Journal of NeuroVirology, 15: 36–50, 2009

ISSN 1355-0284 print / 1538-2443 online
DOI: 10.1080/13550280802385505

informa
healthcare

Proteomic analyses of monocyte-derived macrophages infected with human immunodeficiency virus type 1 primary isolates from Hispanic women with and without cognitive impairment

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The signature for human immunodeficiency virus type 1 (HIV-1) neurovirulence remains a subject of intense debate. Macrophage viral tropism is one prerequisite but others, including virus-induced alterations in innate and adaptive immunity, remain under investigation. HIV-1–infected mononuclear phagocytes (MPs; perivascular macrophages and microglia) secrete toxins that affect neurons. The authors hypothesize that neurovirulent HIV-1 variants affect the MP proteome by inducing a signature of neurotoxic proteins and thus affect cognitive function. To test this hypothesis, HIV-1 isolates obtained from peripheral blood of women with normal cognition (NC) were compared to isolates obtained from women with cognitive impairment (CI) and to the laboratory adapted SF162, a spinal fluid R5 isolate from a patient with HIV-1–associated dementia. HIV-1 isolates were used to infect monocyte-derived macrophages (MDMs) and infection monitored by secreted HIV-1 p24 by enzyme-linked immunosorbent assay (ELISA). Cell lysates of uninfected and HIV-1–infected MDMs at 14 days post infection were fractionated by cationic exchange chromatography and analyzed by surface enhanced laser desorption ionization time of flight (SELDI-TOF) using generalized estimating equations statistics. Proteins were separated by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (1D SDS-PAGE) and identified by tandem mass spectrometry. Levels of viral replication were similar amongst the HIV-1 isolates, although higher levels were obtained from one viral strain obtained from a patient with CI. Significant differences were found in protein profiles between virus-infected MDMs with NC, CI, and SF162 isolates (adjusted *P* value after multiple testing

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The authors would like to thank their patients for supporting this research. Tania Ginebra and Tania de la Torre supported patient outreach, and Dr. Rosa Hechavarría performed neuropsychological testing. The authors thank Drs. Carmen Zorrilla and Hermes García (their clinic directors) for referring patients. The RCMI–Clinical Research Center is thanked for providing staff and supplies for laboratory sample collections. Thanks to Claribel Luciano for support on the gel processing. The authors thank and acknowledge Richard Skolasky for statistical support. They thank Dr. Joseph Bonanno for providing them with the AIF-L antibody. They acknowledge Drs. Howard Gendelman for his critical review of the manuscript, and Edmundo Kraiselburd for his continuous support of this work as part of the Puerto Rico Specialized Neuroscience Program in NeuroAIDS, and Idalí Martínez for her helpful suggestions. This work was supported by U54NS430, MBRS-SCORE-SO6GMO822, RCMI-CRC P20RR11126, RCMI-12-RR03051, and MBRS-RISE GM061838. The NIH AIDS Research and Reference Reagent Program is acknowledged for providing the HIV-1 SF162 viral isolate.

Received 14 August 2007; revised 15 May 2008; Re-revised date of 25 June 2008; accepted 17 July 2008

corrections, or q value <.10). The authors identified 6 unique proteins in NC, 7 in SF162, and 20 in CI. Three proteins were common to SF162 and CI strains. The MDM proteins linked to infection with CI strains were related to apoptosis, chemotaxis, inflammation, and redox metabolism. These findings support the hypothesis that the macrophage proteome differ when infected with viral isolates of women with and without CI. *Journal of NeuroVirology* (2009) **15,** 36–50.

Keywords: HIV-1; HIV-1–associated cognitive impairment; macrophages; proteomics

Introduction

Mononuclear phagocytes (MPs; peripheral blood derived macrophages, perivascular macrophages, and microglia) are cellular reservoirs for human immunodefiency virus type 1 (HIV-1) in the central nervous system (CNS). After HIV-1 enters the CNS, either as a cell free virus or in infected macrophages, it infects perivascular macrophages, eventually causing the cognitive and motor abnormalities characteristics of HIV-1–associated neurocognitive disorders (HAND) and in its most severe form, HIV-1–associated dementia (HAD). HAD is associated with immune activation and trafficking of peripheral HIV-1–infected monocytes across a disrupted blood-brain barrier (BBB). Upon differentiation, infected macrophages release a variety of postinflammatory factors into the brain parenchyma causing neuronal damage (Holguin *et al*, 2007). This process results in psychomotor slowing, memory impairment, and brain atrophy (Anderson *et al*, 2002; McArthur *et al*, 1999). Although the advent of antiviral therapy has diminished the severity of HAD, the disease is still common and presents with variable progression patterns (McArthur, 2004). This poses a challenge for diagnosis. In view of these changes the National Institutes of Health (NIH) and two of its institutes, National Institute of Mental Health (NIMH) and Neurological Disorders and Stroke (NINDS) charged a working group to critically review the definition of HAD (AAN AIDS Task Force 1991). This working group suggested a research nosology for HAND to include three conditions: asymptomatic neurocognitive impairment (ANI), mild neurocognitive disorder (MND), and HAD (Antinori *et al,* 2007).

During HAD, signaling pathways are activated with the release of inflammatory products, chemokines, and neurotoxins (cellular and viral) that attract other cells to the site of infection, resulting in an increase of intraneuronal calcium and subsequently producing neuronal death (Gendelman *et al*, 1997; Li *et al*, 2005; Persidsky *et al*, 1997). In fact, apoptosis of neurons and astrocytes has been detected in autopsy brain tissues from acquired immunodeficiency syndrome (AIDS) patients with HAD (Chen *et al*, 2005; Self *et al*, 2004). The communication between the nervous and the peripheral immune systems is an important pathway for activation of macrophages during HAD (Ballabh *et al*, 2004). The response of the CNS to systemic immune challenge results in brain inflammation caused by infected monocytes trafficking into the brain from the periphery, resulting in the disruption of the BBB (Gartner, 2000; Gonzalez-Scarano and Martin-Garcia, 2005; Reynolds *et al,* 2007).

The HIV-1 replication in monocytes is restricted, but viral replication increases after cell differentiation (Zybarth *et al*, 1999). After HIV-1 infects macrophages, several signals are activated causing morphological and functional changes to the cell (Chertova *et al*, 2006; Ciborowski *et al*, 2007; Kadiu *et al*, 2007; Lee *et al*, 2003). These changes induced by HIV-1 can drive the cell to a permissive state for viral replication (in favor of the virus) or can enhance phagocytosis and intracellular microbial killing. Despite all the confirmed information about the interactions between HIV-1 and macrophages, the exact pathways of macrophage activation after HIV-1 infection remain incompletely understood. We, as well as others, have demonstrated differences in protein profiles of macrophages from HIV-1–infected individuals with cognitive impairment (CI) by using surface enhanced laser desorption ionization time of flight (SELDI-TOF) (Luo *et al*, 2003; Sun *et al*, 2004). We have also found differences in the macrophage proteome (58 proteins up- or down-regulated) after infection with HIV-1 ADA (Carlson *et al*, 2004). This study identified -actin, annexin 5, and L-plastin among others in HIV-1 ADA–infected monocyte-derived macrophages (MDMs). Most recently, we have found that the macrophage secretome is affected by HIV infection (Ciborowski *et al,* 2007) where cystatins B and C, L-plastin, superoxide dismutase, and α-enolase were identified preferentially from infected cells. Taken together, these studies clearly demonstrate the effects of HIV-1 on macrophage activation, structure, and function (Carlson *et al*, 2004; Ciborowski *et al*, 2007).

