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Interacting partners of macrophage-secreted cathepsin B contribute to HIV-induced neuronal apoptosis

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Abstract

Objective—HIV-1 infection of macrophages increases cathepsin B secretion and induces neuronal apoptosis, but the molecular mechanism remains unclear.

Design—We identified macrophage secreted cathepsin B protein interactions extracellularly and their contribution to neuronal death *in vitro*.

Methods—Cathepsin B was immunoprecipitated from monocyte-derived macrophage supernatants after 12 days post-infection. The cathepsin B interactome was quantified by label-free tandem mass spectrometry and compared to uninfected supernatants. Proteins identified were validated by western blot. Neurons were exposed to macrophage-conditioned media in presence or absence of antibodies against cathepsin B and interacting proteins. Apoptosis was measured using TUNEL labeling. Immunohistochemistry of post-mortem brain tissue samples from healthy, HIV-

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Conflicts of Interest

The authors declare no conflict of interests.

Description of the role of each of the authors in the study reported:

Yisel M. Cantres Rosario performed all of the work of this study except mass spectrometry. She wrote the manuscript, performed statistics and IPA analyses. Natalia Hernandez and Karla Negron collaborated in tissue culture and Western blots experiments. Dr. Juliana Perez-Laspiur contributed to the design and procesing of samples for proteomics studies and taught IPA to Yisel Cantres. Dr. John Leszyk performed mass spectrometry experiments and Mascot analyses. Dr. Scott A. Shaffer direct the mass spectrometry facility and contributed to the mass spectrometry analyses and reviewed the manuscript. Dr. Loyda M. Melendez provided the funds for the study, mentored Yisel M. Cantres in the research design, established communications with all authors for manuscript writing and review, selected the Journal of AIDS, provided feedback of the methods and results, reviewed and submitted the manuscript and the responses to reviewers' comments for publication.

infected, and Alzheimer's disease patients was performed to observe the *ex vivo* expression of the proteins identified.

Results—Nine proteins co-immunoprecipitated differentially with cathepsin B between uninfected and HIV-infected macrophages. Serum amyloid p component (SAPC) -cathepsin B interaction increased in HIV-infected macrophage supernatants, while matrix metalloprotease 9 (MMP-9) -cathepsin B interaction decreased. Pre-treatment of HIV-infected macrophage-conditioned media with antibodies against cathepsin B and SAPC decreased neuronal apoptosis. The addition of MMP-9 antibodies was not protective. SAPC was over-expressed in post-mortem brain tissue from HIV-positive neurocognitive impaired patients compared to HIV positive with normal cognition and healthy controls, while MMP-9 expression was similar in all tissues.

Conclusions—Inhibiting SAPC-cathepsin B interaction protects against HIV–induced neuronal death and may help to find alternative treatments for HIV-associated neurocognitive disorders.

Keywords

HAND; macrophage; cathepsin B; SAPC; MMP9; post-mortem brain tissue

II. Introduction

During HIV-1 infection, monocytes cross the blood-brain barrier (BBB) into the central nervous system (CNS) facilitating viral spread into microglia and astrocytes, unleashing a persistent inflammatory response and neuronal damage [1–7]. The inflammation leads to HIV-associated encephalitis (HIVE) and neurological complications known as HIV-associated neurocognitive disorders (HAND). Clinical manifestations range from asymptomatic to the severe form HIV-associated dementia (HAD).

Cathepsin B, a lysosomal cysteine protease, is up-regulated and secreted from HIV-infected macrophages [7–9]. The interaction of cathepsin B with its endogenous inhibitors cystatins B and C is disrupted by HIV infection [7–9]. Notably, cathepsin B present in macrophage-conditioned medium (MCM) contributes to 10–20% of neuronal apoptosis [7]. Cathepsin B has been correlated to amyloid plaque accumulation [10–13], as well as to their degradation [14], being linked to Alzheimer's disease (AD) [15]. Cathepsin B can interact with matrix metalloproteases (MMP) at the extracellular level, suggesting a possible role in cellular migration, tissue remodeling, and cancer [16–19].

We sought to determine if cathepsin B secreted from HIV-infected macrophages interacts with additional proteins at the extracellular level to promote neuronal apoptosis. Cathepsin B was immunoprecipitated (IP) from monocyte derived macrophages (MDM) supernatants at 12 days post-infection (dpi), and the interacting proteins were identified by mass spectrometry (LC-MS/MS). Serum amyloid p component (SAPC) co-immunoprecipitated (co-IP) with cathepsin B from HIV infected supernatants triggering neuronal apoptosis. Cathepsin B and SAPC were over-expressed in the brain of HIV-positive patients at different stages of HAND and in patients with AD. These findings, suggest a common mechanism of cathepsin B – SAPC interaction in neuronal death for both HAND and AD.

III. Methods

Isolation and infection of monocyte-derived macrophages from HIV seronegative donors

Peripheral blood mononuclear cells (PBMC) were isolated from six healthy women by Ficoll® gradient centrifugation. MDM were selected by adherence and inoculated with HIV-1_{ADA} at a 0.1 MOI [7]. Supernatants were collected at 3, 6, 9 and 11 dpi to assess the infection measuring the levels of HIV-p24 antigen concentration by ELISA, following the manufacturer's instructions (Express BioTech, MD).

