HIV-Exposed Seronegative MSM express antiproteases with novel antiviral activity

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INTRODUCTION

•The risk of HIV acquisition through the rectum is significantly higher than other sites of mucosal exposure, which contributes to disproportionate rates of infection in at-risk **men who have sex with men (MSM)** practicing unprotected receptive anal intercourse (URAI).

•HIV susceptibility at the rectal mucosa a result of a thin columnar epithlia, presence of activated T cells within the submucosa, and a tighly associated lymphatic system for easy viral disseminatio(Reviewed in 1).

•However, the immunobiology of rectal mucosa, and factors which affect HIV susceptibility are not well understood and represents a major barrier to the development of prevention technologies. Our recent proteomic analysis of rectal mucosa secretions suggests that this fluid contains hundreds of innate factors important for host defense, and is immunologically distinct from other mucosal compartments and sites of HIV exposure[1].

•Study of **HIV-Exposed Seronegative (HESN)** individuals have shown altered mucosal immune responses in cervical, salivary and foreskin secretions associated with reduced HIV-susceptibility[2-4]. Studying these populations may provide insights into mechanisms of protection against HIV at mucosal surfaces. However, HESN responses have not been investigated in rectal mucosa.

•Our recent research on a HESN MSM population has shown these indivudals to have unique systemic and oral responses to HIV (increased HIV-neutralizing IgA (blood, saliva), increased cc chemokines (saliva) and increased innate proteins (saliva)[2,5,6]. These men have not been evaluated at the rectal muocsa.

Hypothesis: HESN MSM will have unique responses in their rectal proteome that may aid in a reduced susceptibility to HIV at the rectal mucosa.

Applications: This study will provide knowledge on factors that may affect HIV susceptibility, and will broaden our understanding of the immunobiology of the rectal compartment. This information may help guide future rectal microbicide design.

METHODS

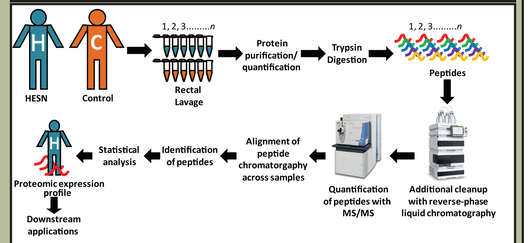


Figure 1. Workflow of the proteomic analysis of rectal mucosal fluid samples from HESN individuals

Mucosal samples from 32 participants (n=9 Ctrl & n=23 HESN) were collected via rectal lavage. Mucosal proteins were eluted, purified via ultracentrifugation/acetone precipitation and digested into peptides. Peptides were cleaned with liquid chromatography and analyzed by label-free MS/MS. Spectra were aligned across samples. Proteins were identified with Mascot Daemon (Matrix Science) and IDs were restricted to 95% peptide and 80% protein confidence and a minimum of 2 peptides ID'd. Normalized protein abundance values were determined to be differentially expressed over time via independent t tests (Benjimani-hochberg corr.). Hierarchical clustering (Cluster 3.0), functional annotation (DAVID Bioinformatics) and GSEA (v2.1.0) were performed. Global protein expression and HESN targets were correlated to epidemiological variables: 1) presence of HIV-neutralizing IgA (t test, p<0.05); 2) frequency of oral exposure (t test, p<0.05); 3) viral load (VL) of partner (Pearsons r, p<0.05).

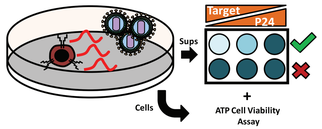


Figure 2. HIV-neutralizaton assay in peripheral blood mononuclear cells (PBMCs)

Correlates of protection identified by proteomic analysis were recombinantly produced and tested for HIV-neutralizaton in PBMC culture. Briefly, fresh PBMCs were isolated from whole blood and PHA stimulated (5 µg/ml) in IL2 supplemented media for 72H (37°C, 5% CO_2). Cells were plated at $5x10^5$ cells/well, AP was added at 40-1 µg/ml and cells were infected at an MOI of 0.05 3B or BaL virus. Input virus was removed after 24H and supernatants were harvested at 5 days and analyzed by p24 ELISA, while cells were analyzed for ATP production by CellTitreGlo (Promega). Percent infectivity and viability was calculated relative to the negative control (no protein added).