Our goal in the current study was to determine the effects of primary HIV-1 isolates (Nieves *et al*, 2007) on the macrophage proteome. The viral isolates were derived from 21 women with or without CI. MDMs from normal donors were infected with the HIV-1 isolates and the cultures were followed for

2 weeks. The macrophage proteome was analyzed by SELDI-TOF protein chip assays to determine the differences in protein profiles among the groups. Following fractionation by cationic exchange chromatography, low abundant proteins were separated by one-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (1D SDS-PAGE) and sequenced by liquid chromatography tandem mass spectometer (LC-MS)/MS. We found proteins unique to macrophages infected with CI viruses. This study demonstrates that HIV-1 variants can affect the macrophage proteome differently and that CI isolates can induce a neurotoxic phenotype in MDMs with decreased antioxidant and more proapoptotic proteins.

Results

Viral replication of primary HIV-1 isolates in MDMs
We compared p24 antigen concentration as a measure of HIV-1 replication in MDMs infected with the HIV-1 isolate from NC (isolate 3), two isolates from patients with CI (isolates 11 and 20), and the laboratory-adapted spinal fluid isolate SF162 derived from a patient with HAD at different time points (days 0, 4, 7, 11, and 14 p.i.). All isolates showed increased infectivity over time in culture (Figure 1A). Significant differences in HIV-1 p24 antigen levels between isolates were found at day 14 p.i. Isolate 20 showed significantly higher HIV-1 p24 antigen levels (mean 223.07 ng/ml) than did the other viruses (SF162 mean 4.61 ng/ml; isolate 11 mean 31.65 ng/ml; Isolate 3 mean 50.37 ng/ml) at day 14 p.i. ($P < .001$) (Figure 1B).

MDM protein profiling
To determine the effect of HIV-1 infection with primary viral isolates and SF162 in the macrophage proteome, we devised the following experimental approach (Figure 2). Briefly, MDM lysates from day 15 p.i. were fractionated by cationic exchange chromatography. Differential protein fingerprints between uninfected and HIV-1–infected macrophages reported previously (Carlson *et al*, 2004) prompted us to further examine the effects of HIV-1 variants from NC and CI on macrophage protein expression. SELDI-TOF protein profiles from pH 9 fraction were chosen for further studies on protein identification based on higher protein concentration and increased number of differences among the groups (data not shown). Spectra from uninfected and infected MDMs with NC (isolate 3), CI (isolates 11 and 20), and HIV-1 SF162 were compared to determine differences in protein profiles (Table 1). When the spectra from MDMs infected with HIV-1 NC were compared to those infected with HIV-1 CI, we found a significant ($q \leq .10$) increase in the mean intensity of the 6617-Da peak in MDMs infected

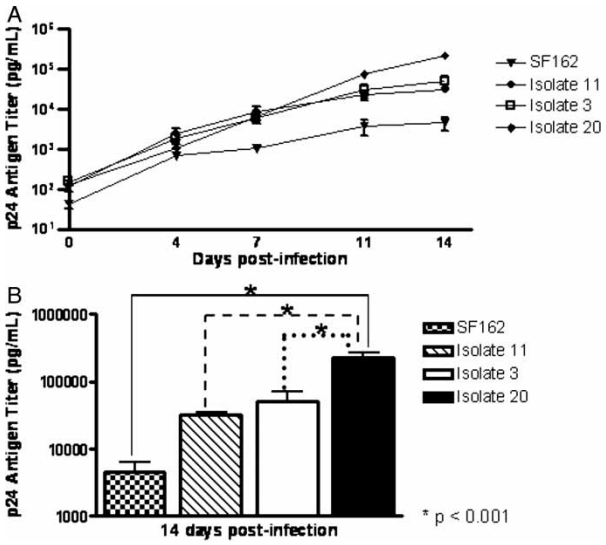

Figure 1 Replication of primary HIV-1 isolates in MDMs. Monocytes were cultured for 7 days for macrophage differentiation and *in vitro* infections. HIV-1 SF162 and primary isolates from Hispanic women with normal cognition (isolate 3) and cognitive impairment (isolates 11 and 20) were inoculated on day 7 and followed for 2 weeks. Culture supernatants were collected and tested for HIV-1 p24 antigen concentration at days 0, 4, 7, 11, and 14 p.i. to monitor virus replication (**A**). Significant differences ($*P < .001$) in HIV-1 p24 titer at day 14 p.i. were observed between isolate 20 as compared with all the others by analysis of variance (**B**).

with the viruses from CI (Figure 3A and B). Intensities of four peaks with m/z of 5300, 5468, 5482, and 8639 were decreased ($q \leq .10$) in MDMs infected with NC virus when compared with MDMs infected with SF162 (Figure 4A to D). The intensity of these four peaks was similar in MDMs infected with NC and CI isolates.

MDM protein identification
After SELDI-TOF protein profiling, pH 9 protein fractions from three individual experiments were selected based on significant spectra differences between groups and separated by 1D SDS-PAGE gels. Bands corresponding to regions of SELDI-TOF spectra were digested with trypsin and the resulting peptides were sequenced by LC-MS/MS. Sixteen (16) proteins were identified with high confidence as common to all groups. The three proteins with higher number of peptides were SH3 domain–binding glutamic acid rich-like protein (seven peptides), Cu/Zn superoxide dismutase (five peptides), and thioredoxin (five peptides). These three proteins have antioxidant activity.

When we compared HIV-1–infected MDMs with the different isolates, three proteins (A-kinase anchor protein 9, annexin V, and ubiquitin) were common to MDMs infected with SF162, CI, and NC viruses (Table 2). Two of these, A-kinase anchor protein 9 and ubiquitin, are important in HIV-1 trafficking, whereas annexin 5 is important in

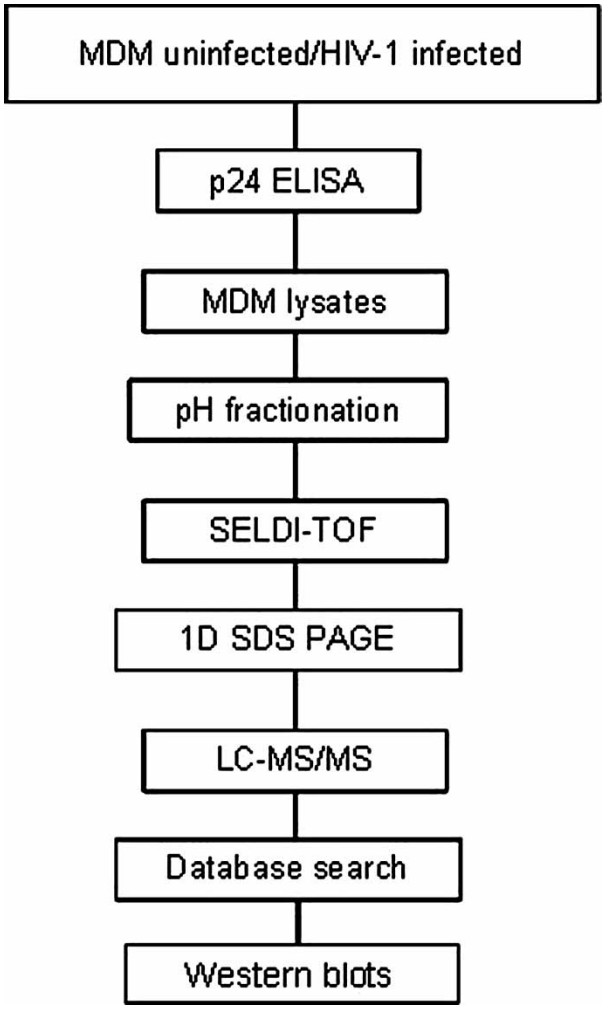

Figure 2 An overview of the experimental design and MDM proteome analyses used in this study.

prevention of HIV infection and apoptosis (Munoz *et al,* 2007). Three proteins (myotrophin, protein kinase C inhibitor protein 1, and thymosin beta-4) were identified only in MDMs infected with SF162 and CI viruses (Table 2). Myotrophin is a cystoplasmic protein related to muscle differentiation whereas protein kinase C inhibitor protein 1 and thymosin beta-4 have a common function in macrophage migration and inflammation. Seven proteins were identified only in MDMs infected with SF162: alpha-1 protease inhibitor, aspartylglucosaminidase, DNA-directed RNA polymerase III subunit, genome polyprotein, heparan sulfate proteoglycan 2, mannose 6 phosphate receptor binding protein 1, and triosephosphate isomerase (Table 2). Most of these are important enzymes involved in cellular metabolism, cell adhesion, and transport of cellular receptors. Six proteins related to cell structure, nucleic acids, and signaling were identified only in MDMs infected with NC virus: chromosome-associated protein E, coactosin-like protein, GTP-binding protein 3, putative methyltransferase NSUN5, teneurin-3, and uncharacterized protein C7orf24 (Table 2).

