coded by DEFA-1 of orangutan. "-"represents termination codons. The * indicates the mutated 17th residue with a termination codon in DEFT-1 and a glutamine (Q) in DEFA-1.

The theta-defensin nascent peptides in rhesus monkeys (RTD) undergo extensive post-translational modification to give rise to the biologically active, mature theta-defensin peptide [136,139,140]. Mature theta-defensins are composed of 18 residues created by the processing and subsequent fusion of two precursor propeptides. Neither the exact mechanism nor the succession of events in this complex pathway is known. Each of the two precursor propeptides generates a nonapeptide containing three cysteines contributing to the three disulphide-bonds in the mature peptide. Also, the two processed nonamers undergo mutual head-to-tail fusion resulting in a macrocyclic final product. There are two types of human theta-defensin pseudogenes that encode two different

nonapeptides (DEFT-1 and DEFT-4) as indicated in **Figure 1.2**. If the premature termination codon in these genes were restored to a conserved glutamine, then there would be three possible retrocyclin peptides that could be expressed from the two precursor polypeptides. These possible retrocyclin peptides and their putative post-translational processing are illustrated in **Figure 1.2**.

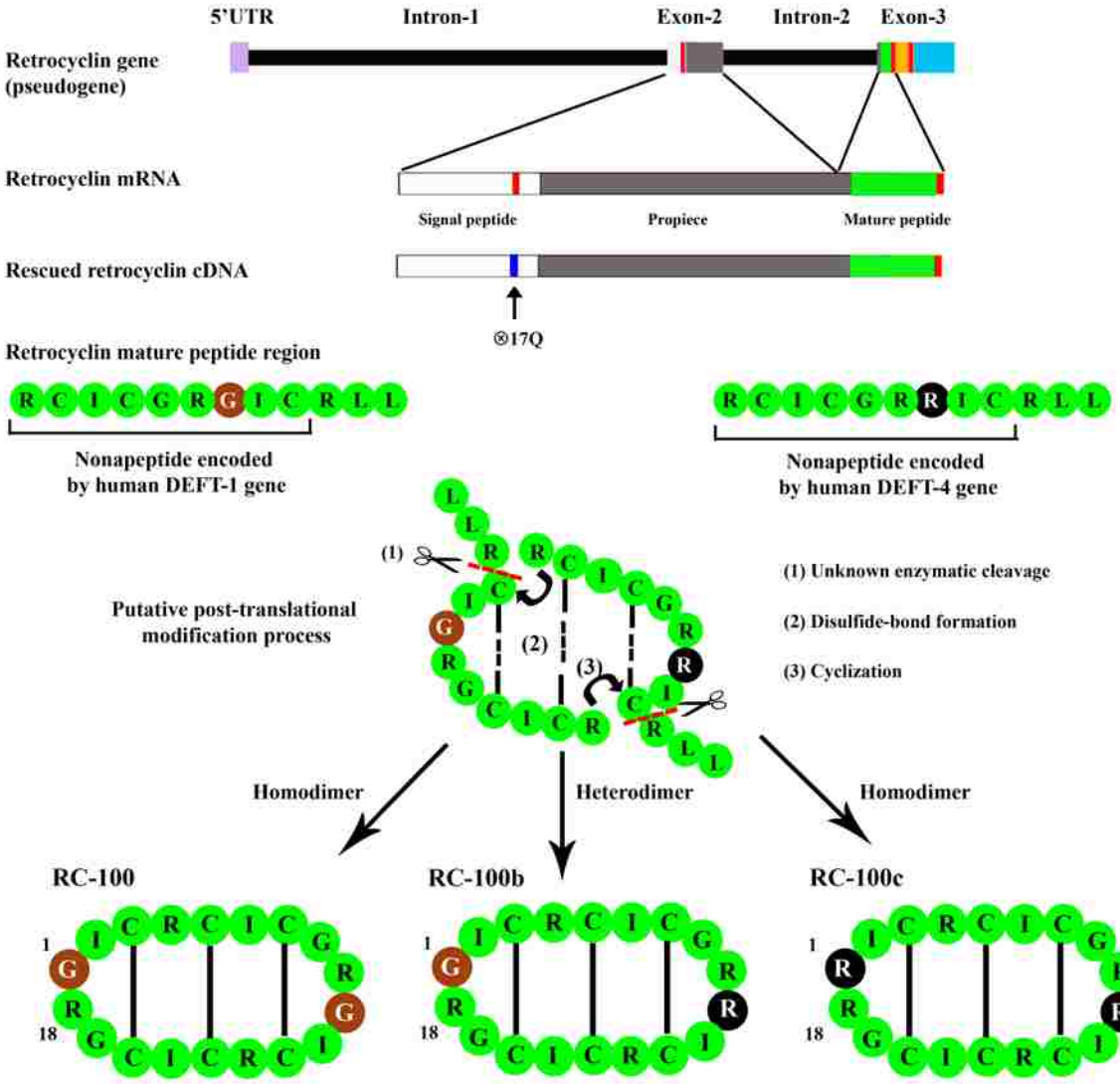

Figure 1.2: Putative post-translational modification of retrocyclin peptides. Figure shows a schematic representation of the retrocyclin pseudogene with the relative position of the exons and the spliced mRNA

transcript encoding the signal peptide, propiece and the mature peptide region. ⊗17Q represents the codon on rescued retrocyclin cDNA in which the mutant termination codon (⊗ /red) was replaced by a conserved glutamine (Q / dark blue). The putative post-translational modification process in which the precursor peptides that contain the nonapeptide undergo 1) an unknown enzymatic cleavage followed by 2) disulfide-bond formation and 3) cyclization by ligation of the N-and C-termini of the two nonapeptides. The three possible mature retrocyclin peptides that could be formed from homodimers of DEFT-1 nonapeptide (RC-100), heterodimers of DEFT-1 and DEFT-4 nonapeptides (RC-100b), and homodimers of DEFT-4 nonapeptide (RC-100c) are indicated.

Synthetic retrocyclins created by solid-state synthesis exhibited properties similar to rhesus theta-defensins [141]. They also were shown to inhibit early stage HIV-1 infection and replication of both X4 and R5 viruses in cell lines (H9) and primary CD4+ lymphocytes [141,142]. Of particular interest was a lysine retrocyclin congener (RC-101) that was twice as active as the native RC-100 peptide, and protected PBMCs completely against HIV-1 infection [135]. Both RC-100 and RC-101 exhibited broad-spectrum and enhanced activity against over two dozen primary isolates from several clades of HIV-1 [128,143]. They are not cytotoxic to human cell lines and when applied topically to organotypic cervicovaginal tissues they induced minimal inflammation and tissue damage [144]. Retrocyclins remain active in the presence of acidic vaginal fluid and likely act in synergy with the other antimicrobial peptides of vaginal secretions. Retrocyclins, due to their many desirable properties discussed above, are potent candidate topical microbicides that could protect against HIV infection.

Retrocyclins prevent HIV-1 entry by inhibiting the fusion of HIV-1 envelope by selectively binding to the C-terminal heptad repeat region of gp41 thereby blocking 6-helix bundle formation [145,146]. A study in which HIV-1 BaL was passaged with sub-inhibitory concentrations of

retrocyclins for 100 days showed that the viral envelope gained modest resistance [147]. It is ironic that DEFT genes are silenced in the human genome, since retrocyclins are not only active against HIV-1, but also against several other viruses including Influenza A [148] and Herpes Simplex virus type I [149]. We envisioned that restoring the endogenous expression of retrocyclins in human cells could help strengthen the innate host defense against many pathogens.

Despite a large body of information available on the properties of retrocyclins, certain questions remain unanswered. If retrocyclin genes were still intact, can active, correctly folded retrocyclins be expressed in human cells? Moreover, will endogenous production of retrocyclins confer protection against HIV-1 infection? To answer these questions, we restored the premature termination codon using site-directed mutagenesis and found that human cells have still retained the machinery to process and create macrocyclic retrocyclin peptides [150]. We next explored alternative ways to activate the endogenous expression of retrocyclins using aminoglycosides that can allow read through of the premature termination codon in human cells [151-155]. Importantly, restoring the expression of retrocyclins in human cells and cervicovaginal tissues conferred protection from HIV-1 entry [150]. These studies have been elaborated in **Chapter 2**.

CHAPTER TWO: REAWAKENING RETROCYCLINS: ANCESTRAL HUMAN DEFENSINS ACTIVE AGAINST HIV-1

2.1 Introduction

Nearly 33 million people are infected with HIV worldwide [156,157], and despite extensive efforts there are no effective vaccines or other countermeasures to protect against HIV transmission [158]. In our attempts to find effective anti-HIV agents, our group determined that certain synthetic θ-defensins called "retrocyclins" are potent inhibitors of HIV-1 infection [128,133,141,143,159]. Retrocyclins belong to a large family of antimicrobial peptides known as defensins, all of which are cationic, tri-disulfide bonded peptides that have important roles in innate host defense. Based on the position of the cysteines and the disulfide bonding pattern, defensins are grouped into 3 subfamilies: α-defensins, β-defensins and θ-defensins [121,160].

θ-Defensins such as retrocyclin have a cyclic peptide backbone, derived from the head-to-tail-ligation of two peptides that each contributes nine amino acids to form the 18 residue mature peptide [136]. θ-Defensins are the only known cyclic peptides in mammals and were originally isolated from rhesus macaque leukocytes and bone marrow [136,139,161]. While θ-defensin peptides are produced in old world monkeys and orangutans, in humans they exist only as expressed pseudogenes [134]. A premature termination codon in the signal peptide portion of human retrocyclin mRNA prevents its translation. The retrocyclin gene is otherwise remarkably intact, showing 89.4% identity with rhesus θ-defensins. Its genetic information was utilized to recreate retrocyclins synthetically and confirm their activity against both X4 and R5 strains of HIV-1 [128,133,141,159].

Retrocyclins inhibit the fusion of HIV-1 Env by selectively binding to the C-terminal heptad repeat region on gp41 blocking 6-helix bundle formation [145,146]. RC-101 is a congener of retrocyclin with a single arginine to lysine substitution that retains structural and functional similarity to retrocyclin [141]. RC-101 exhibited enhanced anti-HIV-1 activity against over two dozen primary isolates from several clades [128,143], and did not induce inflammation or toxicity in organotypic models of human cervicovaginal tissue [162]. Continuous passaging of HIV-1 BaL in the presence of sub-inhibitory concentrations of RC-101 for 100 days induced only minimal viral resistance [147]. Given these beneficial attributes, we envisioned that restoring the endogenous expression of retrocyclins in humans would provide an effective and natural way of combating HIV-1 infection.

In the current study, we restored the translation of this evolutionarily lost retrocyclin peptide by ablating the premature termination codon using site-directed mutagenesis, and analyzed whether human cells can synthesize biologically active retrocyclins. We found that promyelocytic HL60 cells stably transfected with retrocyclin constructs in which the premature termination codon was corrected could express retrocyclins. Application of the expressed retrocyclins to TZM-bl cells, PM1 cells, and PBMCs conferred protection against HIV-1 infection. Moreover, mass spectrometric techniques confirmed the presence of correctly folded mature retrocyclin peptides. We also explored methods to read-through the premature termination codon within the retrocyclin pseudogene. Previous reports revealed that aminoglycoside antibiotics could suppress the termination codon of pseudogenes and disease-associated nonsense mutations [151,153-155,163-165]. In bacteria, aminoglycosides bind strongly to the decoding site on the 16S rRNA, thereby hindering protein synthesis [166]. However, in eukaryotes, aminoglycosides bind to the eukaryotic decoding site with low affinity and induce a low level of translational misreading, which

suppresses the termination codon through the incorporation of an amino acid in its place [167]. Herein, we utilized aminoglycosides to induce translational read-through of the θ-defensin pseudogene, which restored the expression of functional anti-HIV-1 retrocyclin peptides in human cervicovaginal tissue models. Topical application of aminoglycosides to produce endogenous retrocyclins in the vaginal mucosa might soon be an effective preventative to combat sexual transmission of HIV-1.

2.2 Materials and Methods

2.2.1 Maintenance of cells, tissues and viruses

HL60 cells [168,169] obtained from ATCC were cultured in Iscoves's DMEM with 20% FBS, 100 U/ml penicillin and 100 g/ml streptomycin (I20). TZM-bl cells [170] stably expressing CD4, CCR5 & CXCR4, has firefly luciferase gene under the control of HIV-1 promoter (from Dr. Kappes, Dr. Wu and Tranzyme Inc). TZM-bl, HOS-CD4-CCR5 [171,172] (from Dr. Landau), PM1 cells [173], (from Dr. Reitz) and HIV-1 BaL, an R5 tropic strain, were all procured through the NIH AIDS Research and Reference Reagent program. HIV-1 BaL viral stocks were prepared by infecting PM1 cells [147]. Peripheral blood mononuclear cells (PBMC) were isolated from blood drawn from a healthy HIV-1 seronegative donor as per the guidelines of the institutional review board of University of Central Florida. PBMCs were isolated using Lymphosep® (MP biomedicals LLC, Solon, OH), and cultured in RPMI-1640 medium with 10% FBS (R10) supplemented with 50 Units of IL-2 (R10-50U) and 5 g/ml of phytohemagglutinin (PHA) for 3 days. The cells were then resuspended in R10-50U at a density of 0.8 x 10^6 cells/ml and grown for 5 –6 days.

Cervicovaginal tissues (EpiVaginal ™) were obtained from MatTek Corp., Ashland, MA and maintained in proprietary growth medium as per the company's guidelines. The tissues were composed of a full-thickness, stratified vaginal-ectocervical layer intermixed with Langehans cells and underlying lamina propria. The tissues were allowed to grow on transwell cell culture inserts at the air-liquid interface.

2.2.2 Creation of retrocyclin constructs and stably transfected HL60 cells

Retrocyclin cDNA was amplified from human bone marrow cDNA and cloned into pCRII-TOPO vector (Invitrogen, Carlsbad, CA). Two mutations, Termination codon (⊗17)→ Gln (Q17) and Arg (R70)→Lys (K70) were introduced, either ⊗17Q alone (RC-100) or both (RC-101) using Quick change® site-directed mutagenesis (Stratagene, LaJolla, CA) and subcloned in-frame into the phCMV-luc-FSR vector (Genlantis, San Diego, CA) to generate four constructs R1, R3, A1 and A3 (**Figure 2.1A**). Plasmids R1 and A1 encode RC-100 nonapeptide while R3 and A3 encode RC-101 nonapeptide. Constructs A1 and A3 have a longer insert that includes additional downstream residues. HL60 cells (10^7 cells/ 400 l Iscove's DMEM) were co-transfected with 2 g each of linearized R1, R3 or A1, A3 or phCMV-luc vector alone, by electroporation (exponential decay wave mode- 280 V; 975 F) and selected in I20 medium with 300 g/ml G418 sulfate. Stable transfectants thus produced were named according to the constructs used for co-transfection (R1R3, A1A3 or Vector Control: VC). Presence of these constructs in the cells was verified using PCR of genomic DNA (**Figure 2.2A**). PCR conditions used were the following: initial denaturation at 95ºC for 3 min; 30 cycles of 95ºC for 1min, 58ºC for 1 min, 72ºC for 2 min followed by a final extension at 72ºC for 7 min. Sequences of the primers used for the PCR reaction are listed in **Table 2.1**. RNA was extracted from 10^6 cells (HL60, VC, R1R3 and A1A3)

using TRIzol (Invitrogen), cleaned with DNaseI (Ambion Inc., Austin TX) and cDNA synthesized (iScript^{TM}, BioRad, Hercules CA). Expression of recombinant genes was verified by PCR from the cDNA and subsequent restriction digestion using HpyCH4V (New England Biolabs, Beverly, MA) (**Figure 2.2B and 2.2C**).

Table 2.1: Primers used for verification of retrocyclin constructs

Primer Name	Sequence	Accession Number	Primer Location	Template
DEFT_Fwd	TCCTCACTGCCATGCTTCT	AF526271.1	29-47	Genomic DNA
		AF355799	92-110	cDNA
DEFT_Rev	TTATAACAAACGGCAAATTCCT	AF526271.1	897-918	Genomic DNA
		AF355799	285-306	cDNA

2.2.3 Acid extraction and affinity purification of retrocyclin peptides

HL60 cells (control, VC, R1R3 and A1A3) were extracted with 5% acetic acid by vortexing for 20 min, centrifuged for 10 min at 10,000 x g, supernatants were then vacuum-dried and resuspended in 0.01% acetic acid. HL60 acid extracts (equivalent of 20 x 10^6 cells) were affinity purified using anti-RC-101 polyclonal antisera immobilized to a Carbolink™ coupling gel (Pierce Biotechnology Inc. Rockford, IL) prepared according to the manufacturer's instructions. Immunopurified samples were desalted using Sep-Pak C-18 cartridges (Waters, Milford, MA). The eluates were then dried and resuspended in 100 l of 0.01% acetic acid. 100 g of synthetic RC-101 peptide was also affinity purified as positive control (RC-101 IP).

2.2.4 Luciferase-based infection assay to determine anti-HIV-1 activity

TZM-bl cells (4000 cells/well; 96-well plate) were infected with HIV-1 Ba L (2-6.5 ng/ml of

p24^{gag}) in the presence of vehicle (0.01% acetic acid) or HL60 extracts (from 0.25 x 10^6 control or A1A3 or R1R3 cells) or affinity purified extracts (from 0.625 x 10^6 control HL60 or VC or R1R3 or A1A3 cells or RC-101 IP diluted 1:32 times) or RC-101 (20 g/ml) (positive control) for 24 hr. Treatments were then removed and the infection was quantified by measuring luciferase using Bright-Glo reagents (Promega, Madison, WI) in an LMax luminometer (Molecular Devices, Sunnyvale, CA). Cytotoxicity and metabolic activity of cells were verified by a tetrazolium- based MTT assay (R& D systems, Minneapolis, MN) performed on identically treated cells.

