Journal of proteome research

ARTICLE

pubs.acs.org/jpr

# Comprehensive Proteomic Study Identifies Serpin and Cystatin Antiproteases as Novel Correlates of HIV-1 Resistance in the Cervicovaginal Mucosa of Female Sex Workers

A. Burgener,[*,†] S. Rahman,[†] R. Ahmad,[‡] J. Lajoie,[†] S. Ramdahin,[†] C. Mesa,[§] S. Brunet,[§] C. Wachihi,[∥] J. Kimani,[†,∥] K. Fowke,[†,∥] S. Carr,[‡] F. Plummer,[†,§] and T. B. Ball[†,∥,§]

[†]Department of Medical Microbiology, University of Manitoba, Winnipeg, Canada R3T 2N2

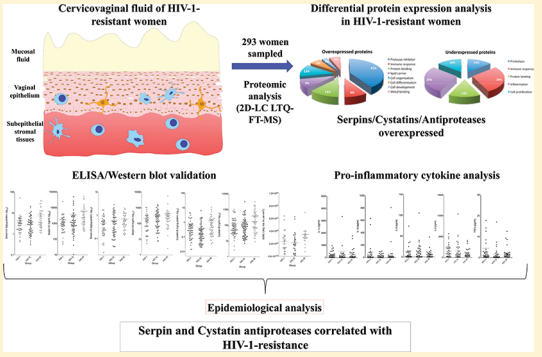
[‡]Broad Institute of MIT and Harvard, Cambridge Center, Cambridge, Massachusetts 02142, United States

[∥]Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya

[§]Public Health Agency of Canada, Winnipeg, Manitoba, Canada

Supporting Information

**ABSTRACT:** Not all individuals exposed to HIV-1 become infected, and evidence from HIV-1 highly exposed seronegative women (HIV-1-resistant) suggests that mucosal factors in the female genital tract, the first site of contact for the virus, are playing a role. To better understand factors mediating protection from HIV-1, we performed a large clinical study using the tools of systems biology to fully characterize the cervicovaginal mucosa proteome in HIV-1-resistant women. Cervicovaginal lavage fluid was collected from 293 HIV-1-resistant, uninfected, and infected sex workers and analyzed by 2D-LC LTQ-FT-MS. Of the more than 360 unique proteins identified, 41 were differentially abundant (>3-fold cutoff) in HIV-1-resistant women. The majority of over-abundant proteins were antiproteases (>40%), some with described anti-inflammatory and anti-HIV-1 activity. Quantification of specific anti-HIV-1 antiproteases Serpin A1, Serpin A3, and Cystatin B and an epithelial antiprotease A2ML1 found them to be significantly over-abundant in HIV-1-resistant women ($p$ = 0.004; $p$ = 0.046; $p$ = 0.0003; and $p$ = 0.04, respectively). Expression levels were not correlated to sexual practices or other epidemiological factors. Mucosal antiprotease levels correlated with pro-inflammatory cytokine concentration ($p$ = <0.0001), but independently of pro-inflammatory cytokine levels in HIV-1-resistant women including TNF-alpha, IL-1 alpha, IL-1 beta, IL-6, and IL-8. This comprehensive systems biology approach identifies mucosal serpins and cystatins as novel correlates of HIV-1-resistance. This represents the first study characterizing these factors in the female genital tract.




**KEYWORDS:** HIV-1, mucosal immunity, vaginal fluid, Kenyan sex workers, innate immunity, infections, HIV resistance, HESN, HIV highly exposed seronegative individuals, immune factors, microbicides, prostitutes, proteomics, mass spectrometry, biomarkers, serine protease inhibitors, cysteine protease inhibitors, serpins, cystatins, cytokines

## INTRODUCTION

Since the beginning of the pandemic, HIV/AIDS has affected over 60 million individuals worldwide and currently infects over 33 million people.[1] The development of a successful vaccine for HIV-1 is thought by many to be the most effective means to stem the HIV-1 pandemic. However, after decades of research and significant investment, there has been a clear lack of progress in this area. The latest trials of candidate vaccines have demonstrated only partial (30%) efficacy,[2] making it clear that HIV-1 is a unique challenge as a vaccine target.[3,4] Further compounding this challenge is that we still lack understanding of what provides natural protection against HIV-infection.[4]

Globally, the HIV-1 pandemic disproportionately affects women due to numerous biological and social factors, and this is especially evident in sub-Saharan Africa and other developing areas, where they constitute more than 60% of new infections.[1] Thus, interest has grown in the development of preventative technologies for women, such as vaginal microbicides.[5] Women are particularly vulnerable to HIV-1 infection, as they cannot control condom use of their partners, making them more susceptible to HIV-1 infection. The development of female-controlled prevention methods, which could be used independently and in regions where HIV-therapeutics is not readily accessible, would be extremely beneficial. However, our lack of knowledge of HIV transmission and pathogenesis and what provides natural protection from infection is a substantial barrier in microbicide research.[4] Therefore, the identification of naturally occurring factors, which mediate protection from HIV infection in women, would be critical to the development of preventative strategies.

**Received:** June 20, 2011
**Published:** October 05, 2011

ACS Publications © 2011 American Chemical Society

**Table 1. Differentially Abundant Proteins in the Cervicovaginal Mucosa of HIV-1-Resistant Sex Workers As Identified by Mass Spectrometry[a]**

| | | over-abundant proteins, ratio (fold change) | | | | | |
|---|---|---|---|---|---|---|---|
| protein | biological function | accession number | sequence coverage (%) | # unique peptides detected | R/U | R/I | R/$(U+I)_{avg}$ |
| Serpin A1 | Elastase inhibitor | P01009 | 71 | 27 | 1.9 | 3.0 | **2.4** |
| Serpin A3 | Elastase/Cathepsin G inhibitor | P01011 | 38.6 | 17 | 7.9 | 20.9 | **11.5** |
| Serpin B1 | Elastase/Cathepsin G inhibitor | P30740 | 52 | 16 | 1.4 | 1.9 | **1.6** |
| Serpin B13 | Cysteine protease inhibitor | Q9UIV8 | 16.3 | 4 | 3.6 | 3.3 | **3.5** |
| Serpin C1 | Anticoagulant, thrombin inhibitor | P01008 | 27.9 | 13 | 9.2 | 11.9 | **10.4** |
| Serpin G1 | Plasma protease C1 inhibitor | P05155 | 13.6 | 6 | 2.9 | 1.2 | **1.7** |
| Cystatin B | Cysteine protease inhibitor | P04080 | 61.2 | 5 | 1.9 | 3.3 | **2.4** |
| Cystatin A | Cysteine protease inhibitor | P01040 | 63.2 | 8 | 2.8 | 1.4 | **1.9** |
| A2ML1 | Elastase inhibitor, broad spectrum antiprotease | Q6ZW52 | 9.4 | 9 | 1.0 | 16.8 | **1.9** |
| Elafin | Elastase inhibitor | P19957 | 26.4 | 2 | 0.8 | 425.4 | **1.6** |
| SPINK 5 | Serine protease inhibitor | Q9NQ38 | 19.6 | 19 | 1.7 | 4.0 | **2.4** |
| Dermcidin | Innate immune response, antimicrobial | P81605 | 18.1 | 2 | 4.0 | 1.2 | **1.8** |
| HSP1B | Protein binding, apoptosis inhibitor | P04792 | 51.7 | 8 | 5.0 | 12.0 | **7.1** |
| ApoA2 | Lipid carrier | P02652 | 69 | 8 | 6.5 | 7.2 | **6.8** |
| ApoA1 | Lipid carrier | P02647 | 79 | 30 | 1.9 | 5.7 | **2.8** |
| ApoE | Lipid carrier | P02649 | 4.7 | 1 | 3.1 | 5.0 | **3.8** |
| S100A7 | Innate immune response, epidermis development | P31151 | 86.1 | 11 | 15.2 | 1.7 | **3.1** |
| IVN | Cell organization | P07476 | 11.4 | 6 | 2.5 | 7.9 | **3.8** |
| SLURP1 | Keratinocyte development, antitumor activity | P55000 | 39.8 | 3 | 1.9 | 4.4 | **2.6** |
| Fibrinogen beta | Cell organization | P02675 | 37.6 | 16 | 1.3 | 4.8 | **2.0** |
| Fibrinogen gamma | Cell organization | P02679 | 24.5 | 8 | 2.0 | 16.6 | **3.6** |
| IGHA1 | Immune response | Q6GMX2 | 31 | 29 | 1.3 | 2.4 | **1.7** |
| Suprabasin | Cell differentiation | Q6UWP8 | 27 | 11 | 2.1 | 3.4 | **2.6** |
| Histidine-rich glycoprotein precursor | Cysteine endopeptidase inhibitor | P04196 | 5.7 | 2 | 1.1 | 3.3 | **1.6** |
| Dyenin 1 chain 2 | Cell organization | Q13409 | 2.1 | 1 | 1.3 | 2.9 | **1.8** |
| Transglutaminase 3 | Protein binding | Q08188 | 33.9 | 17 | 8.2 | 1.3 | **2.2** |
| WAP four-disulfide core domain protein 2 | Serine-endopeptidase inhibitor | Q14508 | 38.7 | 3 | 3.6 | 1.5 | **2.1** |
| Clusterin isoform 1 | Protein binding | P10909 | 14.1 | 5 | 12.1 | 3.3 | **5.2** |
| GAPDH | Cell metabolism | P04406 | 32.2 | 6 | 3.4 | 20.5 | **5.8** |
| Lumican precursor | Protein binding | P15884 | 6.2 | 1 | 2.3 | 8.1 | **3.6** |
| AHNAK | Cell differentiation | Q09666 | 4.2 | 17 | 2.2 | 7.6 | **3.4** |
| Retinol binding protein 4 | Protein binding | P02753 | 27.8 | 3 | 1.7 | 3.8 | **2.3** |
| Hemopexin precurosor | Metal binding, transport | P02790 | 48.4 | 13 | 1.3 | 3.7 | **2.0** |
| Haptoglobin precursor | Metal binding, transport | P00738 | 23.8 | 8 | 9.8 | 3.7 | **5.3** |