Immunoprecipitation of cathepsin B

Uninfected and HIV-infected MDM were replaced with serum-free RPMI-1640 at 11 dpi, incubated overnight. Supernatants were collected, concentrated, and dialyzed overnight at 4°C using QuixSep Micro Dialyzer and Cellu Sep®H1 high-grade tubular dialysis membranes (Membrane Filtration Products, Inc., Seguin, Texas). Total protein concentration was measured by DC™ assay (Bio-Rad, Hercules, CA), and 300μg were used for IP of cathepsin B using Protein G Dynabeads® (Life technologies, Carlsbad, CA) covalently linked to 4μg of anti-cathepsin B monoclonal antibody (Sigma-Aldrich, St. Louis, MO) using 20mM dimethyl pimelimidatex·2HCl. Protein was eluted with 0.1M glycine buffer (pH 2.5) and the pH was restored with tris buffer (pH 7.5). Negative control was prepared performing IP with an irrelevant IgG1 isotype. A cathepsin B positive control was generated performing the IP from MDM lysates.

In-solution digestion of immunoaffinity purified proteins and nanoflow tandem mass spectrometry

Co-immunoprecipitation eluates were processed for mass spectrometry as described [20,21]. Samples were reconstituted in 5% acetonitrile containing 0.1% TFA and analyzed by data-dependent analysis using nanoflow LC-MS/MS on an LTQ Orbitrap Velos mass spectrometer (Thermo Scientific). The resulting data were processed and searched using Mascot (version 2.4.0, Matrix Science, London, UK). The Mascot search utilized the human SwissProt database (version 020613, 20322 entries) and considered peptides at full tryptic specificity, a parent ion mass tolerance of 10.0 ppm and a fragment ion mass tolerance of 0.50 Da. Scaffold (version 4.0.4, Proteome Software Inc., Portland, OR) was used to further validate the peptide and protein identifications. Peptide identifications were accepted at ≥ 80.0% probability as specified by the Peptide Prophet algorithm [22]. Protein identifications were accepted if ≥ 90.0% probability and were assigned by the Protein Prophet algorithm [23]. Proteins that were identified by single peptide matches were included in further analysis. Spectral counts were used as a semi-quantitative tool to compare relevant proteins identified in relation to HIV-1 infection and cathepsin B identification, as postulated [24,25]. Proteins were analyzed as a ratio of spectral counts (HIV/uninfected) by Ingenuity® Pathway Analysis (IPA) software (QIAGEN, Redwood City, CA), to define the potential cathepsin B interactome. Proteins were selected for validation according to the network generated by IPA, functional relevance, and presence or absence between uninfected and HIV-infected replicates.

Western Blots

Protein concentration was measured using a DC assay [7], and 20–40μg of total protein were loaded into 4–20% TGX gels (Bio-Rad). PVDF membranes were probed with: mouse anti-cathepsin B (1:500, Sigma-Aldrich), mouse anti-SAPC (1:250, Abcam, Cambridge, England, UK), mouse anti-cystatin B (1:500, Sigma-Aldrich), rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:250, BIOSS, Woburn, MA), and mouse anti-MMP-9 (1:750, R&D systems, Minneapolis, MN), incubated overnight at 4°C. HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (1:15,000; Sigma-Aldrich) were incubated for 1 hour. For IP samples, a conformation-specific secondary antibody was used (Anti-mouse IgG VeriBlot for IP secondary antibody (HRP); 1:1,000, Abcam). Images were acquired and analyzed using ImageLab™ software (Bio-Rad). Densitometry was performed by dividing the volume (intensity) of each protein by the volume of the GAPDH band for each lane. Membranes were re-probed incubating in Restore Plus Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA) for 30 min at 37°C followed by washing, blocking and probing with a different antibody. MDM lysates from six donors were used.

HIV-positive patients

Retrospective samples (n=12) of MDM supernatants from Hispanic women cohort characterized for cognitive function from 2009 to 2012 were used for this study. Samples were collected as part of the R01 project entitled: “Cystatins and cathepsins in HIV induced neuropathogenesis”, with approval from the University of Puerto Rico Medical Sciences Campus Institutional Review Board (Protocol 0720102) and written consents of participants. Inclusion criteria, viral-immune parameters, and evaluations of cognitive function have been previously described [8,26,27]. According to American Academy of Neurology (m-AAN) criteria patients were classified into normal cognition (NC), asymptomatic cognitive impairment (A), minor cognitive motor disturbance (MC/MD), or HIV-1 associated dementia (HAD).

ELISA

SAPC was measured in MDM supernatants from HIV-1 infected and HIV-seronegative subjects from the Hispanic Cohort characterized for HAND [26], and from supernatants of *in vitro* HIV-infected MDM derived from healthy donors at 6 dpi (n=4 with serum, diluted 1:100) and 12 dpi (n=5 serum-free, diluted 1:1,000), following manufacturers’ instructions (Abcam). Results were normalized using SAPC measured in the culture medium. Pro-cathepsin B, an active precursor of cathepsin B was measured by ELISA (R&D Systems; n=8) in serum-free MDM supernatants at 12 dpi, as well as MMP-9 (R&D Systems; n=5), following manufacturer’s instructions.

Cathepsin B activity

Cathepsin B activity from serum-free MDM supernatants at 12 dpi (n=6) was measured in duplicate using a fluorescent substrate assay kit (Biovision), following manufacturer’s instructions and analyzed in a VersaFluor TM Fluorometer (Bio-Rad) with 400 nm excitation and 505 nm emission filters.

