Published in final edited form as:

Placenta. 2008 December ; 29(12): . doi:10.1016/j.placenta.2008.09.005.

Proteomic analyses associate cystatin B with restricted HIV-1 replication in placental macrophages

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Abstract

Mononuclear phagocytes (MP; monocytes, tissue macrophages, and dendritic cells) are reservoirs, vehicles of dissemination, and targets for persistent HIV infection. However, not all MP populations equally support viral growth. Such differential replication is typified by the greater ability of placental macrophages (PM), as compared with monocyte-derived macrophages (MDM), to restrict viral replication. Since cytosolic protein patterns can differentiate macrophage subtypes, we used a proteomics approach consisting of surface enhanced laser desorption ionization time of flight (SELDI-TOF), tandem mass spectrometry, and Western blots to identify differences between uninfected and HIV infected PM and MDM protein profiles linked to viral growth. We performed the first proteome analysis of PM in the molecular range of 5 to 20 kDa. Importantly, we found that a SELDI-TOF protein peak with an m/z of 11100, which was significantly lower in uninfected and HIV infected PM than MDM, was identified as cystatin B (CSTB). Studies of siRNA against CSTB treatment in MDM associated its expression with HIV replication. These data demonstrate that low molecular weight placental macrophage cytosolic proteins are differentially expressed in HIV infected PM and MDM and identify a potential role for CSTB in HIV replication. This work also serves to elucidate a mechanism by which the placenta protects the fetus from HIV transmission.

1. Introduction

Mononuclear phagocytes (MP; monocytes, tissue macrophages, and dendritic cells) are reservoirs and vehicles for HIV dissemination in the infected human host [1]. Understanding

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HIV dynamics in resident MP is important since viral sequestration in tissue occurs as a consequence of disease progression.

One body tissue in which restricted infection of MP and virus compartmentalization can occur is the placenta [2-4]. Trophoblasts are susceptible to infection but show restricted viral replication [2], whereas productive viral infection occurs in placental macrophages (PM) [5, 6]. Interestingly, the levels of HIV replication in PM are at least 10-fold lower than what is seen in peripheral blood monocyte-derived macrophages (MDM) [7, 8]. Decreased CCR5 expression has been associated with restricted HIV replication in PM [8, 9], but the intracellular mechanisms that affect it are not known. Thus, the current study was designed to apply a proteomics approach to identify cellular protein(s) associated with decreased HIV replication in PM.

Several host factors previously have been associated with HIV restriction in the placenta. Leukemia inhibitory factor (LIF) is a placenta-secreted protein that limits viral replication in the placenta [10]. The pregnancy-related hormone human chorionic gonadotropin beta-subunit (b-hCG) is produced by trophoblasts and upon addition to placenta explants inhibits HIV proteins and progeny virions [11-13]. hCG inhibited HIV RT and p24 antigen from HIV-infected lymphocytes when they were co-cultured with placental trophoblasts [13]. They found a dose-dependent inhibition of HIV infection in hCG-treated tissue explants [12]. They stated that no studies have examined the hCG concentrations in blood and correlated it with viral load or HIV infection [12-13]. However, it is unknown which host factors account for PM infection and viral growth. This study was aimed at comparing the low molecular proteome of HIV infected PM and MDM and identifying protein candidates associated with viral replication. We used a proteomics approach that consisted of surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS), 1D-gel electrophoresis, liquid chromatography tandem MS (LC MS/MS), and Western blot. We identified 12 proteins that correlate with SELDI-TOF protein profiling by LC MS/MS. Of these we chose to validate the expression of cystatin B (CSTB) by Western blot because it was differentially expressed in uninfected and HIV infected PM and MDM. Importantly, previous studies have associated this protein with HIV infected MDM [14]. Lower CSTB was observed in PM than in MDM by Western blot assays. By applying siRNA for CSTB to MDM, we demonstrated that reduced CSTB was associated with decreased HIV replication. Our studies suggest that reduced CSTB contributes to the innate resistance to HIV PM infection and thereby protects against maternal-fetal viral transmission.

