Investigating the Human Immunodeficiency Virus Type One-Infected Monocyte-Derived Macrophage Secretome

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Abstract

Mononuclear phagocytes (bone marrow monocyte-derived macrophages, alveolar macrophages, perivascular macrophages, and microglia) are reservoirs and vehicles of dissemination for the human immunodeficiency virus type-1 (HIV-1). How virus alters mononuclear phagocyte immunoregulatory activities to complete its life cycle and influence disease is incompletely understood. In attempts to better understanding the influence of virus on macrophage functions, we used one-dimensional electrophoresis, and liquid chromatography tandem mass spectrometry to analyze the secretome of HIV-1 infected human monocyte-derived macrophages. We identified 111 proteins in culture supernatants of control (uninfected) and virus-infected cells. Differentially expressed cytoskeletal, enzymes, redox, and immunoregulatory protein classes were discovered and validated by Western-blot tests. These included, but were not limited to, cystatin C, cystatin B, chitinase 3-like 1 protein, cofilin-1, L-plastin, superoxide dismutase, leukotriene A4 hydrolase, and α-enolase. This study, through the use of a unique proteomics platform, provides novel insights into virus-host cell interactions that affect the functional role of macrophages in HIV disease.

Keywords

proteomics; secretome; monocyte-derived macrophages; human immunodeficiency virus; HIV-1-associated dementia

Introduction

Mononuclear phagocytes [(MP): monocytes, dendritic cells, tissue macrophages, connective tissue histiocytes, Langerhans cells of skin, Kupffer cells of liver, and microglial cells of brain]

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comprise the principle elements in the clearance and inactivation for microbial pathogens. Paradoxically, these same cells represent the major target and infectious reservoir for HIV-1 (Bhoopat et al., 2006;Collman et al., 2003;Gartner et al., 1986;Gendelman and Morahan, 1992;Gendelman et al., 1989;Meltzer and Gendelman, 1992). In distinct ways, HIV alters MP innate and adaptive immune functions enabling the virus to persist for long time periods (Kramer-Hammerle et al., 2005;Maslin et al., 2005;Olivetta and Federico, 2006;Palmieri et al., 2004). As a result of circumventing cell-based anti-retroviral mechanisms, reservoirs for HIV are established in bone marrow progenitor cells, circulating monocytes, alveolar and perivascular macrophages, and microglia (Collman et al., 2003;Pomerantz, 2004). Defective phagocytic, intracellular killing, secretory activity, and antigen presentation have been reported in monocytes recovered from virus-infected individuals or following *in vitro* infections (Capsoni et al., 1992;Evans and Wansbrough-Jones, 1996;Gordon and Read, 2002;Kedzierska and Crowe, 2002;Zhou et al., 1998).

MP-virus interactions and immune activation ultimately leads to tissue destruction. This is commonly seen in the brain, gut, lung, and spinal cord where MP soluble factor secretion contributes to viral spread and host tissue injury and inflammation (Ansari, 2004;Giulian, Vaca, and Noonan, 1990;Gordon and Read, 2002;Gupta and Gollapudi, 1993;Ichikawa et al., 2003;Kaul et al., 2005;Lim, Condez, and Poulter, 1993;McArthur, Brew, and Nath, 2005;Satomi et al., 2005;Stevenson and Gendelman, 1994). The latter events can occur by inducing cell death or changes in immune and homeostatic functions as a result of cell secretions. The functional and biological outcomes of HIV-1 MP infection also hinge on cell differentiation, as the viral life cycle is dependent on it. Understanding how such events occur is pivotal in understanding how virus can affect disease while at the same time overcome potent innate anti-retroviral immune responses.

After entering tissue, monocytes differentiate into macrophages where they maintain homeostasis, eliminate microbial pathogens, and clear debris. At the same time the cells respond to a variety of environmental cues. Environmental factors, as well as infection itself, lead to the upregulation of secreted pro-inflammatory cytokines, reactive oxygen species, quinolinic acid, glutamate, arachidonic acid and its metabolites. How these and other cellular factors contribute to a wide range of "primary" HIV-associated diseases is a subject of intense research (Colton, 1994;Colton and Gilbert, 1987;Gelbard et al., 1994;Klegeris and McGeer, 1997). To gain a better understanding of the impact by which HIV-infected macrophage affects its environment we profiled its secreted proteins. A foundation for these studies was made through the establishment of an initial list of both the human macrophage proteome and secretome (Dupont et al., 2004;Mor-Vaknin et al., 2003). We posit that HIV-1 infected macrophages could affect the secretion of new proteins or change protein abundance expressed at otherwise very low levels and under normal physiological conditions. Therefore, our study was designed to profile secretome of infected monocyte-derived macrophages (MDM) and compare the profiles seen with uninfected cells. The intent of these works was to discover proteins that are linked to disease and could, in some manner, be applied as biomarkers for the infected human host.

To achieve these goals, we utilized one-dimensional separation (1D SDS-PAGE) and tandem MS to identify MDM proteins secreted as a consequence of HIV-1 infection. Differential expression of selected proteins was further validated using Western-blot analysis. Presented data provide novel insights into the delicate homeostatic changes within the macrophage evolving from persistent viral replication and cytopathicity.

Results

After 7 days in culture, human monocytes differentiate into macrophage-like cells and readily elicit productive infection following exposure to the macrophage tropic viral strain HIV-1$_{ADA}$. Productive HIV-1 replication was demonstrated by RT activity and reflected progeny virion production released into culture fluids from the infected macrophages (Table 1) (Ciborowski et al., 2004;Gendelman et al., 1988). Another measure of progressive viral infection is formation of multinucleated giant cells (MNGC) expressed as a ratio of the number of nuclei per cell, also called Giant Cell Index (GCI). Fig. 1A illustrates photomicrographs of control and HIV-1-infected MDM. Fig. 1B depicts the GCI index. Formation of MNGC occurs as early as 3 days after infection. On day 10 most MDM form large syncytia. Kinetics, but not susceptibility, of progression of HIV-1 infection is donor dependent. In contrary to T-cells, which are susceptible to apoptosis shortly after being infected with HIV-1, macrophages are more resistant to cell-death and continuously support viral replication (Stevenson and Gendelman, 1994;Vazquez et al., 2005,Wahl, Feldman, and McCarthy, 1996). HIV has been reported to turn-off macrophage apoptotic pathways following infection (Greenway and McPhee, 1997).

Culture supernatants were collected at days 2, 3, 5, 7, 9, 12, and 14 after infection. Removal of the viral inoculum 24 hr after infection was performed by three consecutive rinses. These multiple rinses also removed any proteins that were introduced into the culture with viral inoculum during the initial period of infection. Typically, protein concentration in culture supernatants of HIV-1-infected MDM was ~50% higher than what was observed in controls (data not shown). This difference could reflect changes in cell metabolism. Proteomic analyses were performed on selected culture fluids collected at maximal RT levels which coincided with substantial HIV-1p24 protein secreted into the culture fluids (Fig. 4). This varied amongst donors between days 3 and 9. Although in one instance the RT level was highest on day 12, this and later time points were excluded as cytosolic proteins from dying cells could be present in culture fluids (Kadiu et al., 2006).