SK-N-SH neuroblastoma cell cultures

SK-N-SH neuroblastoma (ATCC® HTB-11™) was cultured in essential modified eagle's medium (EMEM), supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 10% FBS and 1% penicillin-streptomycin. For TUNEL assays, cells were cultured at 1×10^5 cells/well in poly-D-lysine coated 8-well glass chamber slides (Thermo Fisher Scientific), incubated at 37°C, 5%CO_2.

Measurement of apoptosis by TUNEL assay

Neurons were exposed to MDM serum-free supernatants at 12 dpi (macrophage-conditioned media, MCM) diluted 1:4 in plain EMEM and added to neuronal cells at 37°C for 24 hours as described [7]. MCM was pretreated with specific cathepsin B inhibitor CA-074 (Sigma-Aldrich, 10μM) or monoclonal anti-cathepsin B, anti-MMP-9 or anti-SAPC antibodies, either independently or in combination. During apoptosis, fragmented DNA exhibits green fluorescence upon TUNEL labeling. A minimum of three images were acquired for each condition for each donor. Green fluorescent nuclei were counted and divided by the total number of neurons (all DAPI-positive nuclei, blue) to obtain a percentage of apoptotic neurons, using ImageJ software (NIH). MCM were collected from MDM from four donors.

Immunofluorescence of post-mortem brain tissue

Paraffin-embedded post-mortem brain tissue samples from healthy and HIV-infected individuals were provided by National NeuroAIDS Tissue Consortium (NNTC) and processed as described before (Zenon et al, in press). Mouse monoclonal anti-cathepsin B (1:100; Sigma-Aldrich), mouse monoclonal anti-SAPC (1:50; Abcam), rabbit polyclonal anti- ionized calcium-binding adapter molecule 1 (Iba-1) (1:100; Wako), mouse monoclonal anti-MMP-9 (1:65; R&D Systems), rabbit polyclonal anti-amyloid $beta_{1\text{-}42}$(Aβ) (1:50, Abcam), and rabbit polyclonal anti-neurofilament (1:100; Millipore, Billerica, MA) primary antibodies were incubated overnight at room temperature. Alexa Fluor® anti-mouse 488 and anti-rabbit 546 fluorescent secondary antibodies (1:200; Life Technologies) were incubated at room temperature for two hours. All sections were labeled with DAPI (1:500), diluted in Vectashield® (Vector Laboratories, Burlingame, CA). Images were acquired using a Nikon Eclipse E400 fluorescence microscope with a SPOT Insight QE camera and SPOT 5.1 software. A minimum of three images were acquired from each section.

Statistical analyses

Wilcoxon's Signed Rank Test was used to compare the presence or absence of peptides in the samples identified by LC-MS/MS (n=6). Two-tailed unpaired t tests were used for parametric data and Mann Whitney tests were used for non-parametric data derived from ELISA and Western blot densitometry. SAPC ELISA was analyzed by One-Way ANOVA with Tukey's post-test. For neurotoxicity assays, we used two-way ANOVA with Bonferroni post-test. All statistical analyses were done using Prism GraphPad® software 5.0 with $p<0.05$ considered statistically significant.

IV. Results

Identified proteins from uninfected and HIV infected cathepsin B interactome

Cathepsin B was successfully co-IP from serum-free MDM supernatants at 12 dpi (Supp. Figure 1). The procedure was confirmed by identification of cathepsin B peptides in all experimental samples and positive controls and its absence in the negative control by tandem mass spectrometry. A total of 176 proteins had spectral counts with over 90% confidence. From these, 123 proteins that co-IP with cathepsin B were absent from the negative control. Thirty proteins were selected for analyses based on observation rates in the six replicates, when comparing HIV-infected to uninfected MDM supernatants. The spectral counts from 9 proteins, functionally relevant based on the literature, are included in Figure 1A. Many of these proteins were based on single peptide identifications and did not meet the typical two-peptide (per protein) identification criteria. However, many of these single-hit proteins were considered as they were consistently observed across two or more of the six replicates. In most single-hit proteins selected there was a difference of replicates positive for the protein between the conditions (uninfected vs. HIV-infected). The predicted network of interactions generated by IPA software is described in Figure 1B. The proteins that co-IP with cathepsin B in HIV-positive samples and not in uninfected MDM are colored green; proteins colored red were co-IP with cathepsin B in uninfected samples but not in HIV-positive samples. Proteins colored yellow were identified equally in both uninfected and HIV-positive samples. Proteins in blue were not identified in our dataset, but were provided by the software to connect the proteins identified in the network. High density lipoprotein (HDL) and complement proteins are important central hubs of proteins identified. The IPA software catalogued the following diseases related to co-IP proteins: developmental disorders, hereditary disorders, immunological diseases, humoral immune response, inflammatory response, and neurological diseases (Supp. Figure 2). The acute phase response signaling was the most relevant canonical pathway that correlated with cathepsin B functions and the proteins identified. The IPA analysis provided a new link between MDM-derived cathepsin B in HIV infection and the activation of lipids, complement system, and inflammation related proteins that are observed during the infection and may converge in neuronal apoptosis. SAPC co-IP in four of the six HIV-infected MDM supernatants while it was not detected in uninfected MDM supernatants (Figure 1C). Therefore, we hypothesized that SAPC is involved in neuronal death when bound to cathepsin B during HIV-infection. Cathepsin B-MMP-9 co-immunoprecipitate was identified in two of the uninfected samples but not in HIV-positive samples.

Validation of the proteins interacting with cathepsin B by western blot

We validated the co-IP of SAPC in HIV-infected samples by western blot (Figure 2A). Co-IP of cystatin B with cathepsin B was decreased in HIV-infected MDM supernatants, consistent with our previous reports of disrupted interaction after HIV infection of MDM [7]. MMP-9 was detected only in uninfected IP samples, also validating the mass spectrometry results.