| | | under-abundant proteins, ratio (fold change) | | | | | |
|---|---|---|---|---|---|---|---|
| protein | biological function | accession number | sequence coverage (%) | # unique peptides detected | U/R | I/R | $(U+I)_{avg}$/R |
| Complement component 6 | Immune response | P13671 | 2 | 1 | 3.5 | 5.3 | **4.4** |
| Rheumatoid factor D5 light chain | Inflammatory response | A0N5G5 | 43.9 | 5 | 3.3 | 3.1 | **3.2** |
| Pigment epithelium-derived factor | Cell proliferation | P36955 | 26.8 | 10 | 1.2 | 14.5 | **7.8** |
| Granulin | Inflammation, cytokine-like activity | P28799 | 9.1 | 4 | 3.9 | 3.5 | **3.7** |
| Glycodelin isoform 1 | Protein binding | B4EC0 | 32.2 | 5 | 7.4 | 5.8 | **6.6** |
| Cathepsin B | Cysteine protease | P07858 | 10 | 2 | 15.1 | 34.5 | **24.8** |
| Ig kappa chain V−III region NG9 | Immune response | P01621 | 44 | 4 | 41.3 | 5.1 | **23.2** |

[a] Study groups: R, HIV-resistant; U, HIV-uninfected; I, HIV-infected. Sequence coverage %: highest value attained on replicate runs.


dx.doi.org/10.1021/pr200596r |J. Proteome Res. 2011, 10, 5139–5149

Clues to protective mechanisms in women may come from studying HIV highly exposed seronegative (HESN) individuals. As with any infectious disease, not all individuals who are exposed to a virus become infected, and data from the Pumwani Sex Worker Cohort has provided evidence that there are individuals who can be epidemiologically defined as resistant to HIV-1 infection.[6] A subset of approximately 150 women out of a total of over 3500 participants from the Pumwani Sex Worker cohort currently meet this definition, making this one of the largest HESN cohorts available for study.[6,7]

Evidence suggests that HIV-resistant women have novel innate and adaptive immune responses against HIV-1 in the cervicovaginal compartment, the first portal of entry for the virus. These include HIV specific cellular $CD4^+$ and $CD8^+$ immune responses and HIV-1 specific IgA.[8,9] However, IgA was not found to be a correlate of protection in all women.[10] Previously described anti-HIV factors such as RANTES,[11] cc-chemokines SLPI and MIP-$\alpha/\beta$,[12] and Elafin[13] were also found to be elevated in HIV-resistant women. Our pilot study on a small subset of 10 of these women showed differences in protein expression at the mucosal level,[14] supporting the hypothesis that mucosal factors are playing a role in HIV-resistance. However, these studies have been limited by technologies that can observe only a small fraction of the proteome, limited by small population sizes, or taken a reductionist approach by studying only single factors. Indeed, the dynamic properties of a complex system cannot be inferred from characteristics of a single component. Although these studies have increased our understanding of HIV- resistance, we have not yet identified all the correlates of protection in these women and, especially, how this relates to immune activation in the female genital tract.

Novel tools are now available that allow us to take a "systems biology" approach to examine this complex mucosal environment. This is the first large-scale clinical study to characterize the mucosal proteome of the female genital tract to identify factors associated with altered susceptibility to HIV-1 infection. Using a combination of mass spectrometry-based proteomics and traditional immunological methods, we examined the cervical secretions of 293 women, including 76 HIV-resistant, 97 HIV-infected and 120 HIV-uninfected controls, from clinical samples obtained over a 4-year period. In this study, we show that HIV-resistant women have a unique phenotype of cervical mucosa protein expression characterized by an over-abundance of many protease inhibitors, including Serpins, Cystatins, and A2ML1, among 360 unique proteins identified. Cytokine analysis was also performed in parallel to assess the association of local inflammation on the expression of these factors. Many of these are known to be important regulators of the immune response and act to subdue general inflammatory responses in a variety of cell lines and tissues[15−23] and have described antiviral activity against HIV-1 infection of target cells known to be present in the cervicovaginal compartment.[21,24,25] This is the first time these mucosal proteins have been described to be associated to a protective phenotype against HIV-1 infection in the female genital tract *in vivo*.

## RESULTS

### Proteomic Analysis of Cervicovaginal Mucosa Samples and Identification of Differentially Abundant Proteins

To examine the proteome of the CVL samples from HIV-R, HIV-I, and HIV-U subjects, samples were initially analyzed by mass spectrometry to identify differentially abundant proteins. CVL samples were collected from commercial sex workers from the Pumwani sex worker cohort and prepared for proteomic analysis as described in the Materials and Methods. Due to the low amount of protein present in CVL samples, pools were created to increase protein identification coverage by mass spectrometry. Individual CVL samples were pooled (normalized by protein concentration) according to their HIV-status (HIV-resistant, HIV-R; HIV-uninfected, HIV-U; HIV-infected, HIV-I), digested to peptides with trypsin, fractionated by high-PH reverse phase chromatography and then analyzed by a high performance LC−MS/MS using a Fourier transform ion cyclotron resonance mass spectrometer (FT-MS, Thermo) using a label-free approach. Two independent technical replicates were run for each sample, and data was further normalized by total ion current. Spectral counting using Spectrum Mill software was then used to give semiquantitative information on the proteins. Approximately 360 unique proteins were identified on average in each of the samples.

Selection criteria for differentially abundant proteins included the following: (1) showed a >3-fold change in abundance between HIV-R and either of the HIV-U and HIV-I groups; (2) had peptides detected in both technical replicates; (3) the protein was detected in all pooled samples. Table 1 shows the list of proteins that met these criteria, where 34 were over-abundant and 7 were at lower levels in the HIV-R group compared to the average of the other two control groups (HIV-U and HIV-I) (see Supplementary File S1 for complete list of proteins, Supporting Information). This represented ∼10% of the total proteins identified. Some proteins that did not have peptides detected in all samples (below threshold of detection) were manually analyzed on previous association with HIV-resistance from our group, including Elafin and A2ML1.[13,14]

### Gene Ontology of Differentially Abundant Proteins in the Cervicovaginal Mucosa of HIV-1 Resistant Sex Workers

To interpret and determine biological groups and pathways associated with HIV-resistance, the genes associated with the differentially abundant proteins were cross-referenced with the DAVID database for their major known biological function (Figure 1). The most pronounced trend is that majority (>40%) of the over-abundant proteins in HIV-resistant women were antiproteases, with many in the serpin family. These serpins (Serine protease inhibitors) were from multiple clades and include Serpins A1, A3, B1, B13, C1, and G1. Other epithelial-derived antiproteases included Elafin, A2ML1, Cystatin A and Cystatin B. The range of relative abundance was quite broad, where some antiproteases were 3.6-fold over-abundant in HIV-R women (Serpin B13) and others >1 log fold (Serpin C1) from the average of the other two groups. Other antiproteases included SpinK 5, Histidine-rich glycoprotein precursor, and WAP four-disulfide core domain protein 2, which is part of the whey acidic protein family. Many of these antiproteases are involved in the innate defense against microorganisms including HIV-1 infection, either directly or indirectly by inhibiting extracellular proteases, such as Serpin A1, Serpin A3, Serpin C1, Elafin, Cystatin A, and Cystatin B.[19,21,25−28] Another common feature of the over-abundant proteins is that they are involved with the acute phase response, which include Serpin A1, Serpin A3, Apolipoproteins A1/A2, and fibrinogen.[29] However, ApoA1 and Apo A2 are considered negative acute phase response proteins as they decrease in concentration during an acute inflammation event. Many of these antiproteases play roles in moderating inflammation

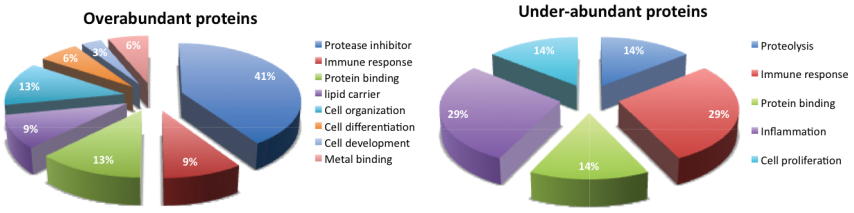



**Figure 1.** Biological functional clustering of differentially abundant proteins in the cervicovaginal mucosa of HIV-resistant sex workers. Proteins were placed into their major functional category according to their gene ontology. Over-abundant proteins in HIV-resistant women are shown on the left, and under-abundant proteins are shown on the right.