Table 1 Summary of differential peak expression between uninfected and HIV-1-infected MDMs

Median m/z	q-value	Median of Intensity (natural scale)			
		Controla	NC	CI	SF162
5300	3.56E-02	1.343*	0.79*	1.31	1.16
5300	6.66E-14	1.343	0.79*	1.31	1.16*
5468	9.60E-03	6.659*	4.244*	5.41	6.41
5468	9.14E-03	6.659	4.244*	5.41	6.41*
5482	5.30E-02	11.585*	11.278	12.43	13.38*
5482	5.30E-02	11.585	11.278*	12.43	13.38*
5642	1.39E-03	7.464*	6.053	5.91*	6.5
6617	1.04E-01	2.585	1.982*	2.81*	2.58
8639	6.82E-02	1.127	0.967*	1.16	1.36*
9956	1.09E-01	14.048*	16.761	16.7*	16.11

[a]Uninfected controls.
*Level of significance determined by adjusted *P* value ($q \leq 0.1$) between selected groups (Storey, 2003).

Twenty proteins were identified in MDMs infected with viral strains related to CI. These included proteins with molecular weights ranging from 5000 to 300,000 Da, many of in a mass range higher than SELDI-TOF m/z values (5000 to 20,000). Identified proteins were categorized into the following four main functions: (1) structural and chemotaxis; (2) protein trafficking; (3) apoptosis; and (4) redox. The first group, structural and chemotaxis, included: L-plastin, myosin RLC, adenine phosphoribosyltransferase, adenosylhomocysteinase, and ADP-ribosylation factor GTPase-activating protein 3. The second group, protein trafficking, included -actin, cathepsin A, conserved oligomeric Golgi complex component 4, Ras-related proteins Rab-7, Rab-18, and Rap-1A, ribosome biogenesis protein BMS1 homolog, RING finger protein 165, and signal recognition particle 54. The third group, apoptosis, included apoptosis-inducing factor 3, DNA fragmentation factor, and pyrin. The fourth group, redox, included ferritin, flavin reductase, and l-lactate dehydrogenase A chain (Table 2). All of the proteins identified in macrophages infected with primary isolates (NC and CI viruses) and SF162 matched with only one peptide with the exceptions of annexin V and β-actin, which matched with two peptides. Although most of the proteins were identified with only one peptide, they met all five criteria and were accepted as ''true positives'' in accordance with the results published by the Plasma Proteome Project performed by the Human Proteome Organization (Omenn *et al*, 2005), which shows ''true positive'' identifications of 25% of the proteins based on the sequence of one peptide.

Protein expression

Western Blots were performed to validate the expression of eight proteins that were identified in MDMs infected with HIV-1 CI virus and selected

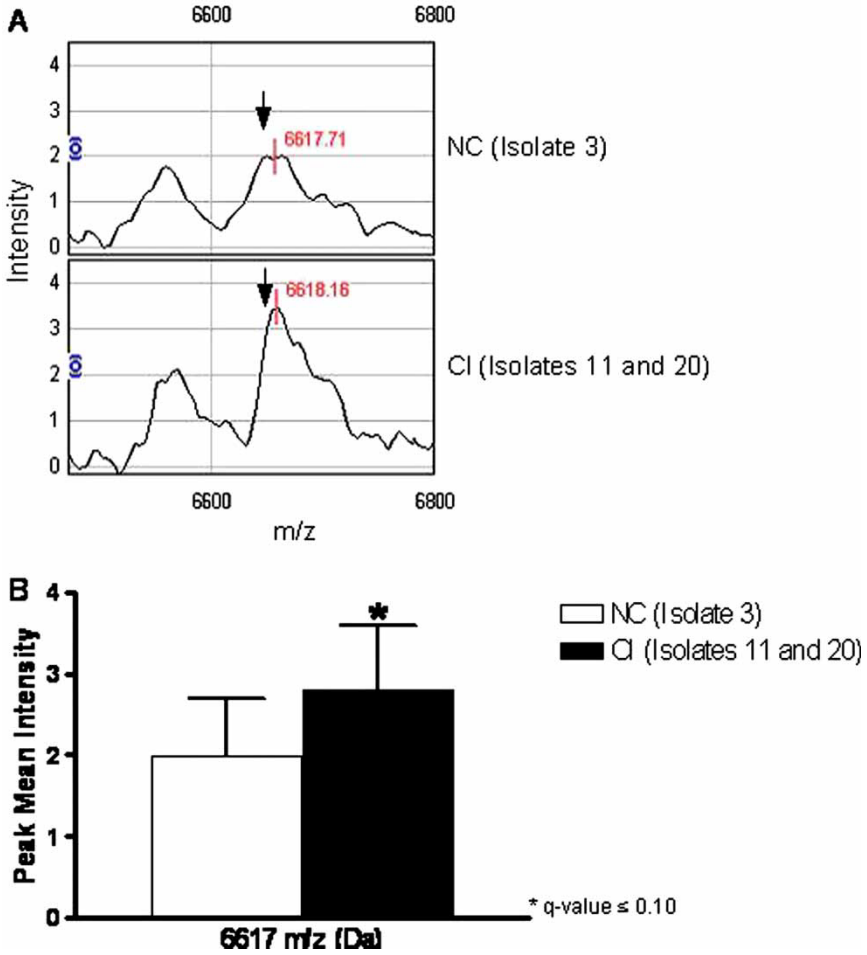

Figure 3 Differential expression of the 6617 m/z peak between MDMs infected with HIV-1 from normal cognition and cognitive impairment. Protein profiles from MDMs after 14 days of infection were compared by SELDI-TOF. Spectra from MDMs infected with a virus isolate from a patient with normal cognition (isolate 3) were compared with spectra from MDMs infected with viruses from patients with cognitive impairment (isolates 11 and 20) for intensity changes (**A**). The bar graph represents the difference between the two groups and the error bars represent one standard deviation for each group, respectively. Following generalized estimating equations with adjusted multiple comparisons, protein peaks with an adjusted *P* value (or a q-value) $<.1$ were considered significantly different among the groups (**B**). Data are representative of three independent experiments.

following the criteria outlined at the Methods. Proteins in gel bands corresponding to molecular weight regions 5 to 20 kDa as indicated by SELDI-TOF experiments were sequenced. As expected, besides high confidence identification, we found proteins represented by one sequenced peptide. Such identifications were considered as low confidence based on the guidelines published by the Plasma Proteome Project (Omenn *et al*, 2005). For all the proteins tested for the different groups, we were only able to determine their relative abundance because Western Blot is only a qualitative method.