Despite exchanges of media with serum-free DMEM prior to sample collection residual serum albumin, immunoglobulins, and/or other most abundant serum proteins were present and likely retained on the cell surface. A representative 12% PAGE gel 24 cm long gel was arbitrarily divided to 20 bands indicated by black boxes (Fig. 2A). Proteins from 20 gel cubes were subjected to in-gel trypsin digestion and subsequently identified by liquid chromatography tandem mass spectroscopy (LC-MS/MS). Table 2 lists 83 proteins identified within high confidence limits. For database searches Seaquest algorithm (Bioworks 3.1SR software, ThermoElectron, Inc.) was employed. Listed proteins represent data combined from four independent experiments. Results of database searches were filtered and confidence hits accepted if sequenced peptides reached or exceeded the criteria of Unified Score of 3000 with Sp>500, Xcorr> 2.5 for doubly charged and 3.5 for triply charged precursor ions, and Delta Cn> 0.3. Identification of protein within high confidence limits was accepted as positive when a minimum of two peptides were sequenced meeting established criteria in at least two independent experiments. Peptides of 30 amino acids or longer with scattered y- or b- series ions that had assigned the Unified Score greater than 3000 but low Xcorr and DeltaCn values were excluded. HIV-1 peptides were poorly fragmented by LC-MS/MS and only few were sequenced (Table 4), which can result from either incomplete trypsin fragmentation or poor ionization. Serum albumin and immunoglobulins were excluded from the summary tables. Proteins which could originate as a residual "contamination" with human serum are marked in the table with "#."

Two "intensely" stained protein bands ~ 49 and 25 kDa (Fig. 2A) represented serum albumin and heavy and light chains of immunoglobulins (data not shown) in concentrated samples.

Therefore, we removed albumin and the other five proteins (IgG, IgA, anti-a-trypsin, haptoglobin, and transferring) from the samples and this region was re-analyzed (Fig. 2B). After removal of high abundant proteins another 27 proteins were identified by LC-MS/MS. One of them, L-plastin, was significantly up-regulated in HIV-1 infected MDM fluids. Table 3 shows the summaries of identified proteins. The total number of identified proteins was 110.

Although our proteomic approach was not quantitative *per se*, we observed differences in the relative abundance of peptides sequenced by LC-MS/MS. This indicated that these proteins could be differentially expressed as a result of viral infection. Based on this interpretation we performed quantitative Western-blot analysis in order to confirm our LC-MS/MS results. This strategy was applied successfully in our previous works (Ciborowski et al., 2004). A few examples of differentially expressed proteins are worth noting. Mass spectrometry indicated cystatin C as differentially expressed and found in culture supernatants of uninfected MDM, but not in infected cells (Table 2). Although Western-blot analysis detected the protein in both control and infected MDM, cystatin C was downregulated in infected cells making it concordant with mass spectrometry data. Cofilin-1 was found upregulated in infected MDM by mass spectrometry and confirmed by Western blot tests (Fig. 3). Using the same paradigm six differentially expressed proteins were found. This included: cystatin B, cystatin C, L-plastin, leukotrine A_4 hydrolase (LTA4H), α-enolase, and chitinase 3-like 1 protein (HC-gp39). Presence of HIV-1 p24 included in Fig. 3 served as an internal control and substantiating virus production. Among selected proteins, levels of HC-gp39 were decreased, and L-plastin, LTA4H and α-enolase were increased following HIV-1 infection. The exception was superoxide dismutase (SOD), a protein shown to be up regulated in HIV-infected MDM and confirmed by Western-blot tests although LC MS/MS showed conflicting results. We posit that such results were seen as SOD co-migrates with other identified proteins such as cofilin-1 and cystatin B when analyzed on 1D SDS-PAGE. Thus, protein co-migration may lead to such discrepancies between LC-MS/MS and Western-blot results.

As mentioned above, the dynamic of viral infection will vary from donor to donor. In consequence, levels of differentially expressed proteins will vary and give rise to substantive standard deviations amongst data sets. Nevertheless, our densitometry analyses (Fig. 4) demonstrated that with the exception of LTA4H, all proteins showed consistent trends in expression with many time points being different with statistical significance ($p<0.05$) and are marked with "*". We also expect that differential expression of more proteins will be identified using this strategy enhanced by additional steps of sample preparation and fractionation.

Discussion

HIV-1 infected MP affect the human host including, most prominently, neuronal dysfunction. This occurs by inflammatory factors and viral protein secretions (Giulian et al., 1993). Nonetheless, little is known about how viral infection triggers immunomodulatory mechanisms affecting its function in disease. The mechanisms of MNGC formation are also not well understood. To gain a better understanding of macrophage function as a consequence of HIV-1 infection, we used profiled secreted proteins by proteomics tests. Our results show that the majority of differentially expressed proteins reported in this study represent a wide range of functional groups and include structural (cytoskeleton), redox, regulatory, and enzymes. In some instances one protein was included in two groups as SOD is an enzyme and is an important component of redox regulatory mechanisms. It is important to note that following HIV-1 infection, the macrophage is stimulated and the number of secreted proteins increased. This observation supports the notion that the virus accelerates production and secretion of macrophage proteins to its advantage, and perhaps represents a coping mechanism to best contain ongoing viral replication.

Using Western-blot analysis, we confirmed differential expression of a number of proteins, which could be linked to viral replication. The roles of these proteins in HIV-1 macrophage infection are not yet fully understood and their contribution to disease can now only be speculated. With regards to HIV-1-associated cognitive impairment, it is well known that virus-infected macrophages and microglia directly contribute to disease as a consequence of inflammatory toxic secretions (Kadiu et al., 2005). The nature of these factors and their relative contribution to disease is complex and involves interactions between primary neuronal cells, glia and blood-borne macrophages (Rostasy, 2005).

Many proteins identified in this study are directly involved or associated with MP inflammatory responses. SOD follows macrophage activation as it is regulated by pro-inflammatory cytokines and independent of substrate concentrations (Ferlat and Favier, 1993;Marikovsky et al., 2003). If mechanism of SOD and other redox enzymes goes out of delicate balance, macrophage are unable to control the levels of toxic radical species which leads to tissue injury. Decreased levels of cystatin C in culture supernatants of *in vitro* HIV-1-infected MDM correlates with previous reports showing decreased levels of this enzyme in cerebrospinal fluid (CSF) during inflammatory processes (Nagai et al., 2000). In CSF of multiple sclerosis patients, the levels of cystatin C were decreased compared to healthy controls (Irani et al., 2006); while in CSF of patients with Alzheimer's or Creutzfeld-Jacobs diseases, cystatin C were increased (Carrette et al., 2003,Sanchez et al., 2004). Taking this together we postulate that the mechanism regulating cystatin C expression is modulated in a disease specific manner rather than being a general characteristic of inflammatory activation of macrophage in the brain. This might be indicative of a compensatory response of macrophage to ongoing productive HIV-1 infection and subsequent inflammation. HC-gp39 is a 42 kDa glycoprotein secreted by articular chondrocytes, synoviocytes and macrophages found to play a role in modulation of cytokine responses following TNF-α and IL-1 stimulation. Therefore, decreased expression of HC-gp39 may lead to enhancement of pro-inflammatory effects and profound toxicities. Leukotrienes are made predominantly by polymorphonuclear leukocytes, macrophages, and mast cells. LTA4H catalyzes the hydrolysis of leukotriene A4 (LTA$_4$) into leukotrine B4 (LTB$_4$). LTB$_4$ is a lipid mediator with roles in immune defense, inflammation, and disease. It is a potent chemo-attractant and mediator of inflammation (Funk, 2001), stimulator of adhesion to endothelium (Tonnesen et al., 1989), and degranulation (Dahlen et al., 1981). It also stimulates generation of superoxide anion (Claesson and Feinmark, 1984) and phagocytosis. Overproduced LTB$_4$ contributes to the pathogenesis of a variety of inflammatory disorders (Goetzl, An, and Smith, 1995).