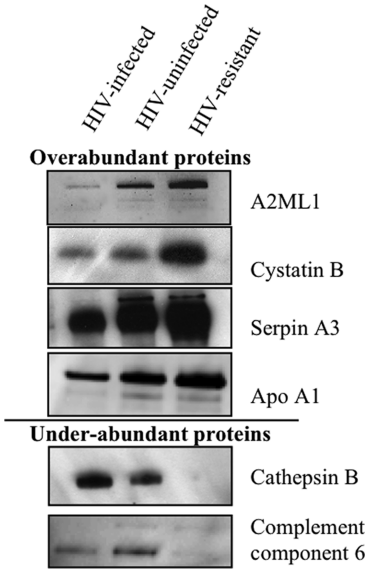



**Figure 2.** Immunoblots of selected differentially abundant proteins in cervicovaginal fluid of HIV-resistant sex workers. CVL protein (1 $\mu$g) from each pooled group used in the MS analysis (HIV-R, $n = 76$; HIV-U, $n = 120$; HIV-I, $n = 97$) was resolved by SDS-PAGE and probed by specific antibodies. The trending patterns of selected over-abundant (above four) and under-abundant (bottom two) proteins identified in HIV-resistant women matched those observed in the mass spectrometry data set.

in a wide variety of cell types and tissues including Serpin A1, B1, and Elafin.[17,23,30] Under-abundant proteins in HIV-1-resistant women included proteases, such as Cathepsin B (cysteine protease), and proteins involved with the inflammation response such as Granulin, complement components, and immunoglobulins. Interestingly, cathepsins are known to inhibit antiviral peptides such as MIP-3$\alpha$, SLPI, and RANTES.[31−33]

From these results, it is clear that HIV-1-resistant women have a unique phenotype of mucosal protein expression which is characterized by increasing abundance of antiviral and anti-inflammatory factors, coupled with lower amounts of inflammatory stimulators and proteases, which can counteract the antiviral response.

## Immunoblots of Differentially Abundant Proteins

To confirm the expression patterns observed in the FT-MS data set, specific proteins were selected (which had commercially available antibodies) and probed by traditional Western blot. Equal amounts of protein from each of the pooled CVL samples (1 $\mu$g) were immunoblotted for the over-abundant proteins A2ML1, Serpin A3, Cystatin B, and Apo A1, and the under-abundant proteins Cathepsin B and Complement component 6 (Figure 2). As there is no known load control for cervical mucosa, samples were normalized by total protein content. Volumetric analysis was performed to obtain a semiquantitative measure of the band intensities for each of the immunoblots and is shown in Table 2. The relative intensity patterns of the immunoblots matched the trending patterns observed in the mass spectrometry data, where the HIV-R group had the highest amount Serpin A3, ApoA1, and the lowest amount of CC6 and Cathepsin B as measured by volumetric analysis. In the case of A2ML1, the HIV-R group had the highest expression ($4.13 \times 10^4$ units), followed by the HIV-U ($2.92 \times 10^4$) and HIV-I ($1.45 \times 10^4$) groups, which did not match the mass spectrometry analysis (the MS data showed the HIV-U group to have equal abundance). This may have simply represented the limitation of accuracy of the label-free MS method for distinguishing smaller differences (<50%) between groups, a routine observation for this technique.[34] However, the immunoblot data was consistent with our earlier studies,[14] and the remaining immunoblots confirmed the MS data.

**Table 2. Volumetric Analysis of Immunoblots of Selected Differentially Abundant Proteins in Pooled Cervicovaginal Lavage Samples**

| | adj. vol. intensity per mm$^2$ | | |
|---|---|---|---|
| protein | HIV-resistant | HIV-uninfected | HIV-infected |
| A2ML1 | $4.13 \times 10^4$ | $2.92 \times 10^4$ | $1.45 \times 10^4$ |
| Cystatin B | $4.47 \times 10^6$ | $3.27 \times 10^6$ | $2.74 \times 10^6$ |
| Serpin A3 | $1.20 \times 10^5$ | $5.77 \times 10^4$ | $1.44 \times 10^4$ |
| Apo A1 | $7.88 \times 10^5$ | $5.65 \times 10^5$ | $3.99 \times 10^5$ |
| Cathepsin B | 41 | $5.27 \times 10^3$ | $1.03 \times 10^4$ |
| Complement component 6 | $2.8 \times 10^3$ | $3.61 \times 10^4$ | $6.85 \times 10^3$ |

## Antiproteases Are Elevated in HIV-1 Resistant Sex Workers As Determined by ELISA and Semiquantitative Western Blot

The proteomics data set was used to generate a general hypothesis about which factors are associated with HIV-resistance and may be playing a role in protecting against HIV-1 infection. Since many proteins were differentially abundant, only a few were selected for confirmation based on their known biological functions. Proteins selected for confirmation included Serpin A1, Serpin A3, Cystatin B, and A2ML1. Serpins A1, A3, and Cystatin B were chosen due to their known anti-inflammatory roles as well as their antiviral activity against HIV-1.[19,25,35] Also, Serpin A1 has been implicated as an early response factor against HIV-1 during viremia in HIV-1 infected individuals.[36] A2ML1 was chosen due to its earlier association with HIV-resistance in our preliminary study[14] and roles in innate immunity.[37] Independent cervicovaginal