2. 2. Materials and Methods

2.1 Isolation and cultivation of PM and MDM

Full-term placentas from HIV-1, HIV-2, and hepatitis B seronegative women were obtained from University Hospital in Puerto Rico. Placentas were transported to the laboratory and perfused for 45 as previously described [6-7, 9]. PM were seeded at a concentration of 1×10^6 cells/mL in a total volume of 30 mL and supplemented with RPMI 1640 containing 20% fetal bovine serum, 10% human serum (Sigma), 100 U/mL pen/strep (Sigma), and 2 mM glutamine (Sigma). Non-adherent cells were removed 7 days after seeding.

Peripheral blood mononuclear cells were obtained from leukopheresis of HIV-1, HIV-2, and hepatitis B seronegative donors as previously described [15]. Monocytes were seeded at the same concentration of the PM. MDM were obtained after 7 days of culture in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% heat-inactivated pooled human sera, 50 µg/mL gentamicin, 10 µg/mL ciprofloxacin (all from Sigma), and 1000 units/mL macrophage colony-stimulating factor (MCSF; a generous gift from Wyeth, Inc., Cambridge, MA).

2.2 HIV infection of PM and MDM

After 7 days in culture, PM and MDM were inoculated with 25 ng of HIV-1_{BaL} per 2×10^5 cells, as previously described [7]. After 1 hour of incubation at 37°C, virus was removed by washing with media. Culture fluids were collected at 0, 2, 4, 6, 9, and 12 days, centrifuged, and stored at -80°C for determinations of HIV-1 p24 antigen levels by ELISA following the manufacturer's instructions (Beckman Coulter, Fullerton, CA). Seven days after infection, cells were harvested for SELDI-TOF assays (see below). Six placentas and three MDM were maintained for 6 days post-infection and 4 PM and MDM from different donors were cultured for 12 days and compared to uninfected controls. Values for HIV infection are presented as log p24 antigen $\pm$ SD. Cultures were monitored for cytopathic effects.

2.3 Preparation of cell lysates

On day 6 after viral infection, cells were washed and cultured for an additional 12-16 hours in media without sera. Cells were washed with cold PBS and incubated on ice for 15 minutes with lysis buffer (5 mM Tris-HCl buffer, pH 8.0, 0.1% Triton X-100, and protease inhibitor (Sigma). Lysates were cleared by centrifugation for 10 minutes at 1,700 rpm at 4°C, and stored at -80°C for further analyses. Protein concentration was measured using BioRad D_C Protein Assay (BioRad Laboratories, Hercules, CA).

2.4 SELDI-TOF analysis

SELDI-TOF was performed to profile proteins of uninfected and HIV infected PM and MDM cell lysates cultured 6 days post-infection. We used weak cation-exchange (CM-10) and the strong anion-exchange (Q-10) (BioRad, Hercules, CA, formerly Ciphergen Biosystems). A total of 50 μ L of protein sample (0.1 μ g/ μ L) was applied per spot and tested in quadruplicates. Binding buffer, sample deposition, and analysis were performed as previously reported [16] with minor modifications. The ProteinChip® reader (PBSIIc system, BioRad) was externally calibrated for each analysis using mixtures of four standard proteins: bovine insulin (5,733 Da), cytochrome C (12,230 Da), superoxide dismutase (15,591 Da), and beta-lactoglobulin (18,363 Da) (all from Ciphergen, Inc.).

2.5 Protein isolation and identification

For protein identification, 20 μ g of protein was hydrated with NuPAGE® LDS buffer (Invitrogen, Carlsbad, CA), and then separated by one-dimensional electrophoresis on a NuPAGE® Novex 10% Bis-Tris (Invitrogen) gel. Proteins were stained with Coomassie BB (BioRad). Bands corresponding to SELDI-TOF spectral regions were cut from the gel with a sterile razor blade. After de-staining with 50% ACN, 50 mM NH₄HCO₃/50% ACN, and 10 mM NH₄HCO₃/50%, the gel pieces were dried and proteins subjected to in-gel trypsin (Promega, Madison, WI) digestion for 12-16 hours. Resulting peptides were extracted using a mixture of 40% H₂O, 60% ACN, and 0.1% trifluoroacetic acid. Samples were dried and resuspended in 12 L of water and 0.1% formic acid prior to LCMS/MS (Proteome X LC-MS/MS system, Thermo Electron Corporation, Waltham, MA) analysis. Data obtained from LC-MS/MS analysis were searched against the NCBI Fasta protein database narrowed to a subset of human proteins using the Sequest algorithm (BioWorks 3.1SR software from Thermo Electron Corporation). Protein identifications were accepted as true positives if the following criteria were met: Xcorr for doubly charged precursor ion >2.5, DeltaCn 0.3, more than 60% of fragment ions per sequenced peptides, and at least two peptides per identified protein [14, 16].