There was no difference in L-plastin, cathepsin A (data not shown), or Rap 1A expression between NC, CI, and SF162 viruses, whereas apoptosis-inducing factor like (AIF-L; data not shown) and DNA fragmentation factor (DFF-40; CAD) showed a trend to increase in isolate 11 (CI). Ferritin heavy chain presented a relative increase in expression with both CI viruses, whereas lactate dehydrogenase (LDH) did not show any significant difference. Representative Western blots for some of these proteins are included in Figure 5A. To summarize, although various monoclonal and polyclonal antibodies were tested against these proteins, their relative abundance was low. Due to the limited amount of sample obtained from all three experiments, Western Blot was not the most suitable method to measure the differential expression of these low-abundance proteins. Other methods such as ELISA could be more suitable, because they require a smaller amount of sample; however, no ELISAs were available for most of the proteins under study. We assayed the expression of superoxide dismutase (Cu/Zn SOD; 16 kDa) by Western Blots because it was identified with five peptides in all

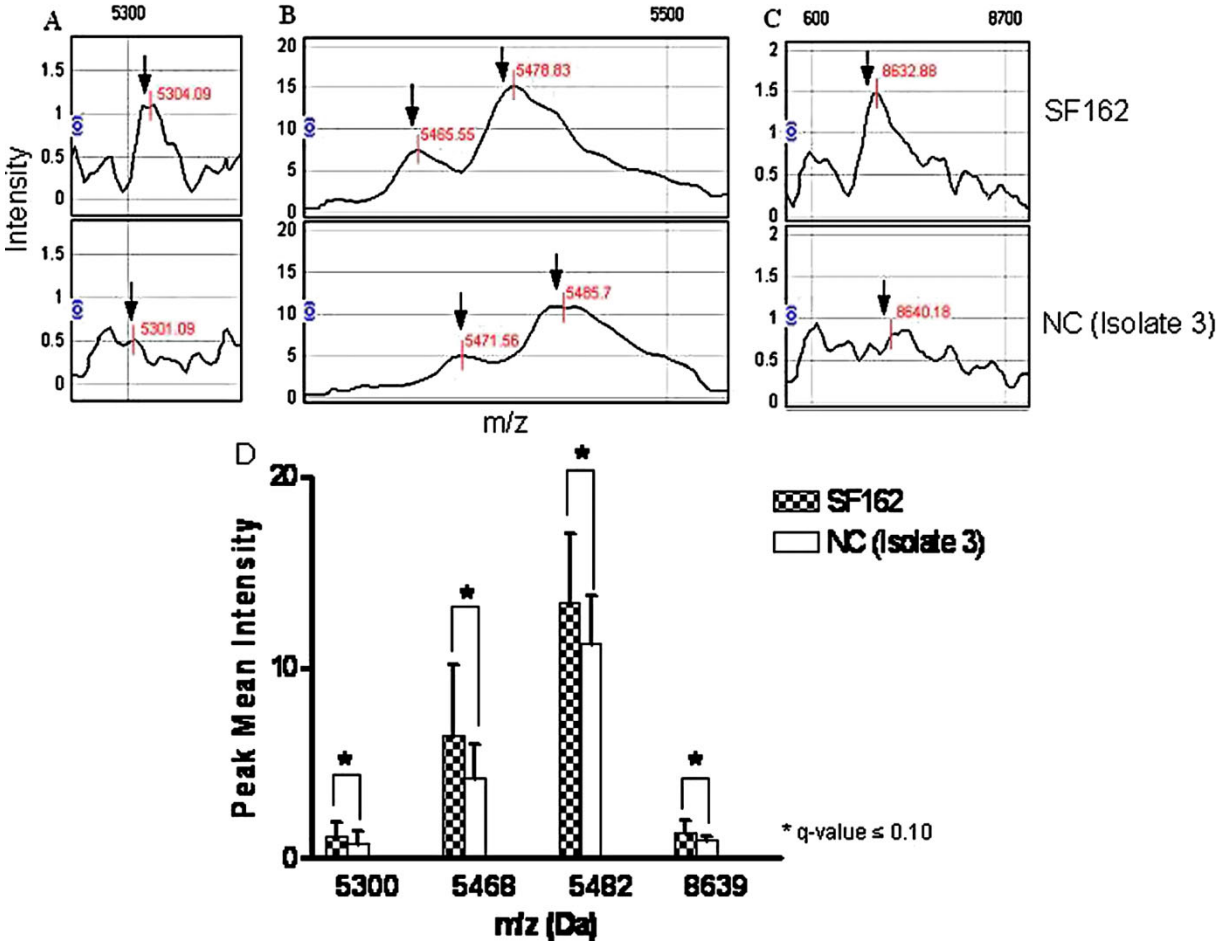

Figure 4 Differential protein profiles between MDMs infected with SF162 and primary isolate from NC. Protein profiles from MDMs after 14 days of infection were compared by SELDI-TOF. Spectra from MDMs infected with SF162 were compared with spectra from MDMs infected with NC (isolate 3) virus for differential protein peak intensities (**A**–**C**). The bar graph represents the comparison between NC and SF162, and the error bars represent one standard deviation for each group, respectively. Following generalized estimating equations with adjusted multiple comparisons, protein peaks with an adjusted *P* value (q-value $<.1$) were considered significantly different among the groups (**D**). Data are representative of three independent experiments.

groups, and found abundant in the CSF of women with CI (Laspiur *et al*, 2007). Surprisingly, we found that Cu/Zn SOD protein expression decreased in MDMs infected with HIV-1 from CI as compared with that from NC (Figure 5B). Following densitometry analysis and normalization against β-tubulin, we confirmed decreased abundance of SOD in MDMs infected with CI ($P<.05$, Kruskal-Wallis chi-square) (Figure 5C).

Discussion

In this study, we identified proteins expressed in macrophages after *in vitro* infection with HIV-1 strains derived from patients with CI. Primary viral isolates induced significant changes in 10 protein peaks between groups: uninfected MDMs and MDMs infected with HIV-1 recovered from NC and CI patients as well as the laboratory adapted SF162 strain. One of these peaks (m/z = 6617) was found to be significantly different between strains isolated from NC and CI subjects. Interestingly, we have found peaks in similar mass range in macrophages (m/z = 6900) and CSF (m/z = 6436, 6695, and 6974) from patients with CI (Wojna *et al*, 2004; Laspiur *et al*, 2007). These findings could suggest similar proteins or disease mechanisms that need to be studied further.

After protein separation by gel electrophoresis and sequencing by tandem mass spectrometry, we identified 16 common proteins among all groups, 20 proteins in MDMs infected with HIV-1 isolates from CI that were not found in cells infected with the NC and SF162 isolates, and only 3 that were shared by SF162 and CI among other comparison groups. These results demonstrate that more proteins were identified at higher molecular weight

