Noncytotoxic Suppression of Human Immunodeficiency Virus Type 1 Transcription by Exosomes Secreted from CD8⁺ T Cells[▽]

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CD8⁺ T cells display a noncytotoxic activity that suppresses transcription of human immunodeficiency virus type 1 (HIV-1) in an antigen-independent and major histocompatibility complex-unrestricted manner. To date, the precise cellular and molecular factors mediating this CD8⁺ T-cell effector function remain unsolved. Despite evidence indicating the dependence of the activity on cell-cell contact, the possibility of a membrane-mediated activity that represses transcription from the viral promoter remains unexplored. We therefore investigated whether this inhibition of HIV-1 transcription might be elicited by a membrane-bound determinant. Using a CD8⁺ T-cell line displaying potent noncytotoxic HIV-1 suppression activity, we have identified a membrane-localized HIV-1-suppressing activity that is concomitantly secreted as 30- to 100-nm endosome-derived tetraspanin-rich vesicles known as exosomes. Purified exosomes from CD8⁺ T-cell culture supernatant noncytotoxically suppressed CCR5-tropic (R5) and CXCR4-tropic (X4) replication of HIV-1 in vitro through a protein moiety. Similar antiviral activity was also found in exosomes isolated from two HIV-1-infected subjects. The antiviral exosomes specifically inhibited HIV-1 transcription in both acute and chronic models of infection. Our results, for the first time, indicate the existence of an antiviral membrane-bound factor consistent with the hallmarks defining noncytotoxic CD8⁺ T-cell suppression of HIV-1.

Noncytotoxic suppression of human immunodeficiency virus type 1 (HIV-1) replication is one of the least understood antiviral mechanisms displayed by CD8⁺ T cells (3, 4, 10, 25, 45, 46). A lack of major histocompatibility complex (MHC) restriction (24, 27, 31, 35) and suppression of HIV-1 replication in heterologous CD4⁺ cell targets (16, 17, 45) distinguish this antiviral mechanism from the classical cytotoxic activity of CD8⁺ T cells. The noncytotoxic mechanism has been shown to suppress the in vitro replication of a broad range of primary clinical and laboratory-adapted isolates and different HIV-1 clades varying widely in genotype as well as HIV-2 and simian immunodeficiency virus (2, 21, 47). A number of studies have indicated that the noncytotoxic action of CD8⁺ T cells specifically inhibits transcription from the integrated HIV-1 long terminal repeat (LTR) promoter (5, 6, 30, 42). This transcriptional repression has been shown to occur in the absence of the HIV-1 transactivating protein, Tat (42). Although culture supernatant from CD8⁺ T cells has been shown to suppress HIV-1 replication (20), cell-to-cell contact between CD8⁺ T cells and HIV-1-infected CD4⁺ cells has been found to be critical for maximal inhibition of viral replication (29, 46), implying the existence of a membrane-associated component for the antiviral activity.

Previous efforts to elucidate the effector molecule(s) for this mechanism have been based on an assumption that the noncytotoxic anti-HIV activity of CD8⁺ T cells is exclusively me-

diated by one or more soluble lymphokine proteins termed CAF (10, 26, 52). However, to date, no CD8⁺ cell-secreted cytokine, chemokine, or inflammatory molecule has been shown to identify with the hallmarks defining the HIV-1 transcription-suppressing activity of CD8⁺ T cells (4, 25, 26). In contrast to soluble factors, the elucidation of a cell membrane-dependent mechanism remains a relatively unexplored avenue for unraveling the noncytotoxic anti-HIV effector function. Studies examining MHC compatibility and CD80/86 costimulatory function indicate that these molecules can augment the noncytotoxic antiviral effect of CD8⁺ T cells, but by themselves they do not direct the actual noncytotoxic action (1, 35). These studies do, however, suggest that a cell membrane-dependent mechanism could potentially regulate the noncytotoxic CD8⁺ T-cell antiviral activity. Such a possibility may consequently extend to the existence of a specific membrane-bound CD8⁺ T-cell molecule that directly represses transcription of the HIV-1 LTR promoter in infected cells.

While a membrane molecule might explain the cell contact dependence of noncytotoxic HIV-1 suppression activity in CD8⁺ T cells, its link to a secreted mediator eliciting the same antiviral activity remains evasive unless intracellular membrane trafficking is considered. In particular, if the putative membrane-bound anti-HIV activity is specifically localized to the luminal space of endosomal membrane, it would facilitate not only cell surface presentation of the activity but also its inclusion into multivesicular bodies of late endosomes, leading to extracellular secretion of the factor and its inclusion into 30- to 100-nm membrane-enclosed vesicles termed exosomes (13, 40, 41). Exosomes are generated intracellularly within the multivesicular structure of late endosomes from a variety of cell

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types, including lymphocytes (37). They are consequently secreted into extracellular fluid upon fusion of late endosomes with the plasma membrane (9, 13, 33, 36, 37, 41, 44).