2.6 Western blot analyses

Twenty micrograms of PM and MDM lysates from 7 different donors were diluted with Laemli sample buffer (BioRad Laboratories, Hercules, CA), and applied to a 4%-20% Tris-

HCl Ready Gel well (BioRad) and transferred to 0.45µm nitrocellulose membranes (BioRad). After blocking with 3% BSA in TBS, the membrane was incubated with goat cystatin B (n=4, 1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) and sheep SOD antibodies (n=3, 1:2000) (Calbiochem, San Diego, CA) followed by incubation with the corresponding secondary antibody conjugated with horseradish peroxidase (HRP). Mouse β-actin antibody (Upstate, Charlottesville, VA) was used as the loading control. The U87 human glioblastoma whole cell lysate was used as the positive control for cystatin B and the HeLa whole cell lysate for SOD (both from Santa Cruz Biotechnology). Signal was detected using the Visualizer™ Chemiluminescent Substrate HRP detection system (Upstate). The density of protein bands was determined using the Versa Doc System with Quantity One Software (BioRad) and normalized against β-actin.

2.7 Small interfering RNA

All the materials used for siRNA studies were obtained from Dharmacon (Dharmacon, Chicago, IL). These included the irrelevant siRNA, used as a negative control (Neg siRNA), the siRNA against CSTB (CSTB siRNA), and Dharmafect 4 as the transfection reagent. The protocol for the transfection was done according to the manufacturer's instructions. The percentage of viable cells in experimental and control cultures was determined prior to *in vitro* infection. Briefly, MDM (1.25×10^5) were cultured in 96-well plates and the viability in Neg siRNA, CSTB siRNA-treated and HIV infected cells were compared to uninfected untreated controls using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). On day 6 after infection, MDM were washed with monocyte media followed by addition of 0.5 µg/mL MTT to each well. Cells were incubated at 37°C for 4 hours and thereafter 100 µL of DMSO was added for 15 minutes at room temperature. Absorbance was measured at 570 nm. Viability for control siRNA, CSTB siRNA, and HIV infected cells were expressed as percentages of the uninfected and untreated cells.

To determine the effect of CSTB on HIV infection, this protein was inhibited from MDM cultures by using small interference RNA (siRNA). Briefly, monocytes were seeded at 1×10^7 cells in 25-mm² plates and differentiated into MDM for 7 days in DMEM containing MCSF. After differentiation, cells were incubated in antibiotic free medium overnight with 5% CO₂ and transfected with 100 nM of Neg siRNA, or CSTB siRNA. Cells were incubated 24 hours in monocyte media after siRNA removal. *In vitro* infection was done with HIV-1_{BaL} at a concentration of 25 ng/mL (MOI of 0.1) after 24-hours of siRNA removal. HIV infection was allowed to proceed for 6 days and supernatant collected to measure HIV p24 antigen levels. Whole cell lysates were prepared as described for Western blotting. To determine inhibition of HIV in silenced CSTB samples, p24 antigen levels from three independent control experiments (Neg siRNA) were determined and expressed as 100% infection. These experiments were performed on MDM cultures from 3 different donors. The mean of each independent p24 antigen value in CSTB siRNA was expressed as a percentage of its respective control siRNA, as reported previously [17].

2.8 Statistical analyses

Analysis of variance (ANOVA) was applied with specimen type and days of infection to compare levels of HIV replication. For SELDI-TOF data analysis, the spectrum obtained was analyzed with an integral statistical software program (Biomarker Wizard) specifically designed for the ProteinChip® (version 3.2). The parameters for peak detection were first pass signal/noise (S/N) ratio = 5, second pass S/N ratio = 2, and mass tolerance = 0.5%. Peaks were labeled automatically by their mass-to-charge ratio (m/z). All peaks were subtracted from baseline, calibrated for mass accuracy, detected and clustered using the Biomarker Wizard® program. Normalized spectra for each of the profiles were compared on the basis of their intensity or normalized peak height. Data from Biomarker Wizard were