2.2.5 Antiviral Infection Assay in suspension cells and HIV-1 p24gag ELISA

Acid extracts of stably transfected HL60 cells were vacuum-dried and resuspended in PBS. PM1 cells (10^5 cells) or PBMCs (10^6 cells) were treated with PBS (vehicle) or HL60 extracts (from 10^4 cells for PM1 and 10^5 for PBMCs) of control cells or A1A3 cells or 10 g/ml of synthetic RC-101 and infected with HIV-1 BaL (2 ng of p24/ml) in 100 l of RPMI medium with 20% FBS (R20) for 2 hr. Cells were then washed with 2 ml of R20, resuspended in fresh medium containing the treatments and cultured for 5-9 days. Subsequently on alternate days culture supernatants were collected and fresh medium with the corresponding treatments was added. Viability of the cells was measured using trypan blue exclusion assay. Amount of HIV-1 virus in the culture supernatants was quantified by ELISA for HIV-1 p24^{gag} (Perkin Elmer, Waltham, MA).

2.2.6 Immuno-dotblot assay

Peptides RC-100, RC-100b, RC-101, RC-101_2K, synthetic protegrin-1 (PG-1), Rhesus theta defensin–1 (RTD-1) and human neutrophil peptides 1-3 (HNP 1-3) or unknown samples were dotted (4 l dot) as indicated on a 0.22 m PVDF membrane (Immobilon-P) that was activated

with methanol and presoaked in TBS. The membrane was then probed with 1:1000 rabbit anti-RC-101 antibody and developed using Immun™- star HRP reagent (BioRad) [162].

2.2.7 Immunostaining of stably transfected HL60 cells using anti-RC-101 antibody

HL60 cells (VC, R1R3 and A1A3) were fixed on slides (100,000 cells/ slide), immersed in 10% Formalin in PBS for 10 min, washed (PBS for 2 min), incubated in Target retrieval solution (Dako North America Inc. Carpinteria, CA) for 20 min at 95°C, cooled to 25ºC, washed, blocked (2% Goat Serum, 0.1% Triton-X, 0.05% Tween-20, antibody buffer (10 mg/ml BSA/1 mg/ml gelatin/PBS) for 20 min and incubated in rabbit pre-immune serum or rabbit anti-RC-101 antibody (1:5000 in antibody buffer) overnight. Slides were washed, incubated in biotinylated goat anti-rabbit IgG antibody (1:20,000 in 1% goat serum/PBS for 30 min), followed by additional washing and treatment with Fluorescein-Avidin D (Vector Laboratories Inc.; 1:500 in PBS for 30 min). Cover slips were mounted using Vectashield fluorescence mounting medium and visualized using a Zeiss Axiovert 200M microscope system.

Tissues for immunofluorescence staining were fixed in 4% paraformaldehyde and slides were prepared by Mass Histology (Worcester, MA). The slides were deparaffinized, washed with TBS, and stained with anti-retrocyclin or pre-immune serum and immunostained the same way as cells. The slides were then visualized on a Zeiss Axiovert 200M microscope system with 450 ms exposure time for all slides.

2.2.8 Separation of proteins from stably transfected HL60 extracts using reverse-phase HPLC

Acid extracts from control HL60 and A1A3 cells (equivalent of 100 x 10^6 cells) were separated by

RP- HPLC using the Alliance HT Waters 2795 Separations Module on a C_{18} Column equilibrated in solvent A (aqueous 0.1% TFA). Elution was done with a gradient of 0- 95 % solvent B (0.08% TFA in acetonitrile), for 75 min, at 1 ml/min. Collected fractions (1 ml each) were vacuum-dried and reconstituted in 100 l of 0.01% acetic acid. Synthetic RC-101 peptide (control) was recovered from the fractions eluting at 26-28 min. A1A3 HPLC fractions (#23-28) were electrophoresed on a 16% Tricine-SDS gel and electroblotted on a 0.22 m PVDF membrane at 180mA for 22 min. The western blot membrane was then processed as described [162] and developed with ChemiGlow reagent (Alpha Innotech, San Leandro, CA). A1A3 RP-HPLC fractions (27-30 min) were pooled and the concentration was determined to be (2.13 ng/ l) by densitometry measurements using Quantity one 1-D analysis (BioRad). A luciferase-based assay was used to verify the activity of A1A3 HPLC fractions (diluted 3 times in D10) against HIV-1 BaL (2 ng p24/ml).

MatTek cervicovaginal tissues treated with PBS (control) or 10 µg/ml tobramycin were extracted with T-PER® reagent (Pierce Biotechnology Inc. Rockford, IL) and separated by RP-HPLC. 20 µg of synthetic retrocyclin (RC-100) was also separated as a positive control. Synthetic RC-100 was eluted in fractions collected at 27-29 min. Tissue samples eluted at 27-29 min were vacuum-dried to near dryness and resuspended in 100 µl of 0.01% acetic acid. HPLC fractions (27-29 min) of MatTek tissue extracts (control or tobramycin-treated) and synthetic RC-100 were analysed by immuno-dotblot analysis.

2.2.9 Mass Spectrometric analysis

A1A3 HPLC Fraction 26, RC-101 and RC-101_2K were reduced, alkylated and treated with Lys-

C protease for 30 min before analyzing by mass spectrometry. In brief, 20 mM Tris [2-carboxyethyl] phosphine (TCEP) was used to reduce (30 min at 25 °C) the samples, alkylated by incubating the samples with iodoacetamide (60 mM; 45 min at 25 °C; pH 8-9) followed by digestion with Lys-C (Wako Chemicals, Richmond, VA; 30 min at 37 °C) and subjected to MALDI-TOF-MS analysis using a model 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA) as described previously [132]. Lys-C digested RC-101 was desalted using C18 ZipTip (Millipore Corp., Billerica, MA) and subjected to Edman degradation on cLC-Procise sequencer (Applied Biosystems).

2.2.10 Aminoglycoside mediated read-through of termination codon

Wildtype and mutant retrocyclin cDNAs were subcloned into phCMV-luc-FSR vector to create unrescued RC-101 and rescued RC-101 C-terminal luciferase fusion constructs, and verified by sequencing. HOS-CD4-CCR5 cells were cultured in antibiotic free growth medium ($D10^-$) and co-transfected with 0.5 g of unrescued or rescued (positive control) RC-101 plasmids along with 0.1 g of phRL-CMV vector (transfection control containing renilla luciferase gene) using Effectene transfection reagent (Qiagen, Valencia, CA). The next day cells were treated for 24 hr with the appropriate aminoglycoside (40 g/ml amikacin or 5 g/ml gentamicin or 10 g/ml tobramycin) or $D10^-$ for control cells. Read-through was determined by measuring luciferase and renilla levels using a dual luciferase assay (Promega).

TZM-bl cells (4000 cells/well; 96 well plate) were cultured in $D10^-$ and treated with vehicle (PBS buffered $D10^-$) or peptides RC-101 or RC-100 (2.5 g/ml each) as positive control or

aminoglycosides as before for 30 min followed by infection with HIV-1 BaL (p24 of 2 ng/ml) at 37°C for 24 hr. Subsequently, viral infection was quantified by measuring luciferase levels using Bright-Glo reagents (Promega). Cellular metabolism was monitored by measuring reduction the ability of cellular dehydrogenases to reduce MTT to formazan (R& D systems).

TZM-bl cells were cultured on coverslips and treated with PBS control or 10 µg/ml of tobramycin for 24 hr. The coverslips were then processed for immunofluorescence staining with anti-retrocyclin (rabbit anti-RC-101 antibody) or pre-immune serum as described above.

For antibody-mediated neutralization experiments, TZM-bl cells (4000 cells/well; 96 well plate) were cultured in $D10^-$ medium and treated with vehicle (PBS) or 10 µg/ml of tobramycin for 24 hr. The next day, cells were treated with either rabbit preimmune or anti-retrocyclin serum diluted 1:10 in $D10^-$ medium containing tobramycin or RC-100 (2.5 µg/ml). Two hours later the cells were infected with HIV-1 BaL (p24 of 5 ng/ml) at 37°C for 24 hr. Viral infection was quantified as described above. An MTT assay was performed to confirm that the treatments were not cytotoxic (data not shown).

2.2.11 Application of aminoglycosides to organotypic cervicovaginal tissue model

Cervicovaginal tissues were treated topically with 100 µl of PBS (control; n = 4) or with 10 µg/ml of tobramycin (n = 8) for 24 hr. Viability was assessed on control and tobramycin-treated tissue (n =1) using MTT assay kit (MatTek Corp., Ashland, MA). Cytotoxicity was measured by quantifying lactate dehydrogenase (LDH) activity in the underlying medium collected 24 hr after

treatment with PBS or tobramycin by using CytoTox96 non-radioactive cytotoxicity assay kit (Promega Corp., Madison, WI).

2.3 Results and Discussion

2.3.1 Creation of promyelocytic cells stably transfected with retrocyclin constructs

θ-Defensins are formed by posttranslational modification of two 12-residue gene products, each of which is processed to give a nonapeptide that contains three cysteines. The N-terminus of one nonapeptide forms a peptide bond with the C-terminus of another nonapeptide, resulting in a cyclic 18 residue peptide with three intramolecular disulfide bonds [134,136]. To determine if human cells have retained the ability to process θ-defensins, we transfected promyelocytic HL60 cells with retrocyclin constructs each encoding a nonapeptide in which the premature termination codon was replaced with a glutamine (⊗17Q).

Four types of constructs were produced: R1, R3, A1 and A3 (Figure 2.1). Aside from the corrected premature termination codon (⊗17Q), all constructs were engineered to contain two termination codons at the end of the gene to ensure read-fidelity. Constructs with an "R" designation terminate after the retrocyclin portion of the gene, while constructs with an "A" designation contain the retrocyclin portion with additional downstream residues that might be critical for translation and/or processing [134,174]. Constructs with a "1" designation do not have any additional residues mutated, while constructs with a "3" designation have the additional Arg to Lys mutation (R70K) encoding the RC-101 nonapeptide.

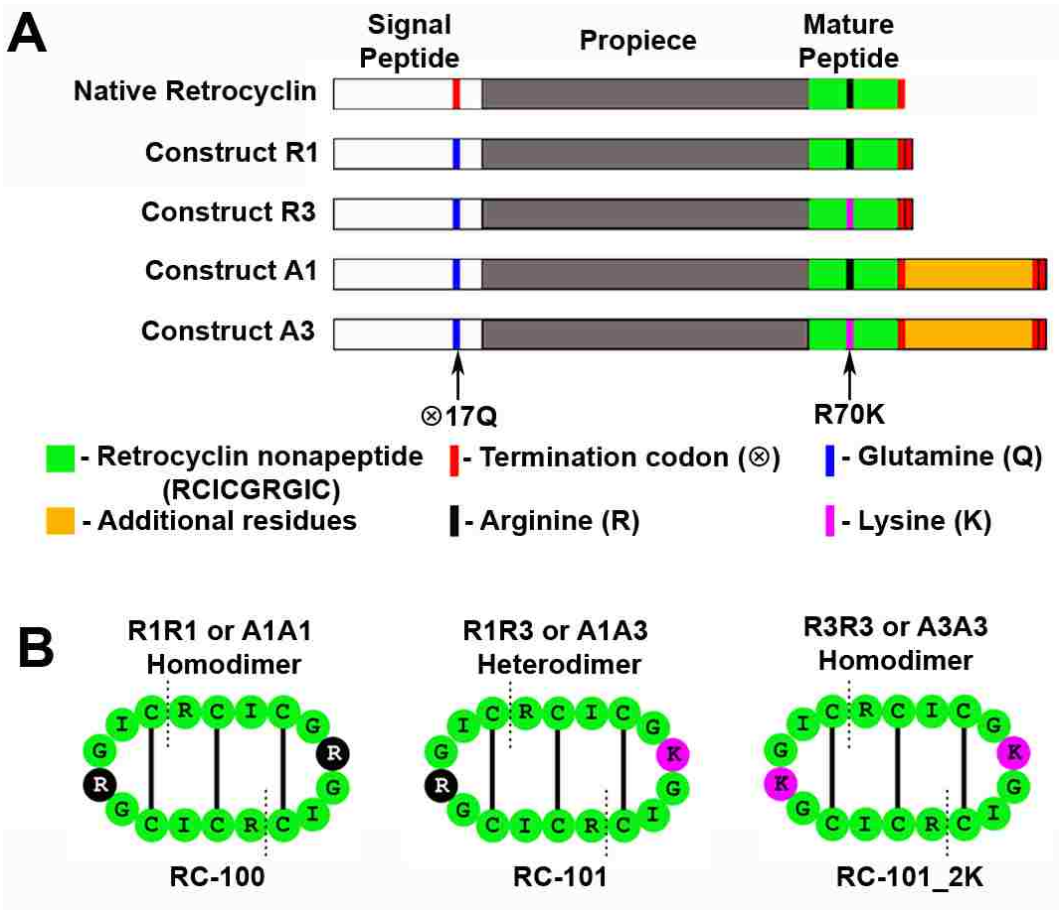

Figure 2.1: Design of retrocyclin constructs. (A) Shows a schematic of the 4 constructs (R1, R3, A1 and A3) used for stable transfections along with native retrocyclin cDNA. All constructs have two termination codons at the end to ensure read-fidelity (red). Constructs A1 and A3 contain additional downstream residues (orange) while constructs R1 and R3 lack them. The two arrows indicate the position at which the two site-directed mutagenesis (⊗17Q and R70K) were performed. (B) Shows the three possible mature retrocyclin peptides that could be formed from the constructs, homodimers of R1 or A1 encoding RC-100 (wild type retrocyclin), heterodimers of A1 and A3 or R1 and R3 encoding RC-101 (single lysine congener) and homodimers of R3 or A3 encoding RC-101_2K (double lysine congener).

HL60 cells were co-transfected by electroporation with either R1 and R3, or A1 and A3, and propagated in the presence of G418 (300 g/ml) to create stably transfected cell lines. Stable transfection was verified by analyzing genomic DNA and mRNA (**Figure 2.2**). Since two different constructs were co-transfected for each condition, combinatorially it would be possible to generate three different retrocyclin peptides as illustrated in **Figure 2.1B**. For example, if cells were co-transfected with the R1 and R3 constructs, they could theoretically generate a heterodimer (R1R3) or homodimers (R1R1 or R3R3).

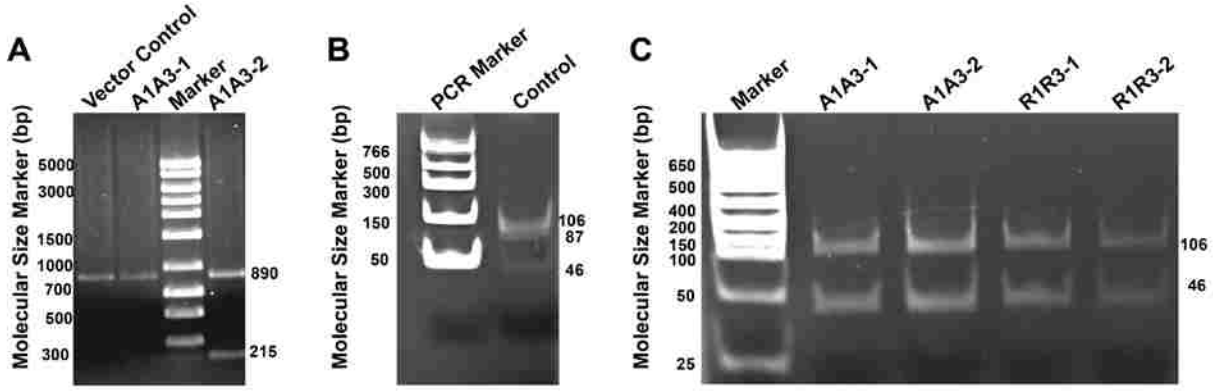

Figure 2.2: Verification of stable transfection of retrocyclin constructs. Analysis of the genomic DNA and RNA of transfected HL60 cells confirms the stable transfection and transcription of rescued retrocyclin constructs respectively. (A) PCR on genomic DNA template from transfected HL60 cells shows a 215 bp fragment representing retrocyclin cDNA construct and a 890 bp fragment of native retrocyclin gene in the genomic DNA of A1A3 clones but not in the Vector control (VC) cells. (B, C) Correction of the premature termination codon of retrocyclin cDNA introduces an additional HpyCH4V restriction site the middle of a 87 bp cDNA fragment. RNA isolated from HL60 cells (control, R1R3 clones 1 & 2 and A1A3 clones 1 & 2) was used to make cDNA. Retrocyclin constructs were amplified by PCR using the cDNA as template and digested using HpyCH4V restriction enzyme. Electrophoresis of the digested PCR products shows the expected 87 bp fragment in control cells (B) and the expected absence of 87 bp fragment in R1R3 and A1A3 clones (C). All the products were also verified by DNA sequencing.

2.3.2 Extracts of promyelocytic cells stably transfected with retrocyclin constructs are active against HIV-1

We next analyzed if correcting the termination codon in the retrocyclin constructs could restore the translation of biologically active retrocyclin peptides. The infection of TZM-bl cells with HIV-1 BaL was significantly reduced when cells were treated with cellular acid extracts of R1R3 cells ($P < 0.004$) and A1A3 cells ($P < 0.002$) **(Figure 2.3A)**. A standard tetrazolium MTT assay revealed that the extracts did not affect cellular metabolism at the concentrations used in the experiment (**Figure 2.3E**). Addition of A1A3 cell extracts to HIV-1 infected PM1 cells (**Figure 2.3B**) and PBMCs (**Figure 2.3C**) showed significant ($P < 0.002$ and $P < 0.004$ respectively) decrease in the viral titer as compared to cells treated with control HL60 cell extract. A trypan blue exclusion assay was performed in PBMCs to monitor cell viability (**Figure 2.3F**). We next affinity purified R1R3 and A1A3 cell extracts using anti-RC-101 antibody and confirmed the antiviral activity in a luciferase-based assay system (**Figure 2.3D**). Interestingly, A1A3 cell extracts were found to be consistently more active than equivalent amounts of R1R3 cell extract, which suggests a role for the downstream residues in retrocyclin processing. These results indicate that biologically active recombinant retrocyclin peptides can be synthesized in human promyelocytic cells. As a next step we tested the presence of retrocyclin in promyelocytic cells using immunostaining.