It is intriguing that cellular proteins, such as cofilin-1 and L-plastin, are found secreted by HIV-1-infected macrophages. Cofilin-1 was not detected or detected at very low levels in culture fluids of control MDM and is present in significant levels in culture supernatants of infected counterparts. Ott and colleagues (Ott, 2002) postulated that structural proteins such as actin, cofilin, ezrin, and moesin are involved in virus assembly and budding, are carried specifically with viral particles, and were not found in vesicles. An H9 T-cell lymphoma cell lines was used as a model for these studies in which virus is released by budding from cell membrane. In our studies, MDM were used where virus budding occurs through the exosomal pathway (Nguyen et al., 2003). Both studies indicate that cofilin-1 is involved in viral assembly and budding suggest that although virus release has different pathways, there are common but yet unknown elements regulating this process. Inhibition of viral assembly utilizing such elements can be used for targeting both types of cells in which HIV-1 replicates.

Presented work provides a systematic proteomic approach leading to a better understanding of the impact of HIV-1 on regulation of MP, which are critical components of innate immunity. In a previously published study of macrophage secretome using 2-dimensional electrophoresis and MALDI-TOF protein fingerprinting, the authors reported 66 identifications based on

accession numbers; but many of these identifications were duplicates such as actin, serum albumin, apolipoproteins, etc. (Dupont et al., 2004). In our current study, we did not find all previously reported proteins; however we found proteins that were not reported by Dupont and colleagues (Dupont et al., 2004). It is important to note that our validation Western-blot analyses showed significant and important differences in expression of several proteins which were identified in samples from infected and control MDM. Identification of functional roles of proteins, which seems to be typical cytoplasmic such as α-enolase and cofilin-1 and which we found to be at relatively higher levels in the extracellular milieu due to HIV-1 infection of MDM, may lead to better understanding mechanisms of how macrophage respond to viral infection. These proteins may have yet unknown functions, specific to extracellular localization, or can be specifically incorporated in HIV-1 particles. Moreover, the fact that proteins belonging to various classes can be regulated in macrophages following viral infection highlights the broad range of cellular processes controlled by the virus.

Proteomic analysis of MDM secretions induced by HIV-1 infection identifies a spectrum of proteins that can be explored for potential use as a broader panel of biomarkers. Multidimensional fractionation protocols of samples lead to identification of constantly increasing number of proteins. Nevertheless, huge dynamic range of proteins concentrations (Anderson and Anderson, 2002) and sensitivity of mass spectrometers remain problems in proteomic studies. Although, in this study we did not detect low abundant proteins such as cytokines and/or chemokines, we did identify differential regulation of many proteins that have not been reported in the literature. Determining whether an individual differentially expressed protein will serve as a biomarker of an inflammatory process in the brain or will be a part of a group of biomarkers with value in predicting clinical outcome will require a larger study using CSF samples from individuals whose clinical status of neuropathology is well defined. Such study is currently undergoing in our laboratory. The application of proteomic approaches are likely to be of continued importance in studies of macrophage function and gene regulation leading to a better understanding of disease processes and the means to combat them.

Material and Methods

Isolation, cultivation, and HIV-1 infection of human MDM

Peripheral blood mononuclear cells were isolated from HIV-1, 2 and hepatitis seronegative donors by leukophoresis and purified by countercurrent centrifugal elutriation (Gendelman et al., 1988). Purified monocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated pooled human serum, 1% glutamine, 50 μg/ml gentamicin (Sigma Chemical Co., St. Louis, MO), 10μg/ml ciprofloxacin (Sigma) and 1000 U/ml highly purified recombinant human macrophage colony stimulating factor (MCSF; a generous gift from Wyeth, Inc., Cambridge, MA). Cells were maintained at 37°C with 5% CO_2 and half medium exchange was performed every two days. After seven days in culture, MDM were infected with HIV-1_{ADA} at a multiplicity of infection of 0.1 or left uninfected as controls. Cells were exposed to viral stock for 4 hr at 1/5 of the total medium volume followed by an additional incubation time of 20 hr in full volume of medium. After 24 hr of exposure to viral stock, inoculum was removed, cells were washed twice, and fresh full medium was added to the cultures. Twenty-four hours prior to media collection for proteomic analyses, full medium was removed, cells were rinsed twice with PBS, and freshly prepared serum-free DMEM was added to the cultures (Ciborowski et al., 2004;Ghorpade et al., 2001).

Cytopathicity

To determine levels of cytopathicity by determining the GCI in HIV-1 infected MDM, monocytes were cultured in 4-well Lab-Tech chamber slides (Invitrogen, Carlsbad, CA) and treated as adherent cultures described above. Giemsa staining was performed on HIV-1_{ADA}

infected and control MDM according to the manufacturer's protocol (Fisher Scientific, Pittsburgh, PA) to assess number of nuclei per cells.

Sample processing

Collected culture fluids were centrifuged at 6000 × g for 10 min to remove any detached cells which might be collected with the fluids. Such prepared culture supernatants were supplemented with a cocktail of protease inhibitors (Sigma, St. Louis, MO) and 0.1% Triton X-100 (Fisher Scientific, Pittsburg, PA). For reverse transcriptase activity measurements, aliquots of culture supernatants were set aside without adding detergent and inhibitors. Protein concentrations were determined using Bio-Rad *DC* Protein Assay (Bio-Rad, Hercules, CA) and then samples were concentrated to ~2 mg/ml with an Ultrafree-CL centrifugal filter device with 5000 Da cutoff membrane (Millipore Corporation, Bedford, MA) unless stated otherwise. Concentrated supernatants were dialyzed 18 hr against water at 4°C using QuixSep® micro dialyzers (Membrane Filtration Products Inc., Seguin, TX) and protein concentrations were measured again using a Bio-Rad *DC* Protein Assay.

Multiple Affinity Column, 4.6 × 50 mm Hu-6 (Agilent Technologies, Inc., Santa Clara, CA) was used to remove the 6 most abundant proteins (serum albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin) from concentrated culture supernatants following manufacturer recommendations. Briefly, 700 μl of concentrated culture supernatant was mixed with Buffer A and loaded onto the column. A flow through fraction was collected and column was regenerated using Buffer B. To collect sufficient amounts of protein for analysis, a total amount of 2.1 ml (3 injections, 700 μl each) was processed for each sample.

Measures of RT activity

RT measured levels measured the numbers of progeny virus and is reflective of productive viral replication. RT activity was determined in culture fluids in triplicates. Reaction mixture consisting of 0.05% Nonidet P-40 (Sigma), 10 μg/ml poly(A) oligonucleotide, 0.25 μg/ml oligo (dT) (Amersham/Pharmacia Biotech, Piscataway, NJ), 5 mM dithiothreitol (Amersham Pharmacia), 150 mM KCl, 15 mM MgCl$_2$, and 10μCi/ml [^{3}H] dTTP (Perkin Elmer, Wellesley, MA) in 50 mM Tris-HCl buffer (pH 7.9) was incubated with samples for 18 hr at 37°C. Radiolabeled nucleotides were precipitated with cold 10% trichloroacetic acid and 95% ethanol using an automatic cell harvester (Skatron Inc., Sterling, VA) and were quantitated by liquid scintillation spectroscopy (Kalter et al., 1991).

