Characterization of plasma exosome protein cargo in HIV patients on ART

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ABSTRACT

Background: Exosomes are microvesicles originating from many cell types including immune cells. Prior studies suggest exosomes play a role in HIV pathogenesis and some comorbidities. The relationship of exosome cargo in peripheral blood to HIV infection, immune responses and comorbidities has potential applications for biomarker discovery and new treatment strategies. Here, we perform a cross-sectional study to characterize protein cargo of circulating exosomes in HIV patients and its relationship to virological and immunological markers.

Methodology: Plasma exosomes were isolated from 67 subjects (n=40 HIV+ from NNTC, age 37-57, 70% male, 42% black, on ART with suppressed viral load [VL<2500 copies] and n=27 HIV- controls matched for age, gender, race). Exosome quality was assessed by dynamic light scattering, transmission electron microscopy and immunoblotting (WB) for exosome markers (HSP70, CD9 and CD63). Proteomic analysis was by LC/MS/MS. Following bioinformatic analysis of hits, proteins were confirmed by WB.

Results: Circulating exosomes were increased in HIV+ subjects compared to controls based on WB for exosomal HSP70, CD9, and CD63 ($p<0.01$). HSP70, CD9 and CD63 were also detected in exosomes released by PBMCs treated with hemin, Antimycin A or LPS (48 hrs). Exosomal Notch4 correlated with kynurenine:tryptophan ratio (K:T ratio, a marker of immune activation) in HIV+ subjects ($p<0.01$). Exosome-associated NOTCH4, but not HSP70, CD9, and CD63 levels, were higher in HIV+ subjects using cocaine vs. non-users, despite similar VL and CD4:CD8 ratios ($p<0.05$). LC/MS/MS proteomics suggested plasma exosomes were mainly derived from myeloid cells (CSF1R) and revealed proteins related to exosomes (EXOSC10, SYNE1, VPS13C), immune activation/inflammation (CRP, DDR1, ADAM33, CSF1R, SEMA4B, LILRB1), Wnt signaling (EPHA4, DDR1, LRP5, LRP8 and NOTCH4), and metabolism (ADIPOQ); many of these were also detected in exosomes released by heme/LPS-treated PBMC. Treatment of THP-1 cells with HIV+ patient-derived exosomes induced modest increases in CXCL10 and other IFN-induced genes, indicating pro-inflammatory effects.

Conclusions: This study demonstrates associations between exosome proteins and disease markers in HIV patients on ART. Circulating exosomes in HIV+ individuals on ART are mainly derived from myeloid cells, carry protein cargo related to immune responses, inflammation, Wnt signaling, and may have pro-inflammatory effects during pathogenesis.

METHODS

- Plasma samples were collected from 40 HIV+ subjects on ART with suppressed or nearly suppressed plasma HIV viral load (VL) between ages 37-57, 70% male, 42% African-American, 45% using cocaine or crack cocaine, from the National NeuroAIDS Tissue Consortium (NNTC), and 27 age/race-matched healthy controls from Bioreclamation (Westbury, NY).
- HIV subjects were further classified as aviremic with VL <400 copies/ml or viremic with low VL between 400-2500 HIV RNA copies/ml.
- Exosomes were isolated from plasma (0.2 ml) using the PureExo kit (101 Bio) and exosome size and quality assessed by dynamic light scattering, transmission electron microscopy, and immunoblotting 25 µg protein for exosome markers (HSP70, CD9, and CD63).
- Plasma exosome fraction was prepared for proteomic analysis by performing three rounds of abundant plasma protein depletion using Proteome Purify 12 and Albusorb, followed by exosome immunoprecipitation. Untargeted LC/MS/MS was performed on an ABSciex 4800Plus MALDI-TOF/TOF mass spectrometer. For peptide mapping, database search was performed using Protein Pilot 4.5b.

RESULTS

Table 1: Demographic and clinical characteristics of the study cohort

	HIV positive (n=40)	HIV negative (n=27)
Age (years)[#]	48 (43.6-51)	53 (48.5-56.5)
Gender (male)	70%	70%
Race		
Black	42.5%	52%
White	32.5%	44%
Other	25%	4%
cART	100%	--
Cocaine use	45%	--
Current CD4 count (cells/ul) [#]	333 (193.7-452.7)	--
Nadir CD4 (cells/ul) [#]	286 (222-450)	--
Plasma HIV Viral Load [#](copies/ml)	339 (40-918.2)	--
Viral Load <400	55%	--
HCV seropositive	33%	--

[#] Median (IQR)

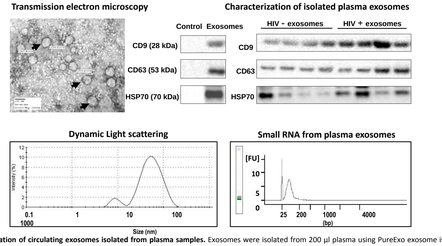

Fig. 1: Characterization of circulating exosomes isolated from plasma samples. Exosomes were isolated from 200 µl plasma using PureExo exosome isolation kit according to the manufacturer's protocol. Exosome size distribution was examined by transmission electron microscopy (top left panel, scale bar=100 nm; arrows indicate exosomes) and dynamic light scattering (bottom left panel). Exosome marker proteins were identified by immunoblotting (25 µg protein) against the exosome markers CD9, CD63, and HSP70 in exosome fraction vs. control samples (top middle) and in HIV- vs. HIV+ plasma exosome samples (top right). Small RNAs were isolated from exosome fractions (miRNeasy kit) and RNA size distribution was assessed with Agilent BioAnalyzer (bottom right panel).