exported for statistical analysis using SAS® software (SAS/STAT Software 9.1, SAS Institute Inc., Cary, NC). Generalized estimating equations (GEE) with adjusted multiple comparisons were used to identify peaks for which there was evidence of statistically significant differences in the distribution of intensity scores among the replicates of uninfected and HIV infected PM and MDM. The raw intensity values were found to be asymmetrical and adjusted prior to analysis using the arsinh transformation: $Y = \log_2 (X + \sqrt{X^2 + 1})$, where 'X' is the observed intensity. This transformation has been used previously to stabilize intensity variance and make data more nearly normally distributed, and it has the advantage over a log-transformation of being able to handle negative intensities [18]. An “adjusted p-value” (a “q-value”) was computed to address the issue of multiple comparisons, by which the false discovery rate was controlled at 0.05 (i.e., no more than 5% expected false positives out of differentially expressed ones) [19].

For Western blot analyses, an un-paired two-tailed Student's t-test was used to compare the densitometry values of the blots obtained from Quantity One Software (BioRad) and illustrated as mean plus SEM. ANOVA was used to compare cell viability results. Unpaired Student's t-test for the HIV p24 antigen levels of the small interfering RNA assays.

3. Results

3.1 Differential HIV replication in PM and MDM

HIV infection of PM and MDM was demonstrated by increased p24 antigen per time in culture. PM showed significantly lower levels of p24 antigen than did MDM regardless of donor ($p = 0.001$; Figure 1A). The differences were ~2 logs from 2 days to 12 days post-infection and confirmed previous observations made in our laboratory [7, 8]. Experiments using MDM and PM cell supernatants cultured for 12 days after infection (Figure 1B) showed that PM remains restrictive for HIV. Cytopathology was not observed in uninfected cultures. We used 6-day lysates from uninfected PM and HIV infected PM for proteomics studies to compare with uninfected MDM and HIV infected MDM at the same time-point.

3.2 SELDI-TOF profiling of PM and MDM

SELDI-TOF profiling with the cationic and anionic chips CM-10 and Q-10, respectively, demonstrated spectral differences between 6-day lysates PM and MDM. Protein spectra of HIV-infected PM and MDM were investigated using CM-10 chips and showed increased peak numbers and higher peak intensities than those observed with Q-10 chips (data not shown). Based on these results, comparisons between the two cell groups following viral infection were done with CM-10 chips. Biomarker Wizard (CIPHERGEN, Inc.) analysis identified a total of 99 peaks between the uninfected and HIV infected cell lysates. Following GEE statistical analyses and multiple comparisons adjustment to control for false positives, 27 proteins were found differentially expressed in uninfected or HIV infected PM and MDM (Table 1). Of these, 12 protein peaks (numbers 4, 6, 7, 9, 11-13, 16-19, and 26) showed lower intensities in both PM and PM-HIV than in MDM and MDM-HIV (Table 1). In addition, seven peaks (numbers 1, 2, 21-24, and 27) had higher intensities in PM and PM-HIV than in MDM and MDM-HIV (Table 1). Two protein peaks (8 and 25) were higher in PM than MDM but were not detected in HIV infected PM or HIV infected MDM (Table 1). Five protein peaks (3, 5, 10, 14, and 15) were lower in HIV infected PM than in HIV infected MDM (Table 1) but were not detected in uninfected cell lysates. One protein peak (number 20) was found only in infected cells and was up-regulated in PM-HIV over MDM-HIV. Representative spectra showing several differentially expressed proteins peaks with m/z of 11101, 10582, 14545, and 10776 are shown in Figure 2. These peaks may represent the same peptide or may represent more than one protein with similar molecular masses given that the SELDITOF technology platform has relatively low resolution. The LC-MS/MS

peptide sequencing is more sensitive and specific, and provides quite accurate information about protein I.D. and relative abundance. Therefore, after discovering significant differences in the protein profiles between uninfected and HIV infected PM and MDM, we proceeded to identify the proteins by LC-MS/MS within the molecular weight range detected from the SELDI-TOF profiling (5-20 kDa).