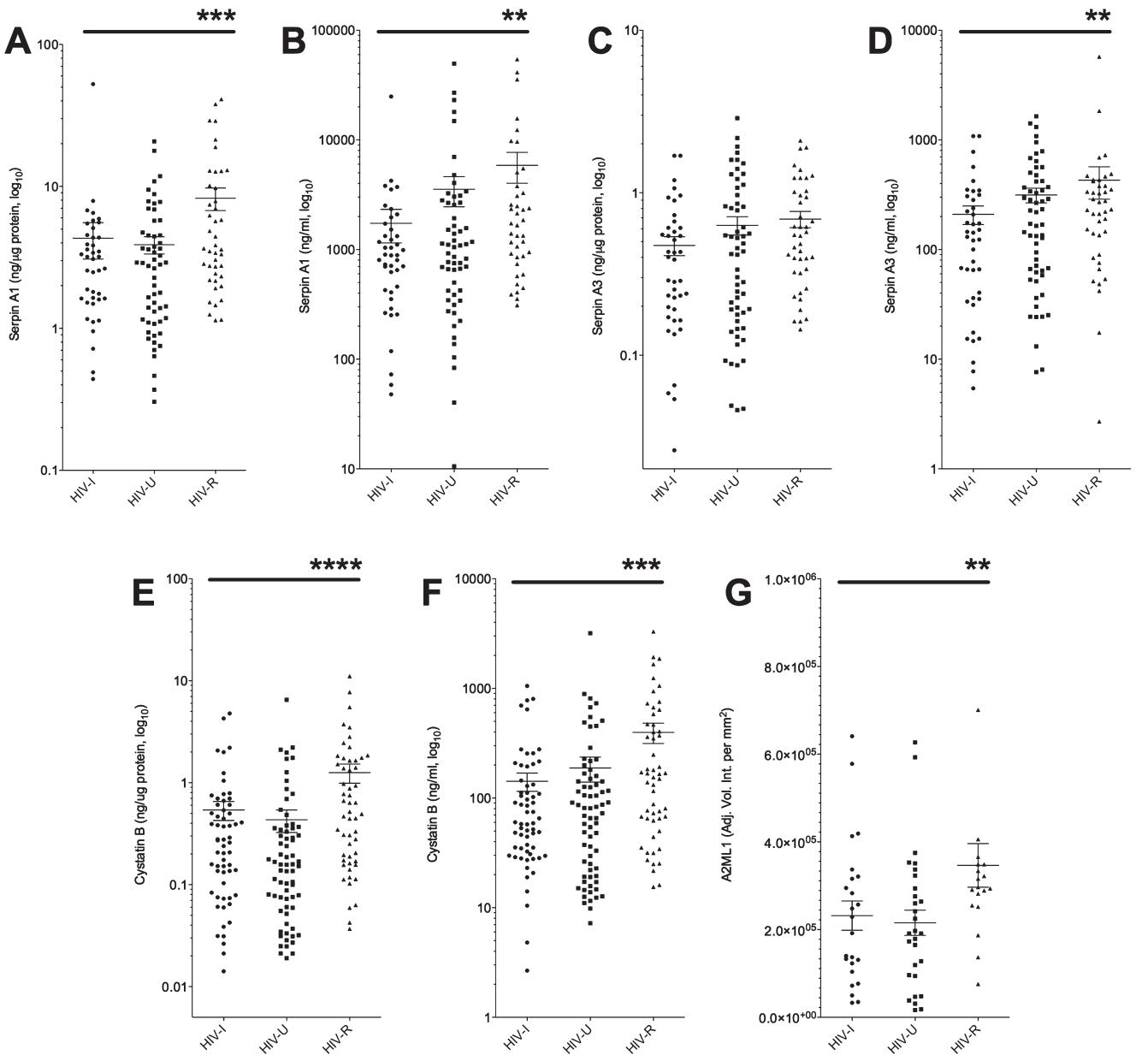



**Figure 3.** ELISA and semiquantitative Western blot of antiproteases Serpin A1, Serpin A3, Cystatin B, and A2ML1 in cervicovaginal lavage samples of commercial sex workers. Cervical lavage samples were grouped based on HIV-status: HIV-I (HIV-infected), HIV-U (HIV-uninfected), and HIV-R (HIV-resistant). This figure shows both specific (ng/$\mu$g of total protein) and total (ng/mL) concentrations of Serpins A1 (A and B), Serpin A3 (C and D) (HIV-R, $n$ = 44; HIV-U, $n$ = 57; HIV-I, $n$ = 42) and Cystatin B (E and F) (HIV-R, $n$ = 54; HIV-U, $n$ = 72; HIV-I, $n$ = 62) as determined by ELISA. A2ML1 (G) was determined by resolving 1 $\mu$g of protein per CVL sample by SDS-PAGE and immunoblotting using an A2ML1 antibody (HIV-R, $n$ = 19; HIV-U, $n$ = 29; HIV-I, $n$ = 24). Relative abundance was determined by volumetric analysis of band intensities. Horizontal bars represent mean levels $\pm$ the standard error of the mean. All samples were run in duplicate. Both of Kruskal−Wallis (1-way Anova) and unpaired two-tailed Mann−Whitney statistical tests were performed to assess expression differences between groups (95% confidence level, or $p < 0.05$). Results of 1-way Anova is shown as follows: **** $p < 0.0005$, *** $p < 0.005$, ** $p < 0.05$.

lavage samples were collected in a follow-up sampling from the same women for quantification of these individual antiproteases. Using a combination of ELISA and semiquantitative Western blot, these antiproteases were measured and the expression patterns are shown in Figure 3.

Statistical analysis was performed on the distribution patterns of these antiproteases between HIV-R, HIV-U, and HIV-I study groups. The HIV-R group had the highest overall expression of all antiproteases including Serpin A1, Serpin A3, Cystatin B, and A2ML1 compared to the HIV-I and HIV-U control groups. Serpin A1 expression was statistically significantly higher in the HIV-R group both in specific concentration (ng per $\mu$g of total protein) ($p = 0.004$) and total concentration ($p = 0.018$) at 8.5 ng/$\mu$g and 5.7 $\mu$g/mL, respectively. This represented over a 100% increase to the other groups (HIV-U: 3.9 ng/$\mu$g, 3.5 $\mu$g/mL; and HIV-I: 4.2 ng/$\mu$g protein, 1.7 $\mu$g/mL). Similarly, Serpin A3 expression was highest in the HIV-R group (0.69 ng/$\mu$g, 420 ng/mL) compared to the HIV-I (0.47 ng/$\mu$g, 205 ng/mL) and HIV-U group (0.64 ng/$\mu$g,


dx.doi.org/10.1021/pr200596r |J. Proteome Res. 2011, 10, 5139–5149

**Table 3. Clinical Data of Commercial Sex Workers in Proteomic Study**

| clinical variable | HIV-resistant | HIV-uninfected | HIV-infected |
|---|---|---|---|
| Age | 41.9 ± 8.1 | 33.9 ± 8.2 | 38.7 ± 6.9[a] |
| Duration of prostitution | 13.7 ± 7.3 | 7.3 ± 7.3 | 12.0 ± 6.8[a] |
| Clients/day | 4.4 ± 3.1 | 4.5 ± 2.7 | 4.4 ± 2.7 |
| Clients/week | 24.3 ± 19.1 | 28.0 ± 21.0 | 26.2 ± 19.3 |
| # Regular clients | 0.7 ± 0.49 | 1.0 ± 1.3 | 0.63 ± 0.88[b] |
| # Condom use/week | 32.8 ± 41.2 | 32.8 ± 24.5 | 32.0 ± 35.9 |

[a] $p$ = <0.0001. [b] $p < 0.05$.

320 ng/mL), and these differences were significant ($p$ = 0.046). Although the HIV-R group had the higher overall expression of Serpin A3, and statistically higher than the HIV-I group ($p$ = 0.01), it did not reach statistical significance over the HIV-U group ($p$ = 0.47). Cystatin B expression had a similar pattern to Serpin A1, where the HIV-R group had highest overall expression (1.26 ng/$\mu$g, 398 ng/mL) over both the HIV-I (0.63 ng/$\mu$g, 142 ng/mL) and HIV-U groups (0.43, 188 ng/mL), representing a 100% and 290% increase in abundance, respectively, which was statistically significant ($p$ = 0.0003). Finally, semiquantitative Western blotting analysis of A2ML1 on a smaller subset of these individuals showed the HIV-R group to have a higher average concentration ($3.5 \times 10^5$ intensity units) compared to the HIV-I and HIV-U groups ($2.1 \times 10^5$ and $2.0 \times 10^5$ intensity units, respectively), which was also statistically significant ($p$ = 0.04).

Antiprotease expression was tightly correlated between Serpin A1, Cystatin B, Serpin A3, and A2ML1 in pairwise comparisons ($p$ = <0.001). Serpin A1 and A3 are acute-phase response proteins in the systemic compartment and tend to increase during an inflammation event, and therefore it is reasonable to assume that this would be true for the mucosal compartment. There is limited information about the regulation of Cystatin B and A2ML1 expression. These results may suggest common pathways of activation or possible costimulation of expression.

## Genital Mucosal Antiproteases Were Independent of Clinical Confounding Factors

Many epidemiological factors could alter the risk of HIV-infection. We did not find any significant differences with respect to sexual practices between study groups (Table 3), which include clients/day, clients/week, and condom usage. However, the HIV-resistant group was slightly older (41.9 years) than the other two groups (HIV-I group: 38.7 yrs, HIV-U group: 33.9 yrs). By our definition for a woman to be HIV-resistant, an individual has to be enrolled in the sex worker cohort for at least 7 years of follow-up and be engaged in sex work for >7 years, so this is an inevitable outcome of this selection criteria. The HIV-uninfected group is the youngest of the study groups as they are new enrollees into the Pumwani cohort. Subsequent analysis showed no association of antiproteases with age, and therefore age was not a contributing factor (aside from negative correlation with Cystatin B, see below). Also a notable difference is the HIV-uninfected group had slightly higher numbers of regular clients, although total clients/day were similar across groups and each group reported a certain number of regular clients.

It is also possible that clinical factors, or sexual practices, could affect antiprotease expression, as it would impact the mucosal environment of the female genital tract.[38] Statistical analysis did not reveal any correlations between antiprotease levels and duration of prostitution, clients/day, clients/week, and condom usage per week (Table 4). The only exception was that Cystatin B was negatively correlated to age ($p$ = 0.01, $r = -0.025$) suggesting a decrease in this protease inhibitor over time. Since the HIV-R group is statistically older than the others this would indicate the difference in Cystatin B levels might be diminished to some degree. ARV use did not affect the levels of these antiproteases and they were not significantly different between those that were on and those that were not taking ARV's in the HIV-I group. Hormonal contraception (oral contraceptives, depovera, etc.), genital hygiene practices (douching), and menstrual cycle (proliferative vs secretive phase) did not vary significantly between the three groups and was independent of the higher abundance of antiproteases in HIV-resistant women. Also, sex workers who reported to be on a "sex break" (a short break from sex work) did not have statistically altered levels of antiproteases to those that were not, suggesting that these factors may be at constitutively higher levels prior to engaging in sex work, and therefore prior to HIV-1 exposure. However, we cannot discount the possibility that these women on sex break were engaged in sexual activity with other partners (boyfriends, etc.) and this data was not collected. Further follow-up with sex workers on prolonged sex-breaks may shed light on this issue.

**Table 4. Correlation Values between Cervicovaginal Antiprotease Levels and Epidemiological Factors**

| | antiprotease (Spearman correlation values) | | | |
|---|---|---|---|---|
| clinical variable | Serpin A1 | Serpin A3 | Cystatin B | A2ML1 |
| Age | 0.65 | 0.57 | 0.01[a] | 0.13 |
| Duration of prostitution | 0.43 | 0.79 | 0.51 | 0.70 |
| Clients/day | 0.59 | 0.70 | 0.78 | 0.83 |
| Clients/week | 0.96 | 0.60 | 0.60 | 0.19 |
| Condom use/week | 0.52 | 0.30 | 0.56 | 0.10 |

[a] Significant association ($p < 0.05$).

Overall, these results indicate that these clinical factors do not influence the concentration of these antiproteases in cervicovaginal mucosa and do not contribute to higher levels observed in HIV-resistant women.

