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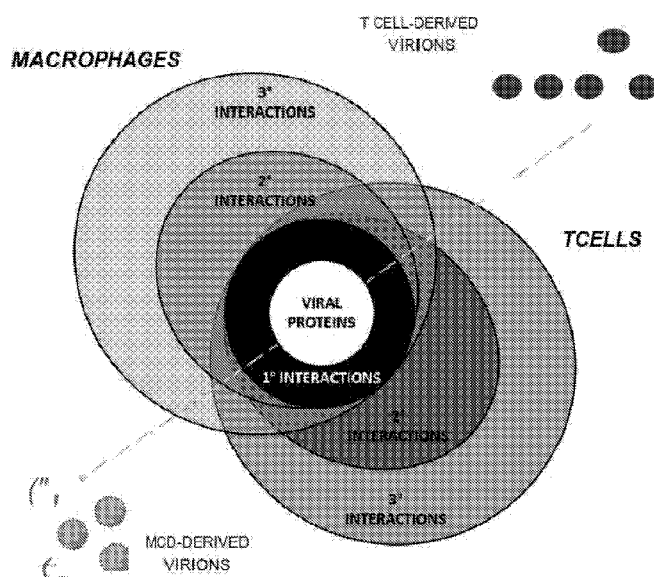


FIG. 2

(57) Abstract: The present invention relates to the field of HIV. More specifically, the present invention provides methods and compositions for determining HIV-1 receptor tropism and cellular reservoirs of HIV-1 replication. Determining cellular tropism of HIV-1 is critical when a patient is on fusion inhibitors to ensure the efficacy of the treatment. Using a combination of affinity capture methods and detection of a panel of cellular markers specific to the cell types HIV-1 has been released from, we are able to unambiguously identify the cellular tropism of HIV-1. Further, by simply changing the order of affinity pull-down, any additional cellular reservoirs of HIV-1 replication can be identified, like the CNS.



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CLINICAL METHODS FOR DETERMINING HIV-1 RECEPTOR TROPISM AND CELLULAR RESERVOIRS OF HIV-1 REPLICATION

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This application claims the benefit of U.S. Provisional Application No. 61/787,752,
filed March 15, 2013, which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENTAL INTEREST

 This invention was made with U.S. government support under grant no. NO1-HV-
28180, awarded by the National Institutes of Health. The U.S. government has certain rights
10 in the invention.

FIELD OF THE INVENTION

 The present invention relates to the field of HIV. More specifically, the present
invention provides methods and compositions for determining HIV-1 receptor tropism and
cellular reservoirs of HIV-1 replication.

15 BACKGROUND OF THE INVENTION

 During HIV replication and packaging, HIV relies on the coordinated interactions
between viral and host proteins.(1) As HIV buds, it incorporates hundreds of cellular host
proteins into the nascent virion, either into its lipid bilayer or inside the HIV virion.(2-4)
Several studies have indicated that host protein incorporation affects both HIV attachment
20 and infectivity.(5, 6) Other proteins, such as cyclophilin A, have been implicated in the
HIV lifecycle;(3, 5, 7-9) however, due to the large number of host proteins reported to be
incorporated into HIV virions, it is difficult to determine the biological relevance, if any,
of many of these proteins.

SUMMARY OF THE INVENTION

25 Presently to determine cellular tropism, predictive sequences of the HIV-1 envelope
gene are used, or infectivity assays of MT-2 cells or other susceptible cell types that are
expressing either CXCR4 or CCR5. In this invention, we are retrospectively interrogating
the source of the virus present in the patient. Thus if virus has been produced from CD4+ T
cells or from monocytes/macrophages, we can identify the cell type by examining unique
30 markers specific to that cell type incorporated into the virion. The invention stems from our
discovery that CD44 a host protein, is common to virus produced from either cell type.
Therefore using affinity purification of virus, we can simply and effectively purify patient

virus and then use a variety of different methods to determine what specific markers identifying the source of the virus is. When an attempt is being made to identify unknown reservoirs of virus production, we perform a series of immune depletions, first using a cocktail of antibodies that will deplete known sites of virus replication, and then finally by capturing residual virus with anti CD44 antibodies.

We have discovered specific markers unique to HIV-1 derived from CD4⁺ T cells, and from Macrophages using mass spectrometry. We have also identified markers common to HIV-1 independent of cell source. Using the common markers to HIV-1, we can use affinity purification to capture HIV-1 and then use markers unique to the specific cell types to identify what cell types the virus has been produced from. In this manner we can determine whether a patient has X4 tropic, R5 tropic or dual tropic HIV-1 infection. To identify unknown sources of HIV-1 replication, a cocktail of specific markers is used to first deplete known sources of HIV-1, and then CD44 is used to pull down remaining HIV-1. Mass spectrometry may then be used to identify markers that can be used in combination with tissue arrays to identify the cell type virus is produced from.

Moreover, we have developed an alternative purification technique using cholesterol that differentially modulates the density of virions and microvesicles (density modification, DM) allowing for high-yield virion purification that is essential for tandem mass spectrometric and quantitative proteomic (iTRAQ) analysis. DM purified virions were analyzed using iTRAQ and validated against Optiprep (60% iodixanol) purified virions. We were able to characterize host protein incorporation in DM-purified HIV particles derived from CD4⁺ T cell lines; we compared this dataset to a reprocessed dataset of monocyte-derived macrophages (MDM) derived HIV-1 using the same bioinformatics pipeline.

Accordingly, in one aspect, the present invention provides methods for treating a patient having an HIV-1 infection. In one embodiment, the method comprises the steps of (a) obtaining a biological sample from the patient; (b) performing an assay to isolate HIV-1 from the biological sample using host proteins that are incorporated into HIV-1; (c) performing an assay on the isolated HIV-1 to identify whether the patient has an X4 tropic, R5 tropic or dual tropic HIV-1 infection using host proteins that are incorporated into HIV-1 and are unique to specific cell types that are infected by HIV-1; and (d) treating the patient based on the type of HIV-1 infection, wherein an X4 tropic infection is treated with a CXCR4 antagonist, an R5 tropic infection is treated with a CCR5 antagonist, and a dual tropic infection is treated with

both a CXCR4 antagonist and a CCR5 antagonist. In a specific embodiment, the host proteins that are incorporated into HIV-1 are one or more proteins listed in Table 3. In another embodiment, the assay of step (b) uses an antibody that binds a host protein that is incorporated into HIV-1.

5 In another embodiment, a method for treating a patient having HIV-1 comprises the steps of (a) obtaining a biological sample from the patient; (b) performing an assay to isolate HIV-1 from the biological sample using an antibody that binds a host protein that is incorporated into HIV-1, wherein the host protein is one or more proteins listed in Table 3; (c) identifying the cell type(s) infected by HIV-1 using one or more antibodies to bind host
10 proteins that are incorporated into HIV-1 and are unique to the cell type that is infected by HIV-1; (d) using polymerase chain reaction to quantify the HIV-1 growing in the cell type(s) and/or identify the location of HIV-1 replication; and (e) treating the patient based on the infected cell type and location of infection. A specific example of this application would be in the identification of virus replicating in the brain of the infected individual. A protein
15 incorporated into the virus from a brain cell type is used to pull down the virus from the plasma (brain parenchyma drains to CSF which drains to plasma); subsequently a PCR amplification method would be used to detect brain specific virus. In this manner a physician might elect to either treat a patient earlier with antiretroviral therapy, or switch antiretroviral therapy to allow for a more brain-penetrant regimen to suppress virus replication in the brain
20 and prevent HIV associated neurological diseases.

In another aspect, the present invention provides a method for identifying an unknown reservoir of HIV-1 production in a host patient comprising the steps of (a) obtaining a biological sample from the patient; (b) immunodepleting known sources of HIV-1 using one or more antibodies that bind to host proteins that are incorporated into HIV-1 and are unique
25 to the cell type that is infected by HIV-1; (c) isolating the remaining HIV-1 using CD44 and/or syntenin-1; (d) performing mass spectrometry to identify host protein that are incorporated into the HIV-1; and (f) using a tissue array to identify the cell type infected by HIV-1. In certain embodiments, the method further comprises the step of treating the patient based on the infected cell type.

30 The present invention also provides methods for determining cellular tropism of HIV. In one embodiment, a method comprises the steps of (a) performing an assay to capture HIV-1 from a sample using biomarkers common to HIV-1; and (b) performing an assay using

biomarkers unique to specific cell types to identify the cell types from which HIV-1 has been produced. In certain embodiments, the assay in step (a) and/or (b) is affinity purification. In particular embodiments, the biomarkers common to HIV-1 comprise one or more biomarkers from Table 3. In a specific embodiment, the biomarkers common to HIV comprise syntenin-1 and CD44. Furthermore, the biomarkers common to HIV comprise one or more additional proteins from Table 3. In other embodiments, the biomarkers unique to specific cell types comprise one or more biomarkers from Table 2. In particular embodiments, it is determined whether the subject from which the sample was obtained has X4 tropic, R5 tropic or dual tropic HIV-1 infection.

In another aspect, the present invention provides methods for purifying HIV virus. In one embodiment, a method for purifying HIV virus from a sample comprises the steps of (a) incubating the sample with cholesterol and 2-hydroxy-beta cyclodextran; (b) filtering the sample on ice; and (c) pelleting through sucrose for about one hour. In a specific embodiment, the sample is an HIV-1 cell line. In a more specific embodiment, the cell line is H9. In an alternative embodiment, the cell line is CEMx174.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Purification of HIV-1 virions using density modification. DM HIV-1 purity was assessed using CD45, a marker of vesicle contamination (Fig 1A). Isolated microvesicles were eliminated with this method (>90% of material was lost) (Fig 1 B, C). HIV-1 infectivity was unchanged (not shown), but virion morphology was substantially altered (Fig 1 D, E).

FIG. 2. Hypothetical schematic of the impact of primary HIV-host protein interactions on virion phenotype. HIV proteins interact with a common set of host proteins that is found in multiple cell types capable of sustaining HIV infection. These common set proteins have secondary and tertiary interactions with both cell-specific and common protein partners and these interactions determine the phenotype of released virions. Thus, despite a limited number of HIV-host protein interactions, viral diversity is driven by the secondary and higher interactions based on cell-type.