3.3 Protein identification by LC MS/MS

One dimensional (1D) SDS PAGE was used to fractionate proteins in the 5 to 20 kDa range. This experiment was designed to identify proteins which molecules masses corresponded to SELDI-TOF peaks showing significant differences in intensities. After 1D protein separation, regions of the gel corresponding to the molecular weights identified in the SELDI-TOF spectra were excised and digested with trypsin. The resulting peptides were sequenced by LC-MS/MS [14, 16] and theoretical molecular weights of identified proteins derived from the NCBI database were compared to SELDITOF peaks (Table 2). This method identified 12 proteins within the mass range of SELDI-TOF profiling with the theoretical molecular weight provided by the NCBI database corresponding to peaks 11, 14, 16-19, and 22-27 in SELDI-TOF analysis (Table 1). These proteins were identified as Cytoskeletal 14-like protein, SH3 glutamic acid rich like 3, protein S-100A8 (also known as calgranulin A), 10kDa heat shock, cystatin B, cytochrome C, SH3 glutamic acid rich like 1, myotrophin, protein S-100A9 (also known as calgranulin B), galectin-1, profilin, and superoxide dismutase CuZn (SOD). Of these, CSTB (MW: 11,139 Da) was selected for further analysis because its differential expression in uninfected and HIV infected PM and MDM correlated well to SELDI-TOF profiling, 1-D gel electrophoresis, mass spectrometry measurements, and because previous studies have identified this protein as increased in HIV infected MDM supernatants when compared to uninfected MDM [14] (Figure 2 and Table 2). We also chose SOD for validation because a peak corresponding to a similar m/z (peak 27, Table 1) was significantly increased in uninfected and HIV infected PM over MDM and because in the same study we identified this protein was up-regulated in supernatants of HIV infected MDM as compared with uninfected controls [14].

3.4 Abundance of CSTB by Western blotting

We performed Western blotting to validate the expression of CSTB and SOD. We found that the relative abundance of CSTB was higher in MDM than PM at 6 and 12 days post-infection (Figure 3A and 3C, respectively). This finding was further confirmed by quantitative densitometry as statistically significant ($p=0.0006$ and $p=0.008$, Figure 3B and 3D, respectively). CSTB was more abundant in HIV infected MDM at 6 and 12 days post-infection than in infected PM (Figure 3A and 3C, respectively), further confirmed by densitometry analysis ($p=0.0002$ and $p<0.0001$, Figure 3B and 3D, respectively). In addition, HIV infected MDM have up-regulated expression of CSTB, as compared with uninfected MDM, at 12 days post-infection ($p=0.009$, Figure 3C and 3D, respectively). However, SOD Western blotting showed no difference between uninfected and HIV infected MDM and PM (Figure 3E). Densitometry further confirmed equal amounts of SOD in the groups (Figure 3F). β -actin, which was used as an internal control, showed an equivalent protein content among all samples. The pattern of CSTB expression found in Western blots (Figure 3A) corresponded to the differentially expressed peak with m/z of 11101 by SELDI-TOF profiling presented in Figure 2A-D for the uninfected and infected lysates, respectively, and the amount of detected peptides in the sequencing (Table 2). These results confirmed the differential expression of CSTB in PM, as compared with MDM.

3.5 Effect of CSTB silencing on HIV MDM replication

To determine the effect of CSTB on HIV replication, MDM were transfected with CSTB siRNA followed by infection for 6 days. Levels of HIV p24 antigen and cell viability were

measured in cells treated with Neg siRNA, cells treated with CSTB siRNA, and untreated HIV infected cells. We compared the levels of HIV between Neg siRNA- and CSTB siRNA-treated cells as previously reported [17, 20] and found that HIV replication decreased significantly in CSTB siRNA-treated MDM ($p=0.01$; Figure 4A). Cell viability was not affected in untreated HIV infected, Neg siRNA-treated, and CSTB siRNA-treated cells ($p=0.2466$) as compared to untreated control (Figure 4B). We measured the expression of CSTB in whole cell lysates to determine if the silencing abrogated protein expression in MDM. As shown in representative Western blot, CSTB siRNA-treated samples showed a decreased protein expression as compared with Neg siRNA-treated controls (Figure 4C). These differences were specific to the cells treated with CSTB siRNA as the β -actin shows similar protein content among all the samples (Figure 4C). Densitometry analysis of Western blot assays confirmed the reduction of cystatin B in CSTB siRNA-treated samples as significant (Figure 4D, $p=0.03$). These results demonstrate that down-regulation of CSTB diminishes HIV replication and confers the MDM low permissiveness to the virus, producing a 40% reduction in p24 antigen levels. These results suggest that low CSTB levels diminish the permissiveness of macrophages to HIV infection.