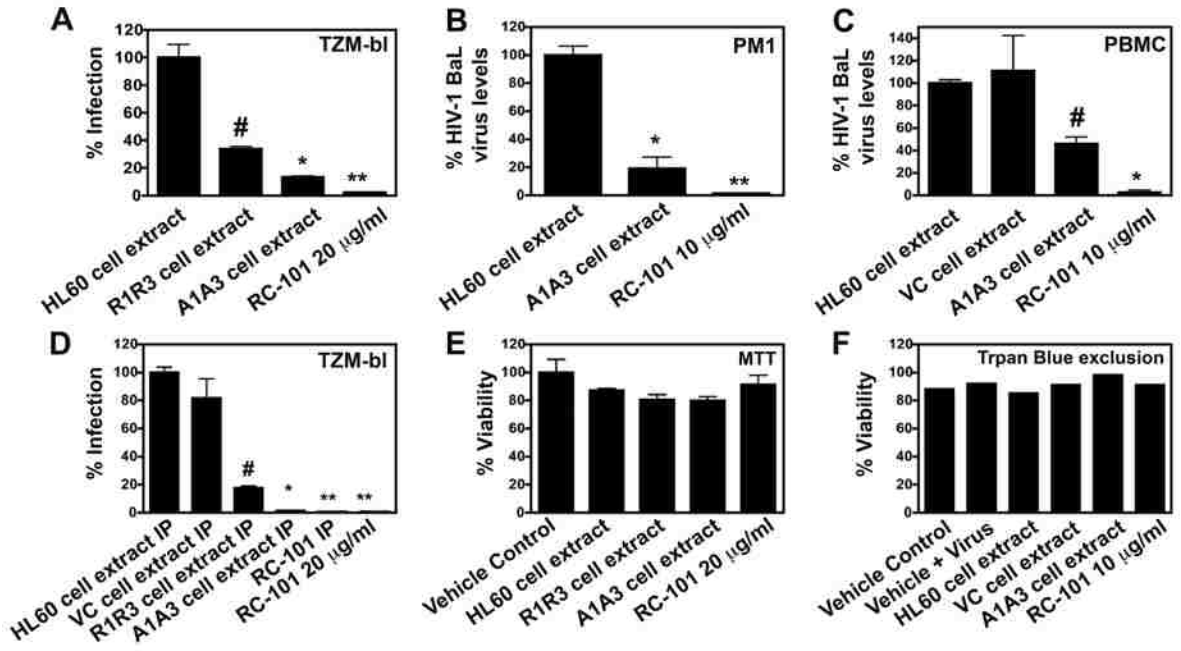

Figure 2.3: Extracts from HL60 cells stably transfected with retrocyclin constructs are active against HIV-1 infection. (A) TZM-bl cells were treated with extracts or peptide as indicated in the figure and infected with HIV-1 BaL (6.5 ng/ml p24) for 24 hr. Infection was measured as percent luciferase activity compared to cells treated with control cell extract (Average RLU of control HL60 extract = 178,200). (B, C) PM1 cells and PBMCs were treated with extracts or peptide as indicated and infected with HIV-1 BaL (2 ng/ml p24) and cultured for 5-9 days. Bars represent percent BaL HIV-1 levels in the supernatants collected on days 5 (B) and 9 (C). The amount of p24 in PM1 cells treated with control extract = 76.85 ng/ml and in PBMCs treated with control extracts = 55.99 ng/ml. (D) TZM-bl cells were treated with immunopurified (IP) extracts or peptides as indicated and infected with BaL HIV-1 (p24 = 2 ng/ml) for 24 hr. Infection was quantified as percent luciferase activity compared to cells treated with control HL60 cell IP extracts (Average RLU = 764,460). Error bars represent SEM. n = 3-4, # $P < 0.004$, * $P < 0.002$, ** $P < 0.0005$. (E) Cellular viability of TZM-bl cells treated with HL60 acid extracts as indicated was determined by measuring the reduction of MTT after 24h (n = 3). Bars represent percent viability as compared to vehicle control and error bars represent SEM. (F) Cell death was monitored in PBMCs treated with the acid extracts by a trypan blue exclusion assay on day 9 (n = 1).

2.3.3 Immunofluorescence staining of stably transfected HL60 cells reveals retrocyclin peptides

Immuno-dotblot analyses revealed that our anti-RC-101 antibody specifically recognized lysine-containing human retrocyclin analogs (synthetic RC-101 and RC-101_2K) and RC-100 (i.e. wild type form) to a lesser extent **(Figure 2.4A)** but not human neutrophil peptides 1-3, or peptides with very similar tertiary structure including rhesus theta defensin-1 (RTD-1) and protegrin-1 (PG-1) **(Figure 2.4B)**. This antibody was used to visualize the expressed retrocyclin peptides in the stably transfected HL60 cells by immunofluorescence staining, which revealed that R1R3 cells and A1A3 cells were brightly stained as compared to Vector Control (VC) cells **(Figure 2.4C)**. Slides treated with pre-immune serum showed no staining (data not shown). Note that the staining of A1A3 was brighter than R1R3 and the morphology of A1A3 cells was smaller than VC cells. Experiments were next designed to purify and confirm the identity of the expressed retrocyclin peptides from the cell extracts.

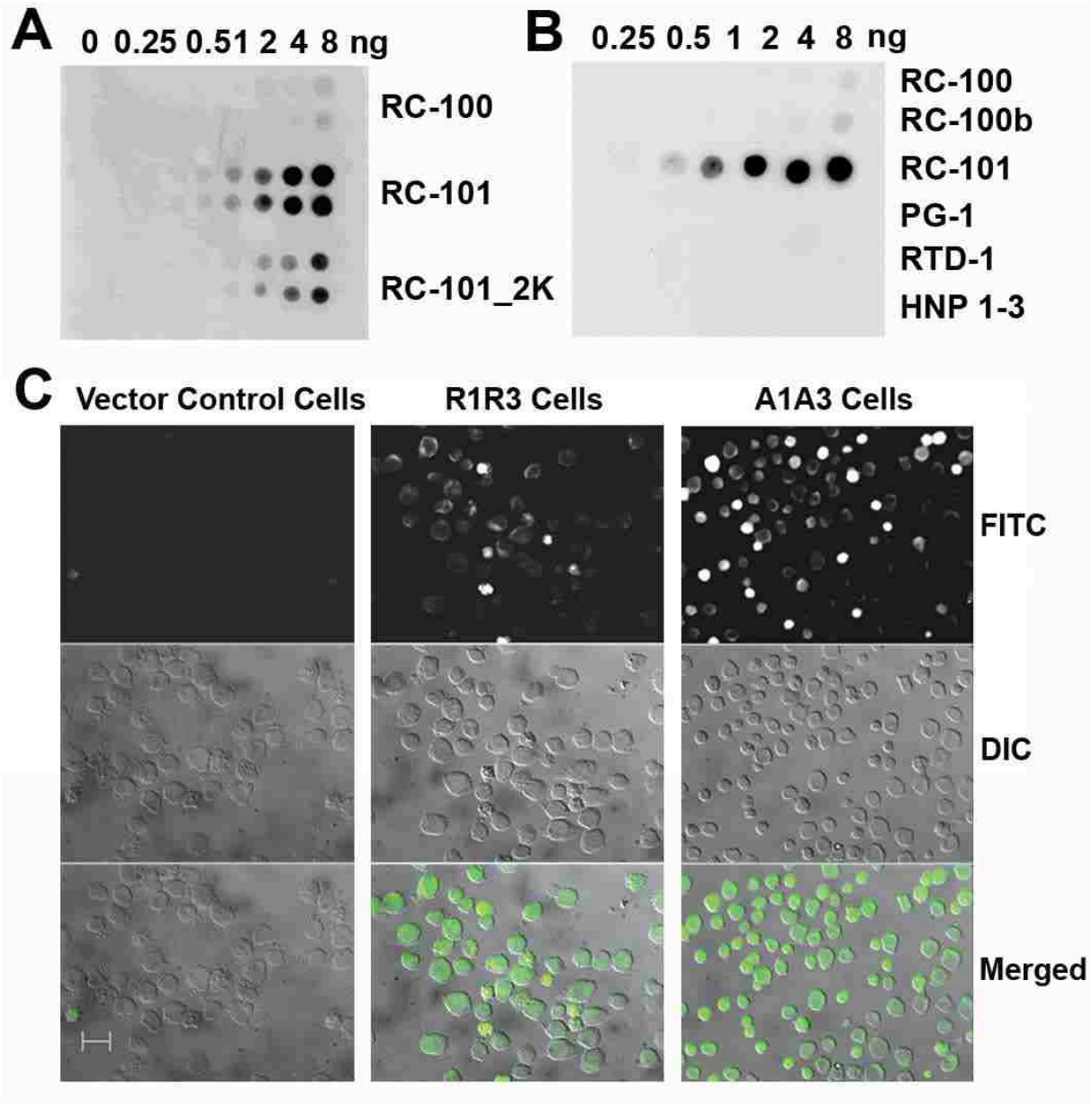

Figure 2.4: Immunofluorescence staining of stably transfected HL60 cells reveals retrocyclin peptides. (A) Retrocyclin peptides RC-100, RC-101 and RC-101_2K peptides (in duplicates) and (B) RC-100, RC-100b, RC-101, protegrin-1 (PG-1), rhesus theta defensin –1 (RTD-1), and human neutrophil peptides 1-3 (HNP 1-3) were dotted (0-8 ng / 4 l dot) on a PVDF membrane and subjected to immuno-dotblot analysis. (C) Vector control, R1R3 and A1A3 (100,000 cells each) were fixed onto glass slides and incubated with rabbit anti-RC-101 antibody

followed by biotinylated goat anti-rabbit IgG secondary antibody and then stained using FITC-avidin. Slides were visualized using Zeiss Axiovert 200M microscope system at 40 X magnification. The three rows show FITC staining, DIC and the merged image respectively. Scale bar represents 20 m.

2.3.4 Stably transfected promyelocytic cells produce retrocyclin peptides

Reverse-phase HPLC (RP-HPLC) was utilized to purify the recombinant retrocyclin peptides from stably transfected HL60 cell extracts. **Figure 2.5A** shows the RP-HPLC trace of A1A3 and synthetic RC-101. Synthetic RC-101 was recovered in fractions collected at 26-28 min. A1A3 HPLC Fractions collected from 23-30 minutes were analyzed on a 16% Tricine-SDS-gel. Control samples did not contain any protein bands at the expected size while fractions from R1R3 cell extracts revealed protein bands of about 6 kDa size (data not shown). Interestingly, A1A3 HPLC fractions revealed multiple protein bands, which we further analyzed by western blot **(Figure 2.5B)**. The western blot analysis revealed bands at sizes corresponding to a monomer, dimer and trimer of retrocyclin. Interestingly, the presence of multimeric forms of retrocyclin has been independently observed by Daly and colleagues [175]. Furthermore, the RP-HPLC purified A1A3 fractions inhibited entry of HIV-1 BaL in TZM-bl cells **(Figure 2.5C)**. The IC_{50} of retrocyclin peptides expressed by A1A3 cells (2 µg/ml) was similar to that of synthetic RC-101 (1.25 µg/ml) [143].

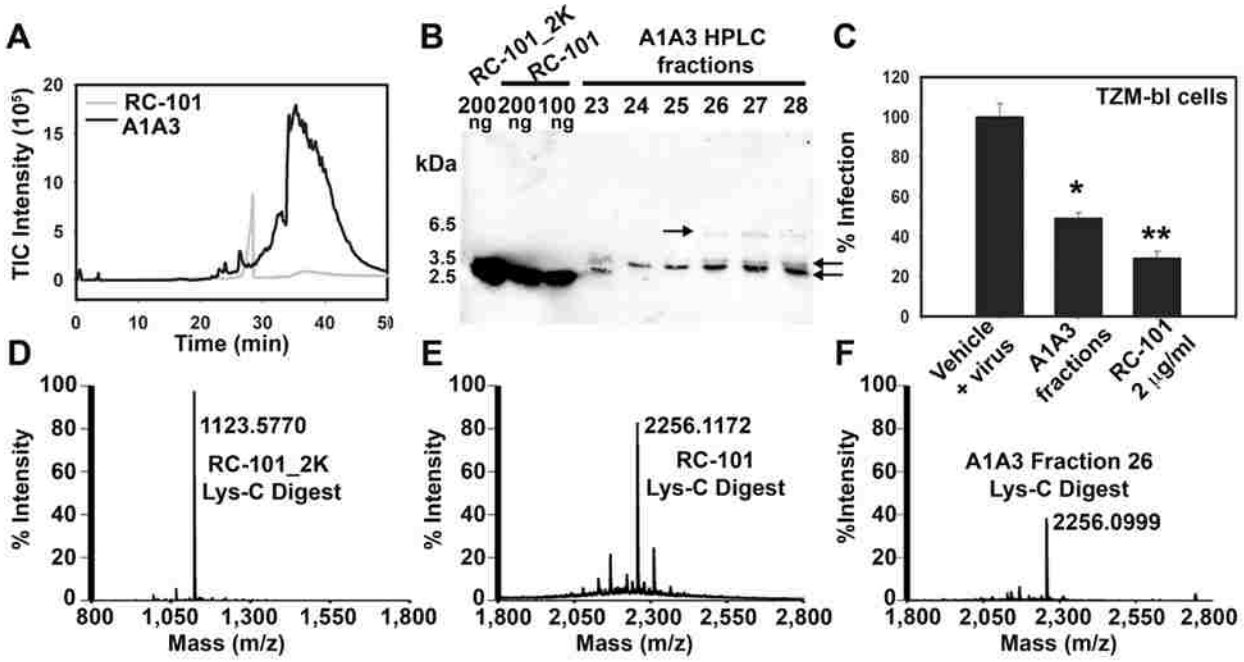

Figure 2.5: Stably transfected promyelocytic cells produce retrocyclin. (A) Shows the RP-HPLC trace of A1A3 cell extract (from 108 cells) and 50 g of synthetic RC-101. (B) Western blot of A1A3 HPLC fractions (23-28 min) using rabbit anti-RC-101 antibody. The arrows indicate the multimeric forms of retrocyclin observed in A1A3 fractions. (C) TZM-bl cells were infected with HIV-1 (p24 = 2 ng/ml) in the presence or absence of pooled A1A3 fractions (final dilution 1:6 in D10) or 2 g/ml of RC-101 for 24 hr. Infection was quantified by luciferase measurement (Average RLU of Vehicle control with virus = 85,450). Error bar represents SEM and n = 3-6, * P < 0.0015 ** P <0.0001. MALDI-TOF MS spectra of Lys-C digested (D) synthetic RC-101_2K, (E) synthetic RC-101 and (F) A1A3 HPLC fraction 26 reveal that A1A3 cells produce RC-101.

To determine the identity of the retrocyclin peptide expressed by A1A3 cells, HPLC fraction 26 was analyzed by mass spectrometric analysis (MALDI-TOF-MS) at the Microchemical and Proteomics Facility, Emory University. Analysis of A1A3 Fraction 26 revealed peaks with masses 1889.775 Da (oxidized) and 1895.890 Da (reduced), which is nearly identical to the expected mass of synthetic cyclic RC-101 (1889.85 Da and 1895.96 Da respectively; data not shown) and is in

agreement with reduction of the three disulfide bridges in the molecule. Furthermore, treatment with iodoacetamide yielded mass species of 2238.081 Da for the A1A3 fraction 26 and 2238.071 Da for RC-101 corresponding to the predicted 6-fold-alkylated form of RC-101 (expected mass = 2238.097 Da). Comparison of spectrum of the Lys-C digest of reduced/alkylated synthetic RC-101_2K (peak at 1123.577 Da; peptide cleaved at two Lys-Gly bonds; **Figure 2.5D**), synthetic RC-101 (peak at 2256.097 Da; peptide cleaved at a single Lys-Gly bond; N-terminal sequence determined as: Gly-Ile-Cys-Arg-; **Figure 2.5E**) and A1A3 fraction 26 (peak at 2256.010 Da) suggests that the A1A3 cells are expressing RC-101 **(Figure 2.5F)**. These data confirmed that correctly folded mature retrocyclin peptides can be expressed by human cells. In the following experiments we explored alternative methods to express the peptide endogenously. Of particular interest was the effect of aminoglycosides in mediating varying degrees of termination codon read-through as previously described [151,153-155,163-165].

2.3.5 Aminoglycosides mediate read-through of termination codon of retrocyclin gene and restore anti-HIV-1 activity

We tested the ability of three commonly used aminoglycosides (gentamicin, amikacin, and tobramycin) to induce termination codon read-through of retrocyclin cDNA. The native retrocyclin gene was fused with a luciferase reporter at the C-terminus to create 2 constructs: unrescued RC-101 and rescued RC-101 (positive control) as shown in **Figure 2.6A.** These constructs were transfected into HOS-CD4-CCR5 cells, grown in the presence of varying concentrations of aminoglycosides, and the degree of read-through quantified by measuring luciferase. Application of tobramycin (10 g/ml) was the most effective, producing a 26-fold increase in read-through ($P < 0.0007$; **Figure 2.6B**).

Having thus established the optimal aminoglycoside concentration required to achieve read-through of retrocylin cDNA, we next determined if aminoglycosides could restore the translation and anti-HIV-1 activity of native retrocyclin peptides. HeLa-derived cells lines such as TZM-bl cells can natively express retrocyclin mRNA (data not shown). We applied aminoglycosides to TZM-bl cells and challenged them with HIV-1 BaL. We found that cells treated with gentamicin and tobramycin significantly ($P < 0.0005$ and $P < 0.0001$ respectively) inhibited HIV-1 infection as compared to untreated cells (**Figure 2.6C**). The effect was modest when compared to inhibition by synthetic peptides. Cell viability, determined by a tetrazolium based MTT assay, was not affected by the application of aminoglycosides at the mentioned concentrations (**Figure 2.6E**).