DETAILED DESCRIPTION OF THE INVENTION

It is understood that the present invention is not limited to the particular methods and components, etc., described herein, as these may vary. It is also to be understood that the terminology used herein is used for the purpose of describing particular embodiments only,

and is not intended to limit the scope of the present invention. It must be noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include the plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to a “protein” is a reference to one or more proteins, and includes equivalents thereof known to those skilled in the art and so forth.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Specific methods, devices, and materials are described, although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention.

All publications cited herein are hereby incorporated by reference including all journal articles, books, manuals, published patent applications, and issued patents. In addition, the meaning of certain terms and phrases employed in the specification, examples, and appended claims are provided. The definitions are not meant to be limiting in nature and serve to provide a clearer understanding of certain aspects of the present invention.

We hypothesized that host proteins that play a significant role in the HIV-1 virion lifecycle or those that significantly affect HIV spread through the host would be conserved in the virus regardless of the progenitor cell type. HIV-1 infects multiple cell types, most prominently macrophages and CD4+ T cells. As these cell types have different protein expression patterns and surface protein composition, it is expected that HIV-1 virions budding from these different cell types carry different sets of host proteins. Further, it is likely that many of the proteins incorporated by the virus are done so through secondary or higher interactions. Here, we define a minimal set of relevant host proteins that are incorporated into HIV-1 virions from multiple cell types.

Mass spectrometry (MS) analysis of purified viral particles is one tool for determining which host proteins are incorporated in HIV virions on a global scale. While there are biochemical and proteomic techniques that can be used to identify HIV-associated proteins, the success of these studies are limited by HIV purification techniques that often result in co-purification of contaminating microvesicles.(10, 11) HIV virions are small, dense particles of approximately 100 nm in diameter; HIV-infected cells produce microvesicles of similar size and density to that of the HIV virion, which have also been shown to share many of the components of HIV.(12) A variety of

techniques have been employed to reduce microvesicle contamination, including CD45 depletion of microvesicles and affinity purification using viral envelope proteins.(13) Due to the large quantities of virions needed, affinity purification of virions or depletion of microvesicles is not a practical option for many biochemical studies.

5 Therefore, we developed a novel strategy for purifying large quantities of HIV-1. By using cholesterol to manipulate the density of the particles in HIV-1 preparations (density modification; DM), we were able to separate virions from contaminating vesicles by centrifugation, making them suitable for analysis by tandem MS. Using this strategy, we were able to characterize host protein incorporation in HIV particles derived from
10 CD4+ T cell lines. We compared our DM purification method with an orthogonal purification approach using inoxidol (OptiPrep) gradients by quantitative proteomics. Lastly, we tested our hypothesis that conserved proteins would reveal critical shared pathways by comparing our dataset of T cell derived HIV-1 virions to a reprocessed dataset of monocyte-derived macrophages (MDM) derived HIV-1 using the same
15 bioinformatics pipeline.

We identified clusters of conserved proteins between MDM and T-cell derived HIV-1. These clusters included an extensive collection of actin isoforms and other core interacting proteins, many of which have previously been documented to interact with viral proteins. (14, 15) These data suggest that a limited number of viral-host protein
20 interactions can explain the phenotypic diversity of HIV-1 virions produced from MDM or T-cells allowing HIV-1 to be incredibly plastic and opportunistic in its final protein composition depending on the cell type it is produced from. The common incorporation of syntenin-1 (a component of tetraspanin enriched membranes, TEMs) and CD44 (hyaluronic acid receptor) is suggestive of a common cellular egress pathway involving
25 TEMs and a common vesicle population that targets hyaluronic acid enriched microenvironments.

Using a novel HIV purification assay we have found a common set of host proteins that are incorporated into virions produced from monocyte-derived macrophages (MDMs) and T cells. DM purification modifies the density of microvesicles, allowing for the
30 purification of large quantities of microvesicle-free viral stocks. This method may not be necessary for virion purification from infected MDMs, since these cells have longer half-lives than T cells, allowing for higher virion yields, and MDMs produce a lower level of

contaminating microvesicles compared to lymphocytes.(2, 10, 11) We found that DM clears >90% of microvesicles using CD45 as a marker protein (by densitometry, not shown). We compared this method to the OptiPrep (60% iodixanol) method for microvesicle-free HIV-1 purification.(16) Results were normalized by CypA, as CypA has been reported to be incorporated into viruses in an approximately 1:10 ratio to gag particles.(7, 8) While using a viral protein for normalization might seem like an adequate normalization tool, viral protein sequence divergence and differential processing by progenitor cell type make it difficult to normalize by these proteins. Both OptiPrep and DM purification methods produce consistent results, reducing levels of proteins known to be incorporated in microvesicles. DM purification proved to be a more stringent approach, as there was a greater reduction in CypA levels coupled with a higher number of significantly reduced proteins compared to OptiPrep methods. Ott and colleagues developed a purification technique based on similar principles, in which proteins in microvesicles are digested with the nonspecific serine protease subtilisin.(14) The subtilisin digestion decreases microvesicle density, allowing for purification of HIV particles by density gradients, allowing for >95% purification of virions.(13) Subtilisin treatment digests membrane proteins, though, and is only suitable for determining the composition of proteins inside the virion.(14)

We cannot rule out that DM purification also modifies viral composition in some manner, as cholesterol has been reported to be an integral component of the viral membrane, and that this may account for some of the protein reduction.(17, 28) Notably, electron micrographs of DM-treated virions show some membrane irregularities, which could impact protein composition of the purified viral stocks. This may explain the absence of some host membrane proteins which have been reported to be incorporated into HIV virions. Of note, tetraspanin proteins were not detected in our analysis. This may not be surprising, though, given that tetraspanin interactions are affected by cholesterol and the DM assay may have disrupted TEMs.(37) It is of note that very few membrane-bound proteins were observed in the common set of proteins, particularly given that TEMs have been shown to be of importance in HIV-1 biology.(38-40) We did detect the PDZ-containing protein syntenin-1 in both MDM- and T cell-derived virions. Syntenin-1 has been shown to have a large variety of interaction partners, including syndecan, Rab 5, Rab 7, CD63, and phosphoinositol lipids. Many of the partners for syntenin-1 are involved in

membrane trafficking, including tetraspanin and TEM-associated proteins.(41, 42) Since TEM components are frequently reported in the viral envelope, but were not detected in this analysis, it is possible that syntenin-1 is involved in the HIV-1-TEM interaction, and that the syntenin-1-TEM interactions were disrupted by our purification process. This may
5 implicate syntenin-1 as an important mediator of viral envelope composition. However, preliminary data with siRNA knockdown of syntenin-1 in HIV-infected Jurkat cells has not demonstrated any effect on virion production (data not shown), so the importance of syntenin-1 in the HIV lifestyle remains speculative.

The incorporation of CD44, a receptor for hyaluronic acid, also provides potential
10 fitness benefits for a lentivirus. HIV replicates in activated T cells, so an attachment to hyaluronic acid may allow the virus to target areas of inflammation, as CD44 induction is a first step of immune activation and also involved in T cell trafficking.(43) Notably, it has been reported that CD44 cell-surface expression is lost in HIV-infected monocytic cell lines, resulting in cell aggregation.(44)

Using quantitative proteomic analyses on DM-purified input, we were able to
15 differentiate between viral stocks prepared from the H9 T cell line and the CEMx174 B cell/T cell hybrid line. There were several proteins which could differentiate between the two stocks, including the B cell activation marker CD48 precursor, which is not unexpected considering the cell line origins(36). These results indicate that proteomic
20 analysis in combination with several purification techniques can be used to differentiate viral stocks from multiple sources. These methods may be applicable to identifying the cell source of virus produced from latently infected cells.

We further compared DM purified viral stocks from H9 cells to a published
25 database of MDM-derived viral stocks. To increase the validity of this comparison, raw data from the Chertova study collected using similar MS instrumentation employed in the current study was reanalyzed using the bioinformatics pipeline developed by our group. This method ensured that both datasets were analyzed using the same stringent search criteria. Using this comparison, we identified a common set of 25 proteins that are
30 incorporated into HIV virions produced in both MDM and T-cell lines. As these proteins are incorporated in virions produced in both cell types, we hypothesize that these proteins may have direct interactions with viral proteins or may be important in the viral life cycle.

The base host protein composition in virions is mainly actin, chaperones, CypA and a handful of other proteins. Many of these have been shown to have functional impact on the HIV lifecycle. The importance of CypA on the viral life cycle has been well documented.(45) CypA is believed to regulate the capsid interaction with host factors, either during uncoating or during other lifecycle processes.(46) The protease α 1-antitrypsin (SERPINA1; AAT) blocks protease cleavage of gp160 and gag polyprotein. Thus, it is not surprising that HIV protease binds and cleaves AAT, and this binding may account for the incorporation of AAT in HIV particles.(47-49) Our results suggest that HIV-1 virions have evolved to target a pathway enriched in ERM family proteins and vesicle trafficking, based on the conserved incorporation of EHD4 in T cell and MDM derived virions. EHD4 has been shown to regulate transport from the early endosome to the recycling endosome and the late endocytic pathway.(50, 51) EH domains bind Rab proteins, which are known to interact with the HIV protein rev.(52) A recent study has suggested a role of HSP90AB in HIV replication. Inhibition of HSP90AB resulted in anti-HIV activity in vitro, with ritonavir-resistant viruses showing hypersensitivity to the inhibitor.(53) Another agent with anti-HIV activity was found to bind HSP90AB and prevent dimerization.(54) Annexin A2, a protein involved in membrane trafficking, likely bridges the gap between the cytoskeletal proteins and the viral membrane. Annexin A2 binds HIV gag and siRNA knockdown of the protein can reduce infectivity of virions generated in MDMs, but this may be cell type dependent.(55-57)

Other identified proteins, including cytoskeletal and HLA proteins have repeatedly been reported in the literature as interacting with HIV proteins.(2, 14, 58, 59) Further, many of the proteins identified in this study, including actin, 2',3'-cyclic-nucleotide 3'-phosphodiesterase, CypA, EEF1A-1, ezrin, annexin 2, HSP70, and HSC71, have also been identified in a quantitative proteomic analysis of an HIV-1 lentivirus vector produced in 293T cells.(60) Thus, this common set of proteins identified in this study is recapitulated by findings by other groups.