4. Discussion

Host cellular proteins influence viral growth [1]. Our study found differences in the levels of CSTB between PM and MDM that are intrinsic to these cell populations and persist after viral infection. CSTB was identified from a large number of potential candidates: 27 protein peaks differentially expressed between uninfected and HIV infected MDM and PM. We decided to focus on CSTB because this protein peak showed significant differences in intensity between uninfected and HIV infected PM and MDM in the SELDI-TOF profiling and correlated with LC MS/MS identification as demonstrated by the number of peptides found. We did not detect any differences between PM and MDM SOD levels by Western blots and LC MS/MS. These results did not correspond to the up-regulation of the 15790 m/z protein peak by SELDI TOF in both uninfected and HIV infected PM, as compared to MDM, thus suggesting that the difference observed in the SELDI-TOF protein peaks could correspond to a different protein with a similar mass as reported for SOD.

In addition, LC-MS/MS data suggested that SOD was present in MDM and PM-HIV (Table 2), with similar number of identified protein peptides. Since the number of peptides identified in a particular sample does not always correlate with the actual protein expression [14], we decided to validate SOD protein expression by Western blots. In previous studies we found SOD differentially expressed in HIV infected MDM supernatants; however, LC-MS/MS data identified this protein only in the uninfected controls. This finding emphasizes the pivotal importance of validation using techniques such as Western blotting or ELISA to perform a quantitative analysis of the protein content.

We also found three additional proteins differentially expressed in uninfected and HIV infected PM and MDM. Some of these has been reported to be associated with HIV infection in both macrophages and T cells. These three are profilin, protein S-100 A9, and SH3 glutamic acid rich-like protein 1. Profilin was incorporated in virions and its secretion increased in parallel to productive HIV replication as determined by increased HIV p24 levels [21-22]. The inhibitor of profilin, poly-L-proline, also inhibited virions progeny production, thus supporting the role of profilin in HIV replication [22]. Using LC-MS/MS we found a similar number of peptides for profilin in all our samples. The SELDI-TOF profiling suggests that profilin was down-regulated in both PM and PMHIV as compared with MDM and MDM-HIV. Future studies on profilin will determine if this protein has a role in HIV infection of PM. Calgranulin B, belongs to the myeloid-related proteins (MRPs) recently found to induce HIV production in a lymphoid cell line [23]. In addition, usually

forms heterodimers with calgranulin A, other member of the MRPs, which was found incorporated in HIV viral progeny [21]. We detected calgranulin A and B proteins by LC-MS/MS in the HIV infected PM and MDM, respectively, suggesting the up-regulation of these proteins upon HIV infection. SH3 glutamic acid rich-like protein 1 belongs to the thioredoxin-like protein superfamily [24]. The thioredoxin system is composed of several proteins, among them the peroxiredoxins, reported to inhibit HIV replication when T cells are treated with recombinant proteins [25]. The exact role of these proteins should be further analyzed in both MDM and PM because previous reports were focused on T cells or on lymphoid cell lines. It remains to be determined what role these three additional proteins play in HIV replication in the placenta.

To our knowledge, this report is the first to show the differential expression of CSTB in PM and MDM macrophages. Cystatin family members are cysteine proteinase inhibitors. Type 1 cystatins, which include forms A and B, are intracellular molecules. In the placenta, cystatins control the protease activity of cathepsins, which are required for placental development and normal embryogenesis [26-28]. In particular, CSTB is responsible for the control of cathepsins B and L, and imbalances in the cathepsincystatin system play an important role in miscarriage [28]. Cathepsins contribute to placental angiogenesis [28], embryo development, and uterine decidualization [29]. Their role is thought to be the regulation of trophoblasts invasion into the maternal decidua, which is necessary for the establishment of the placenta. Cathepsin L produced by trophoblasts is present occasionally in term placenta but at high levels in the first trimester [27]. This protein was found down-regulated in term placenta [28] and its expression associated with macrophages. Because of the close relationship between trophoblasts and PM, we postulate that the down-regulation of CSTB found in our study could naturally be down-regulated at term because of the low levels of the cathepsins. CSTB down-regulation may be an innate mechanism that affects viral permissiveness by yet unknown mechanisms. However, there are no published reports on the exact identity of the cells producing CSTB in placenta.