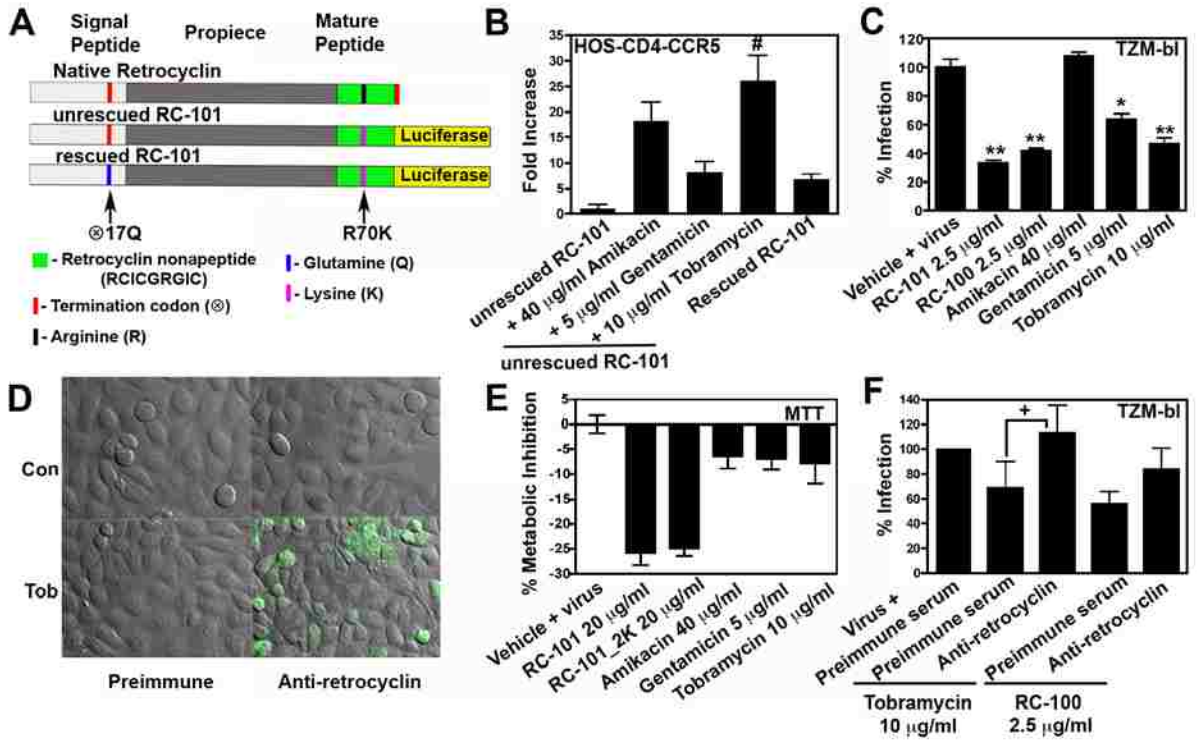

Figure 2.6: Aminoglycosides mediate read-through of the premature termination codon within the retrocyclin gene and promote anti-HIV-1 activity. (A) Shows a schematic representation

of the luciferase fusion constructs unrescued RC-101 and rescued RC-101 along with native retrocyclin cDNA. (B) HOS-CD4-CCR5 cells cultured in antibiotic free medium ($D10^-$) were transfected with unrescued RC-101 (negative control) or rescued RC-101 (positive control) plasmids along with phRL-CMV vector (transfection control). The next day transfected cells were treated with PBS for control cells or aminoglycosides at the indicated concentrations and allowed to grow for 24 hr. Read-through was determined by measuring luciferase levels. Data is expressed as fold increase in luciferase expression normalized to renilla levels. (C) TZM-bl cells grown in $D10^-$ were treated for 30 min with PBS, RC-101 (2.5 g/ml), RC-100 (2.5 g/ml) or aminoglycosides as shown in the figure and infected with HIV-1 BaL (2 ng/ml p24) for 24 hr followed by luciferase measurement. Error bars represent SEM. n = 3-6, # $P < 0.007$ * $P < 0.0005$ ** $P < 0.0001$. (D) TZM-bl cells cultured on cover slips were treated with PBS (Con) or 10 µg/ml tobramycin (Tob) and then immunostained with rabbit pre-immune or anti-retrocyclin serum using a biotinylated secondary antibody FITC-avidin system. (E) Cellular cytotoxicity was assessed by performing an MTT assay on TZM-bl cells treated with indicated amount of peptide or aminoglycosides (n = 3). Bars represent percent metabolic inhibition as compared to control (vehicle + virus). (F) TZM-bl cells, treated with either PBS, tobramycin (10 µg/ml) or RC-100 (2.5 µg/ml), were incubated with preimmune serum or anti-retrocyclin serum as indicated and infected with HIV-1 (p24 of 5 ng/ml). Data is represented as percent infection. Error bars represent SEM. n = 3, + $P < 0.018$ Statistical significance was determined by two-tailed Student t-test.

In order to visualize the retrocyclins expressed by application of aminoglycosides, we performed immunostaining. TZM-bl cells were treated with PBS control or 10 µg/ml tobramycin and stained with anti-retrocyclin antibody or pre-immune serum. Control cells showed no staining while cells treated with tobramycin revealed brightly stained cells suggesting that aminoglycosides can induce the expression of retrocyclin peptides (**Figure 2.6D**).

We next incubated TZM-bl cells with tobramycin (10 µg/ml) for 24 hr, and then treated the cells with preimmune or anti-retrocyclin serum followed by infection with HIV-1. **Figure 2.6F** reveals

that cells treated with preimmune serum showed a modest yet significant reduction in infection as compared to cells treated with anti-retrocyclin antibodies ($P < 0.018$), suggesting that the antibody inhibited the endogenous retrocyclins. These data confirm that the anti-HIV-1 activity observed is due to the endogenous retrocyclin peptides expressed when tobramycin was applied to cells.

2.3.6 Aminoglycosides induce production of retrocyclin peptides in cervicovaginal tissues

We next analyzed the ability of aminoglycosides to induce the expression of retrocyclin peptides in an organotypic model cervicovaginal tissue. Tissues were treated apically with tobramycin or control (PBS) for 24 hr and anti-retrocyclin immunohistochemical analysis was performed. Interestingly, tissues treated with tobramycin alone and stained with anti-retrocyclin antibody revealed brightly stained cells (**Figure 2.7A**) suggesting that production of retrocyclin peptides is induced upon application of aminoglycosides. Lactate Dehydrogenase (LDH) activity in the medium underlying the tissues was performed to determine tissue cytotoxicity. The LDH assay revealed that application of 10 μg/ml tobramycin was not cytotoxic to the tissues (**Figure 2.7B**). In addition, treatment of tobramycin did not affect the metabolic activity adversely, which was determined by an MTT assay performed on one tissue (data not shown).

In order to purify endogenous retrocyclins expressed in the tissues, we utilized reverse- phase HPLC. **Figure 2.7C** shows an HPLC trace of control, tobramycin-treated tissue extracts as compared to synthetic RC-100 peptide. Synthetic RC-100 peptide was recovered in fractions collected at 27-29. Corresponding fractions from control and tobramycin-treated tissues were analyzed by immuno-dotblot analysis using the anti-RC-101 antibody. **Figure 2.7D** shows that

retrocyclin peptides were recovered in fractions 27-29 min in tobramycin-treated tissue samples but not in control tissue samples. The amount of retrocyclin (RC-100) expressed in tobramycin-treated cervicovaginal tissues was estimated by densitometry to be approximately 1.6 µg/tissue. Together these studies show that aminoglycosides are promising molecules to suppress the premature termination codon of retrocyclin transcripts and restore the ability of cervicovaginal tissues to protect cells from HIV-1.

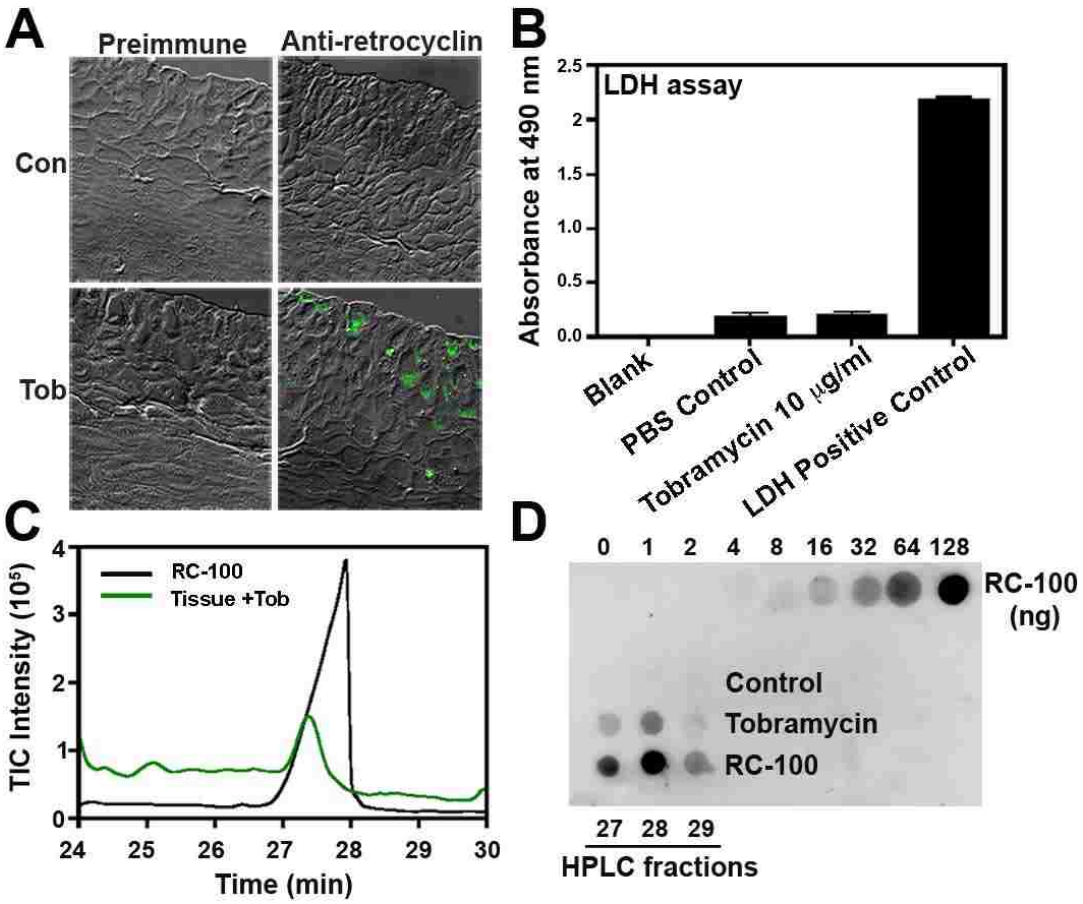

Figure 2.7: Expression of retrocyclins in cervicovaginal tissue model using aminoglycosides. (A) Cervicovaginal tissues were treated with PBS (Con) or 10 µl tobramycin (Tob) and incubated with rabbit pre-immune serum or anti-retrocyclin antibody. The slides were then incubated with biotinylated goat anti-rabbit IgG

secondary antibody and then stained using FITC-avidin. B) Cytotoxicity was determined by measuring Lactate dehydrogenase (LDH) activity in media underlying the tissues treated with PBS or tobramycin as indicated. Bars represent absorbance measured as 490 nm and error bars represent SEM; $n = 6$. (C) HPLC trace of extracts of tissues treated with 10 μg/ml tobramycin (Tissue + Tob) and 20 μg of synthetic RC-100. (D) RC-100 synthetic peptide (indicated amounts), HPLC fractions 27-29 of control, tobramycin-treated and RC-100 were dotted on a PVDF membrane and analyzed by immuno-dotblot.

2.4 Conclusion

Identifying effective drugs to prevent HIV-1 infection and other viral infections is essential for countering the spread of these diseases. Exogenous (synthetic) retrocyclins exhibit full activity in the complex environment of vaginal fluid and the peptide is very well tolerated in organotypic human cervicovaginal tissue models [162]. Moreover, HIV-1 evolves little resistance during continued passaging in the presence of the peptide [147]. For these and other reasons, retrocyclins have emerged as potential topical microbicides to protect against sexually- transmitted HIV-1 infections.

In this study we have taken a different path towards developing θ-defensin therapeutics. The human pseudogenes that encode the demidefensin precursors whose post-translational processing gives rise to mature retrocyclin are expressed at the mRNA level in multiple organs, including the spleen, bone marrow, thymus, testis and skeletal muscle [134] and cervicovaginal epithelia (A.M.C., unpublished data). By transfecting human myeloid cells with plasmids containing retrocyclin genes without a premature termination codon, we demonstrated that the "machinery" needed to process, trim, splice and oxidize retrocyclin precursors was available in human myeloid cells. Two sets of expression constructs were transfected into cells: a shorter form (R1R3) that

terminates at the end of the retrocyclin gene and a longer form that contains (A1A3) additional 3' untranslated residues (UTR). Interestingly, A1A3 cells expressed higher levels of retrocyclin peptides as compared to R1R3 cells indicating a role for additional residues in the translational efficiency of these peptides. This was not altogether surprising as other studies have shown that the length of the 3'-UTR regulates translation efficiency [174,176]. Finally, we showed that aminoglycoside-treated cells and cervicovaginal tissues could produce retrocyclins endogenously by suppressing the premature termination codon in their endogenous mRNA transcript.

Since approximately 30% of inherited disorders may result from premature termination codon mutations, there has been tremendous interest and some progress in developing and applying agents that can read-through premature UAA, UAG or UGA termination codons [155]. Although aminoglycosides, as used in this study, have been most widely investigated, exciting new agents such as PTC-124, have also appeared [177,178]. In a sense, human retrocyclin-deficiency is also an inherited disorder, albeit one with an incidence of 100%. It is caused by a premature termination codon mutation that occurred after human lineage diverged from the lineage we share with orangutans, lesser apes and old world monkeys. Since HIV-1 and other viruses that currently infect humans have evolved in the absence of selective pressure exerted by retrocyclins, the ability to reawaken this ancestral molecule could be used to strengthen the innate immune system's ability to prevent or limit the infections they now induce.

CHAPTER THREE: CATIONIC POLYPEPTIDES ARE REQUIRED FOR ANTI-HIV-1 ACTIVITY OF HUMAN VAGINAL FLUID

3.1 Introduction

Approximately 40 million people have been infected with HIV-1 worldwide according to the 2004 World Health Organization estimates [179]. There has been a dramatic increase in the global spread of human immunodeficiency virus type 1 (HIV-1) especially via the heterosexual mode of transmission [179,180]. At present, nearly 60% of infected individuals are women [181,182]. The natural sexual transmission of HIV occurs through mucosal surfaces such as vaginal or rectal mucosa [183]. Vaginal and rectal subepithelial stromal tissues are densely populated with dendritic cells (DC), macrophages and T-cells that express both CD4 and the HIV-1 co-receptors, CXCR4 and CCR5 [4,184]. Mechanisms whereby HIV-1 journeys across the mucosal epithelia are not completely understood, but may directly involve the epithelial cells [185]. Once the virus reaches the lamina propria, it can either directly infect macrophages or T lymphocytes or adhere to (or infect) dendritic cells, whose traffic to the regional lymph nodes converts them into sites of vigorous viral replication [186,187]. Whereas considerable attention in immunopathogenetic research on HIV-1 has been focused on acquired immunity, only recently has the role of innate immunity surfaced.

A layer of mucosal fluid covers the vaginal epithelium, and is composed of secretions from the cervical vestibular glands, plasma transudate, endometrial and oviductal fluids [188,189]. The fluid covering the vaginal mucosa protects against entry of pathogens into deeper tissues, including periodic sloughing of mucus and underlying cells to remove adherent microbes. The vagina is a host for numerous commensal microorganisms, which release organic acids and antimicrobial peptides to kill pathogenic invaders [112,190]. The vaginal epithelial cells, cervical glands, and

neutrophils contribute antimicrobial peptides to the milieu of the vaginal fluid, including lysozyme, lactoferrin, secretory leukocyte protease inhibitor (SLPI), human neutrophil peptides (HNP-1, -2 and -3) and human β-defensins (HBDs) [111]. We hypothesized that the sum total of these and likely other antimicrobial peptides and proteins contribute to the innate host defense of the vagina.

To date, evidence for the role of antimicrobial polypeptides in vaginal anti-HIV-1 host defense has been largely circumstantial. Lysozyme and lactoferrin have been shown to inhibit the infection by HIV-1 in vitro by preventing the adsorption and penetration of the virus [113,191-193]. Human β-defensins have been shown to inhibit HIV-1 replication [131] through modulation of the CXCR4 coreceptor as well by interacting directly with the virions. Several reports have shown that the level of SLPI is reduced in vaginal fluid of HIV infected persons [117,194]. SLPI has been shown to block HIV-1 infection in monocytes and T-cells by preventing the internalization of the virus prior to reverse transcription [115,117]. However, the action of SLPI is still debatable since other reports suggest that SLPI by itself has no effect on HIV-1 replication [118]. HNP-1-3 inhibit HIV-1 replication in vitro by two mechanisms: in the absence of serum they inhibit HIV-1 replication prior to integration of the virus in CD4+ T cells and in the presence of serum they interfere with the signaling pathways on target cells and block the nuclear import and transcription of HIV-1 genome [128,129,195].

In the current report, we explored the biological role of cationic antimicrobial polypeptides in protecting the vaginal mucosa from infection by HIV-1. We revealed that the cationic proteins in human vaginal fluid inhibited the entry of HIV-1 in human epithelial cell lines and organotypic

cervicovaginal tissues. We utilized a proteomic approach to identify 18 different cationic polypeptides in vaginal fluid, most of which have been previously reported to exhibit antimicrobial properties. While individual polypeptides at physiological concentration did not exhibit antiviral activity against HIV-1 infection, a combination of the peptides partially restored the antiviral activity. Selective depletion of cationic polypeptides from whole vaginal fluid reduced the intrinsic anti-HIV-1 activity. Most importantly, anti-HIV-1 activity of depleted fluid was restored upon repletion with the cationic polypeptide extract. Collectively, these studies suggest that the intrinsic anti-HIV-1 activity of vaginal fluid is an aggregate effect of all its active cationic polypeptide components.