Detectable levels of SAPC and MMP-9 were observed in MDM lysates (Figure 2C) that were secreted to culture supernatants at 12 dpi (Figure 2B). In serum-free supernatants, no

visual difference was observed in any of the proteins. Cystatin B was not detected in MDM serum-free supernatants. None of the proteins tested were statistically significant comparing uninfected to HIV-infected MDM lysates, according to densitometry analyses (Figure 2D).

Cathepsin B secretion is higher in HIV-infected macrophage supernatants but not SAPC or MMP-9

SAPC concentration was not statistically different in MDM supernatants from HIV-seropositive women, compared to healthy women ($p=0.890$) even when subdivided by cognitive status ($p=0.968$), although there seems to be a tendency to increase in cognitively impaired HIV-infected patients (Supp. Figure 3). We did not observe differences when comparing uninfected to HIV-infected MDM supernatants infected *in vitro* at 6 dpi ($p=0.686$).

Cathepsin B secretion is significantly increased in HIV-infected MDM ($p<0.001$) at 12dpi (Figure 3A), which constitute the macrophage-conditioned media (MCM) used for neurotoxicity experiments. However, SAPC and MMP-9 secretion were not altered in HIV-infected MDM ($p=1.00$ and $p=0.691$, respectively) (Figures 3B and 3C). Cathepsin B activity is slightly increased in HIV-infected MDM serum-free supernatants, but was not statistically significant ($p=0.26$, Figure 3D).

Cathepsin B- SAPC interaction in HIV-1 infected MDM secretome contributes to neuronal apoptosis

Neurons were exposed to uninfected or HIV-positive MCM with or without antibodies against cathepsin B, SAPC, and MMP-9. SAPC and MMP-9 antibodies were tested individually or together with cathepsin B antibody, to assess the neurotoxic effect of targeting both proteins simultaneously (Figure 4). We confirmed that productively HIV-infected MCM (Supp. Figure 4) induces higher percentage of neuronal apoptosis than uninfected MCM ($p<0.05$), which is reduced in presence of cathepsin B antibody or CA-074 inhibitor ($p<0.05$). Surprisingly, the pre-treatment of MCM with SAPC antibody also reduced the percentage of neuronal apoptosis ($p<0.05$). The addition of both antibodies to MCM reduced the neuronal apoptosis to the same level as when treated with each antibody separately ($p<0.05$). The effect of pre-treatment of HIV-infected MCM with MMP-9 antibody was variable among the four MDM donors, and was not effective in contrast to the pre-treatment with cathepsin B and SAPC antibodies or cathepsin B inhibitor ($p<0.001$). The pre-treatment of MCM with both cathepsin B and MMP-9 antibodies did not have any effect in uninfected MCM.

Cathepsin B and SAPC are increased in post-mortem brain of HIV-positive neurocognitive impaired and Alzheimer's disease patients

We have reported increased expression of cathepsin B and cystatin B in basal ganglia and hippocampus of a single HIV-infected subject with MCMD and another with AD by immunofluorescence of snap frozen brain tissue [7]. Here we report an overexpression of cathepsin B, cystatin B and cystatin C in the frontal white matter of three patients with HAND (Figure 5A). Cathepsin B and cystatin B (Zenon et al. in press) are also increased in HIV-subsyndromic tissues, while cystatin C is absent at this stage of the disease. Cystatins

have low expression in AD tissues (data not shown). Cathepsin B is increased and shows co-localization with microglia/macrophage marker Iba-1 in AD tissue. MMP-9 co-localized with Iba-1 in HIV-subsyndromic tissue, but was not detected in HAD or AD. SAPC is increased in the brain of HIVE/HAD and AD patients. We observed immunolabeling of Aβ in HIV-subsyndromic and HIVE/HAD tissues in addition to the expected Aβ in the AD tissue. SAPC co-localized with neurofilament in HIVE/HAD to a higher degree than what is observed in AD (Figure 5B). Negative controls demonstrated low unspecific labeling.

V. Discussion

In this study, we aimed to determine the role of secreted cathepsin B interactome in HIV-1 infection and neurotoxicity. We report 9 proteins that co-IP with cathepsin B in uninfected or HIV-infected MDM. The low number of spectral counts found in these proteins is likely due to the low concentration of protein in the final co-IP eluate. However, IPA analysis revealed the connection between these proteins and confirmed functions related to cathepsin B including modification of the extracellular matrix, processing of APP, amyloidogenesis, activation of proteases, and immune response.

From the proteins identified, SAPC co-IP from 4 HIV-infected and none of the uninfected MDM supernatants. SAPC is involved in acute phase response and is linked to amyloid plaque accumulation by stabilizing amyloid fibrils, thus preventing their degradation [28,29]. It is present in AD patients [30], and induced neuronal apoptosis when injected into rat hippocampus [31]. There is proof of efflux of SAPC at the blood brain barrier (BBB) [32], and accumulation of SAPC in the brain of rats with permeabilized BBB, demonstrating its role in neurodegenerative diseases. As the BBB is also compromised in HAND [3,33,34], it is important to understand the link between cathepsin B-SAPC and neurodegeneration during HAND development.