## Antiprotease Levels Were Not Due to Increased Inflammation in the Mucosal Compartment

Given that one of the known biological functions of Serpin A1 and A3 are to counter inflammation and are elevated during the acute-phase response,[23,29] we asked the question if HIV-resistant women were at an increased inflammatory state at the mucosal level. Sex work in general is known to be associated with increased levels of genital inflammation. With respect to Cystatin B and A2ML1, little is known about how these proteins are affected by inflammatory events. Therefore we measured the expression pro-inflammatory cytokines IL-1$\alpha$, IL-1$\beta$, IL-6, 8, and TNF-$\alpha$ in the same CVL samples used in the antiprotease ELISA/immunoblot assays to assess the overall inflammation state at the mucosal surface, and compared between study groups. These are plotted and shown in Figure 4.

With the exception of TNF-$\alpha$, we did not observe any statistical differences in the expression of these pro-inflammatory cytokines between study groups. This included IL-1$\alpha$ (HIV-I, 202.4 pg/mL; HIV-U, 500.9 pg/mL; HIV-R, 241.1 pg/mL), IL-1$\beta$ (HIV-I, 81.8 pg/mL; HIV-U, 60.8 pg/mL; HIV-R, 44.3 pg/mL), IL-6 (HIV-I, 14.7 pg/mL; HIV-U, 12.0 pg/mL; HIV-R,

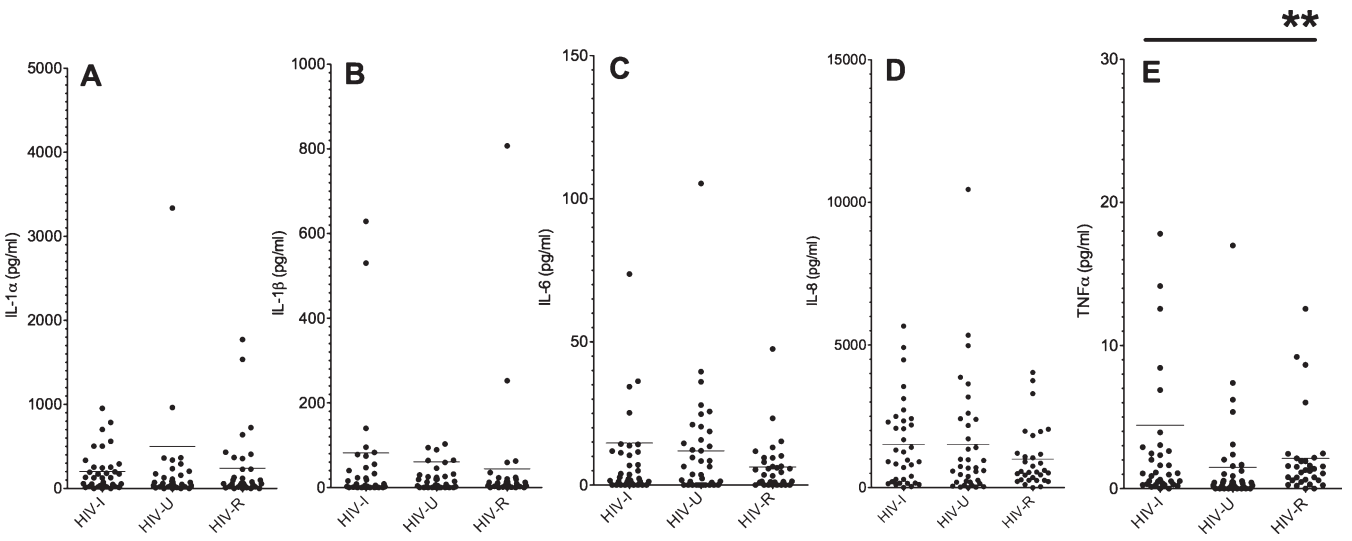



**Figure 4.** Pro-inflammatory cytokine analysis of cervicovaginal lavage samples. A subset of the same CVL samples used for ELISA assays (Figure 3) were assayed by the Luminex platform for cytokines IL-1$\alpha$ (A), IL-1$\beta$ (B), IL-6 (C), IL-8 (D), and TNF-$\alpha$ (E), and grouped based on HIV-status: HIV-I (HIV-infected), HIV-U (HIV-uninfected), and HIV-R (HIV-resistant) (HIV-R, $n$ = 32; HIV-U, $n$ = 36; HIV-I, $n$ = 35). Horizontal bars represent the means of the cytokine concentrations. Both nonparametric Kruskal–Wallis (1-way Anova) and unpaired two-tailed Mann–Whitney statistical tests were performed (95% confidence level, or $p < 0.05$). Results of 1-way Anova is shown as follows: **** $p < 0.0005$, *** $p < 0.005$, ** $p < 0.05$.

**Table 5. Correlation Values between Cervicovaginal Antiprotease Concentrations and Proinflammatory Cytokine Levels in All Women**

| | antiprotease (Spearman correlation values)[a] | | | |
|---|---|---|---|---|
| cytokine | Serpin A1 | Serpin A3 | Cystatin B | A2ML1 |
| IL-1$\alpha$ | $p$ = <0.0001 | $p$ = <0.0001 | $p$ = 0.04 | |
| IL-1$\beta$ | $p$ = <0.0001 | $p$ = <0.0001 | | $p$ = 0.04 |
| IL-6 | $p$ = <0.0001 | $p$ = <0.0001 | | |
| IL-8 | $p$ = <0.0001 | $p$ = <0.0001 | | |
| TNF-$\alpha$ | $p$ = <0.0001 | $p$ = 0.001 | $p$ = 0.004 | $P$ = 0.003 |

[a] Significant association ($p \leq 0.05$).

6.3 pg/mL), IL-8 (HIV-I, 1.52 ng/mL; HIV-U 1.51 ng/mL; HIV-R 1.00 ng/mL). TNF-$\alpha$ was highest in the HIV-U group ($p$ = 0.007), followed by the HIV-U and HIV-R groups (HIV-I 4.70 ng/mL; HIV-U, 1.96 ng/mL; HIV-R, 2.22 ng/mL). With the exception of TNF-$\alpha$, the HIV-resistant women trended *lower* in all cytokines than the other two groups, although this did not reach statistical significance.

Although HIV-resistant women did not have elevated cytokines, antiproteases *did* associate positively with pro-inflammatory cytokine levels in cervicovaginal mucosa in all groups (Table 5). Serpin A1 and A3 associated with all cytokines measured, including IL-1$\alpha/\beta$, IL-6, IL-8 and TNF-$\alpha$ consistent with observations in the literature on systemic expression and *in vitro* studies.[23,29] Cystatin B and A2ML1 also correlated with TNF-$\alpha$ and IL-1$\alpha$ /IL-1$\beta$, respectively. This indicates that mucosal antiproteases are associated with these cytokines in the female genital tract, but it is not known if they are driving this response or merely associated.

Since HIV-resistant women did not have elevated cytokines compared to control groups the increased expression of Serpin A1, A3, Cystatin B, and A2ML1 was not explained by an elevated pro-inflammatory milieu in the female genital tract. Antiprotease expression does associate with pro-inflammatory cytokines, but this is clearly not an explanation for why they are elevated in HIV-resistant women. Overall, this information shows that HIV-resistant women are not at an elevated state of mucosal inflammation (as measured by these cytokines) and increased antiprotease levels are not due to differences in inflammatory status.

## DISCUSSION

In the global fight against HIV-1, attention has been focused on developing new strategies to prevent HIV-1 infection. Therefore the identification of factors that protect against HIV-1 infection via mucosal surfaces will be extremely beneficial in developing new products or therapeutics. Our group has access to one of the largest (>3000 participants) and most comprehensively studied HIV-1-exposed sex worker cohorts,[39] and this has given us a unique opportunity to study natural resistance to HIV-1 infection. We believe that resistance to HIV-1 infection is multifactorial, including specific polymorphism and reduced responsiveness in the IRF-1 gene,[40,41] HIV-1-specific cellular CD4 and CD8 immune responses and HIV-1 specific IgA.[8,9] In this large-scale clinical study, we employed a systems biology approach to fully characterize the cervicovaginal compartment of HIV-resistant and uninfected women to identify novel correlates of altered susceptibility to HIV-1 infection. We demonstrated that the mucosal secretions of HIV-resistant women have a unique phenotype of protein expression characterized by an over-abundance of antiproteases that play important roles in regulating inflammation and providing antiviral/antimicrobial defense. These included many proteins from the Serpin family (A, B, and C-clades), Cystatins (A and B), Elafin, and other broad-spectrum protease inhibitors like A2ML1. This data confirmed and greatly expanded upon our preliminary findings on a smaller subset of HIV-resistant women,[14] and on the small peptide region of the proteome.[13] Follow up CVL samples from the same women confirmed this expression pattern of selected antiproteases, including Serpin A1, Serpin A3, Cystatin B, and A2ML1.


dx.doi.org/10.1021/pr200596r |J. Proteome Res. 2011, 10, 5139–5149

This expression was not due to increased inflammation and independent of clinical confounding factors. This is the first comprehensive *in vivo* finding of these mucosal antiproteases associating with a protective phenotype from HIV-1 infection.