One Dimensional Electrophoresis (1-DE) and Western-blot assays

1-DE SDS-PAGE separations were performed using BioRad Proteome IIxi system and 4-20% gradient gel. For sequencing, a sample of 200 μg of denatured and reduced proteins were loaded per lane. Gels were stained with Brilliant Blue G-Colloidal Coomassie (Sigma), de-stained following the protocol provided by the manufacturer, and digitized using a Typhoon 9410 Phosphoimager (Amersham Biosciences). For Western-blot analyses, 5 to 25μg of protein was loaded per lane of 4-12% NuPAGE gel (Invitrogen, Carlsbad, CA). Electrophoresis followed by transfer and immunodetection was performed as previously described (Ciborowski et al., 2004).

Antibodies

The following primary antibodies were those directed against cystatin B (Biovision, Mountain View, CA), HIV-1 p24 (DakoCytomation, Carpinteria, CA), cystatin C (U.S. Biological, Swampscott, MA), HC-gp39 (R&D Systems, Minneapolis, MN), cofilin-1 (BD Transduction Laboratories, Lexington, KY), L-plastin (Biovision, Mountain View, CA), SOD (Calbiochem, La Jolla, CA), Leukotriene A4 hydrolase (Cayman Chemical, Ann Arbor, MI), and α-enolase

(Santa Cruz, Santa Cruz, CA). Secondary antibodies included HRP-conjugated $F(ab')_2$ fragment of Goat anti-Mouse Fc_γ and Cy5-conjugated $F(ab')_2$ fragment of Donkey anti-Mouse IgG (Jackson Immunoresearch, West Grove, PA), HRP-conjugated anti-Rabbit IgG (Cell Signaling Technology, Beverly, MA), and HRP-conjugated anti-goat IgG and HRP-conjugated anti-Sheep IgG (U.S. Biological). All antibodies were used at concentrations recommended by manufacturers.

In-gel trypsin digestion

Gel pieces excised from 1-DE were cut into 20 fragments and destained at room temperature using 200 μl 50% ACN/50 mM NH_4HCO_3 for 1 hr. Gel pieces were dried, and 1μg trypsin in 50 mM NH_4HCO_3 buffer (Promega, Madison, WI) was added to each piece. Tryptic digestion was carried out overnight at 37°C. Peptides were extracted by washing gel pieces twice with 60% acetonitrile and 0.1% trifluoroacetic acid, transferred to clean vials, dried, and re-suspended in 12 μl of water with 0.1% formic acid for mass spectrometric analysis. All samples were purified using ZipTip® (Millipore Corporation) prior to mass spectrometric analysis.

ESI-LC-Tandem Mass Spectrometry and search parameters

Samples of in-gel trypsin digested proteins were fractionated on microcapillary RP-C_{18} column (New Objectives, Woburn, MA) and resulting peptides were sequenced using ESI-LC-MS/MS system (ProteomeX System with LCQDecaPlus mass spectrometer, ThermoElectron, Inc., San Jose, CA) with a nano-spray configuration. The spectra obtained from LC-MS/MS analysis were searched against the NCBI.fasta protein database narrowed to a subset of human proteins using Sequest search engine (BioWorks 3.1SR software from ThermoElectron, Inc.). In the TurboSEQUEST Search Parameters, Threshold for Dta generation was 10000, Precursor Mass Tolerance for .Dta Generation was 1.4. For .Dta Search, Peptide Tolerance was 1.5 and Fragment Ions Tolerance was 0.00. Charge state was set on "Auto." Database nr.fasta was retrieved from ftp.ncbi.nih.gov and used to create "in-house" an indexed human.fasta.idx with the following 5 key words: Homo, sapiens, human, man, primate and excluding keratins and cytokeratins. We have excluded keratins from our database search based on observations that these proteins can be just contaminants acquired during gels and samples processing. Database was updated every other week throughout of this study. Oxidated methionine was allowed in searches.

Densitometry and statistical analyses

Differential expression of 9 proteins was analyzed by Western-blot (Western Pico, Pierce, Inc. Rockford, IL) and results were transformed into numerical values using densitometry measurement of X-ray films. Densitometry was performed using ImageQuant 5.2 (GE Healthcare, Inc.). Samples were from 3 donors, in infected and control samples on days 1, 3, 5 7, and 10. Sample from one donor was measured twice and the results were averaged and treated as one biologic replicate for analysis. Data was normalized from each analysis by taking the fold change as infected/control on each day for each experiment. The data was then transformed on the log10 scale so that a ratio of 1 on the original scale becomes 0 on the log10 scale, ratios greater than 1 are then positive numbers on the log10 scale and ratios less than 1 are negative on the log10 scale. The experiments from Donor 5 were then averaged giving one set of values to be analyzed. One sample t-tests were then used to compare the average log10 (ratio) to 0 for each day. P-values less than 0.05 are considered to be statistically significant.