Table 2 Identified proteins from macrophages infected with HIV-1 isolates

Proteins	MW[a]	NCBI[b]	SwissProt[c]	Function
Proteins in NC				
Chromosome-associated protein E	135781	30173225	O95347	Central component of the condensin complex, a complex required for conversion of interphase chromatin into mitotic-like condense chromosomes.
Coactosin-like protein	15945	21624607	Q14019	Actin and enzyme binding in a calcium-independent manner.
GTP-binding protein 3	52030	74731069	Q969Y2	Intracellular protein with GTPase activity and involved in tRNA modification.
Putative methyltransferase NSUN5	46692	146289861	Q96P11	May have *S*-adenosyl-L-methionine-dependent methyl-transferase activity.
Teneurin-3	300950	118573058	Q9P273	May function as a cellular signal transducer.
Uncharacterized protein C7orf24	21008	34222503	O75223	Unknown
Proteins in CI				
Adenine phosphoribosyl transferase	19608	4502171	P07741	Catalyzes a salvage reaction resulting in the formation of AMP, which is energetically less costly than de novo synthesis.
Adenosylhomocysteinase	47716	20141702	P23526	Involved in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine.
ADP-ribosylation factor GTPase-activating protein 3 (ARF3)	56928	21263420	Q9NP61	Involved in protein secretion, Golgi vesicle transport and promotes the hydrolysis of the ARF1-bound GTP.
Apoptosis-inducing factor 3 (AIF3 or AIFL)	66791	74732608	Q96NN9	Induces apoptosis through a caspase dependent pathway. Reduces mitochondrial membrane potential.
Beta-actin**	41737	46397333	P60709	Cytoskeletal protein wihich binds to ATP and is involved in cell motility (structural molecule activity).
Cathepsin A	54466	20178316	P10619	Protective lysosomal protein apparently essential for the activity of beta-galactosidase and neuraminidase. Component of the endoplasmic reticulum; Carboxypeptidase, enzyme activator, and intracellular protein transporter activity.
Conserved oligomeric Golgi complex component 4	89095	48429262	Q9H9E3	Required for normal Golgi function.
DNA fragmentation factor (DFF-40 or CAD)	39110	4758150	O76075	Nuclease that induces DNA fragmentation and chromatin condensation during apoptosis. Caspase-activated deoxyribonuclease activity involved in intracellular signaling cascade.
Ferritin heavy chain	21226	56682959	P02794	Plasma membrane protein involved in iron homeostasis. Kinase binding and ferroxidase activity. Involved in the negative regulation of cell proliferation (immune response). Protects DNA from damage.
Flavin reductase (FAD)	22119	4502419	P30043	Role in protecting cells from oxidative damage or in regulating iron metabolism.
L-Lactate dehydrogenase A chain	36689	13786849	P00338	Cytosolic protein involved in the final step of anaerobic glycolysis.
L-plastin	70289	4504965	P13796	Cytosolic actin-binding protein expressed in neutrophils, monocytes, B lymphocytes, and myeloid cells; Calcium ion binding.
Myosin RLC	19794	15809016	P19105	Involved in the regulation of smooth muscle and nonmuscle cell contractile activity.
Pyrin	86444	8928170	O15553	Nuclear or cytoplasmic protein involved in controlling the inflammatory response in myelomonocytic cells at the level of the cytoskeleton organization.
Ras-related protein Rab-18	18023	10880989	Q9NP72	Involved in vesicular trafficking and neurotransmitter release. Plays a role in apical endocytosis/recycling.
Ras-related protein Rab-7	23490	1709999	P51149	Involved in late endocytic transport. Contributes to the maturation of phagosomes.
Ras-related protein Rap-1A	20987	51338607	P62834	Membranal protein with GTPase activity involved in signal transduction.
Ribosome biogenesius protein BMS1 homolog	145807	113420775	Q14692	May act as a molecular switch during maturation of the 40S ribosomal subunit in the nucleolus.
RING finger protein 165	39526	57165361	Q6ZSG1	Unknown.
Signal recognition particle 54 (SRP54)	42779	46577650	P61011	Membranal protein probably involved in the reception and insertion of a subset of proteins at the cytoplasmic membrane.
Proteins in SF162				
Alpha-1 protease inhibitor	46737	50363217	P01009	Angiotensinogen; Serine-type endopeptidase inhibitor activity.
Aspartylglucosaminidase	37194	262567	P20933	Lysosomal protein that cleaves the GlcNAc-Asn bond that joins oligosaccharides to the peptide of asparagine-linked glycoproteins.

Table 2 (*Continued*)

Proteins	MW[a]	NCBI[b]	SwissProt[c]	Function
DNA-directed RNA polymerase III subunit	127785	29428029	Q9NW08	Catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates.
Genome polyprotein	244392	31076989	Q9YLG5	Polyprotein containing capsid proteins VP1, VP2, VP3, and VP4 that forms a closed capsid enclosing the viral positive strand RNA genome. Other subunits (P2A, P3C-cysteine proteases; P2C-associated with viral RNA synthesis of poliovirus and picornavirus).
Heparan sulfate proteoglycan 2 (perlecan)	468830	55665909	Q5VU27	Extracellular matrix component involved in cell adhesion.
Mannose 6 phosphate receptor binding protein 1	47047	20127486	O60664	Cytoplasmic protein required for the transport of mannose 6-phosphate receptors (MPR) from endosomes to the trans-Golgi network.
Triosephosphate isomerase	26669	4507645	P60174	Involved in carbohydrate biosynthesis (gluconeogenesis) and carbohydrate degradation (glycolysis).
Proteins shared by SF162 and CI				
Myotrophin	12895	21956645	P58546	Cytoplasmic protein with a potential role in neuron differentiation. Also, associated to regulation of translation and development of striated muscle.
Protein kinase C inhibitor protein 1	27745	4507953	P63104	Adapter protein implicated in the regulation of general and specialized signaling pathways.
Thymosin beta-4	5053	78103211	P62328	Cytoplasmic protein that plays a role in the organization of the cytoskeleton. Inhibits actin polymerization and the entry of hematopoeitic pluripotent stem cells into the S-phase.
Proteins shared by NC and CI				
Fatty acid-binding protein 5	15164	4557581	Q01469	Cytoplasmic protein involved in fatty acids and protein binding.
HERV-K(III) Pol protein	165184	3702894	P63135	Protein synthesized as Gag-Pro and Gag-Pro-Pol polyprotein precursors. Involved in converting the viral RNA genome into double-stranded viral DNA, early post infection.
Proteins shared by SF162, NC, and CI				
A-kinase anchor protein 9	453667	22538387	Q99996	Binds to type II regulatory subunits of protein kinase A. Scaffolding protein that assembles protein kinases and phosphatases on the centrosome and Golgi apparatus.
Annexin V**	35937	4502107	P08758	Anticoagulant protein that inhibits the thromboplastin-specific complex, which is involved in the blood coagulation cascade.
Ubiquitin	8565	4507761	P62988	Protein modifier which can be attached to target lysines as a monomer or as a lysine-linked polymer (this form leads to their degradation by proteasome). Involved in maintaining chromatin structure, regulation of gene expression, stress response, ribosome biogenesis and DNA repair.

[a]Molecular weight (Da); [b]NCBI accession number; [c]SwissProt accession number.
**These proteins were identified with two peptides, all the other proteins were identified with one peptide.