3.2 Materials and methods

3.2.1 Reagents

Human neutrophil lysozyme and human milk lactoferrin were purchased from Sigma-Aldrich, (St. Louis, MO). Recombinant calgranulin A (S100A8) and calgranulin B (S100A9) were purchased from Abnova Corp. (Taipei, Taiwan). Recombinant Cystatin B and SLPI were purchased from R&D Systems (Minneapolis, MN). Histone H2A was purchased from Upstate USA Inc. (Charlottesville, VA). Cathepsin G was purchased from Bachem Bioscience Inc. (King of Prussia, PA). Recombinant β-defensins, HBD-1 and -2, were generous gifts from Dr. Tomas Ganz (David Geffen School of Medicine, UCLA, CA). The α-defensins, HNP-1, -2 and -3, were purified from human leukocytes and were generous gifts from Dr. Ganz and Dr. Robert I. Lehrer (David Geffen School of Medicine).

3.2.2 Collection and processing of vaginal fluid

Vaginal fluid was collected from post-menarcheal but pre-menopausal healthy female donors after informed consent as per the guidelines of the Institutional Review Board of University of Central Florida. Donors with current or recent vaginal infections and those under antibiotic treatment for any reason were excluded from the study using a questionnaire. To collect undiluted vaginal fluid, an Instead SoftCup (Ultrafem, Inc., La Jolla, CA) was inserted into the vagina as per the manufacturer's instructions, and removed after 30 min. The SoftCup was then centrifuged for 10 min at 1000 × *g* in a 50 ml sterile conical tube to collect the fluid sample [196]. Retrieved samples were then homogenized by sonication on ice using a microtip ultrasound probe (ten 2-3 sec pulses). These "minimally manipulated" whole vaginal fluid samples stored in aliquots at -20°C. This method enabled us to collect approximately 200 µl to 1 ml of vaginal fluid per collection. For most antiviral cell culture assays, the vaginal fluid was not manipulated further. For antiviral cell culture assays the vaginal fluids were extracted with 5% acetic acid for 2 hrs with gentle agitation, and the clarified supernatant was vacuum-dried and resuspended to the original volume in 100 mM sodium phosphate pH 7.4. To prepare the samples for two-dimensional proteomic analyses, the undiluted vaginal fluids were extracted using 5% acetic acid, vacuum dried, and resuspended in 0.1% hexadecyl trimethyl ammonium bromide (CETAB)/10% acetic acid/ 3× acid urea loading dye (9M urea, 5% acetic acid and methyl green).

3.2.3 Selective depletion of cationic polypeptides from vaginal fluid

Carboxymethyl weak cation-exchange resin (CM resin; Bio-Rad, Hercules, CA) was used to deplete cationic polypeptides from vaginal fluid [197]. The CM resin was pre-equilibrated with

vaginal fluid buffer (VFB) (60 mM NaCl and 20 mM KH_2PO_4; pH 6), which has been reported to be similar in electrolyte composition to vaginal fluid [111]. The CM resin was washed with VFB and centrifuged at 10,000 × *g* for 10 min and the overlying VFB was removed. Equal volumes of vaginal fluid from 10-23 donors was pooled, added to an equal bed volume of CM resin pre-equilibrated with VFB, and incubated overnight at 4 °C with gentle agitation. The CM resin was sedimented by centrifugation (16,000 × *g*, 5 min) and the cationic polypeptide-depleted supernatant was collected (hereunto termed "CM-depleted vaginal fluid"). The cationic polypeptides bound to the CM resin were extracted in subsequent 2 hr and 24 hr extractions using 5 resin volumes of 5% acetic acid at 4°C. The extracts were, pooled, vacuum-dried and resuspended to the original volume of vaginal fluid.

3.2.4 Cell lines and viruses

PM1, TZM-bl and H9 cells were obtained from the NIH AIDS Research and Reference Reagent Program. TZM-bl cells are a HeLa-derived cell line that stably expresses CD4 and CCR5 and contains the luciferase gene under the control of the HIV-1 promoter [170]. TZM-bl cells were grown in high glucose Dulbecco Modified Eagle Medium (DMEM) (Mediatech, Herndon, VA) supplemented with 100 U/ml penicillin, 100 µg/ml of streptomycin and 10% FBS. Passages 5-15 were used for experiments, and no change in cell behavior was observed between passages. PM1 cells were maintained at a density of 0.4- 0.8 × 10^6 /ml in RPMI1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 100 mM HEPES and 20% FBS (Gemini Bio-Products, Woodland, CA). H9 cells were cultured in the same manner as PM1 cells except 10% FBS was used. The HIV-1 laboratory strains BaL (R5) and IIIB (X4) were obtained from the NIH AIDS

Research and Reference Reagent Program. HIV-1 BaL was propagated in PM1 cells over 16 days. Supernatants containing virus were collected every other day starting 5 days post-infection, passed through a 0.45 m filter, and stored in aliquots at -80°C. HIV-1 IIIB was propagated similarly using H9 cells. Virus was quantitated by a sensitive commercial ELISA for $p24^{gag}$ (PerkinElmer, Boston, MA).

3.2.5 Assays to determine anti-HIV-1 activity of vaginal fluid and cationic polypeptides

TZM-bl cells were seeded in 96-well dishes (4000 cells/well). After 24 hours, cells were treated in triplicate with 50 µl culture medium containing whole vaginal fluid, CM-depleted vaginal fluid, peptides recovered from CM resin, vehicle control (100 mM sodium phosphate pH 7.4), individual recombinant or purified peptides at physiological concentration (**Table II)**, or combinations thereof. Culture media, or virus diluted in culture media (2 ng/ml p24 for BaL and 5 ng/ml p24 for IIIB), in 50 µl was immediately added to each well, and allowed to incubate at 37 °C/ 5% CO_2 for 24 hrs. Subsequently, luciferase activity was measured with Bright-Glo reagents (Promega, Madison, WI) according to manufacturer's instructions using an LMax luminometer (Molecular Devices, Sunnyvale, CA). Cytotoxicity and the metabolic activity of the cells were verified by a tetrazolium-based (MTT) assay as per the manufacturer's instructions (R&D Systems, Minneapolis, MN).

3.2.6 Two-dimensional gel electrophoresis of vaginal fluid

Acid-extracted vaginal fluid samples were electrophoresed on a 12.5% native acid urea-polyacrylamide gel (AU-PAGE) in the first dimension at 75V for 16-18 hr [197,198]. The gel was then stained with 0.1× amido black (0.04% napthol blue-black, 2.5% isopropanol and 1% acetic

acid) to visualize the protein bands. The entire lane of the first dimension AU-gel was excised, washed twice for 5 min each in dH_2O followed by two 5 min washes with 50 mM Tris pH 8.8, and soaked for 10 min in equilibration buffer (50 mM Tris, 6M Urea, 2% SDS, 20% glycerol, and bromophenol blue *ad libitum*, pH 8.8) containing 10 mg/ml DTT. The gel strips were electrophoresed in a 16% Tricine–SDS-PAGE as the second dimension for 20 hr at 40 mA [199]. Protein spots were visualized by SYPRO Ruby gel stain (Bio-Rad), excised, and stored at 4°C in 1% acetic acid until analyzed by mass spectrometry.

3.2.7 Identification of cationic polypeptides of vaginal fluid

The proteins were then subjected to trypsin digestion and mass spectrometric analysis (MALDI-TOF-MS/MS analysis [200] at the Microchemical and Proteomics facility at Emory University, GA as described earlier [201,202]. GPS Explorer 2.0 software (Applied Biosystems, Foster City, CA) and a MASCOT (http://www.matrixscience.com/) search engine were used for identification of peptide fragments. The NCBI non-redundant database and the *Mammalia* taxonomy were used for the searches.

3.2.8 Human Cervicovaginal Tissue Model

Organotypic "EpiVaginal" cultures of normal human vaginal-ectocervical epithelial cells and immuno-competent dendritic cells were propagated as suggested by MatTek Corporation (Ashland, MA). Each 60 mm^2 tissue adhered tightly atop a microporous membrane insert, and was maintained at the air-liquid interface using 5 ml of maintenance medium (MatTek). Tissues (3 per treatment condition) were pre-treated in with 50 l phosphate-buffered saline (PBS) or 50 l

vaginal fluid diluted 1:1 with PBS for 30 min and then rinsed twice with warm PBS. Tissues were then topically applied with either 100 l of PBS (control), PBS containing 25 ng p24 of HIV-1 BaL, or PBS containing BaL and vaginal fluid (equivalent to 50% of whole fluid), for 24 hr. Treatments were then removed and tissues were washed with 100 l warm PBS, then vaginal fluid (50%) or PBS vehicle were re-applied in 50 l. A one-time dose of 1×10^6 HIV-1 BaL-infected PM1 promyelocytic cells were included underneath the microporous insert to sustain the initial HIV-1 infection, and were removed after 2 days. Basal maintenance media was changed every other day, and the topical (apical) treatments were removed and re-applied on days 3 and 6 post-infection. On day 9 post-infection, DNA was extracted from 2 tissues per treatment condition using Qiagen's DNA Micro kit. Total protein was extracted from the third tissue per treatment condition and assayed by ELISA for HIV-1 $p24^{gag}$ (PerkinElmer).

3.2.9 Detection of HIV-1 provirus in human cervicovaginal tissue

HIV-1 infection of cervicovaginal tissues was assessed by real-time PCR quantitation of the HIV-1 BaL *env* gene (relative to β-actin controls) in total tissue DNA isolated 9 days post-infection. HIV-1 BaL primers used were: 5'- AACACCTCAGTCATTACAC -3' and 5'- TACATTGCTCTTCCTACTTC -3', which amplify a 700 bp region of BaL gp120. β-actin primers used were: 5'- CCTTCCAGCAGATGTG -3' and 5'- GGTGTAACGCA ACTAAG -3', which amplify a 105 bp region of human β-actin. 200 ng DNA was mixed with 2× Sybr Green supermix (Bio-Rad), 200 nM each primer, and dH_2O. Triplicate 20 µl reactions were carried out using the MyiQ real-time PCR detection system (Bio-Rad), and HIV-1 BaL levels were normalized to β-actin. Data was analyzed with iCycler iQ Optical System software. Melt-curve

analysis and gel electrophoresis revealed that single PCR products were amplified for each gene. Moreover, the *env* PCR product was verified by sub-cloning into pCR4-TOPO (Invitrogen), followed by DNA sequence analysis (Biomolecular Sciences Genomics Core Laboratory, UCF, Orlando FL).

3.2.10 Statistics

Luciferase assays were performed in triplicate for each treatment condition in each experiment, with relative light units in vehicle-only control wells set as 100% infection. Each treatment condition was analyzed by one-way ANOVA followed by Tukey pairwise comparison. Mass spectrometric analysis for each polypeptide identified was performed in duplicate, and protein spots with a confidence index (C.I %) greater than 85 percentile, combined with ion scores of ≥40 for one or more peptides matched to each protein, were considered positively identified [200].

3.3 Results

3.3.1 Human vaginal fluid is intrinsically active against HIV-1.

The mucosal layer lining the vaginal epithelial cells is rich in antimicrobial polypeptides that provide a crucial barrier against invading microbial and viral pathogens [111,190]. Although some of these polypeptides have been shown to exhibit antiviral properties [115,129,160,193,203,204], detailed analysis of the intrinsic anti-HIV-1 activity of vaginal fluid has not been reported. Herein, we explored the activity of the cationic polypeptide components of vaginal fluid against HIV-1. TZM-bl cells were treated with either PBS (vehicle control) or vaginal fluid diluted in DMEM high glucose medium with 10% FBS and infected with both R5 (HIV-1 BaL; **Figure 3.1A**) and X4

(HIV-1 IIIB; **Figure 3.1B**) strains of HIV-1. After 24 hours, excess virus was removed and infection was quantitated as a measure of luciferase expression. As compared to vehicle-only controls, vaginal fluid extracts significantly reduced the infection of both viral strains in a dose dependant manner. As measured by a standard MTT tetrazolium assay, the vaginal fluid extracts were not cytotoxic (data not shown). These results indicate that human vaginal fluid intrinsically inhibits the entry of HIV-1 into host cells.

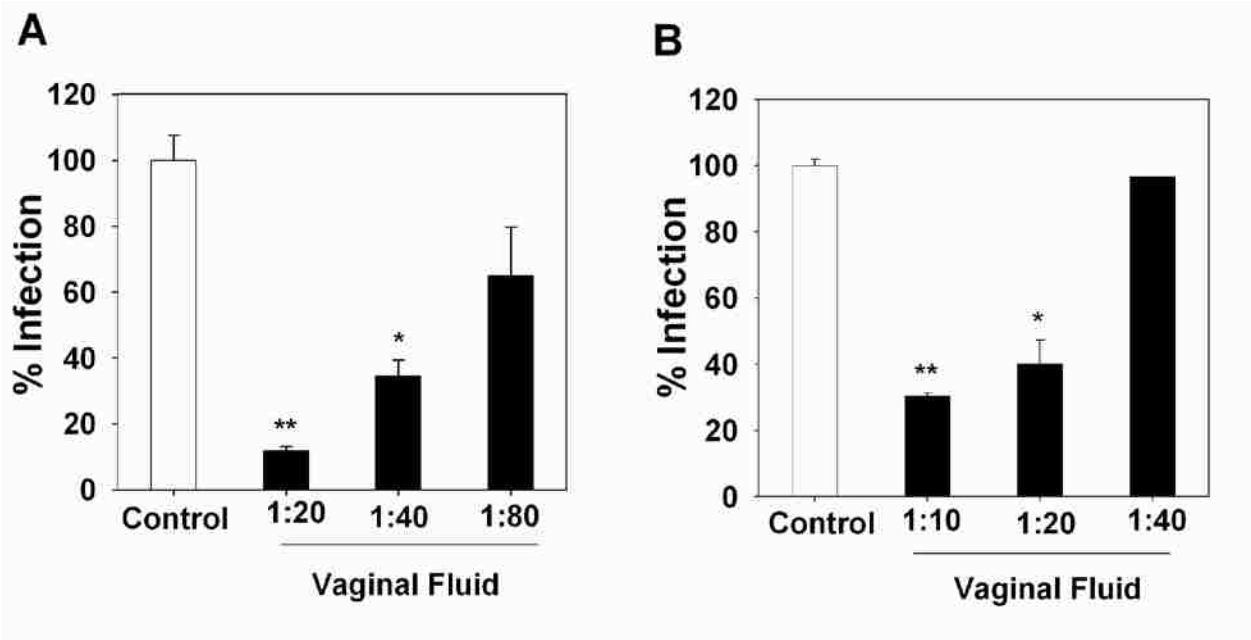

Figure 3.1: Human Vaginal fluid intrinsically inhibits HIV-1 infection. TZM-bl cells were treated with PBS (vehicle control) or vaginal fluid diluted in DMEM high glucose medium to a final concentration as indicated in the figure and were subsequently infected with (A) HIV-1 BaL (2 ng/ml p24) or (B) HIV-1 IIIB (5 ng/ml p24) for 24 hr. Infection was measured as a percent reduction in luciferase activity as compared to infected vehicle-only control (relative light units = RLU). Experiments were performed using 4 different pools of vaginal fluid each in triplicate. As compared with control, double asterisks indicates $P < 0.0002$; a single asterisk indicates $P < 0.0005$. Error bars represent SEM.

3.3.2 Anti-HIV-1 activity resides in the cationic fraction of vaginal fluid.

Experiments were designed to selectively remove the cationic polypeptides from whole vaginal fluid to determine if this depletion reduced the anti-HIV-1 activity of the fluid. Whole, undiluted vaginal fluid was collected from healthy donors using an Instead SoftCup. A weak cation exchange resin, CM-Prep (Bio-Rad), was utilized to deplete the cationic peptides and proteins from vaginal fluid, whilst sparing the concentrations of remaining proteins and electrolytes. We pioneered this technique, and have characterized the selective depletion of cationic polypeptides from nasal fluid [197]. The activity of whole vaginal fluid extract, CM-depleted vaginal fluid, and the polypeptides extracted from the CM resin were tested individually against HIV-1 BaL (**Figure 3.2A**) and HIV-1 IIIB (**Figure 3.2B**) in TZM-bl cells for 24 hours. Cells treated with whole vaginal fluid showed significant reduction in infection as compared with PBS control (P <0.0002; n = 13), while the CM-depleted fluid did not inhibit infection. Similar to whole vaginal fluid, polypeptides extracted from the CM resin exhibited significant anti-HIV-1 activity as compared with both the PBS control and CM-depleted vaginal fluid (P < 0.0002; n = 14). Taken together, these data indicate that the anti-HIV-1 activity of vaginal fluid is contained in the cationic fraction. Whole vaginal fluid, CM-depleted vaginal fluid, and the extracted cationic polypeptides were used in subsequent proteomic and reconstitution assays.

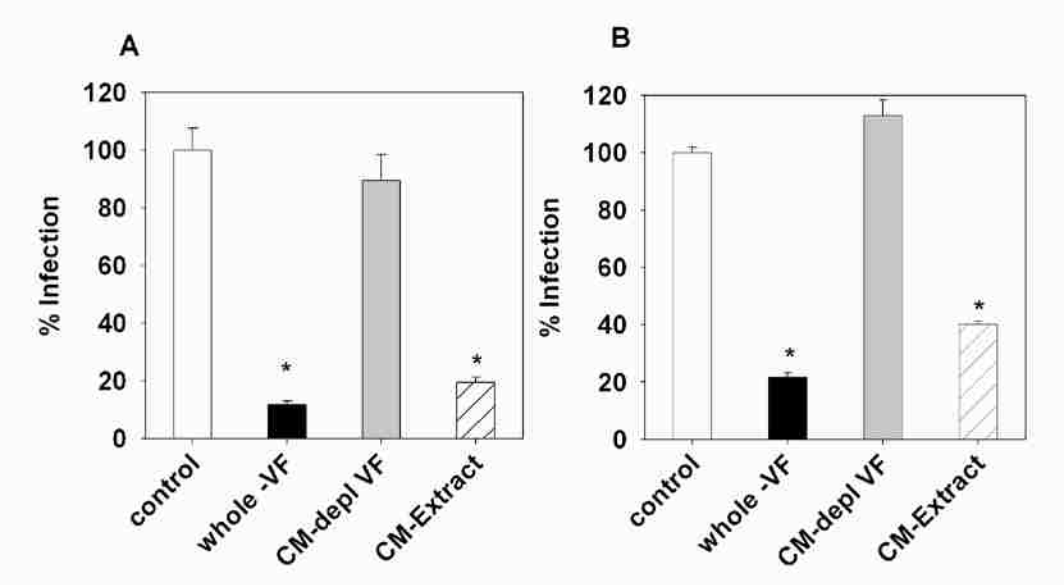

Figure 3.2: Depletion of cationic proteins from vaginal fluid increases HIV-1 infection. TZM-bl cells were treated with vehicle control, whole vaginal fluid extract (Whole-VF), CM-depleted vaginal fluid (CM-depl-VF) or the recovered peptides from the CM-resin (CM-extract) and infected for 24 hrs with HIV-1 (A) BaL p24 = 2ng/ml (B) IIIB p24= 5ng/ml. Luciferase levels were measured and percent infection was calculated as in Figure 1. The results are from experiments performed with 4 different pools of vaginal fluid, each repeated thrice. Asterisk indicates P <0.0002.