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Abbreviations

MP

mononuclear phagocytes

HIV-1

human immunodeficiency virus type-1

MDM

monocyte derived macrophages

SOD

superoxide dismutase

HCgp-39

chitinase 3-like 1 protein

LTA4H

leukotriene A4 hydrolase

DMEM

Dulbecco's Modified Eagle Medium

MCSF

macrophage colony stimulating factor

RT

reverse transcriptase

1-DE

one-dimensional electrophoresis

MS/MS

tandem mass spectrometry

ROS

reactive oxygen species

LTA$_4$

leukotriene A4

LTB$_4$

leukotrine B4

MNGC

multinucleated giant cells

GCI

Giant Cell Index

mAb

monoclonal antibody
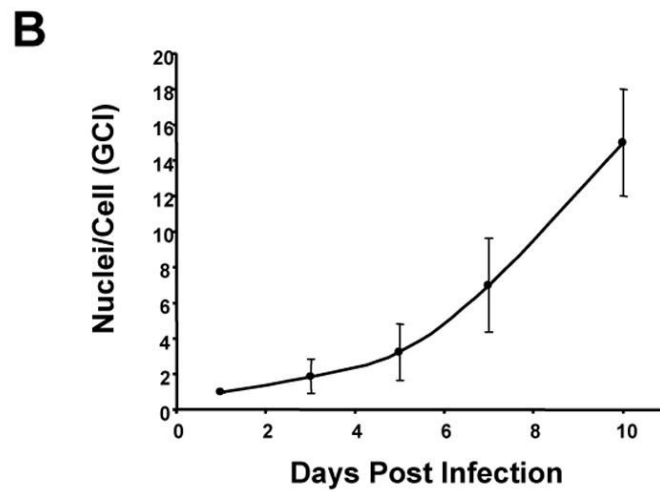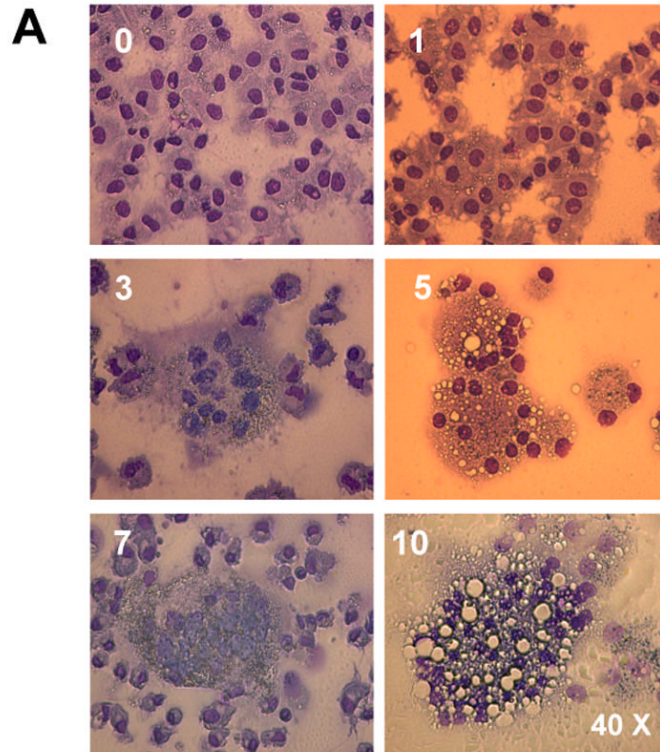
Figure 1.
Cythopatic effects of HIV-1$_{ADA}$$^-$ infected MDM. (A) Photographs of MDM culture infected with HIV-1 showing progressive MNGC formation from initiation of infection to day 10. (B) An index of the number of nuclei per cell increases along with culture progression and secondary infection of cells that were not infected during initial exposure to the virus.
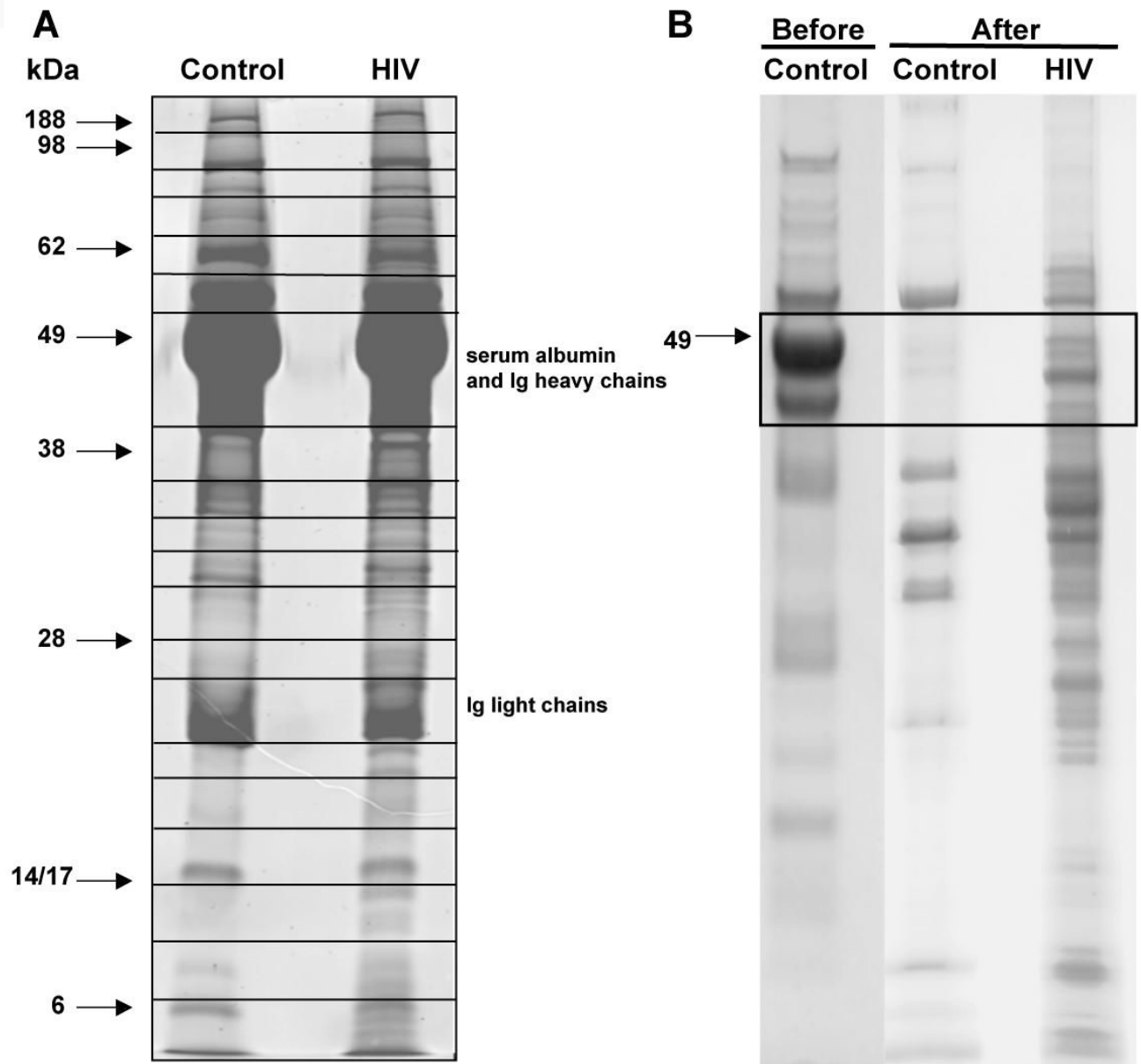
Figure 2.