RESULTS

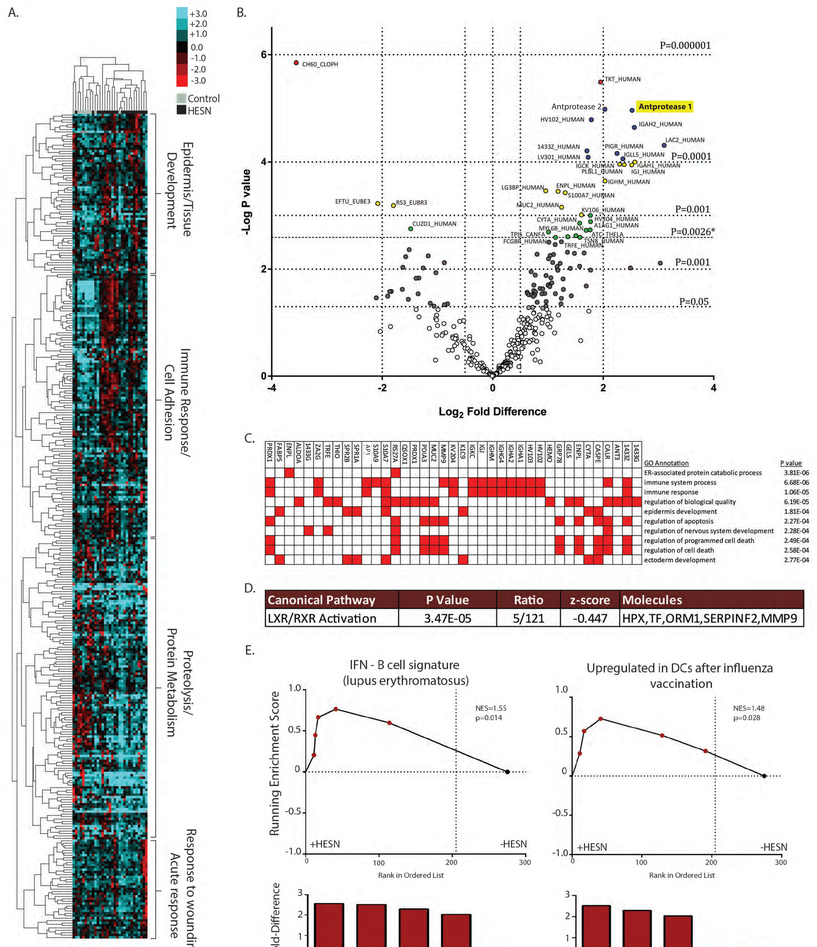
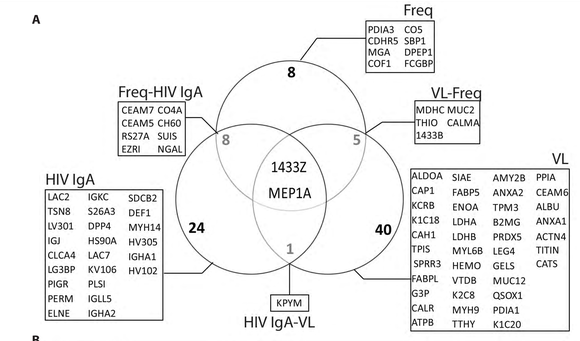


Figure 3: Differential protein expression analysis of rectal mucosal fluid of HESN MSM rectal mucosa identify overexpression of antiproteases and other innate factors

Label-free MS/MS detected 341 proteins in the rectal lavage fluid of HESN MSM and control men. **A:** Hierarchical clustering was able to accurately group HESN from control men with the exception of HESN B5, B16 and B25, which clustered with the control men, and C20, that clustered with the HESNs. Proteins clustered into 4 branches which were each involved in epidermis tissue development, immune response, proteolysis and wound healing. **B:** Thirty proteins were significantly differentially expressed in the HESN phenotype (two-tailed t tests, adjusted p value) and 103 proteins were trending (p<0.05). **C:**DAVID biofunction analysis showed DE (p<0.05) proteins were had roles in immunity ($p=6.68x10^{-6}$), epidermis development ($p=1.18x10^{-4}$), and cell growth/apoptosis($p=2.27x10^{-4}$). **D:** IPA pathway analysis linked HESN protein expression to the decreased LXR/RXR activation (z=-0.447, $p=3.47x10^{-5}$). **E:** GSEA was used to link protein expression to a known immune cell signatures; there was a association of HESN biomarkers with IFN-stimulated B cells (p=0.014) and vaccine-stimulated dendritic cells (p=0.028), though these did not pass multiple comparison correction.