3.3.3 Identification of cationic polypeptides of vaginal fluid.

We next utilized a novel proteomic technique to identify cationic polypeptide components in vaginal fluid. The cationic polypeptide fraction from whole, undiluted vaginal fluid was subjected to AU-PAGE (the first dimension of a two-dimensional gel), which separates polypeptides based on cationic charge density [198,205], A slice from the AU-PAGE was inserted into a Tricine-SDS-PAGE gel (the second dimension) to separate low molecular weight polypeptides by size [69]. Gels were stained with SYPRO Ruby (**Figure 3.3**) or silver (not shown). Spots representing single polypeptides were excised from the gel and the sequence was identified by tandem mass

spectrometry (MALDI-TOF/TOF). Each polypeptide spot was sequenced from samples excised from both silver stained and SYPRO Ruby stained gels.

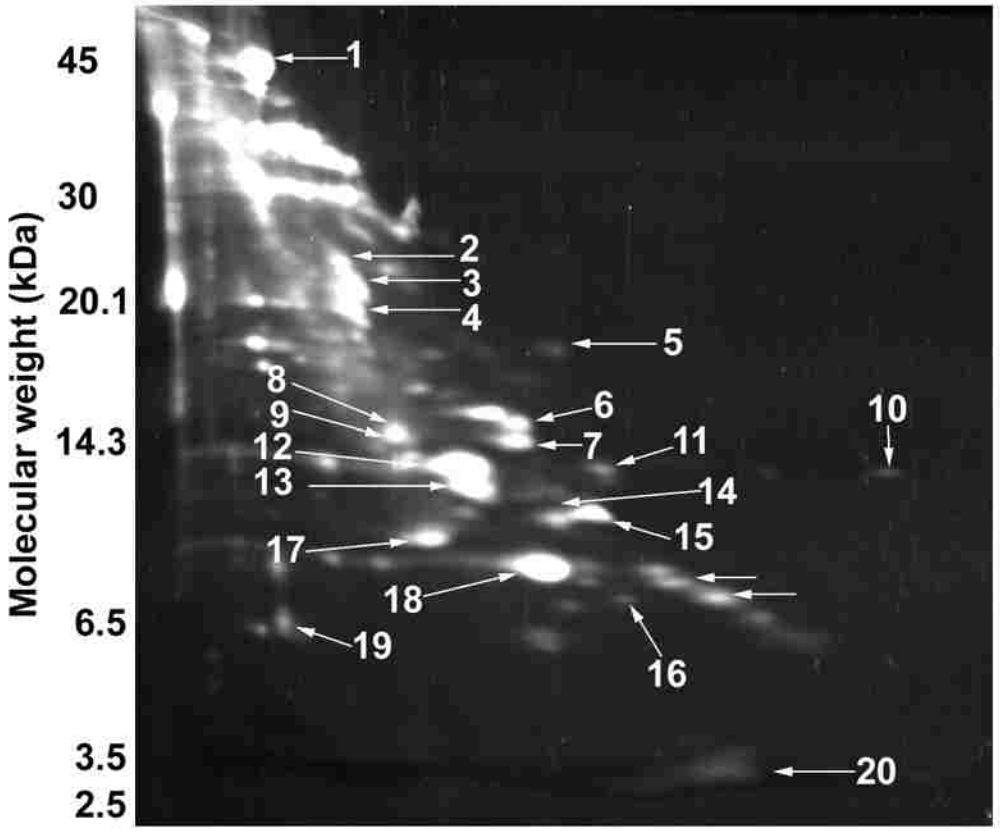

Figure 3.3: Identification of cationic polypeptides in vaginal fluid. Whole vaginal fluid (10 µl) was subjected to first dimensional electrophoresis in an AU-PAGE followed by Tricine SDS PAGE as the second dimension. The SYPRO Ruby stained two-dimensional gel of whole vaginal fluid extract showing the protein spots identified by MALDI-TOF mass spectrometric analysis. Each polypeptide spot and their reported role in host defense are listed in Table I. Unlabeled arrows were identified as fragments of human albumin.

Table 3.1 lists the corresponding protein spots labeled in **Figure 3.3** and indicates how each spot was identified in this study as well as the previously reported biological activity for each identified

polypeptide. Spots with a confidence index (C.I.%) greater than 85% were considered positive. Note that several smaller fragments of albumin were also detected, which are indicated by unlabelled arrows. We also discovered two novel, unnamed polypeptide fragments (**Figure 3.3**, spot #5 and 19) in vaginal fluid. In total, we have positively identified 18 unique cationic polypeptides in vaginal fluid, of which many have reported roles in host defense against HIV-1 infection [113,115,129,131,206,207].

3.3.4 Comparison of proteomic profiles of whole and CM-depleted vaginal fluids reveals cationic polypeptides that contribute to anti-HIV-1 activity.

Two-dimensional gel electrophoresis was next used to characterize the cationic polypeptides that remained in the vaginal fluid after CM depletion, as well as those that were extracted with the CM resin. Figure 3.4 compares 2-D gel electrophoretograms of whole vaginal fluid, CM-depleted vaginal fluid, and the polypeptides extracted from the resin. Among the polypeptides that were absent in CM-depleted fluid yet recovered from the resin include lysozyme, cystatin B, calgranulin B, histone H2A, HNP1-3, lipocalin-2 and cathepsin G (indicated by arrows in Figure 3.4A and 3.4C). Some components are reportedly active against HIV-1 (lysozyme and HNP1-3), while the anti-HIV-1 activities of the others have not been reported. We next explored which of the cationic polypeptide components of vaginal were the principal effectors active against HIV-1.

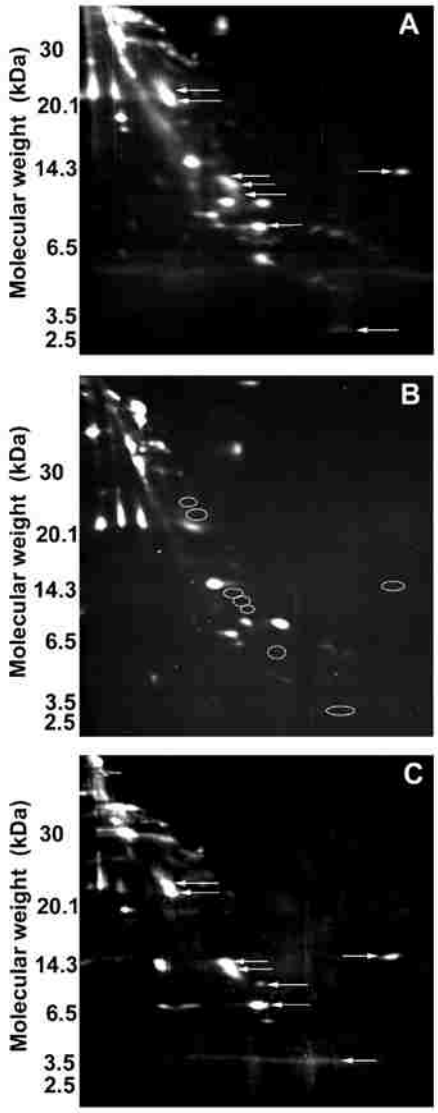

Figure 3.4: Comparison of proteomics profiles of whole vaginal fluid and Depleted vaginal fluid. Two-dimensional gel electrophoresis of 10 µl of (A) whole vaginal fluid, (B) CM-depleted vaginal fluid and (C) the extract of bound proteins from CM-resin was performed. The arrows in panels A and C indicate protein spots that were recovered in the polypeptide extract, and are listed in Table 3.4. The circles in panel B indicate the region where the corresponding cationic proteins in panel A are absent in depleted vaginal fluid.

Table 3.1: Cationic polypeptides identified in vaginal fluid by two-dimensional analysis and MALDI-TOF MS/MS analysis.

	Spot ID	Accession #	MW (Da)	Score[b]	C.I.%[c]	Role in Host Defense
1	Albumin	gi|23241675	45130.4	90	100	Transport of metabolites, drugs and toxins, Transcytosis of myeloperoxidase [208].
2	Neutrophil Gelatinase Associated Lipocalin	gi|4261868	20534.5	51	100	Bacteriostatic, Sequesters Iron siderophores [209,210].
3	Chain A, Cathepsin-G	gi|20664220	26740.9	227	100	Serine protease, Chemoattractant, lymphocyte activator, Inflammation. [211,212].
4	Human Neutrophil Gelatinase Associated Lipocalin	gi|7767000	19976.3	41	100	Antibacterial, sequesters Iron siderophores[209,210].
5	Unnamed protein product	gi|14041892	23795.5	44	100	(Unknown).
6	Histone H2B	gi|184086	11324.1	55	99	Antibacterial [213-216] .
7	H2A histone family	gi|4504251	14086.9	113	100	Antibacterial [213-216].
8	Fatty acid binding protein 5 E-FABP	gi|4557581	15154.5	196	100	Intracellular fatty acid trafficking, Stabilization of leukotrienes, Skin inflammation[217,218].
9	Human Galectin-7	gi|3891470	14934.8	71	100	S-lectin involved in cell-adhesion [219,220], migration immune response [221,222].
10	Lysozyme	gi|3660304	14599.2	100	100	Antibacterial and antiviral [191,197,223,224].
11	Lysozyme (dimer)	gi|14278473	14693.1	90	100	Artifact obtained after extraction from CM-resin [197].
12	Cystatin B-Human	gi|68783	11166.6	54	98	Cysteine proteinase inhibitor, immunomodulator, activates NO synthesis in macrophages [225,226].
13	Calgranulin B	gi|7417329	13102.5	88	89	Antibacterial [227,228]
14	Histone H2A family member	gi|10800144	13927.8	126	100	Antibacterial [213-216]
15	Histone H4	gi|223582	11230.3	127	100	Antibacterial [213-216]
16[d]	H2A histone family member	gi|18105045	13897.8	118	81	Antibacterial [213-216]
17	Cystatin A	gi|4885165	10999.7	120	100	Cysteine proteinase inhibitor, immunomodulator [226,228]
18	Calgranulin A	gi|29888	10930.8	197	100	Antibacterial [227,229]
19	Unnamed protein product	gi|14041892	23795.5	58	100	(Unknown)
20	Human Neutrophil peptide 3 HNP1-3	gi|229858	3489.6	63	99	Antimicrobial, [128,129,230-232]

a The numbers correspond to the labeled spots in **Figure 3.3**
b Ion Score of one or more peptide fragments that match a protein in the database
c Confidence Index percentage
d Note that this spot has a C.I.% index of <85% but was considered positive because it was identified in multiple samples.

3.3.5 Individual cationic peptides and proteins at physiologic concentrations are not active against HIV-1.

We tested the anti-HIV-1 activity of 13 cationic polypeptides that were either purified from natural sources or recombinant proteins. **Table 3.2** lists the physiologic concentration of cationic polypeptides in vaginal fluid from healthy donors as identified in this study and in Valore et al. [111]. Each polypeptide was tested for anti-HIV-1 activity at its measured physiological concentration. TZM-bl cells were treated with individual polypeptides at the final concentrations given in **Table 3.2**, infected with HIV-1 BaL or HIV-1 IIIB, and at 24 hrs anti-HIV-1 activity was measured by quantifying luciferase expression. At physiological concentrations, none of the polypeptides alone inhibited viral entry (data not shown). These data suggest that the antiretroviral activity of vaginal fluid may be a result of two or more cationic antimicrobial polypeptides acting in synergy.

Table 3.2: Physiological concentration of cationic proteins that contribute to anti-HIV-1 activity of vaginal fluid.

Protein	Concentration (μg/ml)	Method of detection	Reference
Calprotectin[ab]	34±7	Semiquantitative Western blot and densitometry	[111,227,229]
Cystatin B[a]	32.16	Densitometry	This study
Lysozyme[a]	13±2	Semiquantitative Western blot and densitometry	[111,223]
Histone H2A[a]	11.04	Densitometry	This study
Cathepsin G[a]	10.88	Densitometry	This study
Lactoferrin	0.9±0.2	Semiquantitative Western blot and densitometry	[111,113,193]
SLPI	0.7±0.1	Semiquantitative Western blot and densitometry	[111,115,117]
HBD-2	0.57±0.13	Semiquantitative Western blot and densitometry	[111,131]
HNP-1-3[ac]	0.35±0.07	Semiquantitative Western blot and densitometry	[111,128,206,233]
HBD-1	0.04±0.02	ELISA	[111,131]

a Represents the arrows indicated in **Figure 3.4A** and **3.4C**
b Calprotectin, heterodimer of calgranulin A and calgranulin B was tested as individual peptides
c HNP-1, HNP-2 and HNP-3 were tested as individual polypeptides

3.3.6 Cationic polypeptides of vaginal fluid synergize to inhibit HIV-1 infection.

The abundance of antimicrobial peptides in vaginal fluid with often overlapping roles in host defense suggests that the anti-HIV-1 activity is not a result of actions from individual peptides. Moreover, our studies also indicate that the individual polypeptides at physiological concentration do not prevent entry of HIV-1 into host cells. We therefore hypothesized that these polypeptides must act in concert to prevent the HIV-1 infection. To test our hypothesis, we prepared a cocktail of 13 available recombinant or natural peptides at physiological concentrations as shown in **Table 3.2**. TZM-bl cells were treated with either the polypeptide mix either alone or the polypeptide mix added to CM-depleted vaginal fluid, and were subsequently infected with HIV-1 (**Figure 3.5**). While the polypeptide mix alone reduced infectivity approximately 40%, this was not significant as compared with vehicle-only control. Moreover, the addition of the polypeptide mix to CM-depleted fluid was also not completely restorative. Due to availability, not every polypeptide identified was represented in the cocktail, which may have contributed to the incomplete restoration of CM-depleted fluid. This hypothesis is supported in our next experiment.

Interestingly, the cationic polypeptide extract (cleaved from the CM resin) was completely restorative to CM-depleted fluid ($P = 0.00012$; n = 14), and the combined anti-HIV-1 activity was equivalent to the activity of whole vaginal fluid (**Figure 3.5**). These data suggest that the anti-HIV-1 activity of vaginal fluid is primarily contained in the cationic fraction, and that the activity is complex and requires the collective polypeptides.

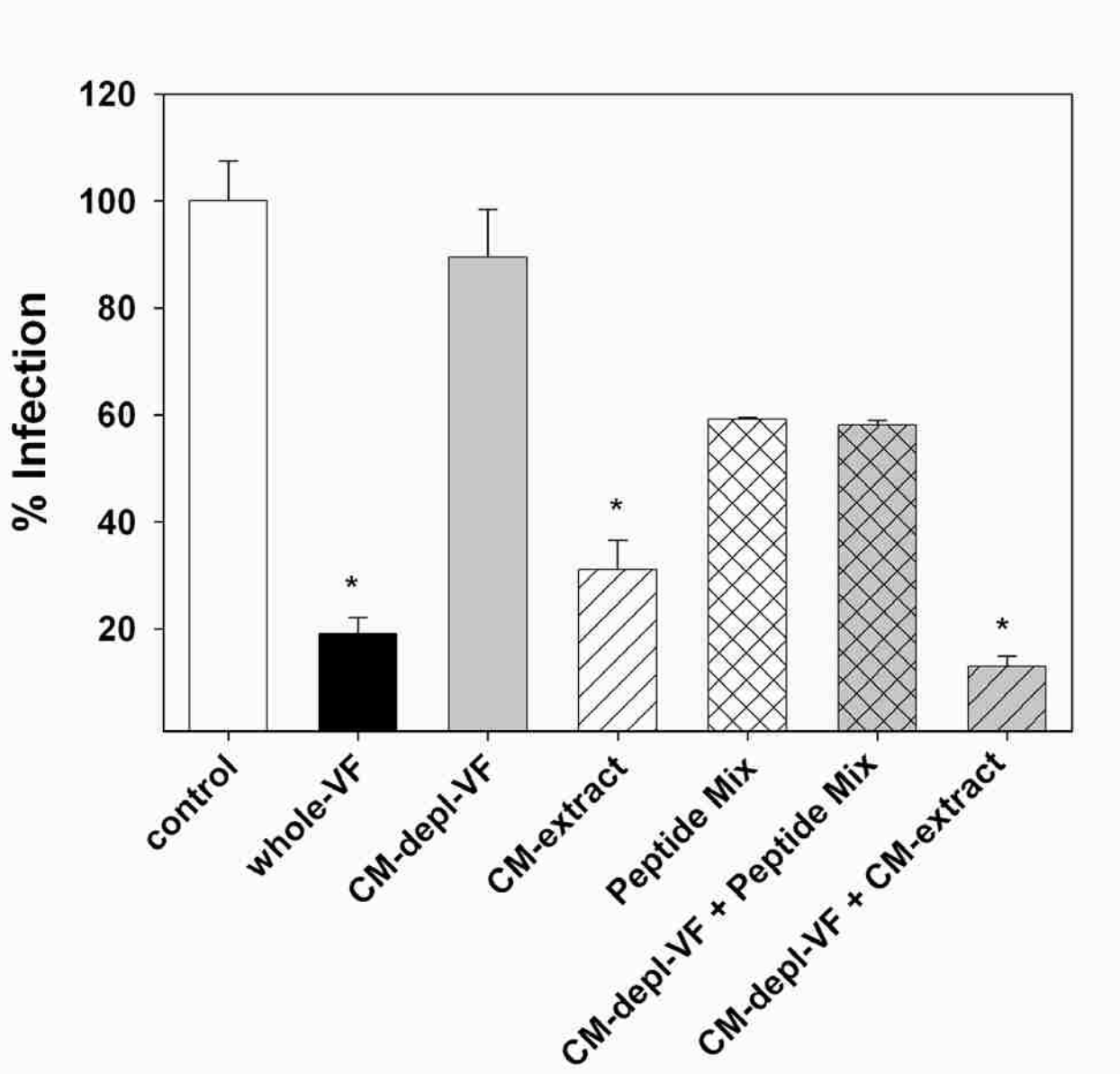

Figure 3.5: Cationic polypeptides of vaginal fluid synergize to inhibit HIV-1 infection. TZM-bl cells were treated as indicated in the figure and infected with BaL (p24 = 2ng/ml) for 24 hr. Luciferase was then measured as described earlier and percent infection was calculated. Asterisk indicates $P < 0.00015$. Experiments were performed in triplicate and error bars represent SEM.