1DE SDS-PAGE analysis of secreted proteins from MDM culture supernatants. Gels were stained with SyproRuby® fluorescent dye. (A) Samples of 100 µg of protein were loaded per lane. 1DE SDS-PAGE analysis of secreted proteins before and after removal of 6 most abundant proteins: serum albumin, IgG, IgA, α-antitrypsin, haptoglobin, and transferring. (B) Samples of 60 µg of protein were loaded per lane. Black box shows region which was re-analyzed after serum albumin was removed.
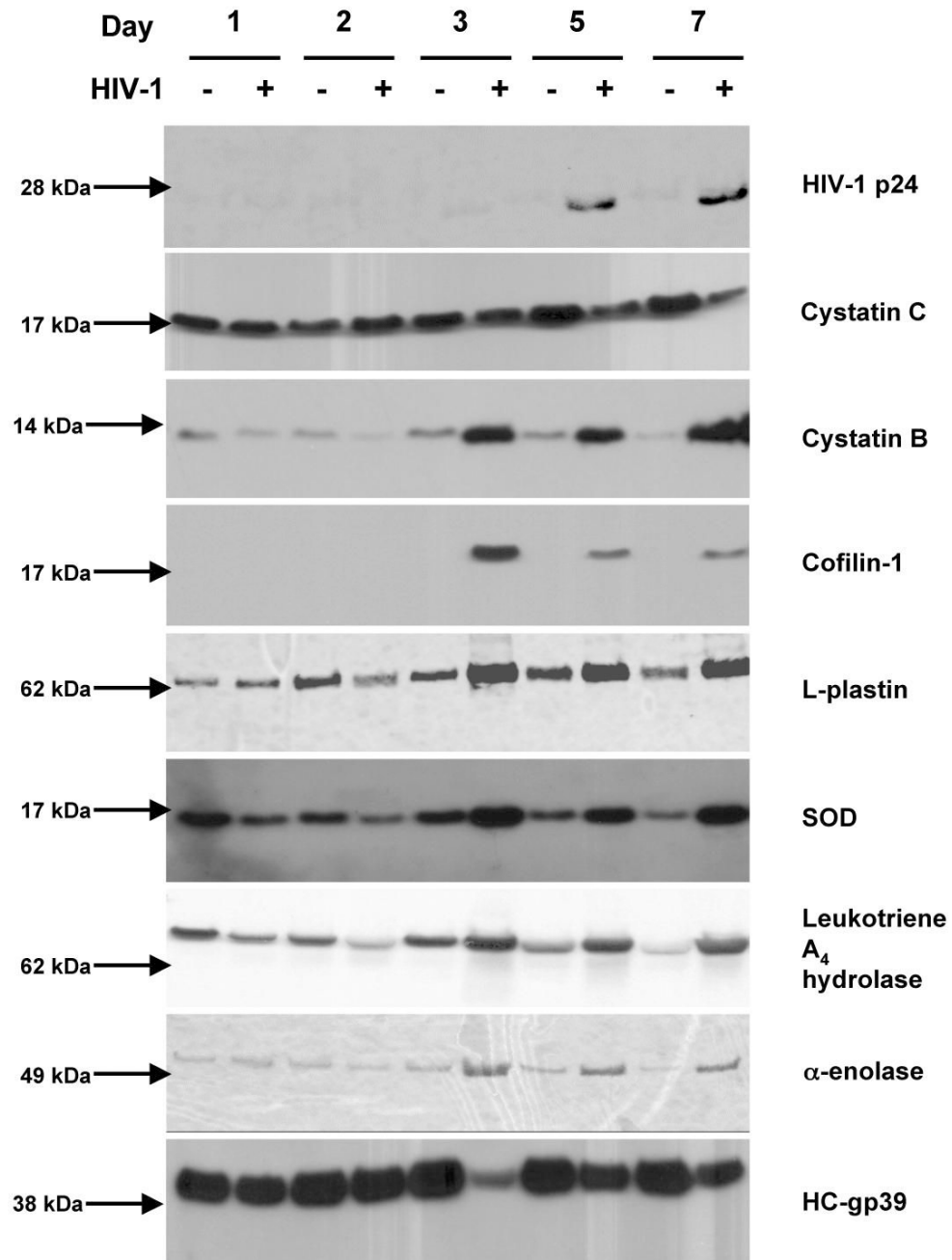
Figure 3.
Western blot analysis of HIVp24, cystatin B, cystatin C, cofilin-1, L-plastin, SOD, LTA4H, α-enolase, and HCgp-39 in culture supernatants of a representative experiment. Samples of culture supernatants from HIV_{ADA}-1-infected and control MDM (5 to 25μg of protein per lane) were loaded on 4-12% NuPAGE gel. Separated proteins were electroblotted onto PVDF membrane and immunodetected as described in the Material and methods.
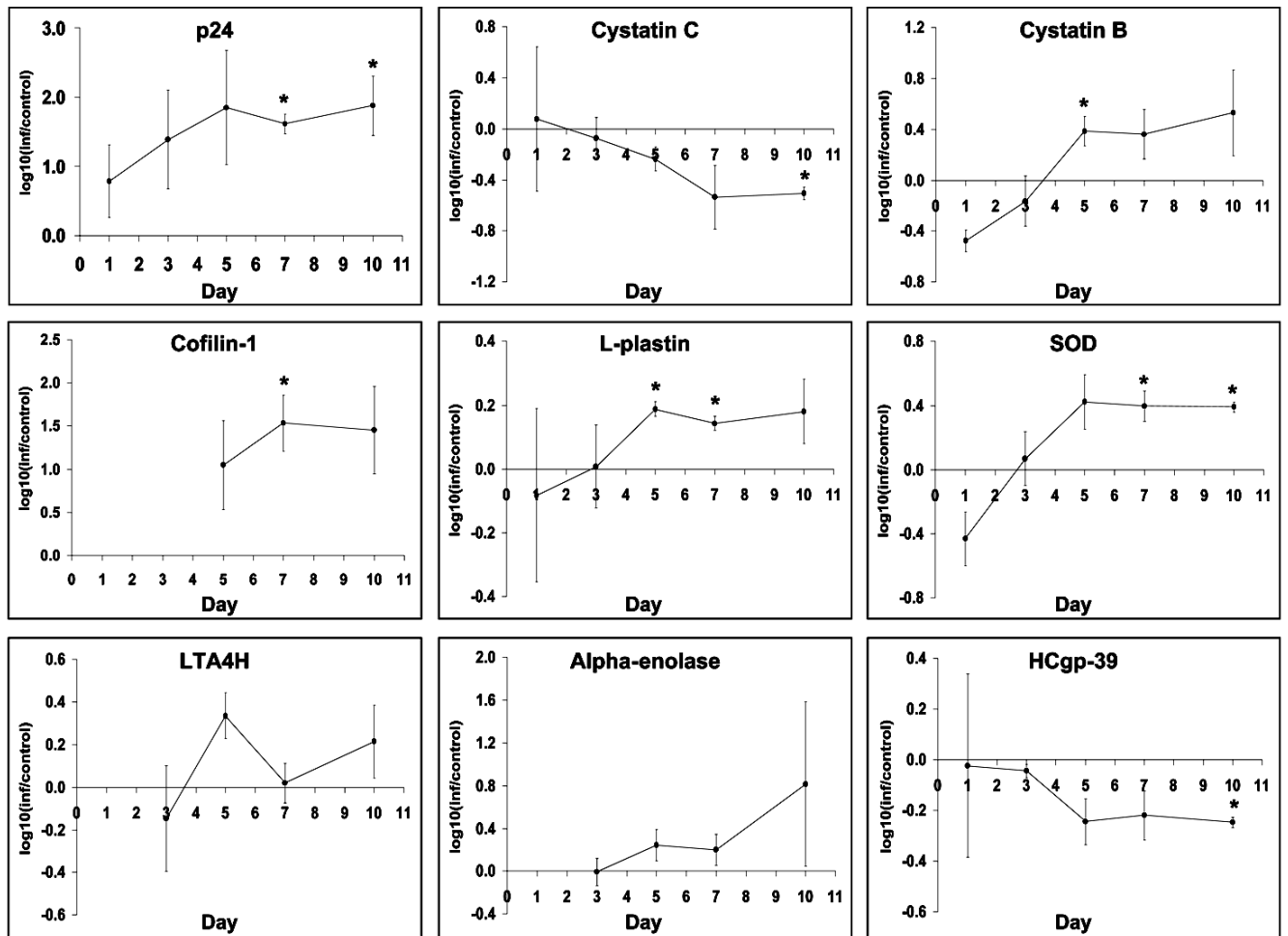
Figure 4.
Quantitative changes in expression of 9 proteins (HIVp24, cystatin B, cystatin C, cofilin-1, L-plastin, SOD, LTA4H, α-enolase, and HCgp-39) in culture supernatants of HIV-1$_{ADA}$ infected MDM. Graph represent densitometric measurements of Western-blots and are expressed as log10 transformed value of change comparing to the corresponding control. Statistical significant differences (P< 0.05) in levels of expression are marked with *.