Given the large number of host proteins incorporated into the virus and the limited number of viral proteins, it is plausible that only a few specific interactions between virus and host proteins allows it to package a large array of host proteins. Host proteins that have a direct interaction with HIV proteins would serve as protein hubs. The 25 proteins identified to be common to the two different cell types are predicted to interact with >1000

related human proteins. Many of these secondary interacting proteins are commonly reported to be incorporated into virions, including ERM proteins and adhesion molecules. By assigning query based network weights (GeneMANIA, see methods), these associational proteins are predicted to interact with 62% and 38% of the proteins common to T cell and MDM derived HIV-1 respectively. However, it is important to note that many more T-cell derived proteins were identified than MDM HIV-1 derived proteins, so this may skew this analysis. Additionally, protein prediction network algorithms are based on protein-protein interactions; other interactions (e.g., lipid or nucleic acid mediated) are not modeled. Thus, only a few direct interactions within the virus may dictate the host protein composition in nearly limitless dimensions. Ultimately, the host protein composition, as well as interaction differences between cell types, may drive virion phenotypic diversity, despite conserved viral protein-host protein interactions between cell types (FIG. 2). While we do not intend to minimize the functional importance of other host proteins incorporated into HIV-1 outside of this minimal set of proteins, it is likely that therapeutic strategies targeting proteins other than these core proteins would result in limited efficacy due to the high degree of plasticity apparent with HIV. Therefore, we would propose that therapeutic or drug development efforts targeting host-virus interactions be focused on interacting proteins showing direct interaction with HIV proteins that are conserved between T cells and macrophages.

Finally, this study demonstrates the critical nature of harmonized data analysis when making inter-study protein comparisons. Existing studies have demonstrated the lab-to-lab and instrument-to-instrument variability in proteomics studies of identical samples, as well as search results from different search algorithms.(61, 62) Our re-analysis of the historical data from Chertova et al using current FDR-driven statistical analysis resulted in a truncated list of virally incorporated host proteins, comparable to what we observed experimentally in our work. This demonstrates the need for archiving of instrument raw data files so they may be subject to reinterpretation as bioinformatics improvements are developed, and highlights the danger of making protein comparisons from tables in the published literature, particularly with regard older data that was not filtered by FDR or another stringent statistical measure.

In summary, using a proteomic analysis approach, this study identifies proteins that are incorporated into the virus in multiple cell types, and many of these proteins have been

shown to be relevant to the HIV life cycle. These proteins may represent important conserved interactions and, therefore, could be targets for interventional strategies.

Strategies for intervention typically include highly active antiretroviral therapy (HAART). A number of HAART regimens are presently used for therapy, alone or in combination, including Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Protease Inhibitors (PI), and/or non-nucleoside reverse transcriptase inhibitors (NNRTIs). In some embodiments, the subject is being treated with a regimen that includes 2 NRTIs plus an NNRTI and/or a PI. In some embodiments, the patient's treatment includes an additional drug or drugs that targets viral integration into genomic DNA (i.e., an integration inhibitor, e.g., raltegravir). The present methods can include the intensification of the patient's treatment by the addition of an additional drug or drugs that inhibits entry of the virus into cells (entry inhibitors).

NRTIs are nucleoside and nucleotide analogs that replace the normal endogenous nucleotides/nucleosides, preventing the reverse transcriptase from transcribing viral RNA. Exemplary NRTIs include COMBIVIR (zidovudine + lamivudine, GlaxoSmithKline), EMTRIVA (emtricitabine, Gilead Sciences), EPIVIR (lamivudine, GlaxoSmithKline), EPZICOM (abacavir + lamivudine, GlaxoSmithKline), RETROVIR (zidovudine, GlaxoSmithKline), TRIZIVIR (abacavir + zidovudine + lamivudine, GlaxoSmithKline), TRUVADA (tenofovir DF + emtricitabine, Gilead Sciences), VIDEX & VIDEX EC (didanosine, Bristol-Myers Squibb), VIREAD (tenofovir disoproxil fumarate (DF), Gilead Sciences), ZERIT (stavudine, Bristol-Myers Squibb), ZIAGEN (abacavir, GlaxoSmithKline), RACIVIR (Pharmasset), amdoxovir (RFS Pharma), apricitabine (Aveixa Limited), and elvicitabine (Achillion Pharmaceuticals).

PIs inhibit the activity of the HIV protease, preventing the production of functional viral particles. Exemplary PIs include AGENERASE (amprenavir, GlaxoSmithKline and Vertex), APTIVUS (tipranavir, Boehringer Ingelheim), CRIXIVAN (indinavir, Merck & Co.), INVIRASE (saquinavir, Hoffmann-La Roche), KALETRA (lopinavir + ritonavir, Abbott Laboratories), LEXIVA (fosamprenavir, GlaxoSmithKline), NORVIR (ritonavir, Abbott Laboratories), PREZISTA (darunavir, Tibotec), REYATAZ (atazanavir, Bristol-Myers Squibb), and VIRACEPT (nelfinavir, Pfizer).

NNRTIs bind to reverse transcriptases and prevent the transcription of viral RNA. Exemplary include INTELENCE (etravirine, Tibotec), RESCRIPTOR (delavirdine, Pfizer),

SUSTIVA (efavirenz, Bristol-Myers Squibb), VIRAMUNE (nevirapine, Boehringer Ingelheim), and rilpivirine (Tibotec).

Entry inhibitors (also known as fusion inhibitors) target the gp120 or gp41 HIV envelope proteins, or the CD4 protein, or CCR5 or CXCR4 receptors on a CD4 cell's surface.

5 A number of entry inhibitors are known in the art, e.g., enfuvirtide (Trimeris and Hoffmann-La Roche, targets gp41); maraviroc (SELZENTRY, Pfizer, targets CCR5); vicriviroc (Schering-Plough Corporation, targets CCR5); PRO 140 (progenies Pharmaceuticals, targets the CD4 protein); Pentafuside (T-20, Hoffman-LaRoche/Trimeris, targets gp41); T-1249 (Hoffman-LaRoche/Trimeris, targets gp41); and NBD-556 and analogs thereof (Madani et
10 al., 16 STRUCTURE 1689-1701 (2008). Additional entry inhibitors include the CXCR4 antagonists AMD 3100 (Johnson Matthey/Anormed Pharmaceuticals), T22 (Seikagaku Pharmaceuticals), and ALX40C-4C (Allelix Pharmaceuticals), and the natural CCR5 ligands (e.g., MIP-1 α ., MIL-1 β , and RANTES), and analogs thereof (e.g., TAK-779 and AOP-RANTES).

15 Inhibitory antibodies targeting the various proteins, e.g., antibodies that specifically bind gp120, gp41, CD4, CCR5, or CXCR4, can also be used, e.g., TNX-355 (Tanox, Inc., targets the CD4 protein).

Dosages, specific formulations, and routes of administration of HIV antiviral drugs are known in the art. See, e.g., Physician's Desk Reference, 63rd edition (Medical Economics
20 Company, Montvale, N.J., 2009); Panel on Antiretroviral Guidelines for Adult and Adolescents. "Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents." Department of Health and Human Services. Nov. 3, 2008; pp 1-139 (available at aidsinfo.nih.gov/ContentFiles/AdultandAdoleacentGL.pdf); and Kuritzkes et al. (1999. AIDS 13:685-694).

25 Without further elaboration, it is believed that one skilled in the art, using the preceding description, can utilize the present invention to the fullest extent. The following examples are illustrative only, and not limiting of the remainder of the disclosure in any way whatsoever.

EXAMPLES

30 The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, articles, devices, and/or methods described and claimed herein are made and evaluated, and are

intended to be purely illustrative and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for herein. Unless indicated otherwise, parts are parts by weight, temperature is in degrees Celsius or is at ambient temperature, and pressure is at or near atmospheric. There are numerous variations and combinations of reaction conditions, e.g., component concentrations, desired solvents, solvent mixtures, temperatures, pressures and other reaction ranges and conditions that can be used to optimize the product purity and yield obtained from the described process. Only reasonable and routine experimentation will be required to optimize such process conditions.

Materials and Methods

Apparatus. MALDI-MS and MS/MS spectrum were obtained using an ABI 5800 MALDI TOF/TOF analyzer (AB Sciex) using a 2 KeV extraction method with CID turned off using dynamic exit.

Reagents. HIVMNC1.4 from either H9 (T-cell line) or CEMx174 (B-cell/T-cell hybrid line) cells was obtained from the AIDS and Cancer Vaccine Program (SAIC-Frederick).

Virus Purifications. HIVMNC1.4 from either H9 (T-cell line) or CEMx174 (B-cell/T-cell hybrid line) cells was obtained from the AIDS and Cancer Vaccine Program (SAIC-Frederick). DM purification was accomplished by incubating virus in 420 µg/ml of cholesterol and 20 mM 2-hydroxy-beta cyclodextrin (βCD) in TNE as indicated, filtration through a 5 µm filter on ice and pelleted through 20% sucrose for 1h at 100K x g. OptiPrep purification was performed as previously described.(16) SDS page, western blotting and EM were performed as described.(17)

Quantitative MS. Virus (normalized by p24) was ultracentrifuged and resuspended in 0.5 M triethylammonium bicarbonate with 1% rapigest, reduced (TCEP) and alkylated (MMTS) and subjected to tryptic digestion as previously described.(18) Peptides were labeled with iTRAQ reagents as follows for HIV-1 derived from CEMx174 cells: m/z 113: Control, m/z 114: DMP, m/z 115: OptiPrep, and for HIV-1 derived from H9 cells: m/z 117: Control, m/z 118: DMP, m/z 115: OptiPrep. Peptides were then subjected to nano rHPLC on a TEMPO-LC MALDI spotting system using a 90 minute gradient from 5% to

80% B (98 % ACN, 0.1 % TFA) at 500 $\mu\text{l min}^{-1}$, Matrix (CHCA, 5 mg / mL in 75 % ACN) was then supplemented to the flow post-column at 500 $\mu\text{l min}^{-1}$, and samples were deposited onto a stainless steel plate at 10 second intervals. MALDI-MS and MS/MS spectrum were obtained using an ABI 5800 MALDI TOF/TOF analyzer (AB Sciex) using a 2KeV extraction method with CID turned off using dynamic exit. ProteinPilot 3.0 was used to search UniProt-SwissProt with contaminants appended (2007.01.23; 254,765 sequences) with peptide threshold of 99.9%, and fixed modifications of iTRAQ (K, N-term), MMTS (C). Due to variable processing of gag, we subsequently normalized virus from different treatments and lines using cyclophilin A, a known Gag interacting host protein(7) using the iTRAQ reporter bias correction feature built into protein pilot.