We therefore investigated the possibility of whether membrane and secreted exosomes from CD8⁺ T cells could mediate the same noncytotoxic antiretroviral suppression activity observed in CD8⁺ T cells. We report evidence for the existence of a membrane-bound anti-HIV factor and its extracellular secretion through endosome-derived exosomes. Secreted exosomes purified from a transformed CD8⁺ T-cell line potentially suppressed replication of R5 and X4 HIV-1 isolates through a protein moiety and repressed transcription of the HIV-1 LTR promoter in both acute and chronic models of infection. Confocal microscopy indicated that interaction of exosomes with CD4⁺ cell targets was limited to the cell surface, with no evidence for their internalization. Our evidence indicates that an intracellular signaling mechanism is involved in exosome-mediated suppression of HIV-1 transcription.

MATERIALS AND METHODS

Cells, virus stock, and plasmid. The CD8⁺ T-cell line, TG, was previously established by herpesvirus saimiri (HVS)-transformation of CD8⁺ T cells from a chronically infected HIV-1-infected subject from the Multicenter AIDS Cohort Study (MACS) (7). Primary CD4⁺ T cells were prepared by depletion of CD8⁺ cells from peripheral blood mononuclear cells (PBMC) using immunomagnetic beads coated with antibody to CD8, as previously described (7, 8). These cells were then activated by incubation with anti-CD3 antibody for 3 days and propagated in RPMI medium supplemented with 20% fetal bovine serum, recombinant interleukin-12 (rIL-2; Roche), and antibiotics for 5 days to selectively expand the CD4⁺ T-cell population. The TZM-bl cell line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, from John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. The 8E5 cell line was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, from Thomas Folks. HeLa Tat cells were also obtained from the AIDS Research and Reference Reagent Program from William Haseltine and Ernest Terwilliger. The fibrosarcoma cell lines U3A and U3AR were a gift from George Stark (Cleveland Clinic, Cleveland, OH). The TG, 8E5, and infected CD4⁺ T cells were cultured in RPMI medium supplemented with 20% fetal bovine serum, 25 mM HEPES, and penicillin/streptomycin. TG cell culture was additionally supplemented with rIL-2. TZM-bl cells were cultured in RPMI medium supplemented with 10% fetal calf serum, and penicillin/streptomycin. A highly defined 40-nm prefiltered fetal bovine serum (HyClone, Logan, UT) devoid of endogenous vesicles was utilized in all cultures. U3A and U3AR cells were grown in 10% Dulbecco's modified Eagle's medium, enriched with 2 mM glutamine. CD8⁺ T cells were isolated from PBMC of two therapy-naïve HIV-1-infected subjects by magnetic bead separation (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. CD8⁺ T cells were grown at a concentration of 1 million/ml in 20% RPMI medium supplemented with rIL-2 (50U/ml) and OKT3 anti-CD3 antibody (1 µg/ml). PBMC samples from HIV-1-positive subjects were obtained with the approval of an Institutional Review Board. Cell growth was monitored weekly by trypan blue exclusion of aliquots taken from cell cultures. Cells were processed for membrane purification when the cell counts of the cultures no longer increased. HIV-1 R5 clinical isolates 33015 and X4 clinical isolate 33074 were obtained from PBMC of HIV-1-infected subjects enrolled in the MACS of the University of Pittsburgh, as described previously (7, 8). HIV-1 R5 isolates ME1 and X4 isolate ME46 were cloned from a MACS patient from primary isolates derived from early (ME1) and late stage (ME46) of disease progression (7). The HIV-1 isolate IN/93/999 is a primary R5-tropic virus of subtype A origin from India. The previously described pSVtat plasmid contains cDNA coding for HIV-1 tat under the control of the simian virus 40 promoter (22).

Purification of cell membranes. Frozen or fresh cell pellets (TG cells or primary CD8⁺ T cells from HIV-1-infected subjects) were thawed; resuspended into a solution of 10% (wt/vol) sucrose, 10 mM Tris-HCl, and 25 mM MgCl₂ (STM buffer); and subjected to three additional freeze-thaw cycles using ethanol-dry ice for freezing and thawing in a 37°C water bath. The disrupted cell suspension was homogenized using a Dounce homogenizer, and the homogenate

was clarified by centrifugation at 800 × g at 4°C to remove organelle matter and larger cellular debris. The organelle-depleted supernatant was extracted and subjected to centrifugation at 20,000 × g for 30 min to pellet the crude cell membrane fraction. The pellet was resuspended in STM buffer, overlaid on a 75% (wt/vol) sucrose density cushion, and recentrifuged at 90,000 × g at 4°C for 1.5 h. The band above the 75% sucrose interface was extracted, washed in STM buffer, repelleted by centrifugation, and resuspended in Hanks balanced salt solution (HBSS) or RPMI medium. Protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, CA). In the case of membrane purification from primary CD8⁺ T cells from HIV-positive (HIV⁺) patients, we used the pellet from centrifugation at 20,000 × g (20,000 × g pellet) without further sucrose gradient refinement due to low sample yield and sample losses during the purification procedure.