B

Correlation	GO Annotation	PValue	Protein
Partner Viral Load	glycolysis	1.38E-10	ALDOA KPYM GSP TPIS MDHC LDHB LDHA ENOA
	generation of precursor metabolites and energy	6.19E-07	ALDOA KPYM GSP TPIS MDHC THIO LDHB LDHA ATPB ENOA
	regulation of biological quality	8.30E-06	ALDOA QSOX1 1433Z GELS PDIA3 THIO ALBU HEMO MUC2 CALR ATPB PRDX5 MYH9 KCRB 1433B ANXA1 ENOA
	actin filament-based process	1.18E-05	ALDOA ACTN4 MYH9 MYL6B GELS CAP1 TITIN CALR
	cytoskeleton organization	7.23E-05	ALDOA ACTN4 MYH9 K2C8 GELS CAP1 K1C20 TITIN CALR
	cellular homeostasis	1.15E-04	ALDOA PRDX5 KCRB QSOX1 PDIA3 THIO HEMO CALR ATPB
	transport	9.27E-04	VTDB FABP5 ACTN4 MYL6B TTHY 1433Z GELS THIO CAP1 ALBU HEMO K1C18 CALR ATPB ANXA1 MYH9 1433B FABPL ANXA2
	cell redox homeostasis	1.12E-03	PRDX5 QSOX1 PDIA1 THIO
HIV-neutralizing IgA	immune system process	1.78E-03	ELNE DPP4 1433Z IGHA1 HV302 IGHA2 IGJ DEF1 CO4A IGKC
	immune response	5.33E-03	1433Z IGHA1 HV302 IGHA2 IGJ DEF1 CO4A IGKC
	negative regulation of apoptosis	7.31E-03	1433Z PERM RS27A
	negative regulation of cell death	7.11E-03	1433Z PERM RS27A
	regulation of cellular component organization	1.70E-02	EZRI MYH14 RS27A
	response to stimulus	1.71E-02	1433Z IGHA1 DPP4 PERM IGJ IGHA2 DEF1 LG3BP IGKC ELNE H590A SUIS NGAL HV302 CO4A
	proteolysis	2.62E-02	ELNE DPP4 MEP1A CO4A RS27A
Frequency of Exposure	regulation of apoptosis	7.30E-06	COF1 1433Z 1433B PDIA3 MUC2 RS27A
	regulation of programmed cell death	7.85E-06	COF1 1433Z 1433B PDIA3 MUC2 RS27A
	regulation of biological quality	1.00E-05	COF1 1433Z 1433B THIO PDIA3 EZRI MUC2 RS27A
	positive regulation of apoptosis	2.08E-05	1433B PDIA3 MUC2 RS27A
	cell morphogenesis	1.13E-04	COF1 EZRI RS27A
	proteolysis	4.14E-04	COF1 MEP1A DPEP1 CO4A RS27A
	cell motion	4.31E-04	COF1 THIO RS27A
	positive regulation of catalytic activity	6.51E-04	COF1 1433B RS27A

C

Epidemiological correlations of Antprotease 1 levels in rectal lavage

HESN Partner Viral Load (highest 0-12mo. prior sampling)
R^2=0.16
p=0.29
Viral Load
Normalized Abundance

Presence of HIV-neutralizing IgA1 in HESN rectal lavage
p=0.52
Normalized Abundance
Present
None

Frequency of unprotected sex with HIV+ partner
p=0.32
Normalized Abundance
Frequent
Seldom

Figure 4: Analysis of proteome expression differences with epidemiological variables indicates Antiprotease 1 (AP1) associates only with the HESN phenotype.