Tandem Mass Spectrometry. 500 μg of capsid equivalents of DM purified HIV-1MNC1.4 /H9virus was desalted and subjected to reverse phase HPLC analysis on a Beckman PF2D system as previously described into 37 fractions, digested and Tandem MS performed as described.(19) Briefly, ESI-MS/MS of tryptic peptides was performed on an LCQ-ion trap-MS/MS instrument using a 60 minute gradient as previously described.(20) Data were acquired using Xcalibur 2.07 (Thermo, San Jose, CA). The three most intense ions (minimum signal of 100,000 ions) were selected for MS/MS fragmentation using a normalized collision energy of 35. Dynamic exclusion was applied for 30 seconds after 1 MS/MS acquisition, with a mass window of 2 Da.

Comparison between MDM derived virus and T cell HIV-1. 17 raw files for the analysis of MDM derived virus from a study published by Chertova et al.(2) were obtained from the authors and analyzed in parallel with 37 DM purified fractions of HIV-1/H9. Briefly, peaks were selected and de-isotoped using DeconMSn for MDM derived LTQ data and using ReAdW (2009v) for LCQ derived HIV-1/H9. The data were then searched using PepArML(21), which uses multiple different search algorithms (OMSSA, X!Tandem with native, k-score and s-score scoring, MASCOT, MyriMatch, and InSpecT) as previously described. (22) Carbamidomethylation was set as a fixed modification and oxidized methionine was set as variable modification. Mass tolerances on precursor and fragment ions were set at 1.5, and .8 Da, respectively and missed cleavage as 1 using a specific search. The database used for search was the UniProt- SwissProt database (version 2010.11.02; 522,019 sequences. Peptides were combined on PepARML using a

random forest approach (Weka) and the results were then parsed into MASPECTRAS 2.0(23) with minimum 2 peptide for a protein and a spectrum false discovery rate of 5%. Peptides assigned to keratin were excluded, and since the analysis was focused on host proteins, viral peptide assignments were excluded. Protein redundancy was removed by MS based evidence clustering.(23) The data analysis pipeline meets all MIAPE standards(23) and raw data and the FASTA database has been uploaded to Tranche (<https://proteomecommons.org/tranche>).

HIV Protein Interactions and Network/Pathway Analysis. Network and pathways analysis was performed using the GeneMANIA gene network tool, which contains 353 human interaction networks based on data from BIND, IntAct and other interaction databases, using association data from protein and genetic interactions, known and predicted pathways, co-expression, co-localization and protein domain similarity.(24) Our analysis was performed using only protein physical interaction data from GeneMANIA with GeneMANIA Cytoscape plugin. Identified host proteins were searched in the HIV-1, Human Protein Interaction Database for reported interactions in the literature.(25-27)

Results

Example 1: DM Virion Preparations Allows for the Separation of Virus from Microvesicles. The DM purification method is based upon our previous studies using beta-cyclodextrin to manipulate cholesterol in HIV-1 particles. (17, 28) As viral purification through a 20% sucrose gradient results in co- purification of HIV and microvesicles of similar density, we differentially modified microvesicle and virion density by adding excess cholesterol to purified viral stocks. Cholesterol is differentially incorporated into microvesicles and virions, resulting in density changes that allow for the separation of highly purified virions from microvesicle contaminants. DM HIV-1 purity was assessed using CD45, a well-defined marker of vesicle contamination, resulting in a >90% reduction of material and elimination of microvesicles (FIG.s 1A-C). DM HIV-1 had a <1 log infectivity decrease (not shown) and virion morphology was substantially altered (FIG. 1D).

To rule out artifact, we validated DM purified samples with an alternative purification method (OptiPrep; 60% iodixanol).(16) The relative abundances of proteins by quantitative proteomics (iTRAQ) from both samples were compared against virus pelleted through a 20% sucrose gradient. Virus preparations were carefully normalized by

capsid protein (p24) by ELISA, and subsequently validated by SDS-PAGE (data not shown) prior to digestion with trypsin and labeling with iTRAQ reagents.

In an iTRAQ experiment, post-hoc corrections for the relative abundance of reporter ions are made to ensure that no single reporter is over-represented in the data analysis.⁽²⁹⁾ This can occur for a multitude of reasons, including variations in manufacturing of reagents or sample preparation conditions during labeling. Recently Brietwieser and colleagues showed that iTRAQ reporter intensities are valid over one log of dynamic range.⁽²⁹⁾ In this study, we manually adjusted iTRAQ reporter bias to ensure that no purification method resulted in a reporter ratio >1 when compared to the control preparation, as protein can only be depleted in the purification process.

For comparisons between viruses (CEMx174 vs. H9 derived HIV-1), we initially attempted to adjust iTRAQ reporter bias based upon spectra assigned to HIV-1 capsid protein (p24). However, p24 resulted in unreliable bias estimation secondary to the extreme sequence divergence of HIV capsid protein and improperly assigned viral peptides by ProteinPilot. Instead, we investigated the use of spectra assigned in control preparations to the host protein cyclophilin A (CypA). The host protein CypA has been reported to be included in virions⁽⁷⁾, but has not been reported to be present in microvesicles except under conditions of extreme cellular stress, such as cellular irradiation.^(30, 31) CypA incorporation has also been reported to be important for maximal HIV infectivity and it has been suggested that the absence of CypA incorporation leads to HIV restriction.⁽⁸⁾ HIV recruits CypA to ~10% of its capsid monomers in newly assembled cores and the CypA binding site on capsid is highly conserved in all primate lentiviruses.^(7, 8) We therefore adjusted iTRAQ reporter bias using CypA peptides, whilst ensuring that the most abundant protein was normalized to a 1:1:1 ratio between different preparations. Indeed, this method did not violate our rule of host proteins in the purification groups being less than control. Final adjustments were minor and accounted for a 25% decrease in CypA for CEMx174 derived HIV-1 and a 35% decrease in CypA for H9 derived HIV-1 using DM purification. Comparatively, a 10% and 40% decrease in CypA were observed using OptiPrep purification for CEMx174 and H9 derived HIV-1, respectively. These results suggest that CypA may indeed be present in microvesicles induced by HIV-1 infection, like other forms of cellular stress.

For virions produced in either H9 or CEMx174 cells, we observed a decrease in protein abundance for both DM and OptiPrep purified virions compared to control methods. DM purification significantly reduced the abundance of 34 proteins for CEMx174-derived HIV-1 virions, whereas OptiPrep purification resulted in significant reductions of 8 proteins (Table 1). Similar results were observed for H9 cells (data not shown). Many proteins that were reduced in quantity for either DM or OptiPrep purification have been shown to be in microvesicles;(32-34) the greater reduction in proteins using DM purification suggests that this method is a more stringent purification measure than OptiPrep purification. However, many of the reduced proteins have also been shown to be incorporated into HIV virions and we cannot rule out the loss of a subset of viral particles in either purification method. (12)

Table 1. Reduced proteins in HIV-1 derived from CEMX174 cells following virion purification using DM or Optiprep.

% of Sequence Coverage	Accession Number	Protein Name	Peptides (95%)	DM: Control	<i>P-val</i>	Optiprep: Control	<i>P-val</i>
42.9	P63104	14-3-3 protein zeta/delta	6	0.5151	0.0371	0.7533	0.0613
56.5	P63261	Actin, cytoplasmic 2	29	0.5099	0.0013	0.92	0.1083
41	P06733	Alpha-enolase Homo sapiens (Human)	9	0.5058	0.0001	0.7502	0.0038
33.3	P07355	Annexin A2	9	0.4459	0.0015	0.8047	0.0188
61.2	P80723	Brain acid soluble protein 1	7	0.523	0.0409	0.7312	0.0264
23.5	P09326	CD48 antigen precursor (B-lymphocyte activation marker BLAST-1)	5	0.4887	0.005	0.949	0.5258
41.5	O00299	Chloride intracellular channel protein 1	5	0.5008	0.0012	1.0572	0.5918
28	P31146	Coronin-1A	4	0.5112	0.0003	0.8208	0.011
30.1	P68104	Elongation factor 1-alpha 1	8	0.5466	0.0006	0.9394	0.4253
50	P15311	Ezrin (p81)	12	0.5889	<0.0001	0.8771	0.0974
26.3	P04406	Glyceraldehyde-3-phosphate dehydrogenase	3	0.4113	0.0231	0.8528	0.5458
51.2	P11142	Heat shock cognate 71 kDa protein	18	0.5481	0.0042	0.9216	0.5634
29.1	P08238	Heat shock protein HSP 90-beta	8	0.5316	0.003	1.0241	0.8397
46.6	P30453	HLA class I histocompatibility antigen, A-34 alpha chain precursor	14	0.6886	0.0117	0.9655	0.7305