Serpins play important roles in regulating inflammation, apoptosis, tissue development, and host defense.[15,16,42,43] They are found many cell types, including epithelial/endothelial cells and a wide variety of immune cells. Their major biological function is to inihibit these serine (and cysteine) proteases (such as cathepsins, elastase, and granzymes), by an irreversible "bait-and-trap" mechanism.[44] Serine proteases, secreted by immune cells, such as cytotoxic T-lymphocytes and neutrophils, are used to kill invading bacteria and combat infections.[45] These proteases in turn trigger immune functions including complement activation, coagulation, enhancing endothelial-leukocyte interactions, and the secretion of inflammatory mediators, to propagate this process.[46] A recent review highlighted the importance that the *balance* between antiproteases and proteases in the female genital tract that could affect the innate defense against microorganisms.[47] The absence of serpins to counterbalance these activities can lead to severe inflammation, tissue damage, and disease.[48,49] Indeed, knockout mice studies have shown that serpins are critical to the mucosal immune system by protecting against protease-mediated inflammatory damage during bacterial infections.[17] With respect to HIV-1, proteases such as cathepsins and elastase can enhance infectivity by either cleaving/inactivating antiviral factors (RANTES, MIP-3$\alpha$, and SLPI),[31,33] act as chemoattractants/stimulants for HIV-target cells,[18,50,51] enhance infection of target cells (CD4+ T cells qand macrophages),[19,52] promote pro-inflammatory cytokine expression (such as TNF-$\alpha$ and IL-1$\beta$), and impair wound healing.[53−56] Many of the serpins found over-abundant in HIV-resistant women are inhibitors of these proteases and can abrogate these processes, such as Serpin A1, A3, B1, B4, and B13,[19,57−59] and to a lesser degree A2ML1 and Elafin. Overall, these effects may be synergistic by reducing effects mediated by excess proteases, such as inhibiting antiviral responses, degradation of the epithelial barrier, or stimulating inflammatory pathways.

Serpins can also affect inflammation by mechanisms *independent* of protease inhibition. For example, Serpin C1 interferes with signal transduction leading to NF-kB activation in monocytes and endothelial cells.[60] Serpin A1 can inhibit TNF-$\alpha$ induced inflammation in endothelial cells,[22] LPS-mediated stimulation of monocytes via regulation of CD14 expression,[61] pro-inflammatory cytokine production in whole blood (IL-8 and TNF-$\alpha$),[23] as well as NF-kB activation through altered ubiquination.[62] Reducing immune activation has been shown to protect from HIV-infection in the female genital tract in animal models.[63] Higher levels of endogenous serpins may be better at preventing inflammation events, or reduce HIV-1-target cell migration and/or susceptibility based on evidence garnered from *in vitro* studies. Determining the impact of serpins on immune activation in this compartment is a logical next step.

The antiviral activity of these factors may also contribute to resistance to HIV-1 infection. Those with direct antiviral activity previously shown *in vitro* include Serpin A1 ($\alpha$-1 antitrypsin) Serpin C1 (antithrombin III), Cystatin A, Cystatin B, and Elafin.[21,24,25,27,35,64,65] The importance of Serpin A1 as an endogenous HIV-1 suppressor is underscored by the fact that lower serum levels leads to faster disease progression.[66,67] We have determined HIV-resistant women to have Serpin A1 in the 50 to 500 $\mu$g/mL range, and ~4 to 40 $\mu$g/mL for Cystatin B, both at biologically relevant concentrations capable of affecting HIV-1 replication based on *in vitro* studies. For Serpin A1 10% inhibition is observed at 50 $\mu$g/mL and 50% at 500 $\mu$g/mL in both primary T-cell and dendritic cells,[62] and Cystatin B demonstrates 20% inhibition against HIV-1 *in vitro* at 1 $\mu$g/mL and 90% at 25−35 $\mu$g/mL,[35,68] well within the physiological range determined in HIV-resistant women. It must be pointed out that both Cystatin B and Serpin A1 were also detected at biologically relevant concentrations to affect HIV-1 replication in the control groups. It is therefore unclear if these differences in concentration would translate into a higher impact against HIV-1 infection for *in vivo* events. However, the combination of so many other elevated antiviral proteins such as Serpin A3, Serpin C1, Cystatin A and Elafin may be synergistic and provide a cumulative advantage for HIV-resistant women. Certainly, the positive association of antiprotease expression supports this hypothesis.

It is not clear, however, if these factors are causal for HIV-resistance, or merely associated with this phenotype. We have not yet determined the expression of all of these factors in a prospective study to evaluate their impact on HIV-acquisition and this is an obvious next step. Also, the male sexual partner responsible for HIV-exposure or transmission was not available for study, and it is possible that partner host factors could impact the mucosal system of these women. As women in each study group reported having multiple sexual partners over many years of sex work this would make this possibility unlikely. We cannot exclude the possibility that other genital infections (papillomavirus, chancroid donovanosis) could have influenced the levels of these antiproteases. However, the women included in this study did not show characteristics of these (ulcers, condylomas), and those with other STI's or URTI's were not included in the study. We did not evaluate the underlying microflora populations during this study and therefore cannot discount the possibility that this played a role in antiprotease expression. However a previous study using *cpn60*-based deep sequencing did not find any significant differences in the vaginal microbiota of HIV-resistant women.[69]

HIV-1 transmission during heterosexual intercourse occurs over a mucosal surface and the risk of infection is affected by many physiological factors. Protection from infection may be mediated by the presence of factors that may limit virus replication and/or dissemination of infected cell populations to the systemic compartment. Indeed, increased inflammation and activation of the immune system in the FGT can be a major contributor to increased susceptibility to HIV-1.[70] Therefore factors that limit the overall inflammatory response, the expansion of infected cell populations, or inhibit HIV-1 replication in the female genital tract might be protective in uninfected individuals. In this study we have shown that HIV-resistant women have a *tipped* balance of elevated protease inhibitors and antiviral factors. Accumulating evidence on Serpins and Cystatins suggest they play an important role in both inflammation and HIV-1 infection.[23,25,36,62,64,66] HIV-resistant women therefore have a highly 'tuned' antiprotease response that may be critical at the front lines of defense during HIV-1 exposure. The biological significance may be that inflammation events are better controlled while at the same time providing a more potent antiviral milieu at mucosal surfaces. This may reduce the risk of spreading infected founder populations in the submucosa to the systemic compartment and allow time for adaptive responses to respond in a controlled manner. We believe this to be a component

or driver of the reduced immune activation phenotype we have observed in these women.[71,72] Examining the complex interplay of each of these factors and how this balance contributes to HIV-resistance will increase our understanding of mucosal immunity and warrants further investigation.

## MATERIALS AND METHODS

### Ethics Statement

The study was performed with the approval of both the University of Manitoba and Nairobi human research ethics board. All subjects gave informed written consent prior to participation in this study.

### Study Population

The Pumwani Sex Worker Cohort was established in 1985 for the study of the immunobiology and epidemiology of sexually transmitted infections (STI's). It is an open cohort that, in addition to research, provides STI and HIV prevention services and antiretroviral treatment for infected members (PEPFAR, President's emergency plan for AIDS relief). The cohort currently comprises over 3800 women, with new enrollment at the rate of approximately 200 women per year. These women have an extremely high risk of HIV-1 infection, with 60% of women being HIV-1 seropositive at enrollment. HIV-1 Resistant Women (HIV-R): We have defined resistance to HIV-1 infection as follow up in the Pumwani cohort for 7 or more years, continuing sex work, and persistently HIV-1 seronegative and negative HIV-1 by PCR. Currently, we have identified over 150 such individuals. HIV-1 Uninfected Nonresistant Sex Workers (HIV-U): HIV-1 uninfected nonresistant sex workers are those women newly enrolled in the cohort who do not meet our definition of resistance, that is, they have been followed for less than three years. A small subset of these women will go on to become resistant, while the majority will eventually seroconvert, in spite of highly effective intervention strategies. Currently, there are over 300 such women enrolled in follow up. HIV-1 Infected women (HIV-I): Currently we have over 400 women who are infected and at different stages of disease progression. Cervicovaginal lavage samples were collected from consenting women enrolled in the Pumwani Sex Worker cohort in Kenya. Current study population: 293 women were included in the study, which were assigned to the following study groups based on HIV-1-status: HIV-1-resistant (HIV-R, $n$ = 76), HIV-1-uninfected (HIV-U, $n$ = 120), HIV-1-infected sex workers (HIV-I, $n$ = 97). All of the women from the sex worker cohort were actively engaged in sex work at the time of cervicovaginal lavage collection. Selection criteria for HIV-1-resistance included enrollment in the cohort for at least 7 years and remaining uninfected (PCR and HIV-1 antibody negative) for more than 7 years while engaged in sex work. Criteria for the HIV-1-uninfected group were women enrolled in the cohort for less than 3 years and are HIV-1 uninfected. Women who had other detectible STI's or bacterial infections of the genital tract at the time of sample collection were not included in the study. Women with concurrent coinfections or STI's such as Chlamydia, *N. gonorrhea*, *T. pallidum*, *H ducreyi*, HSV, or bacterial vaginosis were not included in the analysis. Menopausal and pregnant women, as well as those on menses, were excluded from the analysis.