Down-regulation of CSTB in HIV highly permissive MDM decreased viral replication in MDM, but how this is achieved remains unknown. This study suggests that low levels of CSTB in PM could contribute to HIV restriction in these macrophages. However, other mechanisms also contribute to the low permissiveness of placental macrophages such as the low CCR5 receptor expression reported previously by our group [8] and the presence of known HIV restrictive factors such as LIF [4,10]. Assessment of LIF in PM could provide important information regarding other known inhibitory factors that could be operating in the complex mechanisms underlying PM restriction of HIV. Further studies will be conducted to determine the exact signaling pathways of CSTB in PM restriction of HIV.

Taken together, our results are very significant as they offer the first analysis of the low molecular weight proteome of the PM and suggest a new role for CSTB in HIV replication. Synthetic inhibitors of CSTB could provide important protection against HIV replication, in addition to combined antiretroviral therapies, by blocking primary HIV infection in macrophages and limiting viral spread. If HIV replication is attenuated or decreased by means of interfering with CSTB expression, we could possibly reduce to some extent the infection of macrophage reservoirs and control more efficiently HIV infection. Additional studies of the PM secretome are ongoing in our laboratory in order to fully understand HIV secretory factors in PM and their contribution to the innate mechanism of resistance of the placenta.

Acknowledgments

We acknowledge Dr. Melendez lab and all NeuroAIDS members, for their help. We thank the NIH AIDS Research and Reference Reagent Program for the HIV-BAL isolate. This work was supported by NIH grants: NINDS 1 U54NS430, NIGMS MBRS-SCORE-SO6GMO822, NCRR-RCMI-CRC P20RR11126, RCMI-G12RR03051, and MBRS-RISE GM61838.

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Figure 1.

HIV replication in MDM and PM. (A) *In vitro* HIV infected MDM from three different donors (open circles) and PM from six different donors (solid circles) showed differences in HIV replication 2 days after viral infection. Thirty million cells were inoculated with HIV-1_{BAL} and kept in culture for 7 days. HIV infection was measured in the cell supernatants by HIV-1 p24 viral antigen ELISA. PM showed significantly lower levels of p24 antigen than did MDM starting at day 2 after infection ($p = 0.001$). (B) *In vitro* infection of four different MDM and PM donors were cultured 12 days post-infection, and assayed for p24 viral antigen as previously described. PM showed lower levels of HIV replication than did MDM 6 days post-infection ($p = 0.0005$), demonstrating that they continue to be restrictive to HIV over time in culture. Results are presented as log HIV p24 antigen values $\pm$ SD.

Figure 2.

SELDI-TOF spectra of 6 days uninfected and HIV-infected MDM and PM lysates using weak cation-exchange chips (CM-10). The differentially expressed peaks in MDM and PM with m/z of 10582, 10800, and 11102 are labeled with arrows (panel A). Spectra comparison of MDM (panel A) and PM (panel B) showed that the 11102 m/z peak had significantly lower intensity in PM ($p = 0.0004$). The 11102 m/z peak was also up-regulated in HIV infected MDM (panel C) as compared with HIV infected PM (panel D) ($p = 8.98E-11$).

Figure 3.

Differences in cystatin B expression between uninfected (MDM and PM) and HIV infected (MDM-HIV and PM-HIV). Cell lysates from 4 and 3 different donors were blotted and probed in three independent blots against CSTB (panels A and C) and SOD (panel E) respectively. MDM (panels A and C) had higher CSTB levels than did PM. MDM-HIV (panels A and C) expressed more CSTB than did PM-HIV. Normalized densitometry of 6-day and 12-day lysates (panels B and D, respectively) shows the CSTB protein band is more abundant in uninfected MDM than in PM ($p=0.0008$ and $p=0.008$) and is more abundant in 6-day and 12-day lysates from MDM-HIV than in those from PM-HIV ($p = 0.0003$ and $p < 0.0001$, respectively). In addition, HIV infected MDM have higher CSTB levels than uninfected MDM ($p=0.009$, panels C and D). Blots of SOD (panel E) showed no significant difference between tested samples, and were validated by densitometry of normalized blots (panel F). β -actin (loading control) shows a similar protein content among all the samples. The U87 human glioblastoma whole cell lysate was used as positive control for cystatin B and the HeLa whole cell lysate was used for SOD (both from Santa Cruz Biotechnology). All normalized blots were analyzed by un-paired t-test and are presented as mean $\pm$ SEM.