3.3.7 Vaginal fluid protects against HIV-1 infection of human cervicovaginal tissue.

We next tested whether vaginal fluid could protect organotypic human cervicovaginal tissues against HIV-1 infection. This ex vivo model closely resembles the native mucosae of the ectocervix and vagina, containing full-thickness epithelia composed of vaginal-ectocervical cells that are interspersed with immuno-competent dendritic (Langerhans) cells in the basal and suprabasal layers. To study the role of vaginal fluid in reducing HIV-1 infection and integration of the proviral DNA into the host genome, cervicovaginal tissues infected with HIV-1 BaL in the absence or presence of an apical film of vaginal fluid were compared. The tissues were treated with PBS (vehicle control) or vaginal fluid diluted 1:1 in PBS for 30 min prior to infection with HIV-1 BaL (p24 = 25 ng/tissue) diluted in PBS (control) or in 50% vaginal fluid. 24 hrs post infection, excess virus was removed and PBS control or 50% vaginal fluid was reapplied to the apical tissue surface. Total tissue DNA was extracted 9 days post infection and the proviral DNA levels were assessed by real-time quantitative PCR of the *env* gene of HIV-1 BaL. Compared to tissues topically infected with HIV-1 BaL alone, cervicovaginal tissues that were treated with vaginal fluid for 30 minutes prior to the addition of HIV-1 BaL had approximately 4-fold fewer copies of proviral DNA, although this trend was not statistically significant due to variability in the untreated condition (**Figure 3.6A**). However, viral titer as quantified by $p24^{gag}$ ELISA was significantly lower in cervicovaginal tissues treated with vaginal fluid as compared with control tissues ($P = 0.0091$; n = 2; **Figure 3.6B**). These studies imply that vaginal fluid plays an important role in preventing HIV-1 transmission in the cervicovaginal mucosa.

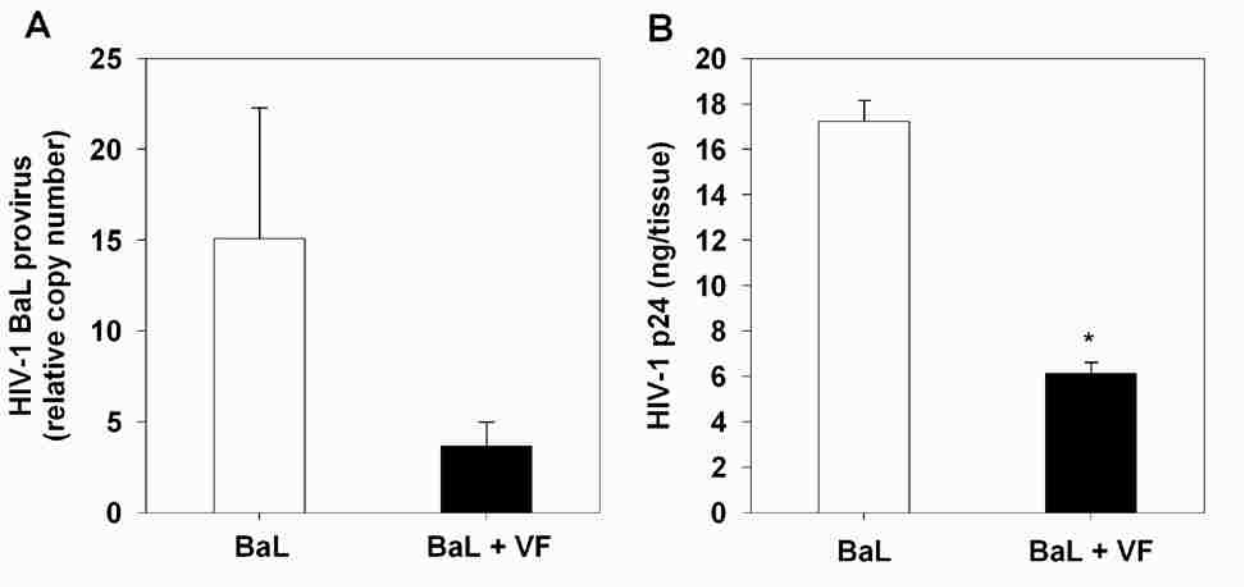

Figure 3.6: Vaginal fluid inhibits HIV-1 infection of human vaginal tissues. Human vaginal organotypic cultures were treated with PBS control or 50% vaginal fluid and infected with HIV-1 BaL. 9 days post infection tissues were harvested for DNA or protein analysis. (A) Realtime quantitative PCR of HIV-1 BaL proviral DNA corresponding to 700bp region of env gene was performed in BaL versus BaL + vaginal fluid (VF) infected tissues. (B) HIV-1 p24 protein levels in BaL versus BaL + VF infected tissues. Asterisk indicates P = 0.0091. Error bars represent SEM.

3.4 Discussion

The mechanisms by which the vaginal mucosa protects against sexually transmitted and other pathogenic infections are not completely understood. While several studies have focused on the adaptive immune system of mucosal surfaces of the female reproductive tract, scant attention has been focused on the innate immune factors in vaginal secretions [234-237]. Evidence is accumulating that vaginal epithelia are more than simple physical barriers to protect against

invading pathogens [111,238,239]. On the contrary, this surface and its overlying fluid are replete with antimicrobial polypeptides that act as effectors of innate host defense.

The current study provides evidence that cationic polypeptides contribute significantly to the intrinsic biological activity of vaginal fluid against HIV-1 infection. Proteomic analysis of the cationic polypeptide fraction of vaginal fluid revealed numerous cationic antimicrobial and host defense polypeptides. Polypeptides with known microbicidal effects that have been identified in our study and previously identified in mucosal secretions include lysozyme, lactoferrin, cathelicidin [240,241], β-defensins, α-defensins and SLPI [111,238]. Although each of the above polypeptides reportedly prevented HIV-1 infection, their activities were realized only when assayed at supraphysiologic concentrations. In the current study we determined that, at physiological concentration, none of the cationic polypeptides tested individually was active against HIV-1. However, a cocktail of the peptides added back to CM-depleted vaginal fluid partially restored the activity. Partial (rather than complete) restoration of activity may be reflective of the following: 1) some of the recombinant proteins may not exhibit the same anti-HIV-1 activity as that of the purified or natural proteins in the secretions, 2) while we created the cocktail with individual polypeptides, the full activity of certain proteins (e.g. calgranulins A and B) may be best realized in their heterodimeric form, and 3) due to availability, several polypeptides that we identified could not be included in the polypeptide cocktail. Any or all of these conditions support the premise that the collective cationic polypeptide fraction is responsible for anti-HIV-1 activity of vaginal fluid. Indeed, when the extracted cationic polypeptide fraction (bound to the CM-resin) was used to reconstitute the CM-depleted fluid, anti-HIV-1 activity was restored completely. Anti-HIV-1 activity of vaginal fluid is likely due to the collective cationic polypeptides acting synergistically.

Whole vaginal fluid was collected from healthy donors using a diaphragm-like device (Instead SoftCup), which enabled the collection of whole *undiluted* cervicovaginal fluid [196]. In contrast, other commonly used methods of cervicovaginal fluid collection, such as extraction from preweighed tampons or vaginal lavage [111,242,243], can suffer from protein adhesion to the tampon or a dilute lavage of unknown protein concentration. Unlike lavage, the Instead SoftCup is convenient and can be self-inserted, thus women are more receptive to donating cervicovaginal fluid. While no one method of collection is perfect, approaches that enable the retrieval of whole, undiluted fluid may afford the best representation of the condition in vivo.

Lactic and other organic acids that result in the low pH of human vaginal fluid (normally pH 3.8-4.5), as well as volatile compounds such as H_2O_2, are thought to contribute to microbial host defense [112,190]. Our studies were designed to minimize or eliminate the effects of these factors, as the acidity of the vaginal fluid was neutralized (pH 7.4) prior to subjecting the fluid to anti-HIV-1 assays. Moreover, although the all of the anti-HIV-1 activity was contained in the collective polypeptide extract, the procedures required for extraction would inactivate or remove H_2O_2 and other volatile compounds. Both whole vaginal fluid and the collective cationic polypeptide fraction were equally active against HIV-1, and thus the activity against HIV-1 was purportedly not a result of ancillary components of the cervicovaginal fluid.

Histones and their fragments were some of the more abundant polypeptides identified in vaginal fluid. Valore and colleagues identified histone H2B in the vaginal fluid of a healthy donor using a specific, yet quite insensitive, antibody [111]. Utilizing a more sensitive proteomic approach, we

identified histone fragments in every vaginal fluid sample tested. Histones have been shown to possess antibacterial properties and are released from activated neutrophils [213,214]. Why are histones present in mucosal secretions – do these proteins confer an active function, or are they just byproducts of cellular decay? Until recently, the latter was the most plausible explanation. However, a current study by Brinkmann and colleagues provided an alternative mechanism behind the presence of extracellular histones [214]. They reported that activated neutrophils release NETs (neutrophil extracellular traps), long elaborations of chromatin and neutrophil elastase that are independent of apoptosis or necrosis. NETs bound and inactivated both gram-positive and gram-negative bacteria, and prevented their dispersal. Moreover, NETs were abundant in experimental dysentery and in spontaneous human appendicitis. It is not known whether inflammatory cells in the cervicovaginal mucosa elaborate NETs and their associated histones as a host defense mechanism, or if histones are released simply as a result of cellular damage.

Other studies of vaginal fluid that specifically searched for the presence of β-defensins using sensitive antibodies were able to immunodetect low levels in vaginal fluid [244]. However, we did not uncover these peptides in our proteomic search, likely due to the scant concentration of these peptides and thus the low chance that they would be identified as a major spot on two-dimensional PAGE. While β-defensins have been shown to inhibit HIV-1 infection (50% inhibition at 20 μg/ml concentration) in human oral epithelial cells [131], the concentrations in vivo are 35- to 500-fold lower (**Table 3.2**) suggesting that they may not play a major role in antiretroviral host defense. Moreover, our studies revealed that HBD-1 and HBD-2 alone failed to inhibit HIV-1 infection at physiological concentration.

Surprisingly, vaginal fluid contains components that are permissive to the transmission of HIV-1. For example, samples of cervicovaginal lavage fluid that contained higher concentrations of calgranulin A have been shown to exhibit greater activation of HIV-1 in latently infected monocytic cells [245]. The human neutrophil-derived serine protease cathepsin G has also been shown to increase the susceptibility of macrophages to HIV-1 infection in vitro [211]. While the mechanism is not known, insights into the role of mucosal polypeptides that increase the probability of transmission and infection of HIV-1 would be critical in the development of effective antiretroviral treatments and preventatives. Taken together the above studies reveal that human vaginal fluid plays a crucial role in innate host defense against HIV-1 transmission.

CHAPTER FOUR: ANALYSIS OF PUTATIVE CLUSTERS OF REGULATORY MODULES IN THE RETROCYCLIN PROMOTER

4.1 Introduction

The expression of many antimicrobial peptides, such as defensins, can be induced in response to pathogenic assaults. Defensins, produced by both leukocytes and epithelial cells, are classified into 3 sub-families (α-,β- and θ-defensins) based on their intramolecular disulfide bonds and tertiary structure. Some α-defensins, such as human neutrophil peptides 1-3 (HNP 1-3), are constitutively expressed and stored in the granules of myeloid cells, while beta-defensins, such as human beta-defensin -1 (HBD-1), are constitutively expressed by keratinocytes [246-248]. On the other hand, certain β-defensins such as HBD 2-4 are inducible by cytokines like interleukin-1 (IL-1), tumour necrosis factor (TNF), or lipopolysaccharide (LPS) [246,249,250].

The third subfamily of defensins known as θ-defensins, exist as pseudogenes in humans due to the presence of a premature termination codon which prevents translation of the protein. However, θ-defensin mRNA has been detected in the human bone marrow, spleen, thymus, testis, skeletal muscle and cervicovaginal epithelial cells [134]. In our recent study we revealed that functional anti-retroviral human theta-defensins (retrocyclins) can be expressed in promyelocytic cells and cervicovaginal tissues [150]. Further, we showed that aminoglycosides allow read–through of the premature termination codon, thereby enabling the successful translation of native retrocyclin transcripts [150]. Having established that functional retrocyclins can be produced by human cells, we set out to study the regulation of this gene.

I*n silico* analysis of the upstream region of the retrocyclin gene (DEFT-1) revealed numerous transcription factor binding elements that are putatively regulated by IFN such as Interferon Regulatory Factors (IRF-1/IRF-2), Interferon Stimulated Gene Factor-3 (ISGF-3), Interferon Consensus Sequence Binding Protein (ICSBP) and others. Our preliminary studies indicated that retrocyclin mRNA expression was upregulated in epithelial cells in response to interferon type-I treatment or HIV-1 infection. Further, we have demonstrated that phosphorylated signal transducers and activators of transcription (STAT-1/-3) complex binds to the ISGF-3 recognition site on the DEFT-1 promoter resulting in upregulation of retrocyclin gene transcription (A.L.C., unpublished studies). Many of the IFN-inducible genes are regulated in a positive and negative manner by cis-acting elements like IRF-1 and IRF-2 respectively [251-253]. Reports by various groups have suggested that ICSBP/IRF-8 acts as a repressor for IRF-1 induced genes and IFN-beta regulated genes [251,254-256]. Some models speculate that ICSBP mediates its repressive effect by interfering with the binding of the ISGF-3 activator complex [257]. In this chapter we reveal that ICSBP may have a role in negative regulation of retrocyclin gene expression.

An independent genome-wide *in silico* study (K.S., unpublished data) based on the presence of 'clusters of regulatory modules' (CRM) revealed the recurring presence of a immune response cluster in the human chromosome 8p23 region. This cluster was defined by the presence of IRF-1/IRF-2, ISGF-3, AP-1 and NF- κB sites within a 250 bp region. Such a cluster was found at about 1.5kb upstream of the retrocyclin genes. We have examined the activity of these putative regulatory clusters by cloning them into enhancer trap reporter vectors and examining reporter expression in transfected cells.

4.2 Materials and Methods

4.2.1 Maintenance of cell lines

HeLa cells were obtained from ATCC and cultured using DMEM with 10% FBS, 100 g/ml of penicillin and 100 U/ml of streptomycin. HL60 cells were procured from ATCC and were cultured in Iscoves's DMEM with 20% FBS, 100 U/ml penicillin and 100 g/ml streptomycin (I20).

4.2.2 Prediction of retrocyclin promoter region

Genome contigs of the human 8p23 region were downloaded from NCBI database. Theta-defensin genes (DEFT) were identified by BLAST analysis. A 3kb region upstream of the transcription start site was extracted for further analysis. Putative transcription factor binding sites were predicted using TESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess)

4.2.3 Construction of plasmids

Human genomic DNA was isolated from HL60 cells using DNeasy kit (Qiagen, Valencia, CA) as per the manufacturer's instruction. Promoter fragments were PCR amplified as indicated in **Figure 4.2**. PCR products were electrophoresed on an agarose gel using 1X TBE buffer and verified for size. PCR fragments were then restriction digested with enzymes KpnI (New England Biolabs, Beverly, MA) and SacI (New England Biolabs) and ligated into appropriately linearized pGL3-promoter vector (New England Biolabs) using T4 DNA ligase. The ligation reactions were then transformed into TOP 10 chemically competent *E.coli* cells (Invitrogen, Carlsbad, CA) and

selected using 100 g/ml of ampicillin. Positive clones were verified for sequence integrity at the Florida State University sequencing center, Tallahasse, FL or Genewiz Inc. North Brunswick, NJ.

4.2.4 Transfection and luciferase assay

HeLa cells were seeded at a density of 4000 cells /well in a 96-well plate and allowed to grow for 2 days. Cells were then transfected with 100 g of positive control (pGL3-control), vector control (pGL3-promoter), or promoter constructs using Effectene reagent (Qiagen) as per the manufacturer's instructions. Cells were stimulated with IFN-beta (1 Unit/ml), or treated with DEX (10^{-5} M) (Sigma-Aldrich, St. Louis, MO), or both as indicated. Twenty-four hour post-transfection, cells were lysed and luciferase activity was measured using Bright Glo luciferase assay kit (Promega, Madison, WI) as instructed by the manufacturer's guidelines.

4.2.5 Statistics

Data is represented as fold increase over base line. Raw data was normalized to pGL3-basic vector. The threshold was set to the activity of control vector (pGL3-prom). Experiments were repeated four times. Statistical significance was determined by Wilcoxon-rank sum test. All statistical analyses were performed using the statistical package *R* (www.r-project.org).

4.3 Results

4.3.1 Computational analysis of retrocyclin promoter region

The DEFT genes were located on the chromosome 8 by BLAST analysis and the sequence of a 3 kb region upstream of the transcription start site was analyzed. Transcription factor binding sites

were predicted using Transcription Element Search Software (TESS) in the TRANSFAC database. The search revealed the binding sites for transcription factors IRF-1/IRF-2, ISGF-3, ICSBP or IRF-8 that are IFN response elements. In addition, we also predicted NF- κB and AP-1 binding sites that are located on numerous genes involved in immune response [258,259]. **Figure 4.1** shows the transcription factor binding sites located in the 3 kb region upstream of DEFT gene.