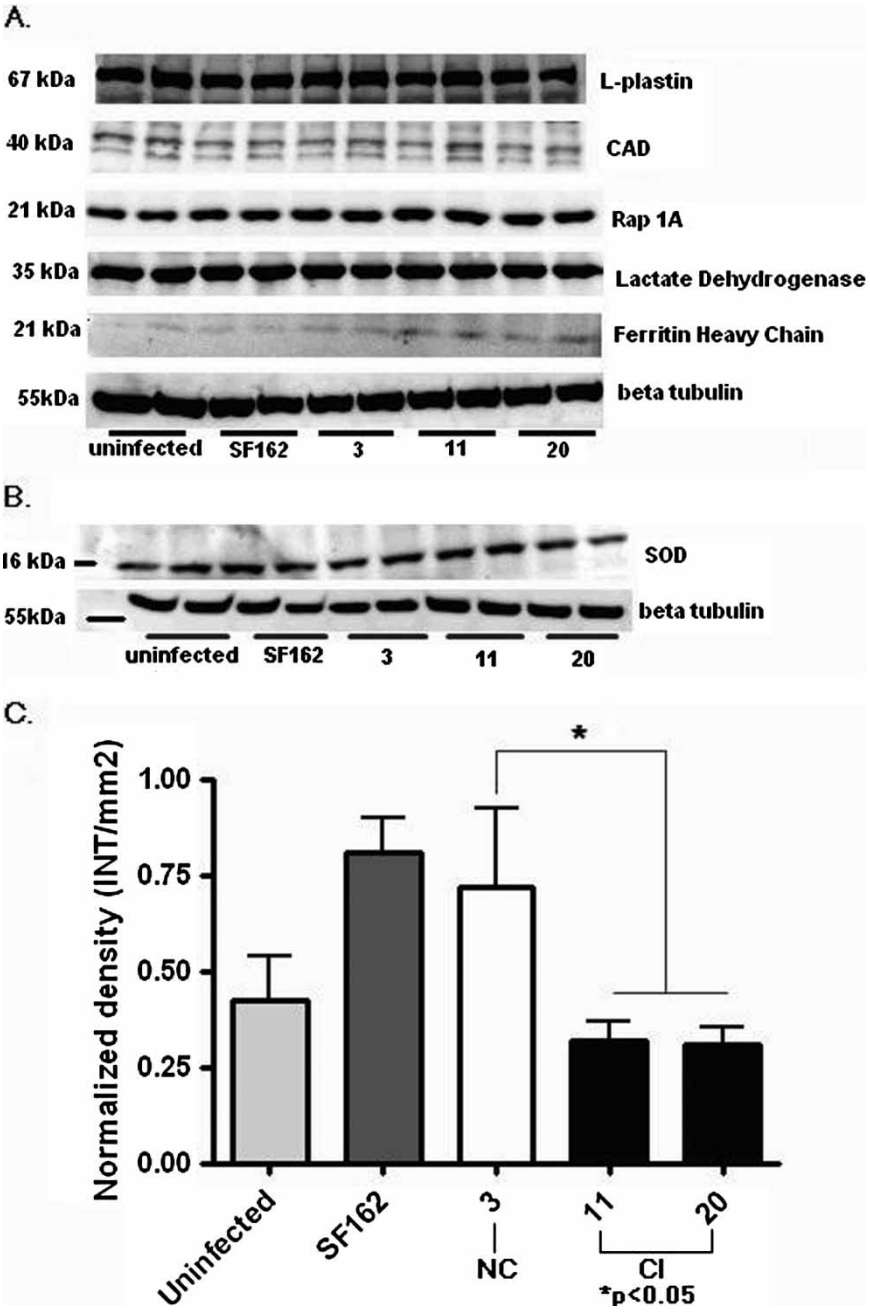

Figure 5 Protein expression of MDMs infected with HIV-1 SF162, NC, and CI isolates by Western blots. Representative Western blots of uninfected controls and MDMs infected with HIV-1 SF162, NC, and CI isolates (**A**). SOD-1 expression in uninfected MDMs and MDMs infected with HIV-1 SF162, CI, and NC (**B**). Densities of protein bands were normalized to beta-tubulin expression on the same blot (**C**). MDMs infected with primary isolates from CI showed significant decrease in SOD expression when compared with NC ($P < .05$). Data analyzed correspond to the densitometry of three different experiments.

range by tandem mass spectrometry than peaks observed in SELDI-TOF spectra. Proteins identified by tandem mass spectrometry did not matched with the SELDI-TOF data. This could be due to the fact that in SELDI-TOF experiments we investigated proteins within an m/z range of 5000 to 20,000 Da under non-reducing conditions. Aggregation of proteins thus having total mass higher than 20,000 m/z cannot be detected. Tandem mass spectrometry is done from gel-tryptic digested proteins. In SDS-PAGE electrophoresis disruption of protein complexes is facilitated by heat denaturation, therefore, proteins may not migrate at the same mass owing to different charges or conformational states.

Among the 16 common proteins found in uninfected MDMs and MDMs infected with isolates NC,

CI, and HIV-1 SF162, the most abundant proteins belonged to the antioxidants group. These included SH3 domain–binding glutamic acid protein (identified with seven peptides), Cu/Zn superoxide dismutase (SOD; identified with five peptides), and thioredoxin (identified with five peptides). These results reflect the importance of antioxidants in cell viability and function. Interestingly, the 20 proteins identified in MDMs infected with CI HIV-1 isolates included functions related to structure, chemotaxis, trafficking, apoptosis, and redox. All of these, except two structural proteins, were identified with a single peptide. In order to determine the relative abundance of these proteins in MDMs infected with CI viruses and given the limited concentration of proteins available from these experiments, we chose to study eight proteins among these four groups. The criteria for selecting these proteins included number of peptides, antibody availability, and functional group as described in the Materials and Methods section. Among the functional groups, for *structural* we tested L-plastin; *for trafficking* we tested cathepsin A and Rap 1A; for *apoptosis* we tested apoptosis inducing factor like (AIF-L) and DNA fragmentation factor (DFF-40; CAD); and for *redox* we tested ferritin heavy chain (FHC) and lactate dehydrogenase (LDH). From our results we were able to detect expression of these enzymes in uninfected MDMs and MDMs infected with isolates NC, CI, and HIV-1 SF162. We found a relatively higher expression of apoptotic inducing factor like (AIF-L), CAD, and the antioxidant protein FHC in MDMs infected with CI viruses after normalization. The AIF-L is a homologous protein of the apoptosis-inducing factor protein (AIF) that induces apoptosis through the caspase 3 activation pathway (Xie *et al*, 2005). Even though HIV-1–infected macrophages live for prolonged time in tissue compartments, acting as viral reservoirs and promoting virus replication, signal transduction of apoptotic genes has been reported (Vazquez *et al*, 2005; Wahl *et al*, 2003). These include the apoptotic protein TRAIL and TRAIL-over-expressing macrophages in the brain of HAD patients (Zhang *et al*, 2001). Apoptosis-inducing factor-like and DNA fragmentation factor could be inducing apoptotic pathways in the macrophage and this may be due to HIV-1 infection. Ferritin heavy chain, which was found increased in MDMs infected with CI, sequesters the excess of free iron and mediates the recently discovered ''antioxidant activities'' of nuclear factor (NF)-κB, and by these means suppresses the generation of reactive oxygen species (Bubici *et al*, 2006; Tsuji, 2005).

We selected to determine expression of another antioxidant protein, Cu/Zn SOD, by Western blots. Although, this protein was common to all the samples, we wanted to test differences between NC and CI groups because it was identified with a high number of peptides. Recently, we found that this protein is decreased in monocytes from Hispanic women with CI (Velásquez *et al*, 2007). Interestingly, after densitometry and statistical analyses, we found a significant decrease of this protein in MDMs infected with CI viruses. These results suggest that HIV-1 variants from CI could induce oxidative stress by reducing SOD-1 expression in infected MPs.

Because we could not isolate viruses from the CSF of HIV-1–infected women due to HAART (Nieves *et al,* 2007), the HIV-1 SF162 was chosen to compare with the HIV-1 isolates from NC and CI. We obtained common and unique protein profiles in MDMs infected with SF162 and the viruses from NC and CI. Protein identification by LC-MS/MS also revealed common and unique proteins in MDMs infected with NC, CI, and SF162, demonstrating that there are common and distinct changes in the MDM proteome induced by viral isolates from CI.

Taking these observations together, our findings suggest that the HIV-1 variants isolated from patients with NC and CI can activate different pathways in macrophage protein trafficking, apoptosis, and oxidative stress. The differences in protein expression found in macrophages infected with primary isolates from NC and CI suggest a specific virus-cell interaction that activates diverse signaling events and cellular pathways that affect cellular function. It is important to emphasize that the presence of these proteins was not related to HIV-1 replication levels. All the cultures were inoculated with at the same MOI and these proteins were not detected in macrophages infected with HIV-1 BaL, a laboratory-adapted macrophage-tropic virus with high replication level (data not shown). There are several limitations of this study, which include the small number of HIV-1 primary macrophage-tropic isolates obtained from NC and CI groups and the limited concentration of protein obtained from virus-infected MDMs for Western blots. Because most of the study patients were on HAART, the viral isolation and growth proved to be a challenging task.