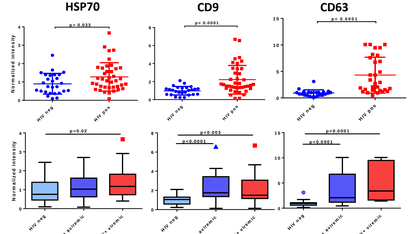

Fig. 2: HSP70, CD9, and CD63 are increased in plasma exosome fractions from aviremic or viremic HIV+ subjects on ART compared to controls. Exosomes isolated from plasma of HIV- and HIV+ subjects (aviremic with < 400 copies/ml or viremic with 400-2500 copies/ml) were probed for exosome-associated proteins by immunoblotting 25 µg protein with antibodies against HSP70, CD9, and CD63.

Biological Classification	Control (n=2)	HIV (n=2)
Myeloid markers	CSF1R	CSF1R
IL production	DDR1, LAMP1, CXCL16	AKAP13, **CRP**, DDR1, LAMP1
Interferon production / response	**LILRB1,** CXCL16, (TAP2)	**LILRB1,** (TICAM1)
Response to viral infection	**LILRB1**	**LILRB1,** (TICAM1)
Chemokine production	AKAP13, CXCL16	AKAP13
Natural killer cell mediated immunity	**LAMP1,** LILRB2, **LILRB1**	**CRP, LAMP1, LILRB1**
Immune activation, inflammation	**DDR1**, ADAMTS7, **ADAM33**, CXCL16, **SEMA4B**, LILRB2, **LILRB1**, **CSF1R,** (TAP2)	**CRP**, DDR1, **ADAM33**, AKAP13, **SEMA4B**, **LILRB1**, **CSF1R**
Vesicles	**OTOF,** (SYNE1, TAP2)	VPS13C, **OTOF,** (EXOSC10)
Fatty acid/lipid metabolism	**DPEP3,** SLC27A5, CYP4A11, LRCOL1	**HGFAC, LRCOL1, DPEP3, ADIPOQ,** (TM6SF2, FADS2)
Wnt signaling	DDR1, LRP5, **LRP8**, EPHA4, **NOTCH4,** CORIN	**NOTCH4,** DDR1, **LRP8**
Neurological process	PCSK9, KCTD3, SYNE1	AKAP13, (BCL2L1)
Cellular stress response	ITPR3 ,ING1, CDH1	**CRP,** AKAP13, CDH1, (BCL2L1)

Table 2: Proteomic analysis of exosomes isolated from plasma. Abundant plasma proteins were depleted from plasma exosome fractions (n=2 each for HIV+ and control subjects) using Proteome Purify 12 and Albusorb. Exosomes were immunoprecipitated using the Exoflow™ exosome purification kit. Following exosome elution and protein precipitation, untargeted LC/MS/MS was performed using the ABSciex 4800Plus MALDI-TOF/TOF mass spectrometer. For peptide mapping, database search was performed using Protein Pilot 4.5b and peptides with highest coverage (95%) score were selected for downstream GO group mapping and functional annotation using the Biobase TRANSFAC tool. Proteins identified by at least 2 unique peptides at greater than 95% confidence are shown in bold, and low confidence (1 peptide scoring below 95%) are shown in parenthesis; Red font indicates proteins detected only in HIV+ plasma exosome samples and green font indicates proteins detected only in HIV- samples.

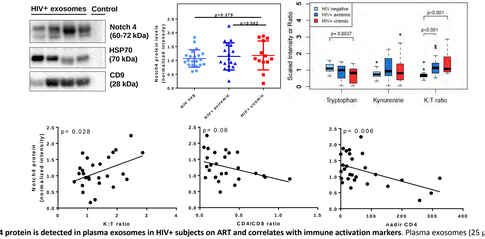

Fig.3: Notch4 protein is detected in plasma exosomes in HIV+ subjects on ART and correlates with immune activation markers. Plasma exosomes (25 µg protein) were immunoblotted with Notch4 and exosome marker antibodies (top left). Top middle panel shows Notch4 protein levels in exosomes from control vs. HIV+ aviremic and viremic subjects. K:T ratio, an immune activation marker is increased in aviremic and viremic HIV+ subjects as compared to controls (top right). Notch4 levels in plasma exosomes correlated positively with K:T ratio and negatively with increasing CD4/CD8 ratio and nadir CD4 count as indicated by Pearson correlations (bottom panels).