31.1	P01903	HLA class II histocompatibility antigen, DR alpha chain precursor	7	0.4474	0.007	0.9419	0.4304
52.6	P13760	HLA class II histocompatibility antigen, DRB1-4 beta chain precursor	7	0.5259	0.0458	0.9599	0.7407
41.7	P13761	HLA class II histocompatibility antigen, DRB1-7 beta chain precursor	7	0.5595	0.001	0.9605	0.6705
25.9	P05362	Intercellular adhesion molecule 1 precursor	3	0.5685	0.0489	0.8179	0.1331
57.5	P26038	Moesin	19	0.5428	0.0012	0.9082	0.0732
18.4	Q09666	Neuroblast differentiation-associated protein AHNAK	2	0.4589	0.0143	0.7738	0.025
19.2	P43007	Neutral amino acid transporter A	5	0.3368	0.0098	0.8818	0.5752
32.2	Q06830	Peroxiredoxin-1	5	0.3815	0.0157	0.7814	0.0282
23	P13796	Plastin-2 (L-plastin)	4	0.5221	0.0042	0.8782	0.1899
16.4	Q8WUM4	Programmed cell death 6-interacting protein	2	0.5772	0.0397	0.8987	0.5737
16.2	P30101	Protein disulfide-isomerase A3 precursor	1	0.4702	0.0423	0.7946	0.3432
23.4	P14618	Pyruvate kinase isozymes M1/M2	5	0.5098	>0.0001	0.926	0.2772
26.6	P15153	Ras-related C3 botulinum toxin substrate 2 precursor	2	0.5057	0.0433	0.963	0.811
24.5	P62834	Ras-related protein Rap-1A precursor	1	0.4817	0.0063	0.7928	0.0465
81.8	P62328	Thymosin beta-4	3	0.4805	0.0215	1.0323	0.7312
31.6	P61586	Transforming protein RhoA precursor	2	0.4166	0.0123	0.9393	0.5786
37.1	P67936	Tropomyosin alpha-4 chain	3	0.506	0.0248	0.8225	0.3176

17.6	Q9BQE3	Tubulin alpha-6 chain	3	0.4275	0.0074	0.8795	0.2247
72.4	P62988	Ubiquitin	3	0.4191	0.0037	0.9396	0.5418

Example 2: iTRAQ Analyses Can Differentiate HIV-1 Virions Derived from a T Cell line and a B Cell/T Cell Hybrid Cell Line. Viral stocks produced from different cell lines displayed unique phenotypes, and virion composition reflected the progenitor cell type. DM purified viral stocks derived from CEMx174 and H9 cells were compared. Table 2 shows that 15 proteins can be used to differentiate between the cell lines. Notably, virions produced from CEMx174, which is a T-cell/B-cell hybrid,(35) contained higher levels of CD48 antigen precursor, a marker of B-cell activation.(36)

Table 2. Proteins able to differentiate between HIV-1 derived from CEMX174 or H9 cells

% of Sequence Coverage	Accession No.	Protein Name	Peptides (95%)	CEM:H9	P value*
61.2	P80723	Brain acid soluble protein 1	7	6.1243	0.042
23.5	P09326	CD48 antigen precursor	5	1.8935	0.0377
45.8	P23528	Coffin-1	6	2.1352	0.0266
29.4	P04075	Fructose-bisphosphate aldolase A	4	2.2855	0.0409
33	P04899	G(i), alpha subunit	5	2.3068	0.0296
46.6	P30453	MHC class I antigen A*34	14	3.3001	0.0184
16.9	P04233	HLA class ii histocompatibility antigen gamma chain	2	4.2203	0.0346
47.9	P07737	Prolifin-1	4	2.4426	0.0169
23.4	P14618	Pyruvate kinase isozymes M1/M2	5	1.8639	0.016
21	P05023	Sodium/potassium transporting ATPase alpha-1 chain precursor	7	2.0799	0.0344
81.8	P62328	Thymosin beta-4	3	2.4172	0.0273
37.1	P67936	Tropomyosin alpha-4 chain	3	2.7548	0.0016
17.6	Q9BQE3	Tubulin alpha-6 chain	3	1.9302	0.0058
19.1	P07437	Tubulin beta chain	3	1.6285	0.0268

Example 3: Shotgun Analysis of DM Modified HIV-1 Identifies 283 Host Proteins.

While our iTRAQ experiments provided us with a powerful method of determining our relative purification efficiency, no multidimensional protein separation strategies were used for this experiment. Therefore, to extend our coverage of the DM HIV proteome, we performed HPLC separation of DM-HIV-1, and collected 37 fractions that were then

subject to analysis by MS/MS on LCQ-duo equipped with an Agilent nano-HPLC system. The resulting spectra were pooled and searched on our MS-analysis pipeline. We found that the peptides were assigned to >1800 proteins, including redundant assignments; these proteins clustered to 283 individual host proteins using a spectrum false discovery rate of 5%.

Example 4: MDM and T Cell-Derived HIV-1 Virions Incorporate a Limited Number of Shared Host Proteins. To determine which host proteins were incorporated to the virion both in T- and in macrophage-cell types, we compared our T cell dataset to the MDM-derived HIV-1 dataset generated by Chertova and colleagues.(2) To ensure that the datasets were comparable, the Chertova dataset was reanalyzed using our data analysis pipeline. 136 (38 clustered) proteins were unique to the macrophage derived virus, and 1339 (241 clustered) proteins were unique to the T-cell derived virus. 680 (79 clustered) proteins were shared between the MDM derived and T-cell derived dataset. Of the 79 common proteins, many of these proteins were isoforms of the same protein with different peptides identified (Table 3). The majority of these proteins were distinct actin isoforms. Other proteins of note were ERM proteins, the dynamin domain containing protein EH4, a phosphodiesterase, CypA and heat shock proteins. The only conserved membrane proteins identified were syntenin-1 (a TEM protein) and CD44 (hyaluronic acid receptor), a marker presently used in commercial kits to enrich HIV.

As the conserved set of proteins may represent important cellular partners for the HIV virion, we conducted a literature search to determine whether the identified host proteins have been reported to be relevant in the HIV lifecycle and interact with viral proteins (Table 4). Out of the 25 protein clusters reported, 15 have previously been described in association with HIV-1, and 10 represent previously undefined associations.

To determine if the conserved set of proteins between MDM and T-cell derived HIV-1 could be used to reconstruct the protein composition of each virus, we seeded the GeneMANIA human network database with the core set of proteins and allowed for the 1000 most-related interacting partners. We found that 29% and 53% of host proteins from T cell derived or MDM derived HIV-1, respectively, could be explained by primary interactions.

Table 3. Identified proteins common to MDM- or T-cell derived HIV-1 virions.

Gene Name	Gene Symbol	% of Sequence Coverage	No. of Unique Peptides	No. of Unique Spectra
2',3'-cyclic-nucleotide 3'-phosphodiesterase	CNP	11.17	5	7
6-phosphogluconate dehydrogenase, decarboxylating	Pgd	9.94	3	5
Actin (37 isoforms)	act1	12	827	8432
Alpha-1-antiproteinase	SERPINA1	32.22	17	38
Alpha-2-H	AHSG	25.35	9	16
Annexin A2	ANXA2	52.22	21	62
CD44 antigen	CD44	7.28	4	14
Cell division control protein 42 homolog	CDC42	36.65	6	26
EH domain-containing protein 4	EHD4	24.59	9	12
Elongation factor 1-alpha (4 isoforms)	eEF1a	10.9	6	78
Ezrin	EZR	18.44	9	23
Heat shock 70 kDa protein (11 isoforms)	HSPA	15.78	132	516
Heat shock cognate 71 kDa protein	HSPA8	39.63	26	126
Heat shock protein H	HSP90AB1	24.45	15	52
Hemoglobin fetal subunit beta	-	60.69	10	101
Hemoglobin subunit beta (3 isoforms)	HBB	27.9	4	18
HLA A (MHC class I)	HLA-C	19.13	10	39
HLA DR (MHC class II) (3 isoforms)	HLA-DRB1	21.06	5	28
Moesin	MSN	23.4	15	70
P (Ankyrin Repeat Containing)	POTEE	10.33	16	168
Peptidyl-prolyl cis-trans isomerase A (cyclophilin A)	PPIA	56.37	14	103
Phosphoglycerate kinase 1	PGK1	32.86	12	22
Pyruvate kinase isozymes M1/M2	PKM2	40.12	16	40
Ras-related C3 botulinum toxin substrate 2	Rac2	30.73	6	7
Syntenin-1	SDCBP	10.2	7	9
Ubiquitin (Fragment)	-	39.69	4	27

Table 4. HIV-1-human protein interactions reported in the literature.(25-27)

Host Protein	HIV Protein Interaction
2',3'-cyclic-nucleotide 3'-phosphodiesterase	Activated by: tat
Actin	Inhibited by (multiple isoforms): env Binds (multiple isoforms): gag, nef Interacts with (multiple isoforms): gag Associates with (multiple isoforms): nef rearrangement induced by (multiple isoforms): nef, tat Cleaves (multiple isoforms): pol Associates with (beta-actin): rev
Alpha-1-antiproteinase	Interacts with: env
Annexin A2	Colocalizes with, interacts with (isoform 1): gag Downregulated by (isoform 2): tat
CD44	Downregulates: vpr
Cell division control protein 42	Inhibited by, interacts with, upregulated by: nef

Eukaryotic translation elongation factor 1 alpha 1	Inhibited by: gag Binds: gag, pol Interacts with: tat
Ezrin	Binds: env Interacts with: env, gag Incorporates: gag Upregulated by: vpr
heat shock protein 70kDa	Interacts with (protein 5), upregulated by (multiple proteins), inhibits (multiple proteins): env Incorporated by (multiple proteins), stimulates (multiple proteins), inhibits (protein 8): gag Regulates (multiple proteins): tat Inhibits (protein 1a), binds (protein 1a), competes with (multiple proteins): vpr
MHC Class I	Interacts with, complexes with: env Binds: env, gag, nef, pol Upregulated by: env, tat Colocalizes with, inhibited by, modulated by: nef Downregulated by: nef, tat, vpu
MHC Class II	Associates with, incorporated by: env Upregulated by: env, tat Inhibited by, interacts with: env, nef Colocalizes with, relocalizes, relocalized by: gag Downregulated by: gag, nef, tat
Moesin	Binds, relocalized by: env Incorporated by: gag
Peptidyl-prolyl cis-trans isomerase A (cyclophilin A)	Inhibited by, required by: env Incorporated by, modulates, interacts with, stabilized by: gag Binds: gag, nef, vif Isomerizes: gag, vpr
Ras-related C3 botulinum toxin substrate	Interacts with: nef Activated by, downregulated by: tat
Syntenin-1	Upregulated by: env
Ubiquitin	Ubiquitinates: gag, rev, tat

No interactions have been reported for 6-phosphogluconate dehydrogenase, decarboxylating; Alpha-2-H; EH domain-containing protein 4; HSP90AB; Hemoglobin fetal subunit; HBB; P (Ankyrin Repeat Containing); Phosphoglycerate kinase 1; Pyruvate kinase isozymes M1/M2

5 *Example 5: Host proteins identified in neurotropic/neurovirulent strains of SIV.* To extend our initial studies examining T-cell versus Macrophage Tropic viruses, we have now examined known neurotropic/neurovirulent strains of SIV. Table 5 shows the host proteins that were identified in these virions by mass spectrometry. Importantly, CD44 was identified in these viruses as well, supporting our claims that there are conserved sets of host proteins
10 incorporated into viruses from all cell lines studied. CD20 also identified the cell line that these viruses were produced from (CEMx174 cells, a B-cell-T-cell hybrid cell line). Thus, by

identifying cell type specific markers that are enriched, with a combination of proteins, we can identify the source of HIV/SIV production.