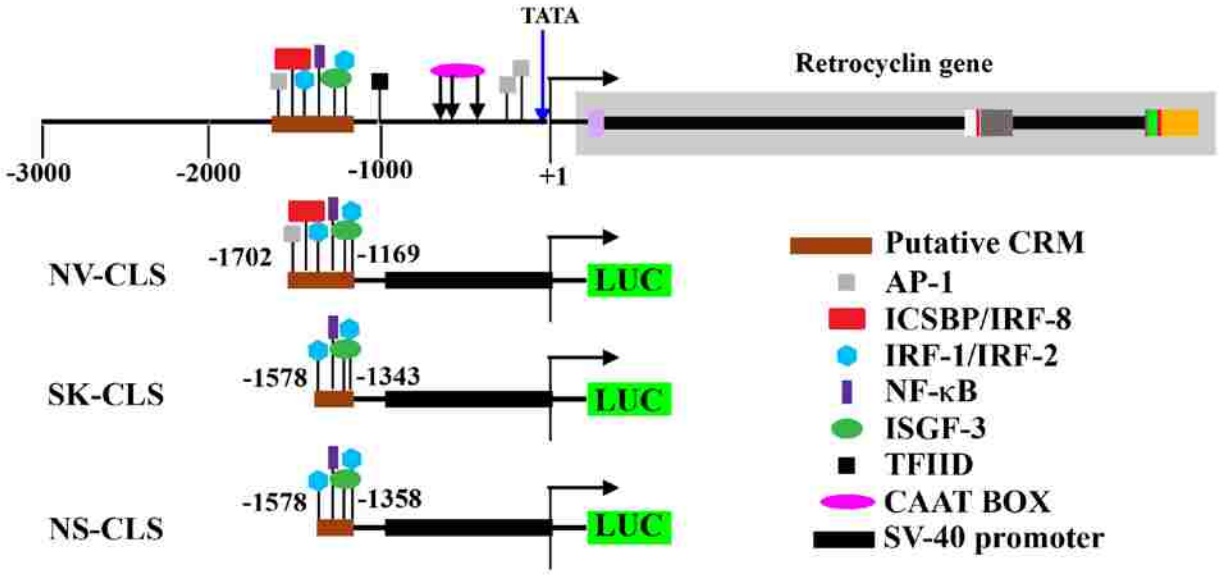

Figure 4.1: Schematic representation of the putative cluster on retrocyclin promoter. Figure illustrates the native retrocyclin gene along with a 3 kb upstream region. The transcription factor binding sites located on the putative cluster of regulatory module (CRM) are marked. The region amplified to create constructs NV-CLS, SK-CLS and NS-CLS are shown.

Most of the predicted clusters were present within 1.4-1.7 kilobases upstream of the gene start site. This 1.5 kilobase separation was representative of the distance between a distal CRM and the gene start site by computational analysis (K.S. personal communication). From the regions that contained the clusters, we amplified and cloned two different overlapping fragments into an enhancer trap vector containing a luciferase reporter. Primary amongst the tested fragments was

NV-CLS, a 533 bp region containing the predicted cluster. Similarly, the computationally identified cluster (SK-CLS) was also cloned into the enhancer trap vector as shown in **Figure 4.1**. It is important to note that the SK-CLS cluster is located upstream of a copy of another retrocyclin gene in the chromosome 8p23 region and is 81.5% similar to the sequence shown in **Figure 4.2**.

Figure 4.2: Sequence of an enhancer cluster upstream of retrocyclin gene. Figure shows the sequence of the region -1750 bp to -1150 bp upstream of the retrocyclin gene. The transcription factor binding sites predicted using TESS is highlighted. The black arrows represent the NV-CLS fragment and the blue arrows indicate the SK-CLS and NS-CLS fragments.

4.3.2 IFN response elements contribute to activity of retrocyclin promoter

Our preliminary analysis on the activity of SK-CLS and NV-CLS revealed that SK-CLS fragment showed higher expression of luciferase than NV-CLS when transfected into HeLa cells (**Figure 4.3A**). However, this was observed in a cell lineage specific manner, and only certain HeLa cell lines allowed the expression of reporter from the SK-CLS fragment. In order to explain the

observed differences in expression, we compared the transcription factor binding sites in SK-CLS and NV-CLS fragments. We identified an ICSBP/IRF-8 binding site in NV-CLS, but not in SK-CLS. ICSBP/IRF-8 has been shown to be a repressor for IFN-inducible genes [254-256,260]. In order to understand the role of ICSBP/IRF-8 in the regulation of this cluster, a deletion construct NS-CLS (**Figure 4.1**) was created using a forward primer located downstream of the ICSBP binding site and cloned into pGL3-prom vector **(Figure 4.2)**.

4.3.3 Role of ICSBP/IRF-8 in the regulation of putative clusters of regulatory modules located on retrocyclin promoter.

Constructs SK-CLS, NV-CLS and NS-CLS or pGL3-prom (vector control) were transfected into HeLa cells and treated with vehicle control **(Figure 4.3A**), 10^{-5} M Dexamethasone (DEX), an inhibitor of ICSBP/IRF-8 (**Figure 4.3B**), 1 Unit/ml of IFN-β (**Figure 4.3C**), or DEX and IFN-β (**Figure 4.3D**). Twenty-four hours post-transfection, cells were lysed and luciferase was quantified. Treatment with DEX in cells transfected with NV-CLS, but not SK-CLS or NS-CLS increased the expression of luciferase (1.5 fold) as compared to vector control, suggesting a role for ICSBP/IRF-8 in the regulation of retrocyclin gene expression. Although the application of 1 Unit/ml of IFN-β to transfected cells showed only a modest increase in the activity of the enhancer fragments after 24 hr, addition of DEX to cells primed with IFN-β showed an increase in the activity of the NV-CLS fragment. A Wilcoxon rank sum statistical analysis of the activity of NV-CLS was determined to have a *p*-value = 0.05 (**Figure 4.3B, 4.3C and 4.3D**). This shows that the retrocyclin gene expression may be tightly regulated by the ISRE elements and ICSBP/IRF-8 transcription factor.

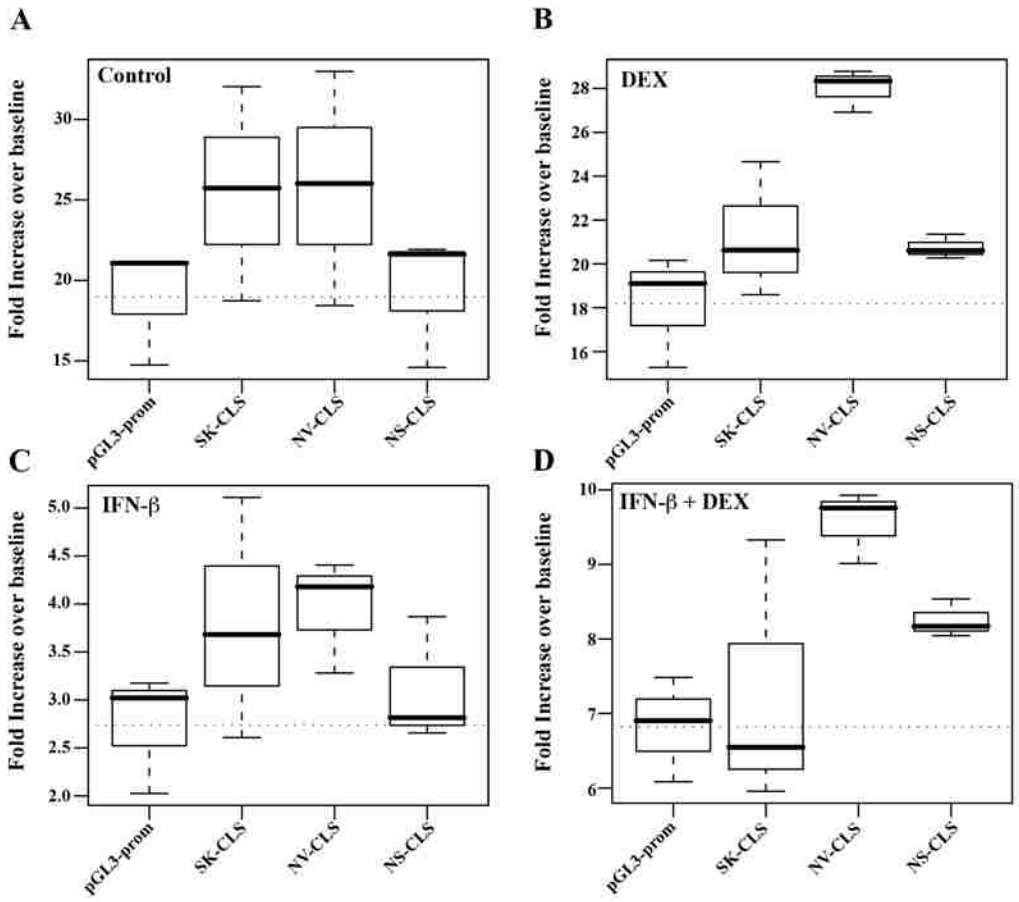

Figure 4.3: Activity of retrocyclin enhancer clusters. HeLa cells were transfected with pGL3-prom (vector control) or SK-CLS or NV-CLS or NS-CLS and treated with vehicle control or 10-5 M Dexamethasone (DEX) or 1 Unit/ml of IFN-β for 24 hr. Activity of the enhancer clusters was measured by quantifying luciferase. Data is represented as fold increase over baseline (pGL3-basic). The dotted line represents the activity of the SV40 minimal promoter of control vector.

4.4 Discussion

In this study, we have identified CRM's that respond to a cohort of transcriptional regulators (IRF-1/2, ISGF-3, ICSBP, NF-κB and AP-1) upstream of the retrocylin genes. Further, we have evidence that ICSBP plays a role in down-regulation of retrocyclin gene expression. Originally it

was thought that the expression of ICSBP was restricted to cells of lymphoid origin, but some groups have isolated active ICSBP from epithelial cells [261,262]. In our hands, we found that application of dexamethasone, an inhibitor of ICSBP increased the activity of the retrocyclin enhancer cluster in cervicovaginal epithelial cells. Activating the transcription of retrocyclin genes using interferons and restoring the translation using aminglycosides might provide the host cell with a potential shortcut in the activation of its antiviral defense.

Interestingly, despite being a pseudogene, retrocyclin gene transcription is responsive to HIV-1 infection and treatment with IFNs in cell lines (A.L.C., unpublished studies). This raises interesting possibilities about the gene itself since it resides in the highly polymorphic 8p23 region in the human genome [134,137,263,264]. It is known that genomes give birth to novel genes in polymorphic regions that witness high levels of recombination, called the 'gene nurseries' [265]. Given that the 8p23 region is polymorphic and the retrocyclin gene itself is lost only recently in evolution (in hominids), it is possible that the region is currently undergoing changes [134]. Following this line of reason, one can expect the current state of retrocylin genes (expressed pseudogenes) to be nothing but a snap shot in evolutionary history, and a single point mutation could reverse these dormant genes into active genes.

In conclusion, we have demonstrated that the retrocyclin genes are under the control of a complex, tightly regulated cluster with Interferon being an important hub in this regulatory network. Knowledge of retrocyclin gene cluster regulation would enable us to exploit their interferon mediated activation for therapeutic purposes.

CHAPTER FIVE: CONCLUSION

Sexual transmission of HIV-1 continues to be the most common mode of spread of HIV-1 infection [3,4]. Despite decades of research efforts, there are no effective drugs to treat HIV-1 infection. Development of HIV-1 vaccines has been difficult due to the rapid mutation rate of the virus. Therefore, there is an urgent need for effective prophylactic measures active against HIV-1 to curb the spread of the AIDS pandemic. Prevention methods such as condom use are effective to prevent HIV-1 entry. However, women who are at greatest risk of acquiring HIV infection often are unable to negotiate condom use, which is under the control of the male sexual partner. Topical vaginal microbicides are a viable HIV-1 prevention strategy that provides women with control over their sexual health [71,266,267].

In our studies, we have taken a simple approach to translate a human theta-defensin pseudogene, retrocyclin and activate an innate antiviral mechanism in human cells. Theta-defensins are 18-residue macrocyclic peptides formed by the head-to-tail ligation of two nona-peptides, and are potent inhibitors of HIV-1 infection. Whilst theta-defensin genes (DEFT) are intact in Old World monkeys, they exist as pseudogenes in humans [134]. In this work, we revealed that, upon correction of the premature termination codon in DEFT pseudogenes, human myeloid cells produce cyclic, antiviral retrocyclins peptides indicating that these human cells have retained the intact machinery to make cyclic peptides. Further, we exploited the ability of aminoglycoside antibiotics to suppress the premature termination codon in retrocyclin transcripts to produce functional peptides active against HIV-1. Since tobramycin and other aminoglycosides could restore the endogenous production of retrocyclins in human cervicovaginal tissues,

aminoglycoside-based topical vaginal microbicides might be useful in preventing sexual transmission of HIV-1 [150].

The primary sites of HIV-1 infection are the vaginal mucosal surfaces that are replete with antimicrobial proteins or polypeptides that are effectors of innate host defense [111]. Previous studies by other groups have analyzed the role of one or more antimicrobial peptides in host defense. However, the anti-retroviral activity of the antimicrobial peptides present in human vaginal fluid at physiological concentrations has not been not clearly understood. Our study indicates that cationic polypeptides contribute to the majority of the intrinsic anti-HIV-1 property of vaginal fluid. We identified 18 different cationic polypeptides using a proteomic approach in minimally manipulated vaginal fluid. When these cationic peptides were tested individually at physiological concentration they failed to prevent HIV-1 infection, but a mixture of commercially available cationic peptides partially restored the anti-HIV-1 activity. Moreover, depletion of cationic polypeptides from vaginal fluid reduced the anti-HIV-1 activity, while addition of the cationic polypeptide extract restored the anti-HIV-1 property. This suggests that the intrinsic property to inhibit HIV-1 entry resides in the cationic polypeptide fraction of human vaginal fluid [132].

In spite of the presence of numerous antimicrobial peptides, prevalence and transmission of HIV-1 continues to rise. Reduction in the expression of antimicrobial peptides in vaginal fluid may be associated with increased risk of HIV-1 infection [111,132]. Therefore, enhancing the expression of antiviral peptides such as retrocyclins during these periods could help in preventing HIV-1 infection. We analyzed the regulation of expression of retrocyclin gene and identified putative

clusters of regulatory modules located in the gene upstream region. This regulatory cluster contained various transcription factor binding sites that are involved in IFN response pathway. One of the transcription factor binding sites: Interferon Stimulated Gene Factor -3 (ISGF-3) was determined to be associated with activated signal transducers and activators of transcription (STAT) complex and upregulate the expression of retrocyclin (A.L.C. unpublished study). Another factor called Interferon Consensus Sequence Binding Protein (ICSBP) acted as a negative regulator of retrocyclin gene expression. Our data suggest that despite being a pseudogene, retrocyclin expression is regulated in a positive and negative manner. Understanding the regulation of retrocyclins by interferons would help in enhancing the expression of retrocyclins. A combination of interferons and aminoglycosides could help to activate and express endogenous retrocyclin peptides to prevent HIV-1 infection.

In conclusion, we have shown that if the premature termination codon in retrocyclin transcript is restored human myeloid cells can express cyclic active retrocyclin peptides [150]. Moreover, the premature termination codon can be readthrough by aminoglycosides to express endogenous anti-retroviral retrocyclins [150]. The desirable properties of retrocyclins and its congeners, such as broad spectrum activity against various strains of HIV-1 [141-143], activity in the acidic pH of human vaginal fluid [162], low cytotoxicity to human cells and ectocervical tissues [150,162] makes them promising molecules to develop as topical microbicides to prevent HIV-1 infection.

APPENDIX : INSTITUTIONAL REVIEW BOARD – APPROVED CONSENT FORM FOR HUMAN SUBJECTS

University of Central Florida Institutional Review Board Office of Research & Commercialization
12201 Research Parkway, Suite 501 Orlando, Florida 32826-3246 Telephone: 407-823-2901, 407-882-2012 or 407-882-2276
www.research.ucf.edu/compliance/irb.html

EXPEDITED CONTINUING REVIEW APPROVAL NOTICE

From : **UCF Institutional Review Board**
FWA00000351, Exp. 10/8/11, IRB00001138

To : **Alexander M. Cole** and Co-PIs: **Amy L. Cole**

Date : **January 05, 2009**

IRB Number: **BIO-07-04219**

Study Title: **Retrocyclins: Circular defensins active against HIV-1**

Dear Researcher,

This letter serves to notify you that the continuing review application for the above study was reviewed and approved by the IRB designated reviewer on **1/5/2009** through the expedited review process according to 45 CFR 46 (and/or 21 CFR 50/56 if FDA-regulated).

Continuation of this study has been approved for a one-year period. The expiration date is 1/4/2010. This studywas determined to be no more than minimal risk and the categories for which this study qualified for expedited revieware:

3. Prospective collection of biological specimens for research purposes by noninvasive means.

4. Collection of data through noninvasive procedures (not involving general anesthesia or sedation) routinelyemployed in clinical practice, excluding procedures involving x-rays or microwaves. Where medical devices are employed they must be cleared/approved for marketing.

Use of the approved, stamped consent document(s) is required. The new form supersedes all previous versions, which are now invalid for further use. Only approved investigators (or other approved key study personnel) may solicit consent for research participation. Subjects or their representatives must receive a copy of the consent form(s).
All data must be retained in a locked file cabinet for a minimum of three years (six if HIPAA applies) past thecompletion of this research. Any links to the identification of participants should be maintained on a password-protected computer if electronic information is used. Additional requirements may be imposed by your fundingagency, your department, or other entities. Access to data is limited to authorized individuals listed as key study personnel.

To continue this research beyond the expiration date, a Continuing Review Form must be submitted 2 – 4 weeks priorto the expiration date. Use the Unanticipated Problem Report Form or the Serious Adverse Event Form (within 5 working days of event or knowledge of event) to report problems or events to the IRB. Do not make changes to thestudy (i.e., protocol methodology, consent form, personnel, site, etc.) before obtaining IRB approval. Changes can be submitted for IRB review using the Addendum/Modification Request Form. An Addendum/Modification Request Form **cannot** be used to extend the approval period of a study. All forms may be completed and submitted online at https://iris.research.ucf.edu .

On behalf of Tracy Dietz, Ph.D., UCF IRB Chair, this letter is signed by:

IRB Coordinator

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