To examine the clinical relevance of SAPC in HAND, its concentration was measured in supernatants of macrophages from HIV-infected women under anti-retroviral therapy. SAPC did not show differences when stratified by cognitive function. However, inhibition of SAPC, cathepsin B, or both proteins from HIV-infected MCM before addition to neurons was neuroprotective. Previous reports indicated that SAPC possess neurotoxic properties [31], including the stabilization of amyloid fibrils, which is the hallmark of AD neurodegenerative process [30]. These results suggest that HIV-1 infection is promoting the association of SAPC with cathepsin B more than a change in SAPC expression and/or secretion to increase neurotoxicity.

MMP-9 co-IP with cathepsin B only from uninfected supernatants. Inhibition of MMP-9 from HIV-infected MCM was not neuroprotective. MMP-9 can degrade extracellular amyloid peptides [35–37], which might explain the negative effect of inhibiting this protein in MCM before exposure to neurons.

The *ex vivo* expression of SAPC in the brain was tested by immunofluorescence. Cathepsin B, SAPC, cystatin B and cystatin C are overexpressed in deep frontal white matter from HIV-positive patients with neurocognitive impairment, and absent in HIV-positive normal cognition and in HIV-negative subjects. SAPC and cathepsin B expression is higher than

cystatins. Moreover, SAPC and cathepsin B are overexpressed in AD patients compared to healthy controls. Cathepsin B co-localized with Aβ peptides in AD tissue, supporting evidence of its role in the disease. We are reporting a novel role for SAPC in HAND with its co-localization with neurofilament in HIVE/HAD tissues. Recently, a mechanism of intraneuronal amyloid accumulation was described in a transgenic mice model expressing HIV-1 gp120, amyloid precursor protein (APP) and presenilin-1 (PS1). In this model, gp120 triggers accumulation of APP and beta-secretase (BACE1) with subsequent accumulation of Aβ peptides in the lysosomes of neurons [38]. Amyloid aggregation has been described in the brain of HIV-infected patients carriers of the apolipoprotein E allele 4 (ApoE4) [39]. The expression of APP has been observed in the surface of circulating monocytes from HIV-infected patients, but it was not a successful biomarker for neurocognitive impairment [40]. Since cathepsin B and SAPC have been linked to amyloid plaque formation and stabilization [10,12,13,28,29,39,41–43], both proteins might be triggering amyloid plaque formation among other molecular imbalances in HIV-positive patients, therefore contributing to neuronal dysfunction and death. Another possible mechanism is the interaction of SAPC with Fcγ receptors [44] which are involved in Aβ-mediated neurotoxicity and development of AD [45,46].

In future studies, we plan to determine if SAPC and MMP-9 interactions with cathepsin B are present in microglia supernatants, since cathepsin B secreted from HIV-infected microglia is also neurotoxic *in vitro* (Zenon et al, in press). Second, all our studies have been conducted using women donors to compare with our Hispanic women cohort characterized for HAND. Additional studies will be performed using male PBMCs. In our study, we did not use growth factors to differentiate monocytes. While GM-CSF differentiates macrophages into a pro-inflammatory state (M1 type), M-CSF differentiates macrophages into an anti-inflammatory state (M2 type), with different cytokine profiles [47–51]. The secretion of cathepsin B and interacting partners can be studied by inducing a specific polarization. In this study, we decided to allow the macrophages to adhere in culture and observe the changes in morphology induced by HIV-1 infection. In agreement with the literature, we observed a mixture of round and spindle shaped macrophages in cultures from all healthy PBMC donors [47] and similar concentration of cathepsin B in MDM supernatants across the cultures. Finally, we used the HIV-1_{ADA} strain, which productively infects MDM *in vitro*, to elucidate mechanisms triggered by the infiltration of macrophages into the CNS of HIV-positive patients. However, it is in our interest compare these results to those obtained when microglia or MDM are infected with a viral strain isolated directly from the brain [52].