Table 5

<i>Name</i>	<i>Pep (95%)</i>
40S ribosomal protein S16 - Aplysia californica (California sea hare)	1
60S acidic ribosomal protein P0 - Homo sapiens (Human)	2
60S ribosomal protein L13 - Homo sapiens (Human)	2
60S ribosomal protein L6 - Homo sapiens (Human)	3
A2M protein - Bos taurus (Bovine)	1
Alpha 2-tubulin - Laodelphax striatellus (small brown planthopper)	5
ARHA protein - Homo sapiens (Human)	2
B2M protein - Homo sapiens (Human)	2
Beta 5-tubulin - Macaca mulatta (Rhesus macaque)	5
Brain cDNA, clone: QccE-15075, similar to human glucosidase, alpha; neutral AB - Macaca fascicularis (Crab eating macaque) (Cynomolgus monkey)	1
Brain cDNA, clone: QflA-13958, similar to human ribosomal protein L18 - Macaca fascicularis (Crab eating macaque) (Cynomolgus monkey)	1
Calnexin - Homo sapiens (Human)	1
CALR protein - Homo sapiens (Human)	3
CD20 - Callithrix jacchus (Common marmoset)	2
CD44 molecule - Homo sapiens (Human)	5
CD48 protein - Homo sapiens (Human)	3
CDNA FLJ45706 fis, clone FEBRA2028457, highly similar to Nucleolin - Homo sapiens (Human)	4
cDNA FLJ75881, highly similar to Homo sapiens transferrin receptor - Homo sapiens (Human)	1
cDNA FLJ75977, highly similar to Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide - Homo sapiens (Human)	3
cDNA FLJ76178, highly similar to Homo sapiens ATP synthase, H ⁺ transporting, mitochondrial F1 complex, beta polypeptide - Homo sapiens (Human)	6
cDNA FLJ76227, highly similar to Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide - Homo sapiens (Human)	2
cDNA FLJ76921, highly similar to Homo sapiens protein tyrosine phosphatase, receptor type, C - Homo sapiens (Human)	3
cDNA FLJ77524, highly similar to Homo sapiens prostaglandin E synthase 3 - Homo sapiens (Human)	1
cDNA FLJ77645, highly similar to Homo sapiens splicing factor, arginine/serine-rich 1 - Homo sapiens (Human)	1
cDNA FLJ77837 - Homo sapiens (Human)	2
cDNA FLJ77860, highly similar to Homo sapiens histone 2, H2be - Homo sapiens (Human)	10
cDNA FLJ77950, highly similar to Homo sapiens alpha-2-HS-glycoprotein - Homo sapiens (Human)	2
cDNA FLJ78244, highly similar to Homo sapiens eukaryotic translation initiation factor 4A, isoform 1 - Homo sapiens (Human)	1
cDNA FLJ78336, highly similar to Homo sapiens peptidylprolyl isomerase A - Homo sapiens (Human)	3
cDNA FLJ78442, highly similar to Homo sapiens phosphoglycerate kinase 1 - Homo sapiens (Human)	4
cDNA FLJ78691, highly similar to Homo sapiens heat shock 90kDa protein 1, alpha - Homo	4

sapiens (Human)	
CDNA: FLJ21717 fis, clone COL10322 - Homo sapiens (Human)	3
Chaperonin containing TCP1, subunit 3 - Homo sapiens (Human)	1
Cofilin isoform - Homo sapiens (Human)	1
Coproporphyrinogen oxidase variant - Homo sapiens (Human)	2
Cytoplasmic actin - Oikopleura longicauda	24
DRB1 protein - Homo sapiens (Human)	5
Elongation factor 1-alpha - Homo sapiens (Human)	6
Enolase - Homo sapiens (Human)	9
Eukaryotic translation elongation factor 1 gamma - Homo sapiens (Human)	1
Fructose-bisphosphate aldolase - Macaca fascicularis (Crab eating macaque) (Cynomolgus monkey)	2
Glyceraldehyde 3-phosphate dehydrogenase - Homo sapiens (Human)	11
H2A histone family, member Y - Bos taurus (Bovine)	1
Hemoglobin, gamma - Bos taurus (Bovine)	7
Heterogeneous nuclear ribonucleoprotein C - Macaca fascicularis (Crab eating macaque) (Cynomolgus monkey)	3
Histone 1, H2bi - Bos taurus (Bovine)	13
Histone H2A - Homo sapiens (Human)	2
Histone H3 - Mesocapnia sp. BYU_PL010	5
Histone H4 - Nematostella vectensis (Starlet sea anemone)	16
HLA-DPA1 protein - Homo sapiens (Human)	2
HMG-1 - Homo sapiens (Human)	7
HSP90AB1 protein - Homo sapiens (Human)	3
HSPA5 protein - Homo sapiens (Human)	10
HSPA9 protein - Homo sapiens (Human)	1
HSPD1 protein - Bos taurus (Bovine)	4
Lectin, mannose-binding, 1 variant - Homo sapiens (Human)	1
LOC783373 protein - Bos taurus (Bovine)	1
LSP1 protein - Homo sapiens (Human)	1
Major histocompatibility complex, class II, DR alpha - Homo sapiens (Human)	4
MHC class I antigen - Homo sapiens (Human)	7
MHC class I antigen - Homo sapiens (Human)	6
MHC class I antigen - Homo sapiens (Human)	5
MHC class II antigen - Homo sapiens (Human)	2
MHC class II antigen - Homo sapiens (Human)	2
MHC class II HLA-DR-beta mRNA - Homo sapiens (Human)	7
Muscle-specific actin 3 - Aedes aegypti (Yellowfever mosquito)	13
Myosin-IG - Homo sapiens (Human)	2
Na+/K+-ATPase alpha 3 subunit variant - Homo sapiens (Human)	3
NCL protein - Homo sapiens (Human)	3
Non-muscle myosin heavy polypeptide 9 - Homo sapiens (Human)	3
OTTHUMP00000016411 - Homo sapiens (Human)	4
Pancreatic thread protein - Bos taurus (Bovine)	2
PC4 protein - Homo sapiens (Human)	2

Peptidyl-prolyl cis-trans isomerase - Homo sapiens (Human)	3
Peroxiredoxin 1 - Macaca fascicularis (Crab eating macaque) (Cynomolgus monkey)	1
Phosphoglycerate mutase 1 - Homo sapiens (Human)	2
PLEK protein variant - Homo sapiens (Human)	1
PLG protein - Bos taurus (Bovine)	10
Profilin 1 - Homo sapiens (Human)	2
Protein disulfide isomerase family A, member 3 - Papio anubis (Olive baboon)	1
Putative uncharacterized protein - Homo sapiens (Human)	3
Putative uncharacterized protein - Homo sapiens (Human)	4
Putative uncharacterized protein - Homo sapiens (Human)	1
Putative uncharacterized protein - Macaca fascicularis (Crab eating macaque) (Cynomolgus monkey)	5
Putative uncharacterized protein DKFZp459D1928 - Pongo pygmaeus (Orangutan)	10
Putative uncharacterized protein DKFZp459F231 - Pongo pygmaeus (Orangutan)	1
Putative uncharacterized protein DKFZp468I1928 - Pongo pygmaeus (Orangutan)	5
Putative uncharacterized protein DKFZp468J1717 - Pongo pygmaeus (Orangutan)	6
Putative uncharacterized protein DKFZp469B1212 - Pongo pygmaeus (Orangutan)	1
Putative uncharacterized protein DKFZp469L1516 - Pongo pygmaeus (Orangutan)	3
Putative uncharacterized protein DKFZp469O2211 - Pongo pygmaeus (Orangutan)	5
Pyruvate kinase - Homo sapiens (Human)	6
Ribosomal protein L1 - Homo sapiens (Human)	3
Ribosomal protein L10 - Homo sapiens (Human)	3
Ribosomal protein L15 - Homo sapiens (Human)	2
Ribosomal protein L18a variant - Homo sapiens (Human)	2
Ribosomal protein L32 - Ovis aries (Sheep)	2
Ribosomal protein L4 variant - Homo sapiens (Human)	2
Ribosomal protein L5 variant - Homo sapiens (Human)	2
Ribosomal protein L7a - Homo sapiens (Human)	2
Ribosomal protein S2 - Homo sapiens (Human)	3
Ribosomal protein S3A - Homo sapiens (Human)	2
Ribosomal protein S6 - Homo sapiens (Human)	2
Ribosomal protein S8 - Homo sapiens (Human)	3
Ribosomal protein S9 - Papio anubis (Olive baboon)	1
RPL14 protein - Homo sapiens (Human)	2
RPL7 protein - Homo sapiens (Human)	2
RPLP2 protein - Homo sapiens (Human)	2
Serine hydroxymethyltransferase - Homo sapiens (Human)	1
Serum albumin - Bos indicus (Zebu)	11
Similar to beta 2-microglobulin - Bos taurus (Bovine)	2
Similar to Drosophila melanogaster CG8415 - Drosophila yakuba (Fruit fly)	2
Similar to Elongation factor 2b - Homo sapiens (Human)	5
Similar to ribosomal protein L17 - Bos taurus (Bovine)	3
Similar to ribosomal protein S14 - Bos taurus (Bovine)	3
Similar to ribosomal protein S18 - Bos taurus (Bovine)	1
Similar to sp P51401 Saccharomyces cerevisiae RPL9B 60S ribosomal protein L9-B - Yarrowia	1