Purification of exosomes. Exosomes were harvested from culture supernatants by adaptation of previously described methods involving serial centrifugation of the culture supernatant, followed by sucrose density gradient purification (18, 37). Culture fluid from TG cells or primary CD8⁺ T cells from HIV-1-infected subjects was harvested by centrifugation at 300 × g for 10 min to deplete cells. Cell-free culture supernatant was subjected to serial centrifugations of increasing centrifugal force to derive supernatants and pellets at 800 × g for 30 min, 6,000 × g for 30 min, 15,000 × g for 30 min, and 60,000 × g for 1 h, with all spins performed at 4°C. The 15,000 × g pellet was then resuspended in HBSS and subjected to a discontinuous sucrose density gradient centrifugation at 90,000 × g at 4°C through a 40% sucrose (1.14 g/ml) layer overlaid over a 60% sucrose (1.21 g/ml) cushion. After centrifugation, the membrane fractions banded over the 40% and 60% sucrose interfaces were removed, diluted in HBSS, and recentrifuged at 18,000 × g to purify the fractions. The membrane pellet was resuspended in HBSS and analyzed for the presence of exosomes. Exosome protein concentration was measured using a Bradford assay (Bio-Rad, Hercules, CA).

Quantitative acute HIV-1 replication suppression assay. A quantitative acute HIV-1 replication suppression assay was performed as described previously (8). Briefly, CD4⁺ T cells acutely infected with HIV-1 isolate 33015 were incubated in the presence or absence of CD8⁺ T cells, membrane, or exosomes for 5 days at 37°C in 5% CO₂. On day 5, the extent of productive HIV-1 replication was assayed by measuring the concentration of extracellular p24 protein in culture supernatant using a standardized enzyme-linked immunosorbent assay (ELISA) kit (Perkin Elmer). Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (34).

Electron microscopy. Formvar-coated copper grids (200 mesh) were floated on a drop of a highly concentrated exosome sample for approximately 30 s. The grids were removed, and excess sample solution was wicked away with filter paper and then placed on a drop of 0.45-µm-pore-size filtered 1% uranyl acetate in deionized double-distilled H₂O for 30 to 60 s. Excess stain was wicked away before imaging. Exosomes isolated on anti-MHC class II antibody-coated immunomagnetic beads were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 1 h and pelleted in a 1.5-ml microcentrifuge tube at 500 × g. The supernatant was removed, and pelleted beads were resuspended in 10 to 15 µl of 3% gelatin in PBS (gelatin heated to 36°C prior to resuspension). Spheroids were pelleted in this solution to concentrate them at the tip of the gelatin plug. The gelatin-bead plug was hardened at 4°C for 30 min, and then the entire plug was fixed in 2.5% glutaraldehyde in PBS for 15 min. Exosome pellets were washed three times in PBS and then postfixed in 1% OsO₄ plus 1% K₃Fe(CN)₆ for 1 h. Following three additional PBS washes, the pellet was dehydrated through a graded series of 30 to 100% ethanol, followed by infiltration into a Polybed 812 epoxy resin (Polysciences, Warrington, PA) for 1 h. After several changes of 100% resin over 24 h, the pellet was embedded in a final change of resin and cured at 37°C overnight, followed by additional hardening at 65°C for two more days. Ultrathin (60 nm) sections were collected on 200-mesh copper grids and stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min. All grids were imaged using a JEOL JEM 1210 transmission electron microscope ([TEM] Peabody, MA) at 80 kV, fitted with a bottom mount AMT 2k digital camera (Advanced Microscopy Techniques, Danvers, MA).

Flow cytometric analysis of exosomes. Flow cytometry analysis of exosomes was adapted from methods previously described (9). An aliquot containing 2.5 × 10⁵ anti-MHC class II antibody-coated beads was incubated with exosomes at a concentration of 80 µg/ml in 250 µl of fluorescence-activated cell sorter (FACS) buffer (4% fetal calf serum-PBS). Bead-captured exosomes were washed twice with FACS buffer, followed by monoclonal antibody incubation at room temperature for 30 min. Monoclonal biotin-conjugated immunoglobulin G1 antibodies to MHC class II, CD9, CD63, and CD81 were purchased from R&D Systems (Minneapolis, MN). Monoclonal phycoerythrin-conjugated immunoglobulin G1 antibodies to CD3, CD8, and CD4 were purchased from BD Biosciences (San Jose, CA). Exosomes labeled with biotinylated antibody were

washed twice, followed by incubation with streptavidin-phycoerythrin conjugate (Invitrogen, Carlsbad, CA) for 15 min. After labeling, samples were subjected to two additional washes in FACS buffer, followed by analysis of exosome-bound beads on a Beckman Coulter EPICS XL-MCL flow cytometer.