Cathepsin B inhibitors CA-074 and E64d are currently under study as therapeutic candidates for AD treatment, reduction of amyloid plaque formation, and improvement of cognitive function [12,41]. These results might shed light on mechanisms shared by AD and HAND, for which common pathways are currently under study [[53,54] and reviewed in [55]]. Targeting cathepsin B-SAPC complex upon HIV-1 infection may represent a novel strategy for drug development against HAND.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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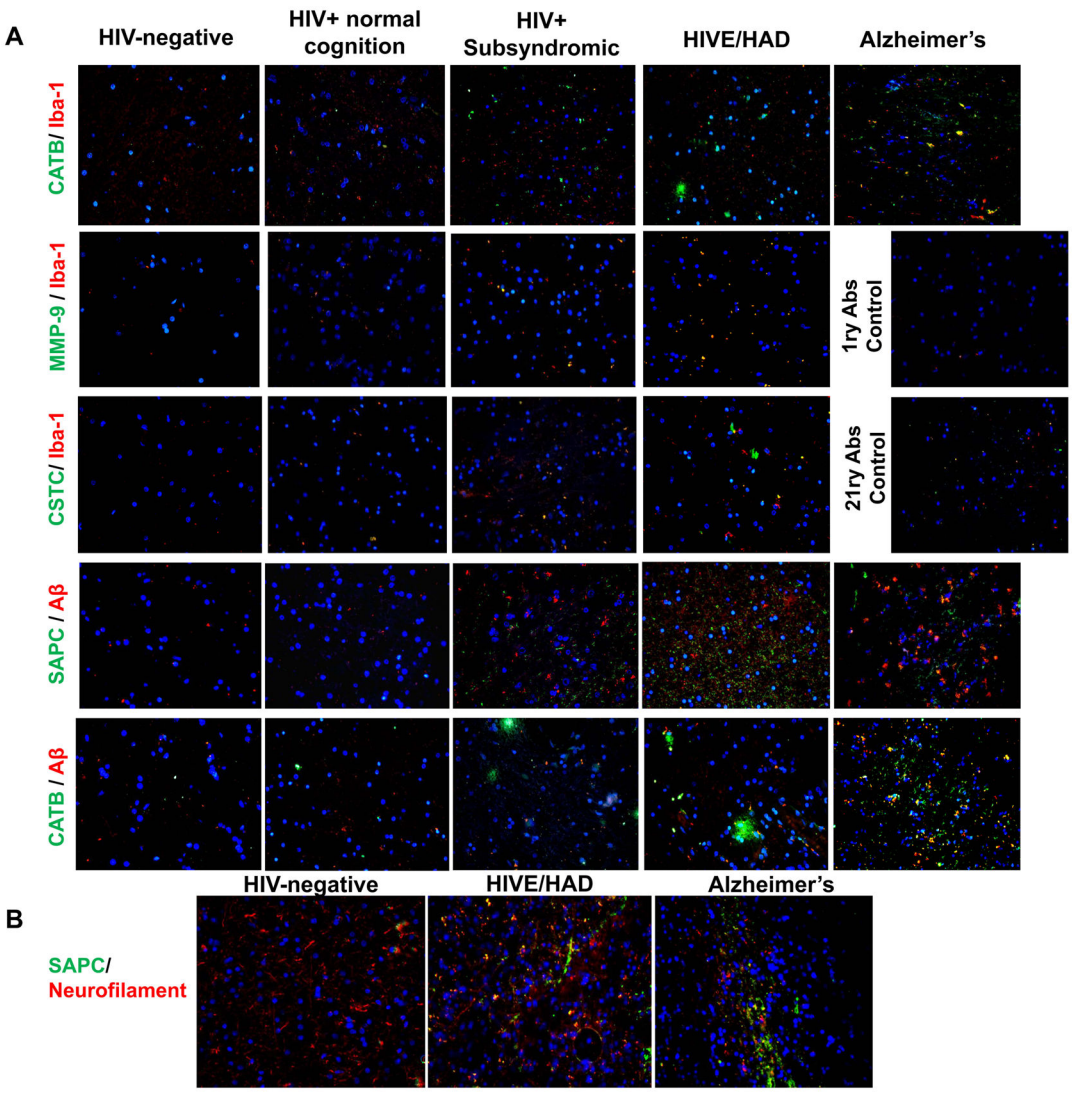

Figure 1. Predicted protein network of cathepsin B interacting proteins from spectral count ratios of HIV-infected to control MDM supernatants

A. Proteins differentially co-IP and identified in uninfected and HIV-infected MDM supernatants after cathepsin B immunoprecipitation followed by LC-MS/MS. All the proteins were identified differently in two or more uninfected or HIV-infected samples. Average spectral count for each protein in all the samples (n=6) from each variable are reported. The numbers in the bars indicate the number of samples in which the peptides were identified. B. LC-MS/MS spectral counts ratios of HIV-infected to uninfected IP samples were entered into IPA software for analysis of possible interactions and networks involving the proteins identified. Proteins colored in green represent the proteins that co-IP with cathepsin B in HIV-positive samples and not in uninfected controls. Proteins colored in red, were co-immunoprecipitated in uninfected samples but not in HIV-positive samples. Proteins colored in yellow were identified equally in both uninfected and HIV-positive samples. Proteins in blue are provided by the software although not identified in our dataset, to connect the proteins identified by the proteomics procedure. C. Green and red colored

proteins were then selected for testing by Wilcoxon's test to determine a statistical tendency and select proteins for validation. Based on literature and Wilcoxon's signed rank tests ($p = 0.06$), only SAPC was selected for subsequent experiments.

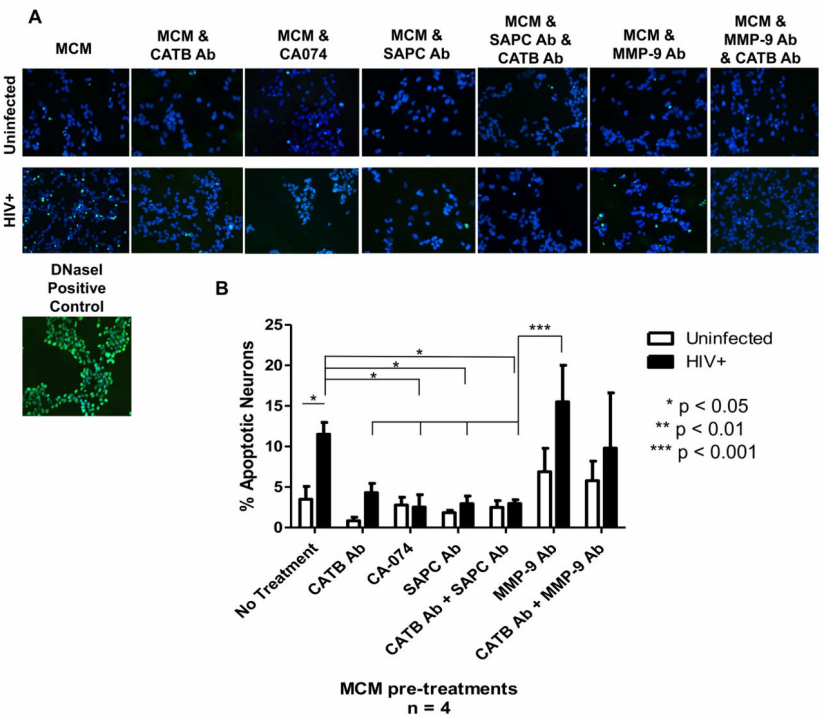

Figure 2. Validation of intracellular and extracellular cathepsin B interacting proteins in uninfected and HIV-infected MDM at 12dpi