lipolytica (<i>Candida lipolytica</i>)	
SLC25A5 protein - <i>Homo sapiens</i> (Human)	2
Splicing factor - <i>Canis familiaris</i> (Dog)	1
Testis cDNA clone: QtsA-14092, similar to human histone 2, H2aa - <i>Macaca fascicularis</i> (Crab eating macaque) (<i>Cynomolgus</i> monkey)	5
Testis cDNA clone: QtsA-17287, similar to human kinesin family member 23 - <i>Macaca fascicularis</i> (Crab eating macaque) (<i>Cynomolgus</i> monkey)	1
Thymosin-like 4 - <i>Homo sapiens</i> (Human)	2
TRB@ protein - <i>Bos taurus</i> (Bovine)	39
Triosephosphate isomerase - <i>Homo sapiens</i> (Human)	2
Tubulin, beta 2C - <i>Homo sapiens</i> (Human)	5
Tumor necrosis factor - <i>Homo sapiens</i> (Human)	1
Tumor necrosis factor receptor superfamily member 5 - <i>Homo sapiens</i> (Human)	2
Tumor rejection antigen - <i>Homo sapiens</i> (Human)	3
Ubiquitin - <i>Ciona savignyi</i> (Pacific transparent sea squirt)	2
Uncharacterized protein ANXA6 - <i>Homo sapiens</i> (Human)	12
Uncharacterized protein MSN - <i>Homo sapiens</i> (Human)	8
Vitronectin - <i>Bos taurus</i> (Bovine)	8
Table 1. Proteins identified from neurotropic/neurovirulent viruses.	

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We claim:

1. A method for treating a patient having a human immunodeficiency virus type 1 (HIV-1) infection comprising the steps of:
 - 5 a. obtaining a biological sample from the patient;
 - b. performing an assay to isolate HIV-1 from the biological sample using host proteins that are incorporated into HIV-1;
 - c. performing an assay on the isolated HIV-1 to identify whether the patient has an X4 tropic, R5 tropic or dual tropic HIV-1 infection using host proteins that are
10 incorporated into HIV-1 and are unique to specific cell types that are infected by HIV-1; and
 - d. treating the patient based on the type of HIV-1 infection, wherein an X4 tropic infection is treated with a CXCR4 antagonist, an R5 tropic infection is treated with a CCR5 antagonist, and a dual tropic infection is treated with both a CXCR4 antagonist and a CCR5 antagonist.
- 15 2. The method of claim 1, wherein the host proteins that are incorporated into HIV-1 are one or more proteins listed in Table 3.
3. The method of claim 1, wherein the assay of step (b) uses an antibody that binds a
20 host protein that is incorporated into HIV-1.
4. A method for treating a patient having HIV-1 comprising the steps of:
 - a. obtaining a biological sample from the patient;
 - b. performing an assay to isolate HIV-1 from the biological sample using an
25 antibody that binds a host protein that is incorporated into HIV-1, wherein the host protein is one or more proteins listed in Table 3;
 - c. identifying the cell type(s) infected by HIV-1 using one or more antibodies to bind host proteins that are incorporated into HIV-1 and are unique to the cell type that is infected by HIV-1;
 - 30 d. using polymerase chain reaction to quantify the HIV-1 growing in the cell type(s) and/or identify the location of HIV-1 replication; and
 - e. treating the patient based on the infected cell type and location of infection.

5. A method for identifying an unknown reservoir of HIV-1 production in a host patient comprising the steps of:
- a. obtaining a biological sample from the patient;
 - 5 b. immunodepleting known sources of HIV-1 using one or more antibodies that bind to host proteins that are incorporated into HIV-1 and are unique to the cell type that is infected by HIV-1;
 - c. isolating the remaining HIV-1 using CD44 and/or syntenin-1; and
 - d. performing mass spectrometry to identify host protein that are incorporated
 - 10 into the HIV-1; and
 - e. using a tissue array to identify the cell type infected by HIV-1.
6. A method for determining cellular tropism of HIV comprising the steps of:
- a. performing an assay to capture HIV-1 from a sample using biomarkers
 - 15 common to HIV-1; and
 - b. performing an assay using biomarkers unique to specific cell types to identify the cell types from which HIV-1 has been produced.
7. The method of claim 6, wherein the assay in step (a) and/or (b) is affinity purification.
- 20 8. The method of claim 6, wherein the biomarkers common to HIV-1 comprise one or more biomarkers from Table 3.
9. The method of claim 6, wherein the biomarkers common to HIV comprise syntenin-1
- 25 and CD44.
10. The method of claim 9, wherein the biomarkers common to HIV comprises one or more additional proteins from Table 3.
- 30 11. The method of claim 6, wherein the biomarkers unique to specific cell types comprise one or more biomarkers from Table 2.

12. The method of claim 6, wherein it is determined whether the subject from which the sample was obtained has X4 tropic, R5 tropic or dual tropic HIV-1 infection.

13. A method for purifying HIV virus from a sample comprising the steps of:

- 5 a. incubating the sample with cholesterol and 2-hydroxy-beta cyclodextran;
 b. filtering the sample on ice; and
 c. pelleting through sucrose for about one hour.