Table 1

Levels of reverse transcriptase (RT) activity in 6 culture supernatants

	Donor	RT ×104					
		1	2	3	4	5	6
Days	1	*ND**	ND	ND	ND	ND	ND
	2	-	-	-	29.72	11.42	18.21
	3	-	-	-	46.61	13.27	47.71
	5	-	-	-	6.50	32.23	52.76
	7	5.43	44.32	52.73	3.90	34.95	57.16
	9	14.53	85.12	44.35	-	-	-
	12	17.30	64.02	36.61	-	-	-
	14	15.81	49.24	20.46	-	-	-

*ND** not detected

- not tested

Table 2

Proteins identified in secretome of HIV-1-infected and control MDM (LC-MS/MS identification)

HIV- infected N - uninfected COM - common	Protein	NCBI	Swiss Prot	M.W.	Postulated Function
HIV	14-3-3 gamma protein	5726310	P61981	28,171	Signal Transduction, Cytoskeletal organization and biogensis
HIV	14-3-3 protein zeta/delta	33872678	P63104	27,745	transcription factor binding
HIV	14-3-3 protein/cytosolic phospholipase A2	4262000	Q6LD62	7,957	protein domain specific binding
HIV	3-monooxgenase/tryptophan 5-monooxygenase activation protein	31543976	P61981	28,171	regulation of mitosis, regulation of signal transduction
COM	Actin various forms	*	*	*	
COM	Aldolase A protein	28595	P04075	39,289	fructose-bisphosphate aldolase activity, catalytic activity
COM#	Alpha 2 macroglobulin	46812315	P01023	163,278	intracellular protein transport
COM	Alpha enolase	2661039	P06733	47,038	phosphopyruvate hydratase activity, plasminogen activator activity
COM#	Alpha-1 antitrypsin	28637	Q13747	22,828	serine-type endopeptidase inhibitor activity
COM	Amyloid P component, serum	30582339	P02743	25,387	chaperone-mediated protein complex assembly, acute-phase response,
COM	Annexin A2	16306978	P07355	38,473	protein folding
COM	Apo-B100 precursor	28780	P04114	515,563	phospholipase inhibitor activity
COM#	Apolipoprotein A-I	37499465	Q6Q785	30,778	lipid transporter activity, receptor binding
COM#	Apolipoprotein A-II preproprotein [Homo sapiens]	4502149	P02652	11,175	lipid transporter activity, protein binding
COM	Apolipoprotein A-IV precursor [Homo sapiens]	178757	P06727	45,371	lipid transporter activity, protein binding
N	Apolipoprotein C-II precursor [Homo sapiens]	32130518	P02655	11,284	lipoprotein lipase activity
N	Apolipoprotein C-III precursor [Homo sapiens]	4557323	P02656	10,852	lipoprotein lipase activity
COM	Apolipoprotein D precursor [Homo sapiens]	4502163	P05090	21,276	lipid metabolism, transport
COM	Apolipoprotein E precursor [Homo sapiens]	4557325	P02649	36,154	lipid transporter activity, lipid binding, tau protein binding
COM	Apolipoprotein H (beta-2-glycoprotein I) [Homo sapiens]	32165624	P02749	38,298	lipid transporter activity, heparin binding
HIV	Cap-G [Homo sapiens]	63252913	P40121	38,518	actin binding
COM	Cathepsin B preproprotein [Homo sapiens]	22538437	P07858	37,822	intracellular degradation and turnover of proteins
HIV	cathepsin D preproprotein [Homo sapiens]	4503143	P07339	44,552	aspartic-type endopeptidase activity
HIV	cathepsin S [Homo sapiens]	179957	P25774	37,510	cysteine-type endopeptidase activity
COM	Ceruloplasmin (ferroxidase) [Homo sapiens]	4557485	P00450	122,205	ferroxidase activity, copper ion transporter activity, oxidoreductase activity
COM	Chitinase 3-like 1 [Homo sapiens]	25058041	P36222	42,613	extracellular matrix structural constituent, hydrolase activity, hydrolyzing O-glycosyl compounds, chitinase activity
HIV	cofilin 1 (non-muscle) [Homo sapiens]	30582531	P23528	18,371	actin binding, protein binding
HIV	cofilin 2 [Homo sapiens]	33946278	Q9Y281	18,737	protein binding
COM	Cystatin B (stefin B) [Homo sapiens]	68783	Q76LA1	11,140	endopeptidase inhibitor activity, cysteine protease inhibitor activity
N	Cystatin C precursor [Homo sapiens]	4503107	P01034	15,799	cysteine protease inhibitor activity, protein homodimerization activity
HIV	cytosolic malate dehydrogenase [Homo sapiens]	5174539	P40925	36,295	oxidoreductase activity; malate dehydrogenase activity
HIV	cytovillin 2 [Homo sapiens]	6063151	Q9UIZ2	18,419	cytoskeletal protein binding
HIV	ezrin [Homo sapiens]	6063147	Q9UIZ6	19,237	cytoskeletal protein binding
HIV	filamin [Homo sapiens]	1203969	P21333	280,761	actin binding
HIV	filamin 2 [Homo sapiens]	8885790	Q14315	290,959	actin binding

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HIV- infected N - uninfected COM - common	Protein	NCBI	Swiss Prot	M.W.	Postulated Function
HIV	filamin A [Homo sapiens]	53791221	Q60FE5	278,226	actin binding
N	FNHU fibronectin precursor [validated] - human	279675	P02751	262,607	extracellular matrix structural constituent, protein binding
HIV	fructose-1,6-bisphosphatase [Homo sapiens]	3293553	P09467	36,683	carbohydrate metabolism
HIV	gag protein [Human immunodeficiency virus 1]	55416335	%	%	
HIV	gag protein [Human immunodeficiency virus 1]	33145913	%	%	
HIV	gag protein [Human immunodeficiency virus 1]	33145909	%	%	
HIV	gag-pol polyprotein [Human immunodeficiency virus 1]	21671035	%	%	
COM	Gelsolin isoform a [Homo sapiens]	4504165	P06396	85,697	actin filament polymerization, calcium ion binding
COM	Gelsolin isoform b [Homo sapiens] (cytoplasmic)	38044288	P06396	85,698	actin filament polymerization, calcium ion binding
HIV	glutathione S-transferase omega 1-1 [Homo sapiens]	55925946	Q5TA03	27,566	glutathione transferase activity
HIV	glyceraldehyde-3-phosphate dehydrogenase [Homo sapiens]	31645	P04406	35,922	glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity
COM	GM2-activator protein [Homo sapiens]	31857	P17900	20,822	sphingolipid activator protein activity
COM[#]	Haptoglobin [Homo sapiens]	1620396	P00739	39,008	serine-type endopeptidase activity, hemoglobin binding
COM[#]	Haptoglobin [Homo sapiens]	3337390	P00738	45,205	serine-type endopeptidase activity, hemoglobin binding
N	Histidine-rich glycoprotein precursor [Homo sapiens]	4504489	P04196	59,578	cysteine protease inhibitor activity, heparin activity
HIV	hp2-alpha [Homo sapiens]	296653	Q6LBY9	41525	serine-type endopeptidase activity, hemoglobin binding
HIV	Human Thioredoxin	1827674	P52566	11,705	Rho GDP-dissociation inhibitor activity
HIV	ITH4_HUMAN Inter-alpha-trypsin inhibitor heavy chain H4 precursor	13432192	Q14624	103,358	endopeptidase inhibitor activity
COM	Lactate dehydrogenase A [Homo sapiens]	5031857	Q6ZMR3	36,507	L-lactate dehydrogenase activity, oxidoreductase activity
HIV	lactate dehydrogenase B [Homo sapiens]	4557032	Q9BYZ2	41,979	L-lactate dehydrogenase activity
HIV	L-lactate dehydrogenase	34527427	P00338	36,558	L-lactate dehydrogenase activity
HIV	Macrophage capping protein	21730367	P40121	38,524	actin binding
COM	MMP9 [Homo sapiens]	10443048	P14780	78,427	macrophage differentiation, metalloendopeptidase activity
HIV	moesin [Homo sapiens]	4505257	P26038	67,689	cytoskeletal protein binding
HIV	NADP-dependent isocitrate dehydrogenase [Homo sapiens]	11374664	O75874	46,659	isocitrate dehydrogenase (NADP+) activity
COM[#]	Orosomucoid 1, precursor [Homo sapiens]	20070760	P02763	23,512	modulating the activity of the immune system during the acute-phase reaction
COM[#]	Orosomucoid 2 [Homo sapiens]	4505529	Q5T538	23,603	signal transduction
COM	Peroxiredoxin 1	55959887	Q06830	22,110	oxidoreductase activity, peroxidase activity, antioxidant activity
HIV	phosphoglycerate kinase 1 [Homo sapiens]	4505763	P00558	44,483	phosphoglycerate kinase activity
COM	PREDICTED: similar to FKSG30	51493213	Q9BYX7	42,016	structural constituent of cytoskeleton, protein binding, ATP binding
COM	Profilin 1	30582841	Q53Y44	15,054	cytoskeleton organization and biogenesis
HIV	proteasome activator subunit 1	5453990	Q06323	28,723	proteasome complex
HIV	proteasome activator subunit 2	30410792	Q9UL46	27,230	proteasome complex
HIV	prothymosin alpha [Homo sapiens]	307352	Q15203	8,161	unknown
HIV	Rho GDP dissociation inhibitor beta [Homo sapiens]	20379030	P52566	22,988	Rho GDP-dissociation inhibitor activity
COM	Serine (or cysteine) proteinase inhibitor	30584253	P01009	55,299	endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity, protein binding