Co-immunoprecipitation of serum amyloid p component, matrix metalloprotease-9 and cystatin B with cathepsin B from MDM supernatants at 12dpi (A). Serum-free supernatants from uninfected and HIV-infected MDM at 12dpi and human serum as positive control for SAPC (B). Intracellular MDM expression of SAPC, cathepsin B, MMP-9, cystatin B, and GAPDH as loading control. Human serum as positive control for SAPC and (C). Densitometry of proteins in MDM lysates normalized against GAPDH. Two-way ANOVA statistical test comparing all the bands (D). Representative of three independent experiments with MDM from six different donors. Data represented as mean ± standard error measurement (SEM).

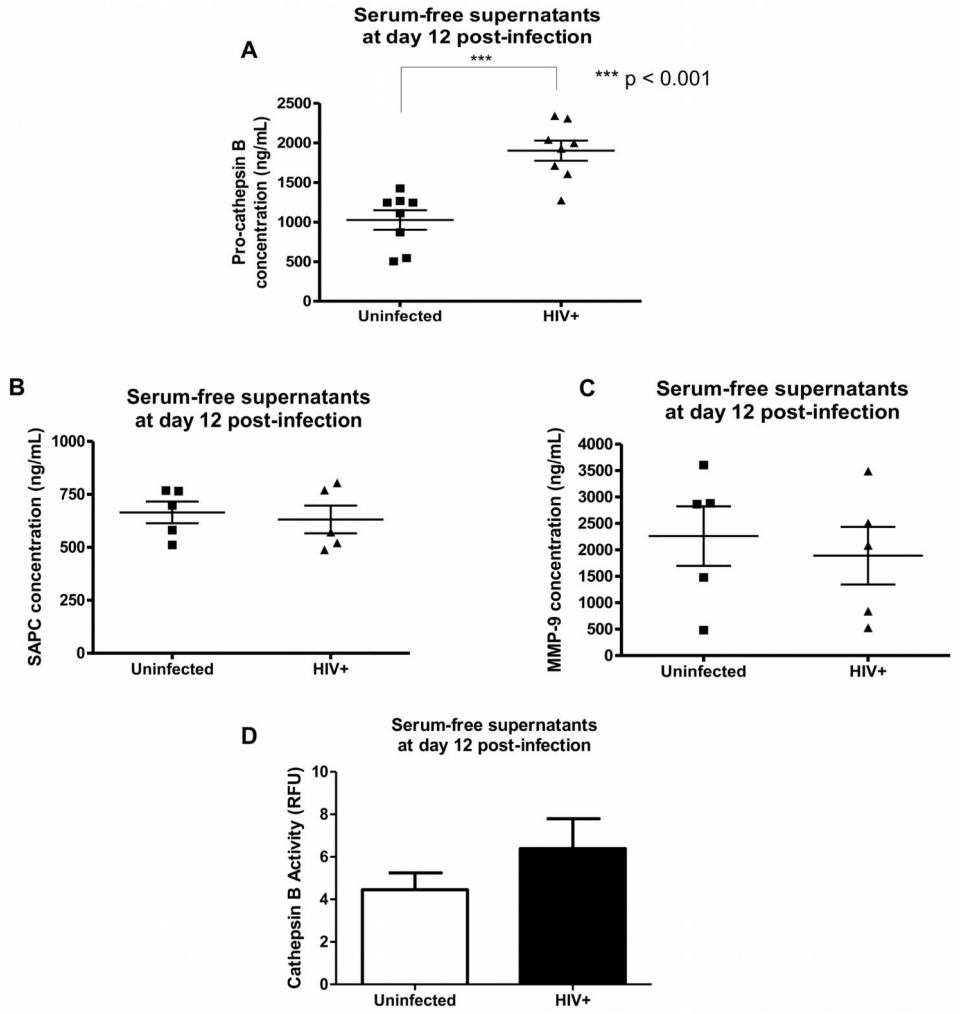

Figure 3. Concentration of cathepsin B and interacting partners in uninfected and HIV-infected MDM supernatants at 12 dpi

Pro-cathepsin B (A), SAPC (B) and MMP-9 (C) concentration in uninfected and HIV-infected MDM supernatants from 12dpi was measured by ELISA. Cathepsin B activity was measured using a fluorometry-based assay (D). Mann Whitney and unpaired t tests were used to analyze the results (***p<0.001). Data represented as mean ± SEM.