14. The method of claim 13, wherein the sample is an HIV-1 cell line.

10

15. The method of claim 14, wherein the cell line is H9.

16. The method of claim 14, wherein the cell line is CEMx174.

15

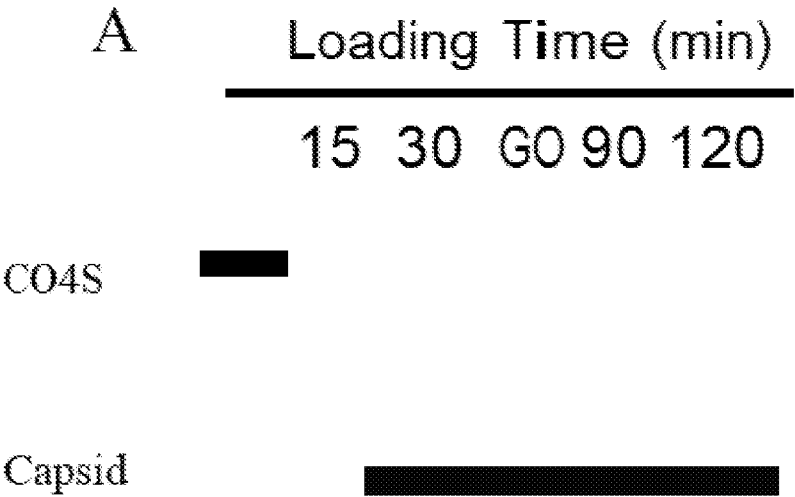


FIG. 1A

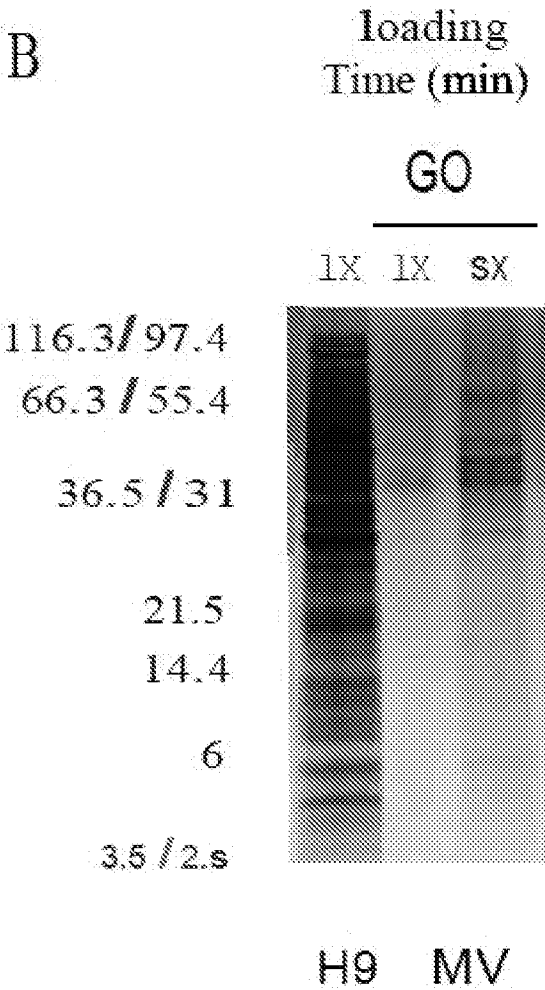


FIG. 1B

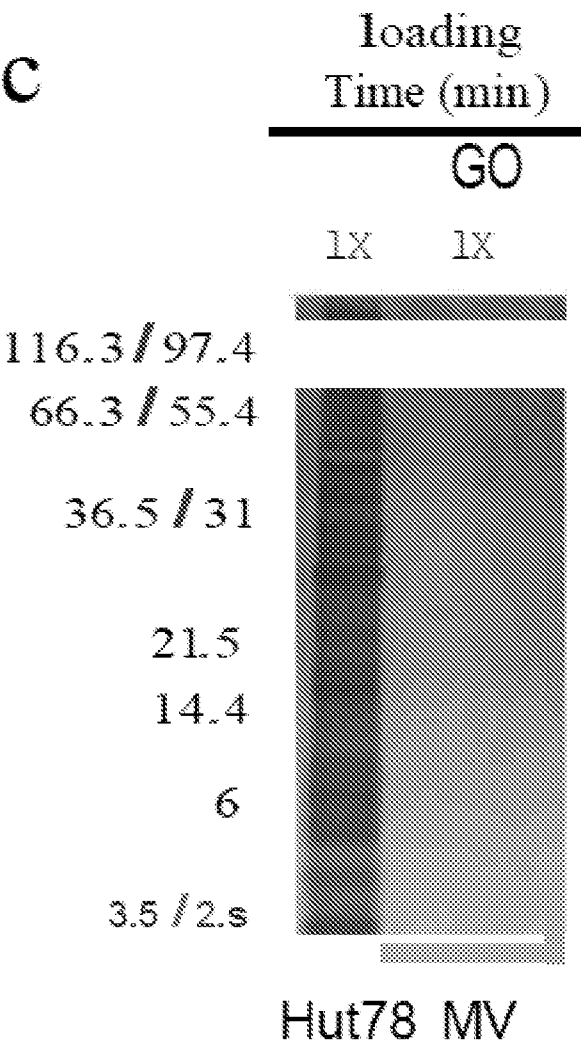


FIG. 1C

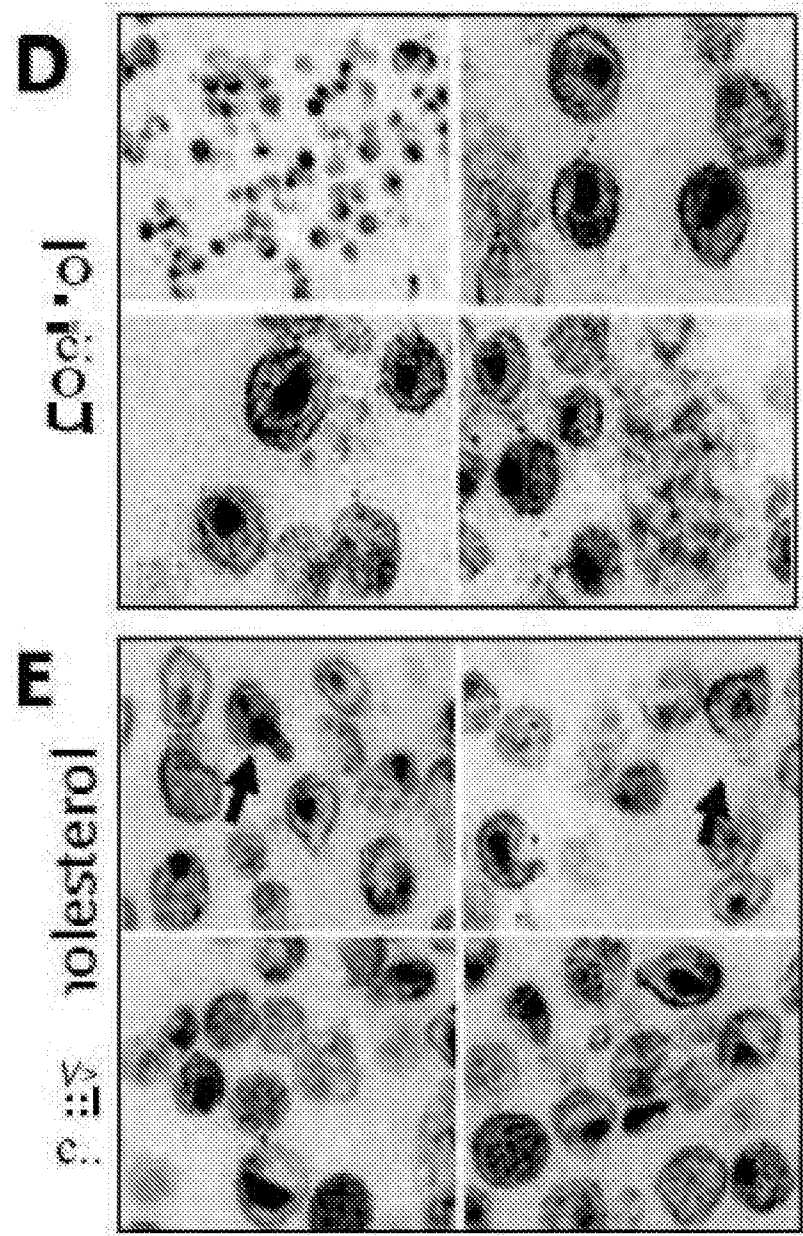


FIG. 1D

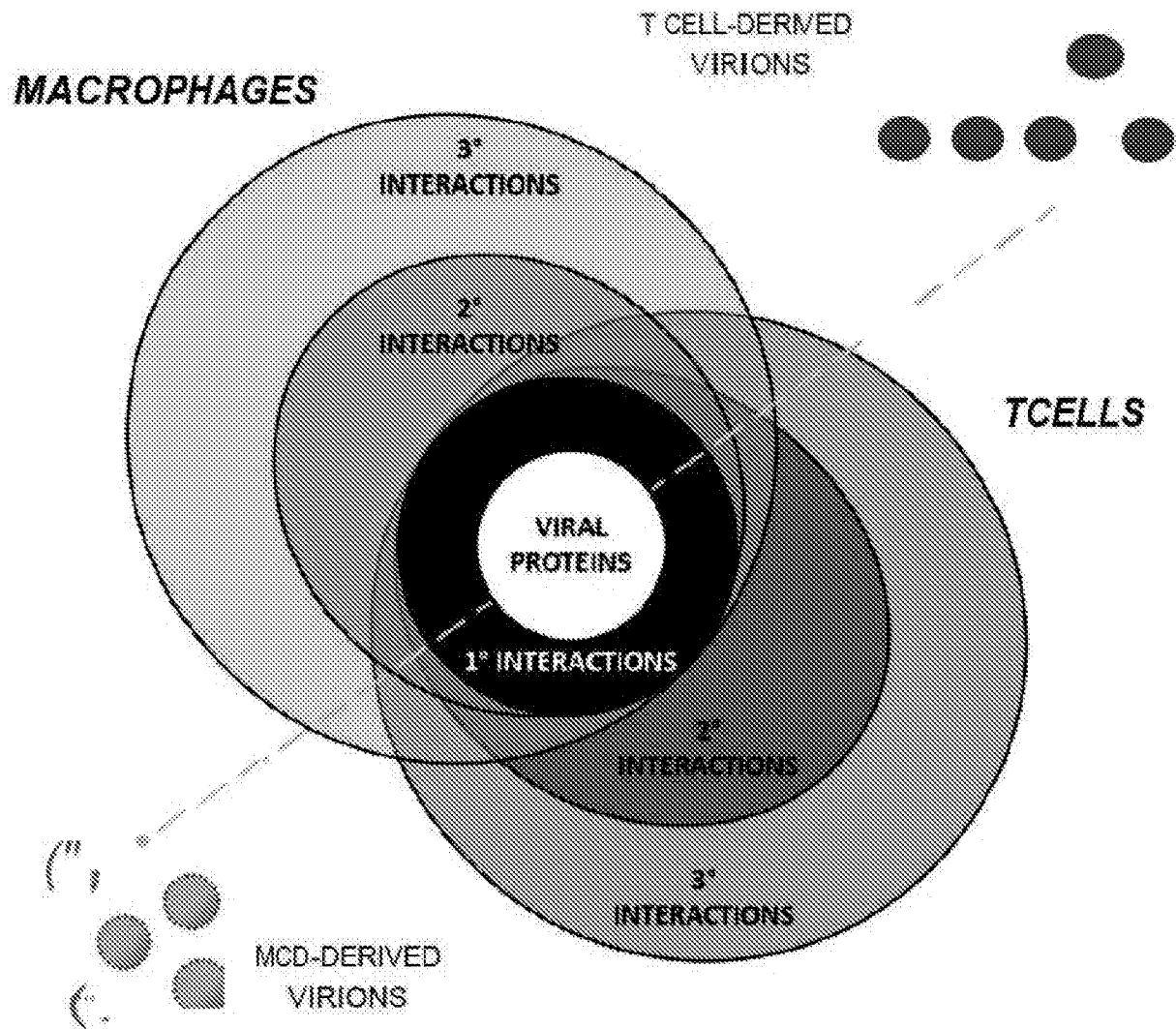


FIG. 2

A. CLASSIFICATION OF SUBJECT MATTER**A61K 39/21(2006.01)i, C07K 14/16(2006.01)i, A61P 31/18(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 39/21; C12P 19/34; C12Q 1/70; C12Q 1/68; C07K 14/16; A61P 31/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: HIV, biomarker, cellular tropism, purification, cholesterol, 2-hydroxy-beta cyclodextrin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LINDE, MICHAEL E. et al., `The conserved set of host proteins incorporated into HIV-1 virions suggests a common egress pathway in multiple cell types`, Journal of Proteome Research, 22 February 2013, Vol. 12, pp. 2045-2054 See abstract, pages 2046-2051, tables 2-3.	6-11,13-16
A	CHERTOVA, ELENA et al., `Proteomic and biochemical analysis of purified human immunodeficiency virus type 1 produced from infected monocyte-derived macrophages`, Journal of Virology, 2006, Vol. 80, No. 18, pp. 9039-9052 See abstract, page 9040, left-column, page 9042, left-column, table 1.	6-11,13-16
A	WO 2009-111042 A2 (CENETRON DIAGNOSTICS) 11 September 2009 See abstract, claim 3.	6-11,13-16
A	US 2003-0207294 A1 (CLAVEL, FRANCOIS et al.) 06 November 2003 See abstract, claims 1, 21.	6-11,13-16
A	CANTIN, REJEAN et al., `Discrimination between exosomes and HIV-1: Purification of both vesicles from cell-free supernatants`, Journal of Immunological Methods, 2008, Vol. 338, pp. 21-30 See abstract, page 23, right-column.	6-11,13-16



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

18 August 2014 (18.08.2014)

Date of mailing of the international search report

19 August 2014 (19.08.2014)

Name and mailing address of the ISA/KR

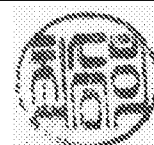
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INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/030173**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-5, 12
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-5, 12 pertain to methods for treatment of the human body by surgery or therapy, as well as diagnostic methods practiced on the human body, and thus relate to a subject matter which this International Searching Authority is not required to search (PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv)).
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/030173

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2009-111042 A2	11/09/2009	WO 2009-111042 A3	01/07/2010
US 2003-0207294 A1	06/11/2003	AU 2002-223052 B2	20/09/2007
		AU 2002-23052 A1	21/05/2002
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		US 2004-0053219 A1	18/03/2004
		US 2004-0101828 A1	27/05/2004
		WO 02-38792 A2	16/05/2002
		WO 02-38792 A3	25/09/2003