Figure 4.

Effect of silencing of CSTB by siRNA on HIV replication in MDM. Levels of p24 viral antigen decreased significantly in HIV infected MDM CSTB siRNA as compared with HIV infected MDM control siRNA (panel A, $p=0.01$). The silencing procedure did not decrease cell viability in HIV infected MDM and in parallel cultured cells treated with Neg and CSTB siRNA (panel B, $p=0.2466$). Panel C shows representative Western blots of HIV infected MDM Neg siRNA- and CSTB siRNA-treated samples. Densitometry analysis further demonstrated that HIV infected MDM CSTB siRNA-treated samples have a lower CSTB protein expression than HIV infected Neg siRNA-treated controls (panel D, $p=0.03$). β -actin loading controls were similar in all the samples tested (panels E and F). Samples from panel B were analyzed by ANOVA. The blots in panels A, C, and F were analyzed by un-paired t-test, and panel D is presented as mean $\pm$ SEM.

Table 1

SELDI-TOF protein peaks in uninfected and HIV infected PM

Peak Number	Peak Median m/z ^a	Peak Intensity in PM ^b	Peak Intensity in PM-HIV ^c
1	5037	↑	↑
2	5232	↑	↑
3	5644	-	↓
4	5680	↓	↓
5	5815	-	↓
6	5913	↓	↓
7	7699	↓	↓
8	7890	↑	-
9	9193	↓	↓
10	9550	-	↓
11	9905	↓	↓
12	10098	↓	↓
13	10216	↓	↓
14	10330	-	↓
15	10582	-	↓
16	10776	↓	↓
17	10959	↓	↓
18	11101	↓	↓
19	11289	↓	↓
20	11878	-	↑
21	12214	↑	↑
22	12344	↑	↑
23	12430	↑	↑
24	13623	↑	↑
25	14545	↑	-
26	15013	↓	↓
27	15790	↑	↑

^A Mass to charge ratio

^B SELDI-TOF statistical analyses of protein profiles in uninfected PM compared to MDM. All peaks showing ↓ relationship have decreased intensity and those ↑ have increased intensity and all have an adjusted p (a “q value”) ≤ 0.05. Samples labeled - have a q value > 0.05 and were not considered significant.

^C SELDI-TOF statistical analyses of protein profiles in HIV infected PM compared to HIV infected MDM

Table 2

Comparison of SELDI protein peaks and LC MS/MS sequencing data

SELDI -TOF m/z ^a	Molecular weight ^b	Total Peptides Detected in Sequencing ^c				Protein name ^d
		MDM	PM	MDM- HIV	PM-HIV	
9905	9987	NF	2	NF	NF	Cytoskeletal 14-like protein
10330	10438	2	NF	NF	NF	SH3 glutamic acid rich like protein 3
10776	10835	NF	NF	2	NF	Protein S-100 A8 (Calgranulin A)
10959	10932	2	2	2	2	10 kDa heat shock protein
11101	11139	4	2	4	3	Cystatin B
11289	11749	2	2	2	2	Cytochrome C
12344	12774	4	NF	4	NF	SH3 glutamic acid rich like protein 1
12430	12895	NF	2	2	NF	Myotrophin
13623	13242	NF	NF	2	2	Protein S-100 A9 (Calgranulin B)
14545	14716	NF	NF	2	NF	Galectin-1
15013	15054	5	6	5	5	Profilin
15790	15936	2	NF	NF	2	Superoxide dismutase CuZn

^A SELDI-TOF m/z significantly detected peaks between groups^B Theoretical molecular weight according to ExPASy Protein Database^C NF: not found in sequencing^D Total proteins peptides detected in protein identification by LC MS/MS in two independent experiments. All peaks meet the criteria of Unified Score of 3000 with Sp>500, XCorr>2.5 for doubly charged and 3.5 for triply charged precursor ions, Delta Cn>0.3, more than 60% of fragment ions per sequenced peptides and at least two peptides per identified protein