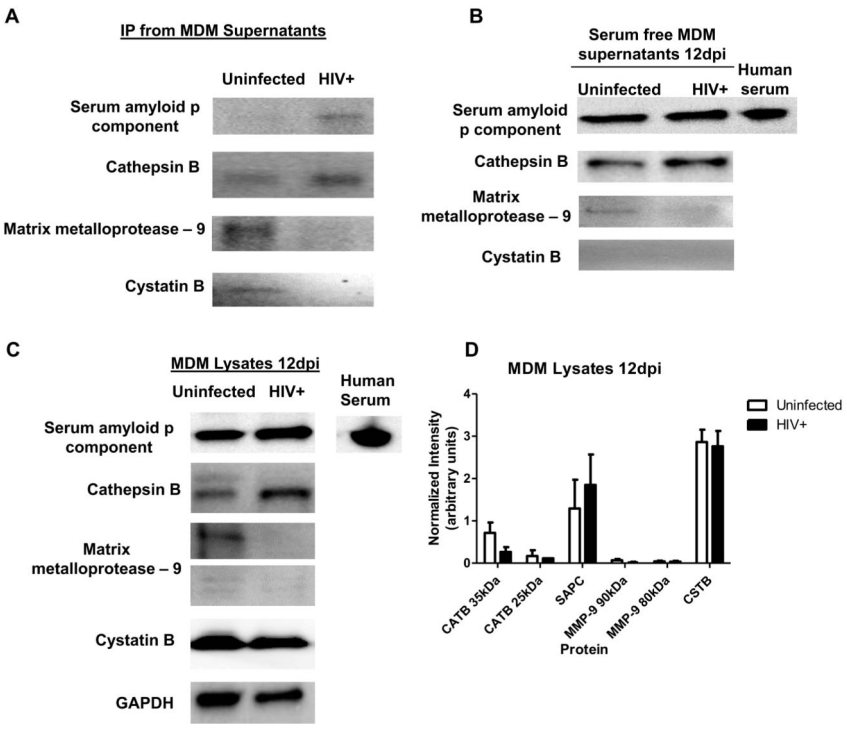

Figure 4. Neurotoxic potential of cathepsin B extracellular interactome measured by TUNEL labeling

Neuronal apoptosis measured by TUNEL labeling for DNA fragmentation upon uninfected or HIV-infected macrophage-conditioned media exposure for 24 hours. MCM was pre-treated with cathepsin B (CATB) antibody (Ab), cathepsin B inhibitor (CA074), serum amyloid p component (SAPC) antibody, matrix metalloprotease 9 (MMP-9) antibody, CATB and SAPC antibodies together or CATB and MMP-9 antibodies together (A). A positive control for apoptosis TUNEL labeling was generated by treating neurons with DNAse I enzyme to induce strand breaks. TUNEL-positive cells are shown in green and all nuclei stained with DAPI are shown in blue. Green nuclei percentage of the total (blue) nuclei were quantified for each condition and statistically analyzed by two-way ANOVA (B). Data represented as mean ± SEM.

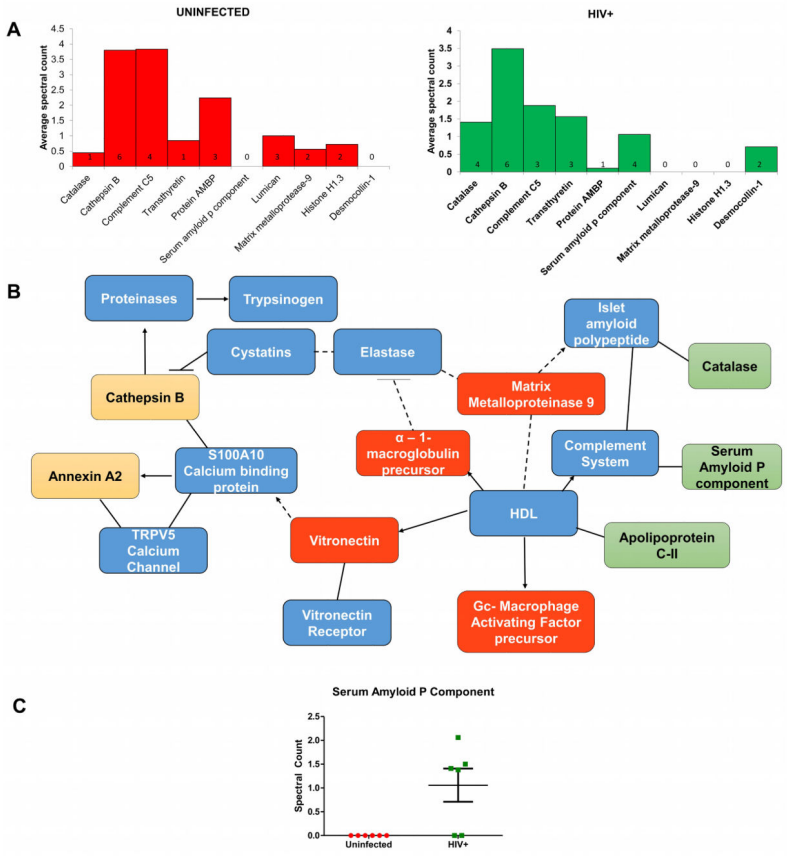

Figure 5. Expression of cathepsin B, cystatins B and C and MMP-9 in deep frontal white matter post-mortem brain tissue from HIV-infected and Alzheimer's patients

Double immunofluorescence labeling of paraffin-embedded deep frontal white matter tissue samples from healthy subjects (n=3), patients diagnosed with HIV-infection with normal cognition (n=3) or with HIV-associated neurocognitive impairment (n=4) and AD patients (n=2). Cathepsin B, cystatin B, cystatin C and MMP-9 proteins were labeled together with Iba-1 as a microglia and monocyte marker. Negative controls were obtained incubating tissues with only primary antibodies or only secondary antibodies Cathepsin B and SAPC were labeled together with Aβ peptides (A). Tissues from healthy, HIVE/HAD and AD patients were labeled for SAPC and Neurofilament (B). All nuclei were labeled with DAPI. Magnification 400X.