### Cervicovaginal Lavage (CVL) Sample Collection and Preparation

CVL samples were collected from each woman during two separate visits to the clinic. The first sample was used for mass spectrometry analysis and the follow-up sample for the validation experiments (ELISA's, Western blots) and Luminex assays. The ectocervix was briefly washed with 2 mL of sterile 1x phosphate buffered saline (PBS), followed by collection of the lavage from the posterior fornix. Samples were placed into a 15 mL conical tube, centrifuged to remove cellular debris, and the supernatant stored at −70 °C until analysis.

### Mass Spectrometry Analysis and Label-free Relative Quantitation

Peptide samples were fractionated by reverse phase liquid chromatography, and analyzed by a high performance LC−MS/MS using a Fourier transform ion cyclotron resonance mass spectrometer (FT-MS, Thermo) using a label-free approach. Two independent replicate MS analysis was carried out per sample. See Supporting Information for detailed protocols, protein preparation, digestion to peptides, and subsequent fractionation prior to mass spectrometry analysis.

### Western Immunoblotting

Protein (1 $\mu$g) from each sample was resolved by SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). Bands were developed with Immobilon detection reagent (Millipore), and volumetric analysis performed using Quantity One software (BioRad). See Supporting Information for detailed protocols and antibody information.

### ELISA Assays of Cystatin B, Serpin A1, Serpin A3

A sandwich ELISA was developed to quantify Cystatin B, and Serpin A1/Serpin A3 were measured using commercially available human A1AT and Alpha 1-Antichymotrypsin ELISA kits (Immunology Consultants Laboratory, Newberg, OR), according to the manufacturer's protocol. All samples were run in duplicate and blinded to the study group of which they belonged. See Supporting Information for more details.

**Cytokine Measurement.** Cytokine levels were determined in CVL samples using the Milliplex MAP multiplex kit and analyzed on the Bio-Plex-200 platform (Biorad). CVL were prepared and analyzed according the overnight protocol given by the manufacturer (Milliplex). Lower detection limit (LDL) was 6.4 pg/mL for IL-1$\alpha$, 0.7 pg/mL for IL-1$\beta$ and Il-6, 0.3 pg/mL for Il-8 and 0.1 pg/mL for TNF-$\alpha$. Sample below the LDL was assigned a value of 0 pg/mL.

### Statistical Analysis

Statistical analysis was performed using Prism Statistical software (version 5). The ELISA data show the mean and standard error of aggregate values. Across group variations for protein expression data (ELISA's, Western blots), clinical information, and cytokine expression, were calculated by nonparametric Kruskal−Wallis statistical test (1-way Anova) and intergroup variations were analyzed using unpaired two-tailed Mann−Whitney tests. Significance differences were those that had p values of $<0.05$. Associations between protein expression information (ELISA's, Western blots) and clinical data sets were calculated using nonparametric Spearman correlation tests (at 95% confidence intervals), where p values $<0.05$ were deemed significant.

## ASSOCIATED CONTENT

### Supporting Information

Supplementary methods and table. This material is available free of charge via the Internet at http://pubs.acs.org.

## AUTHOR INFORMATION

### Corresponding Author

*E-mail: burgener@cc.umanitoba.ca.

## ACKNOWLEDGMENT

We thank the women from the Pumwani Sex Worker cohort for their dedicated and consistent participation in this study. This work was funded through the Bill and Melinda Gates Foundation (GC-6: the Grand Challenges in Global Health program, Frank Plummer, PI, Blake Ball, Co-PI; and GC-13: Population Health Metrics Research Consortium Project, Chris Murray, PI, Steven Carr, Co-PI), the Canadian Institute for Health Research (CIHR), the Manitoba Research Health Council (MHRC), and the Public Health Agency of Canada (PHAC).

## REFERENCES

(1) UNAIDS. *Report on the global AIDS epidemic*; 2009.

(2) Rerks-Ngarm Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* **2009**, *361*, 2209–2220.

(3) Garber, D. A.; Silvestri, G.; Feinberg, M. B. Prospects for an AIDS vaccine: three big questions, no easy answers. *Lancet Infect. Dis.* **2004**, *4* (7), 397–413.

(4) Virgin, H. W.; Walker, B. D. Immunology and the elusive AIDS vaccine. *Nature* **2010**, *464*, 224–31.

(5) Shattock, R. J.; Moore, J. P. Inhibiting sexual transmission of HIV-1 infection. *Nat. Rev. Microbiol.* **2003**, *1* (1), 25–34.

(6) Fowke, K. R.; et al. Resistance to HIV-1 infection among persistently seronegative prostitutes in Nairobi, Kenya. *Lancet* **1996**, *348* (9038), 1347–51.

(7) Simonsen, J. N.; et al. HIV infection among lower socioeconomic strata prostitutes in Nairobi. *Aids* **1990**, *4* (2), 139–44.

(8) Kaul, R.; et al. HIV-1-specific mucosal IgA in a cohort of HIV-1-resistant Kenyan sex workers. *Aids* **1999**, *13* (1), 23–9.

(9) Kaul, R.; et al. HIV-1-specific mucosal CD8+ lymphocyte responses in the cervix of HIV-1-resistant prostitutes in Nairobi. *J. Immunol.* **2000**, *164* (3), 1602–11.

(10) Horton, R. E.; et al. Cervical HIV-Specific IgA in a Population of Commercial Sex Workers Correlates with Repeated Exposure But Not Resistance to HIV. *AIDS Res. Hum. Retroviruses* **2008** .

(11) Iqbal, S. M.; et al. Elevated T cell counts and RANTES expression in the genital mucosa of HIV-1-resistant Kenyan commercial sex workers. *J. Infect. Dis.* **2005**, *192* (5), 728–38.

(12) Hirbod, T.; et al. HIV-1 neutralizing activity is correlated with increased levels of chemokines in saliva of HIV-1-exposed uninfected individuals. *Curr. HIV Res.* **2008**, *6* (1), 28–33.

(13) Iqbal, S. M.; et al. Elevated elafin/trappin-2 in the female genital tract is associated with protection against HIV acquisition. *Aids* **2009**, *23* (13), 1669–77.

(14) Burgener, A.; et al. Identification of differentially expressed proteins in the cervical mucosa of HIV-1-resistant sex workers. *J. Proteome Res.* **2008**, *7* (10), 4446–54.

(15) Mangan, M. S.; Kaiserman, D.; Bird, P. I. The role of serpins in vertebrate immunity. *Tissue Antigens* **2008**, *72* (1), 1–10.

(16) van Gent, D.; et al. Serpins: structure, function and molecular evolution. *Int. J. Biochem. Cell Biol.* **2003**, *35* (11), 1536–47.

(17) Benarafa, C.; Priebe, G. P.; Remold-O'Donnell, E. The neutrophil serine protease inhibitor serpinb1 preserves lung defense functions in Pseudomonas aeruginosa infection. *J. Exp. Med.* **2007**, *204* (8), 1901–9.

(18) Chertov, O.; et al. Identification of human neutrophil-derived cathepsin G and azurocidin/CAP37 as chemoattractants for mononuclear cells and neutrophils. *J. Exp. Med.* **1997**, *186* (5), 739–47.

(19) Moriuchi, H.; Moriuchi, M.; Fauci, A. S. Cathepsin G, a neutrophil-derived serine protease, increases susceptibility of macrophages to acute human immunodeficiency virus type 1 infection. *J. Virol.* **2000**, *74* (15), 6849–55.

(20) Janciauskiene, S.; et al. Inhibition of lipopolysaccharide-mediated human monocyte activation, in vitro, by alpha1-antitrypsin. *Biochem. Biophys. Res. Commun.* **2004**, *321* (3), 592–600.

(21) Elmaleh, D. R.; Brown, N. V.; Geiben-Lynn, R. Anti-viral activity of human antithrombin III. *Int. J. Mol. Med.* **2005**, *16* (2), 191–200.

(22) Subramaniyam, D.; et al. TNF-alpha-induced self expression in human lung endothelial cells is inhibited by native and oxidized alpha1-antitrypsin. *Int. J. Biochem. Cell Biol.* **2008**, *40* (2), 258–71.

(23) Pott, G. B.; et al. Alpha-1-antitrypsin is an endogenous inhibitor of proinflammatory cytokine production in whole blood. *J. Leukoc. Biol.* **2009**, *85* (5), 886–95.

(24) Congote, L. F. The C-terminal 26-residue peptide of serpin A1 is an inhibitor of HIV-1. *Biochem. Biophys. Res. Commun.* **2006**, *343* (2), 617–22.

(25) Shapiro, L.; Pott, G. B.; Ralston, A. H. Alpha-1-antitrypsin inhibits human immunodeficiency virus type 1. *FASEB J.* **2001**, *15* (1), 115–122.

(26) Challacombe, S. J.; Sweet, S. P. Oral mucosal immunity and HIV infection: current status. *Oral Dis.* **2002**, *8* (Suppl 2), 55–62.