Our study is novel in the field of NeuroAIDS and our findings are very significant as they show for the first time that different HIV-1 viral strains provoke changes in the macrophage's proteome that most likely lead to differences in function during infection. This finding could define a neurotoxic role for the macrophage determined in part by HIV-1 variants that could influence the development of neurotoxicity and cognitive impairment.

Materials and methods

Patient cohort

This study was conducted as part of the NeuroAIDS Specialized Neuroscience Research Program (SNRP) at the University of Puerto Rico, Medical Sciences Campus (UPR, MSC). HIV-1–seropositive women

were recruited from primary HIV-1 clinics at UPR, MSC. The study had the approval of the Institutional Review Board (number 0720102) and was conducted with the informed consent of all participants. The inclusion and exclusion criteria, recruitment, and evaluation have been described previously (Wojna *et al*, 2006). Plasma and cerebrospinal fluid (CSF) viral loads were determined with use of an Ultrasensitive RNA Roche Amplicor at an AIDS Clinical Trial Group (ACTG)-certified laboratory with a detection range of 50 to 75,000 copies of RNA/ml. Cognitive impairment was determined in accordance with the American Academy of Neurology HIV-1 associated dementia criteria (1991, 1996) (AAN criteria) (American Academy of Neurology AIDS Task Force, 1991, 1996), modified (m-AAN criteria) to include an asymptomatic cognitively impaired group (Wojna *et al*, 2006). The asymptomatic cognitively impaired group is defined as patients with abnormal neuropsychological tests (1 SD in two or more tests or 2 SD in one or more tests below the normal control group) but who presented neither functional/emotional nor neurological findings. According to the m-AAN criteria, patients were classified as having normal cognition (NC), asymptomatic cognitive impairment, minor cognitive motor disturbance (MCMD), or HAD. For our study, patients with asymptomatic cognitive impairment, MCMD, or HAD were grouped together under the term cognitively impaired (CI) and were compared with patients having normal cognition (NC) as described (Nieves *et al*, 2007). All patients included in this study were negative for other infectious diseases and toxicology.

Monocyte isolation

Peripheral blood mononuclear cells were isolated from HIV-1–, HIV-2–, and hepatitis-seronegative healthy donors by leukopheresis and purified by countercurrent centrifugal elutriation (Gendelman *et al*, 1988). Monocytes were >95% pure as determined by CD14-FITC (fluorescein isothiocyanate) staining and flow cytometry analysis. Cells were seeded at a concentration of 1×10^6 cells/ml on monocyte medium (Dulbecco's modified Eagle's Medium [DMEM], 1000 U macrophage colony-stimulating factor [M-CSF; a generous gift from Wyeth, Cambridge, MA], 10% human serum, 1% l-glutamine, 50 μg/ml gentamicin, and 10 μg/ml ciprofloxacin) and seeded in 6-well plates for 7 days. Medium was half-exchanged every 2 to 3 days. At day 7, the supernatant was removed and cells were washed twice with phosphate-buffered saline (PBS) at room temperature prior to HIV-1 infection.

Preparation of viral stocks

Primary isolates were obtained from the peripheral blood of 62 HIV-1–seropositive women characterized for cognitive function who were using highly active antiretroviral therapy (HAART) as described (Nieves *et al,* 2007). HIV-1–infected peripheral blood mononuclear cells (PBMCs) from these women were cocultured with mitogen-activated PBMCs from normal donors for 2 weeks and supernatants recovered for detection of virus by HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA). Out of 62 viral cultures, 21 HIV-1 isolates were recovered and characterized for tropism in lymphocytes and macrophages. From these, three viruses were identified that replicated in macrophages and used the CXCR4 and CCR5 coreceptors (isolates 3, 11, and 20). HIV-1 isolates 11 and 20 were derived from women with CI and isolate 3 was derived from one woman with NC. We compared the peripheral blood primary isolates with HIV-1 SF162, a spinal fluid isolate derived from a patient with HAD and acquired from the NIH-AIDS Reagent Program, National Institutes for Allergy and Infectious Diseases. Virus stocks were prepared from these four viruses (3, 11, 20, and SF162) by high-speed centrifugation of supernatants at 19,000 rpm, −10°C, for 2 h, resuspended in monocyte medium containing 20% of human serum, and titrated in MDMs by limited dilution analyses using HIV-1 p24 ELISA as previously described (Arroyo *et al,* 1996; Renta *et al,* 1997).

HIV-1 infection of MDMs

MDMs from eight different donors (2×10^5) were inoculated with 25 ng of HIV-p24 antigen of each viral isolate per well in 6-well plates in triplicate cultures. This was equivalent to a multiplicity of infection (MOI) of 0.1. Cells were incubated with virus for 4 h at 37°C, and thereafter each well was washed twice with monocyte medium at room temperature to remove unbound virus. After three medium washes, cells were resuspended in monocyte medium without M-CSF and cultured at the same concentration. Supernatants were collected on days 0, 4, 7, 11, and 14 post infection (p.i.), and viral infection was monitored by HIV-1 p24 antigen ELISA (Coulter) according to the manufacturer's instructions. On day 14 p.i., cells were washed and resuspended in serum-free medium for 24 h prior to cell lysis for proteomic analyses. The three experiments with productive HIV-1 infection were selected for proteomics studies.

Preparation and fractionation of MDM lysates

After overnight incubation with serum-free medium, on day 15 after infection, cells were washed twice with cold PBS. Adherent MDMs were incubated for 25 min on ice with cold lysis buffer (5 mM Tris-HCl buffer at pH 8.0, 0.1% Triton X-100, and 0.05% of protease inhibitor solution) at a ratio of 100 μl of buffer/1×10^6 cells. Cell lysates were collected, vortexed, and centrifuged at 4°C for 10 min at $500 \times g$ (1500 rpm). The protein concentration of resultant MDM lysates was determined using the BioRad DC protein assay (Richmond, CA) following

the manufacturer's instructions. Cell lysates from triplicate wells from each individual experiment were pooled and stored in aliquots at −80°C for proteomics analyses. Fractionation of MDM lysates was performed to positively detect low abundant proteins by cationic exchange chromatography using Ciphergen CM Ceramic HyperD spin columns (C540-0026; Ciphergen Biosystems). These columns isolated positively charged proteins that bound to the resin from the unbound negatively charged proteins that were eluted. In brief, columns were washed three times with 200 μl of equilibration buffer (0.1 M ammonium acetate pH 4.0) and centrifuged at 80 × *g* for 30 s to remove the buffer. From each sample, 300 μg of protein was added to the spin columns and incubated for 35 min with shaking. Two washes were performed with 200 μl of equilibration buffer followed by vortex and centrifugation (30 s) of the columns. Subsequent washes were performed with 200 μl of buffer with pH 9, 7, 5, 4, and 3, and with an organic buffer consisting of 33.3% isopropanol/16.7% acetonitrile/0.1% trifluoroacetic acid. These washes were followed by incubation for 10 minutes with shaking. After columns were centrifuged for 30 s, fractions were collected and stored at −80°C for subsequent experiments.