HESN MSM reported frequency of exposure to partner's HIV through oral unprotected sex, viral load of HIV+ partner was measured within 0-12 months prior to sampling and the presence of HIV-neutralizing IgA in the HESN rectal mucosal sample was assayed. **A:** Two tailed t tests (p<0.05) showed that 23 proteins were differentially expressed between HESN MSM practicing unprotected oral intercourse often/always (n=13) and sometimes/seldom (n=7); two individuals did not respond to the question. Further, 29 proteins were differentially expressed between HESN MSM who had HIV-neutralizing IgA in their rectal lavage (n=7) and those that did not (n=15). A noticeable effect was observed when protein expression was correlated to measured partner viral load; 48 proteins showed a trending correlation (p<0.05, Pearsons correlation) with partner viral load >50 (n=9). **B:** VL correlates held functions in energy metabolism ($p=1.38x10^{-10}$), IgA correlates held functions in immune response ($p=1.78x10^{-3}$) and proteins that varied with frequency of exposure were largely apoptotic factors ($p=7.03x10^{-6}$). **C:** One of our strongest correlates of protection, Antiprotease 1, did not correlate with these factors.

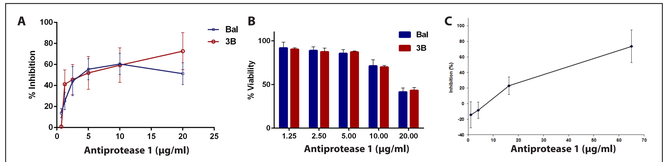


Figure 5: Evaluation of HIV-neutralization capacity of antiprotease 1 in both PBMC and rectal tissue explant assay.

HIV-neutralization assays were completed with recombinant AP1 in both PBMC and colorectal explant culture. HIV infection was measure-through p24 ELISA, while PBMC cell viability was measured with a luminescent assay sensitive to cellular ATP production. **A and B:** AP1 was able to inhibit HIV infection in PBMC culture at up to 80% (3B) and 50-60% viability while maintaining >50% viability (20 µg/ml AP). PBMCs experienced high levels of cytotoxicity past 20 µg/ml of AP (data not shown). **C:**Inhibitions of HIV in colorectal explants were also performed (performed by Carolina Herrera). AP1 was able to inhibit infection by 65% at high concentrations (70 µg/ml of protein).

SUMMARY

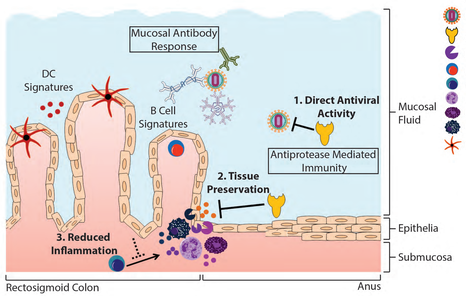


Figure 6. Proposed mechanisms for the role of antiproteases and other innate factors in rectal mucosa in the context of HIV infection.

1. Proteomic analysis of HESN MSM found 30 differentially expressed soluble immune factors that may be important in limiting HIV infection through the rectal mucosa.
2. Functional enrichment and canonical pathway analysis described a state of lowered immune activation in HESN MSM. This may be beneficial in HIV immunity[6,7].
3. Increased antiprotease expression in HESN MSM agrees with our previous findings in the female genital tract secretions of HESN women[8,9].
4. We were able to show that one antiprotease (AP1) had anti-HIV activity in both PBMC and colorectal explant culture, which further supports a protective role for these proteins in HIV-immunity.
5. Epidemiological correlations showed that partner VL influenced expression of cell energy metabolism and cell division proteins in fluid; frequency of oral exposure was associated with differences in apoptotic factors; HIV neutralizing IgA was associated with total Ig expression, and other innate factors.
6. AP1 did not associate with epidemiological variables related to exposure.

Limitations: The Venhälsan cohort is primarily orally exposed to HIV with limited rectal exposure via URAI and are not exposed as highly as other documented HESN sex worker populations; however, this cohort has both documented systemic and oral HIV-neutralizing IgA , suggesting protection is not limited to the oral mucosa. Furthermore, the population size is not large, limiting statistical power

Conclusion: HESN MSM overexpress antiproteases with previously undescribed antiviral activity, which may contribute to reduced susceptibility to HIV in the rectal compartment. This is likely a result of innate differences rather than HIV-exposure. Our findings overlap with previous studies of showing an overabundance of antiviral factors in the cervical secretions of HESN women, supporting further study into their roles in HIV infection. This knowledge is may be important for the development of safe, effective HIV-prevention technologies for MSM.

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CIHR IRSC

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