(27) Ghosh, M.; et al. Trappin-2/Elafin: a novel innate anti-human immunodeficiency virus-1 molecule of the human female reproductive tract. *Immunology* **2009**.

(28) Bingle, C. D.; Vyakarnam, A. Novel innate immune functions of the whey acidic protein family. *Trends Immunol.* **2008**, *29* (9), 444–53.

(29) Jensen, L. E.; Whitehead, A. S. Regulation of serum amyloid A protein expression during the acute-phase response. *Biochem. J.* **1998**, *334* (Pt 3), 489–503.

(30) Li, Q.; et al. Recombinant human elafin protects airway epithelium integrity during inflammation. *Mol. Biol. Rep.* **2009**.

(31) Taggart, C. C.; et al. Cathepsin B, L, and S cleave and inactivate secretory leucoprotease inhibitor. *J. Biol. Chem.* **2001**, *276* (36), 33345–52.

(32) Hasan, L.; et al. Function of liver activation-regulated chemokine/CC chemokine ligand 20 is differently affected by cathepsin B and cathepsin D processing. *J. Immunol.* **2006**, *176* (11), 6512–22.

(33) Lim, J. K.; et al. N-terminal proteolytic processing by cathepsin G converts RANTES/CCL5 and related analogs into a truncated 4−68 variant. *J. Leukoc. Biol.* **2006**, *80* (6), 1395–404.

(34) Bantscheff, M.; et al. Quantitative mass spectrometry in proteomics: a critical review. *Anal. Bioanal. Chem.* **2007**, *389* (4), 1017–31.

(35) Venkataraman, N.; et al. Cationic polypeptides are required for anti-HIV-1 activity of human vaginal fluid. *J. Immunol.* **2005**, *175* (11), 7560–7.

(36) Kramer, H. B.; et al. Elevation of intact and proteolytic fragments of acute phase proteins constitutes the earliest systemic antiviral response in HIV-1 infection. *PLoS Pathog.* **2010**, *6* (5), e1000893.

(37) Chen, C. H.; et al. The essentiality of alpha-2-macroglobulin in human salivary innate immunity against new H1N1 swine origin influenza A virus. *Proteomics* **2010**, *10* (12), 2396–401.

(38) Anderson, B. L.; Cu-Uvin, S. Clinical parameters essential to methodology and interpretation of mucosal responses. *Am. J. Reprod. Immunol.* **2011**, *65* (3), 352–60.

(39) Horton, R. E.; et al. Cohorts for the study of HIV-1-exposed but uninfected individuals: benefits and limitations. *J. Infect. Dis.* **2010**, *202* (Suppl 3), S377–81.

(40) Ball, T. B.; et al. Polymorphisms in IRF-1 associated with resistance to HIV-1 infection in highly exposed uninfected Kenyan sex workers. *Aids* **2007**, *21* (9), 1091–101.

(41) Su, R. C.; et al. A transient IRF1 response in HIV-exposed, seronegative individuals differs from that in HIV-susceptible controls and is governed by epigenetic mechanisms. *Blood* **2011**.

(42) Silverman, G. A.; et al. Serpins flex their muscle: I. Putting the clamps on proteolysis in diverse biological systems. *J. Biol. Chem.* **2010**, *285* (32), 24299–305.

(43) Silverman, G. A.; et al. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J. Biol. Chem.* **2001**, *276* (36), 33293–6.

(44) Whisstock, J. C.; et al. Serpins flex their muscle: II. Structural insights into target peptidase recognition, polymerization, and transport functions. *J. Biol. Chem.* **2010**, *285* (32), 24307–12.
(45) Ashton-Rickardt, P. G. Serine protease inhibitors and cytotoxic T lymphocytes. *Immunol. Rev.* **2010**, *235* (1), 147–58.
(46) Heutinck, K. M.; et al. Serine proteases of the human immune system in health and disease. *Mol. Immunol.* **2010**, *47* (11−12), 1943–55.
(47) Fahey, J. V.; et al. New approaches to making the microenvironment of the female reproductive tract hostile to HIV. *Am. J. Reprod. Immunol.* **2011**, *65*, 334–343.
(48) Law, R. H.; et al. An overview of the serpin superfamily. *Genome Biol.* **2006**, *7* (5), 216.
(49) Askew, D. J.; Silverman, G. A. Intracellular and extracellular serpins modulate lung disease. *J. Perinatol.* **2008**, *28* (Suppl 3), S127–35.
(50) Wilson, T. J.; Nannuru, K. C.; Singh, R. K. Cathepsin G recruits osteoclast precursors via proteolytic activation of protease-activated receptor-1. *Cancer Res.* **2009**, *69* (7), 3188–95.
(51) Sambrano, G. R.; et al. Cathepsin G activates protease-activated receptor-4 in human platelets. *J. Biol. Chem.* **2000**, *275* (10), 6819–23.
(52) El Messaoudi, K.; et al. HIV-1 infectivity and host range modification by cathepsin D present in human vaginal secretions. *Aids* **1999**, *13* (3), 333–9.
(53) Hashemi, F. B.; et al. Myeloid-related protein (MRP)-8 from cervico-vaginal secretions activates HIV replication. *Aids* **2001**, *15* (4), 441–9.
(54) Herrick, S.; et al. Up-regulation of elastase in acute wounds of healthy aged humans and chronic venous leg ulcers are associated with matrix degradation. *Lab. Invest.* **1997**, *77* (3), 281–8.
(55) Ashcroft, G. S.; et al. Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. *Am. J. Pathol.* **1999**, *155* (4), 1137–46.
(56) Ashcroft, G. S.; et al. Secretory leukocyte protease inhibitor mediates non-redundant functions necessary for normal wound healing. *Nat. Med.* **2000**, *6* (10), 1147–53.
(57) Remold-O'Donnell, E.; Chin, J.; Alberts, M. Sequence and molecular characterization of human monocyte/neutrophil elastase inhibitor. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89* (12), 5635–9.
(58) Schick, C.; et al. Squamous cell carcinoma antigen 2 is a novel serpin that inhibits the chymotrypsin-like proteinases cathepsin G and mast cell chymase. *J. Biol. Chem.* **1997**, *272* (3), 1849–55.
(59) Jayakumar, A.; et al. Inhibition of the cysteine proteinases cathepsins K and L by the serpin headpin (SERPINB13): a kinetic analysis. *Arch. Biochem. Biophys.* **2003**, *409* (2), 367–74.
(60) Oelschlager, C.; et al. Antithrombin III inhibits nuclear factor kappaB activation in human monocytes and vascular endothelial cells. *Blood* **2002**, *99* (11), 4015–20.
(61) Nita, I. M.; Serapinas, D.; Janciauskiene, S. M. alpha1-Antitrypsin regulates CD14 expression and soluble CD14 levels in human monocytes in vitro. *Int. J. Biochem. Cell Biol.* **2007**, *39* (6), 1165–76.
(62) Zhou, X.; et al. HIV Replication in CD4+ T Lymphocytes in the Presence and Absence of Follicular Dendritic Cells: Inhibition of Replication Mediated by {alpha}-1-Antitrypsin through Altered I{kappa}-B{alpha} Ubiquitination. *J. Immunol.* **2011**, *186* (5), 3148–55.
(63) Li, Q.; et al. Glycerol monolaurate prevents mucosal SIV transmission. *Nature* **2009**, *458* (7241), 1034–8.
(64) Munch, J.; et al. Discovery and optimization of a natural HIV-1 entry inhibitor targeting the gp41 fusion peptide. *Cell* **2007**, *129* (2), 263–75.
(65) Congote, L. F. Serpin A1 and CD91 as host instruments against HIV-1 infection: are extracellular antiviral peptides acting as intracellular messengers? *Virus Res* **2007**, *125* (2), 119–34.
(66) Bryan, C. L.; et al. HIV infection is associated with reduced serum alpha-1-antitrypsin concentrations. *Clin. Invest. Med.* **2010**, *33* (6), E384–9.
(67) Potthoff, A. V.; et al. HIV infection in a patient with alpha-1 antitrypsin deficiency: a detrimental combination? *Aids* **2007**, *21* (15), 2115–6
(68) McNeely, T. B.; et al. Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *J. Clin. Invest.* **1995**, *96* (1), 456–64.
(69) Schellenberg, J. J.; et al. Molecular definition of vaginal microbiota in East African commercial sex workers. *Appl. Environ. Microbiol.* **2011**, *77* (12), 4066–74.
(70) Miller, C. J.; Shattock, R. J. Target cells in vaginal HIV transmission. *Microbes Infect.* **2003**, *5* (1), 59–67.
(71) McLaren, P. J.; et al. HIV-exposed seronegative commercial sex workers show a quiescent phenotype in the CD4+ T cell compartment and reduced expression of HIV-dependent host factors. *J. Infect. Dis.* **2010**, *202* (Suppl 3), S339–44.
(72) Card, C. M.; et al. Decreased immune activation in resistance to HIV-1 infection is associated with an elevated frequency of CD4(+)CD25-(+)FOXP3(+) regulatory T cells. *J. Infect. Dis.* **2009**, *199* (9), 1318–22.