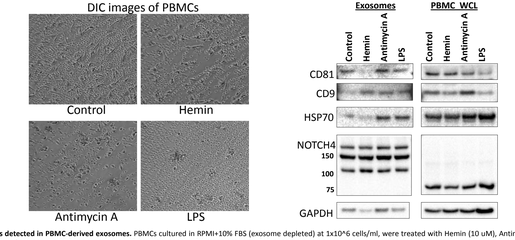

Fig.4: Notch4 is detected in PBMC-derived exosomes. PBMCs cultured in RPMI+10% FBS (exosome depleted) at 1x10^6 cells/ml, were treated with Hemin (10 uM), Antimycin A (5uM), LPS (0.5 ug/ml), or with PBS for a period of 48 hrs (left panel, 10X magnification). Cell supernatant was collected and exosomes were precipitated using Exoquick TC reagent. 25 µg of protein extracted from exosomes or whole cells was loaded onto SDS PAGE gels and immunoblotted for exosome markers, GAPDH, and Notch4 proteins (right panel). Uncleaved forms of Notch4 (bands at 230, 150 and 110 kDa) were detected in exosomes, while the cleaved form (70 kDa) was detected in PBMC cell lysates.

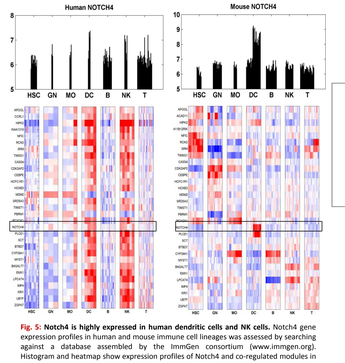

Fig. 5: Notch4 is highly expressed in human dendritic cells and NK cells. Notch4 gene expression profiles in human and mouse immune cell lineages was assessed by searching against a database assembled by the ImmGen consortium (www.immgen.org). Histogram and heatmap show expression profiles of Notch4 and co-regulated modules in human and mouse immune cell lineages. Notch4 mRNA is expressed in dendritic cells in both human and mouse, whereas expression in NK cells was detected only in humans.

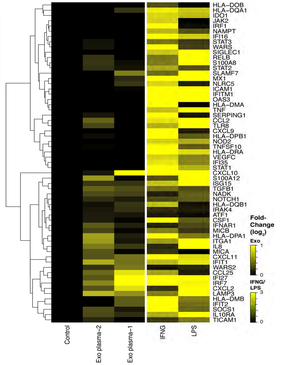

Fig. 7: Plasma exosomes from HIV+ subjects induce gene expression changes indicative of immunomodulatory effects and immune activation in THP-1 monocytic cells. THP-1 cells were treated with plasma exosomes (10 ug/ml) from 2 HIV+ subjects for 48 hrs (left heatmap panel) compared to IFN-γ (100 IU/ml) or LPS (0.5 ug/ml) stimulation (right heatmap panel). mRNA expression was measured using the Nanostring nCounter PanCancer Immune Profiling Panel and custom codeset of 30 additional genes to quantify expression of a total of 800 genes. Left heatmap shows unsupervised hierarchical clustering of gene expression changes induced by treatment with exosomes relative to control. Treatment of THP-1 cells with HIV+ plasma exosomes induced gene expression changes which partially overlap those induced by IFN-γ and LPS.

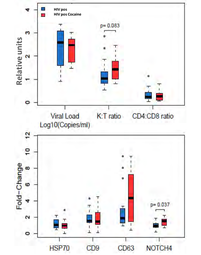

Fig. 6: Exosomal Notch4 protein is increased in plasma from HIV+ subjects using cocaine compared to HIV+ non-users. Kynurenine:tryptophan (K:T) ratio, a marker of immune activation, but not viral load, was higher in HIV+ cocaine users vs. non-users, consistent with other studies. Exosomes isolated from plasma from HIV+ cocaine users or non-users were probed by immunoblotting (25 µg protein) with antibodies against the exosome markers HSP70, CD9, and CD63 or Notch4. Exosomal Notch4, but not other exosome-associated proteins, was elevated in cocaine users vs. non-users.

CONCLUSIONS

- Exosome marker proteins HSP70, CD9, and CD63 are elevated in plasma from aviremic and viremic HIV+ subjects on ART compared to HIV- controls
- Proteomic analysis of plasma exosomes by mass spectrometry revealed peptides mapping to proteins associated with inflammation, immune activation, interleukin and interferon production, Wnt signaling, and stress responses.
- Kynurenine:tryptophan (K:T) ratio, an immune activation marker, was elevated in HIV+ subjects compared to HIV- controls, and in HIV+ cocaine users vs. non-users.
- Notch4 protein was detected in plasma exosomes, correlated positively with K:T ratio and inversely with nadir CD4 counts and CD4:CD8 ratio, and was detected at higher levels in plasma exosomes from HIV+ cocaine users vs. non-users
- Circulating plasma exosomes from HIV+ subjects on ART carry protein cargo related to immune responses and immune activation, and may have pro-inflammatory effects during HIV pathogenesis that may be further augmented by cocaine use and oxidative stress.