HIV - infected N - uninfected COM - common	Protein	NCBI	Swiss Prot	M.W.	Postulated Function
N	SH3 domain binding glutamic acid-rich protein like 3 [Homo sapiens]	55960189	Q5T123	9,381	secreted protein
HIV	smooth muscle and non-muscle myosin alkali light chain isoform 1 - 4	17986262	P60660	16,799	motor activity; calcium ion binding
N	Superoxide Dismutase [Homo sapiens]	27065835	P00441	15,805	superoxide dismutase activity
HIV	transaldolase 1 [Homo sapiens]	5803187	P37837	37,540	carbohydrate metabolism
COM#	Transferrin	37747855	P02787	77,050	ion transport; iron ion binding
HIV	transferrin receptor	4507457	P02786	84,901	transferrin receptor activity
HIV	Triosephosphate isomerase 1	173898815	P60174	26,538	triose-phosphate isomerase activity
HIV	tropomyosin 3	55665780	Q5VU59	27,175	cytoskeletal protein binding
HIV	Tropomyosin 4	12803959	P67936	28,391	cytoskeletal protein binding
COM	TTR	48145933	Q6IB96	15,873	thyroid hormone-binding protein
HIV	tyrosine 3/tryptophan 5 -monooxygenase activation protein	21735625	P63104	27,745	transcription factor binding
HIV	VIM	47115317	P08670	53520	protein binding; structural constituent of cytoskeleton

%
for details see Table 4

& and $ - NCBI and SwissProt accesion numbers respectively.

*
Multiple accesion numbers of various forms (including fragments) were obtained from dadabase searches.

#
Top 12 most abundant proteins found in human serum

Table 3

Proteins identified in the range of 50 to 60 kDa in 1DE after removal of 6 most abundant proteins.

HIV - infected; N - uninfected; COM - common	Protein	NCBI&	SwissProt§	M.w.	Postulated functions
N	Alpha 2 macroglobulin	46812315	P01023	163,278	Endopeptidase inhibitor activity, enzyme binding, Il-1 binding, Il-8 binding, TNF binding, protein homooligomerization
HIV	ATPase, H+ transporting, lysosomal 70kDa	15341906	P38606	68,304	Proton-transporting two-sector ATPase complex
HIV	BiP	1143492	P11021	72,333	ATP binding, calcium ion binding, caspase inhibitor activity, negative regulation of caspase activity, anti-apoptosis
COM	Complement component 3	40786791	P01024	187,164	G-protein coupled receptor protein signaling pathway, immune response, signal transduction
COM	Gelsolin	38044288	P06396	85,698	Actin filament polymerization, actin filament severing, barbed-end actin filament capping.
HIV	Heat shock 70kDa protein 8 isoform 2	24234686	Q53HF2	53,500	ATP binding, nucleotide binding, protein folding, response to unfolded protein
HIV	Heat shock 90kDa protein 1, alpha	83699649	Q2PP14	98,143	ATP binding, nitric-oxide synthase regulator, protein homodimerization, TPR domain binding, mitochondrial trans regulation of nitric oxide biosynthesis, protein refolding, signal transduction Nitric-oxide synthase regulator, TPR domain binding, positive regulation of nitric oxide biosynthesis, protein refolding, unfolded protein
HIV	Heat shock 90kDa protein 1, beta	56204416	Q5T9W7	83,264	Unfolded protein binding, male meiosis, response to unfolded protein, spermatid development
HIV	Heat shock protein	4204880	P54652	70,021	
HIV	HSP70-2	4529892	P08107	70,052	Anti-apoptosis, mRNA catabolism, response to unfolded protein
HIV	Interferon-induced Mx protein	4505291	P20591	75,403	GTP binding, defense response, induction of apoptosis, signal transduction
HIV	IQGAP1 protein	40674640	Q6P1N4	107,539	Mediates VE-cadherin-based cell-cell contacts and VEGF signaling at adherence junctions
HIV	Karyopherin beta 1	19923142	Q14974	97,170	Zinc ion binding, NLS-bearing substrate import into nucleus, protein import into nucleus, translocation
COM	Leukotriene A4 hydrolase	4505029	P09960	69,154	Epoxide hydrolase activity, peptidase activity, zinc ion binding, inflammatory response, leukotriene biosynthesis
COM	L-plastin	14043359	P13796	70,289	Actin binding, calcium ion binding
HIV	MARCKS protein	33877895	Q6NVI1	14,859	Interacts with the metabotropic glutamate receptor type 7 and modulates G protein-mediated constitutive inhibition
COM	Matrix metalloproteinase 9	22532481	P14780	78,427	Collagenase activity, gelatinase B activity, zinc ion binding, macrophage differentiation
HIV	Moesin	5419633	P26038	67,689	Structural constituent of cytoskeleton, cell motility, receptor binding
HIV	Myristoylated alanine-rich protein kinase C substrate	56203645	P29966	31,423	Actin filament binding, calmodulin binding
HIV	Radixin	28436809	P35241	68,564	Binding barbed end of actin filaments, binds SLC9A3R1
HIV	SEC23-related protein A	38202214	Q15436	86,147	Vesicle-mediated transport
N	Transferrin receptor	4507457	P02786	84,901	Transferrin receptor, activity iron ion homeostasis
COM	Transketolase	31417921	Q53EM5	67,906	Oxygen-independent glucose metabolism
HIV	UNC-112 related protein 2 long form	41281905	Q86UX7	75,953	Cell adhesion
HIV	Vacuolar sorting protein 35	9622850	Q96QK1	91,707	Retrograde transport, endosome to Golgi
HIV	Villin 2 (ezrin)	66347778	Q4VX75	69,413	Cytoskeletal protein binding
HIV	Zyxin	58530845	Q15942	61,277	Cell adhesion, cell-cell signaling, signal transduction

& and $ - NCBI and SwissProt accesion numbers respectively

Table 4

LC-MS/MS identified HIV-1 proteins and found in culture supernatants of HIV-1 secreted proteins.

Protein	NCBI accession number	Peptide sequence	MH$^+$	z	XCorr	DeltaCn
Reverse transcriptase	gi|55709508|gb|AAV58679.1|[55709508]	YQYNVLPMGWXGSPAIFQSSMTXVLEPFR	3346.90	3	2.621	0.322
Envelope glycoprotein	gi|62906511|gb|AAY20909.1|[62906511]	QAYCNINATEWNNTLQEVGRELEK	2825.06	3	2.937	0.353
Gag protein	gi|5668939|gb|AAD46087.1|AF077336_1[5668939]	QLQPSLQTGSEELK	1558.72	2	3.920	0.397