SELDI-TOF

Proteomics analysis was performed by SELDI-TOF protein chip assay using the weak cationic exchange chip, CM10 (BioRad, Inc. former Ciphergen Biosystems, CA). A total protein concentration of 0.05 μg/ml from samples derived from each one of the three experiments was applied to the chips in quadruplicates. Briefly, each protein sample was diluted in binding buffer (100 mM ammonium acetate pH 4 with 0.1% Triton-X) to a final volume of 250 μl. Before sample loading, each CM10 spot was equilibrated with binding buffer. An aliquot of 50 μl of the protein sample was loaded on each spot and incubated using a Bioprocessor (Ciphergen) for 30 min at room temperature with shaking. Spots were rinsed twice with binding buffer followed by a wash with high-performance liquid chromatography (HPLC)-grade water to remove unbound proteins. Spots were air-dried and the matrix sinapinic acid (SPA) diluted to 50% in the solvent (30% acetronitrile, 15% isopropanol, 0.5% trifluoroacetic acid [TFA] 1%, 0.05% Triton-X100, and HPLC-grade water) was applied (1 μl) twice. The molecular mass/charge (m/z) ratios of proteins were detected with a ProteinChip reader (PBS IIc series; Ciphergen). The ProteinChip reader was externally calibrated for each analysis by mass using a mixture of five standard proteins: bovine ubiquitin (5,733.6 Da), bovine cytochrome *c* (12,230.9 Da), bovine superoxide dismutase (SOD) (15,591.4 Da), equine cardiac myoglobin (16,951.5 Da), and beta-lactoglobulin (18,363.3 Da). The spectra obtained from each sample were analyzed using the Biomarker Wizard ProteinChip software 3.2 (Ciphergen). The following parameters for peak detection were used: first pass signal/noise (S/N) ratio = 5, second pass S/N ratio = 2, mass tolerance = 0.5%.

Protein separation by 1D SDS-PAGE

Samples from pH 9 fraction were selected based on the most abundant protein differences and 20 μg of each protein sample were dehydrated using Speed Vac, rehydrated with NuPAGE LDS buffer (Invitrogen, Carlsbud, CA) and separated by 1D electrophoresis on a NuPAGE Novex 10% Bis-Tris (Invitrogen) gel. Proteins obtained from HIV-1–infected macrophages derived from three different donors were isolated in three different gels and stained with Coomassie Brilliant Blue (BioRad, Hercules, CA). Protein bands from each one of the three gels ($n = 34$), for a total of 102 bands corresponding to the molecular weights observed in SELDI-TOF (m/z of 5 to 20 kDa), were excised using a sterile razor blade. Gel pieces were destained with 50% acetonitrile (ACN), 50 mM NH_4CO_3/50% ACN, and 10 mM NH_4HCO_3/50%. After gel pieces were dried, proteins were digested with trypsin (Promega, Madison, WI) by overnight incubation at 37°C. Peptides were extracted with 0.1% trifluoroacetic acid/60% ACN, dried, and purified using ZipTip (Millipore) before liquid chromatography–mass spectrometry (LC-MS)/MS protein analysis.

Protein identification

Resulting peptides were resuspended in 0.1% formic acid/HPLC-graded water. The ionized peptides were detected on a LTQ LC-MS/MS system (Thermo Electron, Waltham, MA) as previously described (Ciborowski *et al*, 2007; Laspiur *et al,* 2007). Data obtained from the LC-MS/MS analysis were searched against the human NCBI.fasta protein database to identify the matching protein using BioWorks 3.1SR (ThermoElectron). Protein identifications were accepted as true positives according with previously described parameters (Omenn *et al*, 2005). The criteria used for data mining were as follows: BioWorks unified score ≥3000, Xcorr for singly charged precursor ion ≥2, Xcorr for doubly charged precursor ion ≥2.5, Xcorr for triply charged precursor ion ≥3, DeltaCn ≥0.3, and more than 55% of fragment ions per sequenced peptide. Proteins with one peptide were considered candidates for identification if the peptide met all the parameters. These proteins were accepted as ''true positive'' identifications in accordance with the results published by the Plasma Proteome Project performed by the Human Proteome Organization (Omenn *et al*, 2005). Their results showed that as many as 25% of identifications based on the sequence of one peptide were confirmed as ''true positive.''

Protein expression
Western Blot analysis was performed in order to determine the relative abundance of several proteins identified in MDMs infected with viruses from CI. Due to sample limitation, we selected eight proteins based on the following criteria: (1) number of peptides identified, (2) antibody availability, and (3) functional group in relation to CI. All proteins assayed were identified with only one peptide with the exception of Cu/Zn superoxide dismutase, which was identified with five peptides. The proteins were classified in four groups, according to their major function: structural (L-plastin), apoptotic (apoptosis inducing factor like [AIF-L] and DNA fragmentation factor subunit beta [DFF-40; CAD]), trafficking (cathepsin A and Rap 1A), and redox (l-lactate dehydrogenase A chain, ferritin heavy chain [FHC] and Cu/Zn superoxide dismutase). Protein lysates and controls were resolved by SDS-PAGE (in duplicates), transferred to nitrocellulose membranes, blocked with 3% bovine serum albumin (BSA), and incubated overnight at 4°C with the corresponding primary antibody. Immunoblot detection was performed for selected proteins from the different functional groups with the following antibodies: goat anti-human L-plastin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-human apoptosis inducing factor like (AIF-L) was provided by Dr. Joseph Bonanno, Indiana University (Xie *et al,* 2005), mouse anti-DNA fragmentation factor subunit beta (DFF-40; CAD) (Novus Biologicals, Littleton, CO), mouse anti-cathepsin A (R&D Systems, Minneapolis, MN), mouse anti-Ras–related protein Rap 1A (BD Biosciences, Franklin Lakes, NJ), goat anti-l-lactate dehydrogenase A chain (LDH) (Santa Cruz Biotechnology), rabbit anti-ferritin heavy chain (FHC) (Novus Biologicals), and sheep anti-Cu/Zn superoxide dismutase (SOD-1) (Calbiochem, San Diego, CA). All membranes were incubated for 1 h at room temperature with the corresponding secondary antibody conjugated to horseradish peroxidase enzyme (HRP). Expressed proteins were detected using the Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific–Pierce Biotechnology, Rockford, IL), enhanced chemiluminescent substrate. Images were obtained using a Versa Doc Imaging System (BioRad Laboratories, Hercules, CA) and densitometric analysis of images was performed using the Quantity One software. All data obtained were normalized against a monoclonal anti β-tubulin antibody (Sigma Aldrich, St. Louis, MO). ELISA for SOD-1 (Calbiochem) was determined from a 1:200 dilution of uninfected and HIV-infected cell lysates.

Statistical analysis
For HIV-1 replication studies, we used a linear regression model to determine significant differences in HIV-1 replication in MDMs at day 14 p.i. Comparisons of the p24 antigen levels between the isolates at day 14 p.i. were analyzed using analysis of variance in SAS software version 9.1 (SAS Institute, Cary, NC). The significance level was determined using a type I error rate of 0.05. SELDI-TOF protein profiles were initially analyzed using the Biomarker Wizard software 3.2. All peaks in spectra were baseline subtracted, calibrated on mass accuracy, and normalized with the Biomarker Wizard program. Data were exported to Microsoft Excel for analysis using SAS software. Profiles from uninfected MDMs and MDMs infected with HIV-1 (SF162, CI, and NC primary isolates) were compared on the basis of normalized peak height. Generalized estimating equations (GEEs) with adjusted multiple comparisons were used to identify peaks with significant differences in the distribution of intensity scores among the groups. Specifically, the raw intensity values were found to be asymmetrical and were adjusted prior to analysis by means of the following transformation: $Y = \log_2 (X + SQRT[X**2 + 1])$, where 'X' is the observed intensity. This transformation has been used previously to stabilize the intensity variance and make the data more normally distributed, and it has the advantage over a log-transformation of being able to handle negative intensities (Beyer *et al*, 2006; Huber *et al*, 2002). An ''adjusted *P* value after multiple testing corrections'' (a 'q-value') was computed and we chose a cutoff (10%) that controlled the expected number of false positives (Storey, 2003). Therefore, there were no more than 10% false positives among the selected significant differences between groups. These comparisons were made using GEE statistics. Western blot experiments were then conducted to verify those differentially expressed proteins. An unpaired Student's *t* test was used to analyze densitometry data from Western blots to determine statistically significant differences between the compared groups at a significance level of .05.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on iFirst on 26 December 2008.