Biochemical treatment of exosomes. For all biochemical treatments, purified exosomes were first pelleted by centrifugation at $45,000 \times g$ for 1 h. For sodium carbonate treatment, pellets were resuspended in 0.1 M Na_2CO_3 for 30 min at 4°C and neutralized with 1 M HEPES buffer, followed by pelleting and resuspension in cell culture medium. For protease treatments, exosome pellets were resuspended in 1 ml containing 5 $\mu\text{g}/\text{ml}$ trypsin plus 5 $\mu\text{g}/\text{ml}$ chymotrypsinogen A for 12 h, followed by pelleting and resuspension in RPMI medium. For organic solvent extraction, pellets were dissolved in a 2:1 mixture of chloroform/methanol and vortexed vigorously, after which the resolved phases (methanol, chloroform, and precipitated proteins) were extracted, evaporated, and resuspended in RPMI medium to assay for HIV-suppressive activity. For acetone delipidation, exosome pellets were dissolved in cold acetone (-20°C), vortexed vigorously, and incubated at -20°C for 1 h, and proteins were precipitated by $18,000 \times g$ centrifugation. The supernatant was discarded, and precipitated proteins were air dried, dissolved in RPMI medium, and recentrifuged at $18,000 \times g$ to remove insoluble particulates; soluble proteins were extracted with the RPMI medium supernatant.

Acute HIV transcription suppression assay. An assay for measurement of LTR promoter inhibition was adapted from previously described methods (5). TZM-bl cells were seeded at 25,000 cells per well in a 96-well plate and cultured at 37°C for 24 h. Cells were incubated with exosomes or medium only for up to 24 h at 37°C . After incubation, cells were washed twice with medium prior to LTR activation. For gene reporter expression induced by virus infection, TZM-bl cells were infected with HIV-1 isolate 33015 and supplemented with 8 $\mu\text{g}/\text{ml}$ DEAE-dextran for 1 h, washed with medium, and incubated at 37°C for 24 h after infection. For Tat-transactivated LTR induction, TZM-bl cells were liposome-transfected with the Tat-expressing plasmid pSVtat using the Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). For mitogen activation of the LTR promoter, TZM-bl cells were incubated with 100 ng/ml phorbol myristate acetate (PMA; Invitrogen, Carlsbad, CA) for 8 to 12 h. The extent of LTR-induced gene expression of β -galactosidase was measured using a β -GLO Assay (Promega, Madison, WI).

Quantification of mRNA by real-time RT-PCR. TZM-bl cells were plated at 75% confluence in 10% RPMI medium. TZM-bl cells were incubated with medium only or with medium supplemented by HIV-1-suppressing exosomes. After 16 h of incubation of cells with exosomes or medium only, LTR activation by viral infection with HIV-1 isolate 33015 was performed. Cellular RNA was isolated from these TZM-bl cells using RNeasy (Tel-Test Inc., TX) according to the manufacturer's instructions. Two micrograms of RNA from each replicate was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, CA) and then subjected to real-time quantification for β -galactosidase mRNA using the following primers: Fwd, 5'-ATCAGGAT ATGTGGCGGATGA-3'; Rev, 5'-TGATTGTGTAGTCGGTTTATGCA-3'. The probe used was 6FAM-CCGTGACGCTCTCGT-MGBNFQ (where FAM is carboxyfluorescein and MGBNFQ is molecular groove binding nonfluorescent quencher). The endogenous normalization control used for the experiment was real-time reverse transcription-PCR (RT-PCR) for 18S rRNA. Specific primers and probe (VIC/MGB) for 18S rRNA real-time RT-PCR were obtained from Applied Biosystems. All PCRs were performed in a real-time PCR 7500 system. Gene expression quantification using TaqMan Gene Expression Assays was performed as the second step in a two-step RT-PCR. Assays were done in 30- μl singleplex reaction mixtures containing TaqMan Universal PCR Master Mix, 20 $\times$ TaqMan Gene Expression Assay Mix, and cDNA according to the manufacturer's instructions (Applied Biosystems, CA). Reaction conditions consisted of preincubation at 50°C for 2 min, 95°C for 10 min, and then cycling for 50 cycles of 95°C for 15 s and 60°C for 1 min.

Chronic HIV-1 transcription suppression assay. 8E5 cells were cultured in the presence or absence of TG exosomes over a time course of 25 days. Cell concentration was adjusted every 5 days to maintain continuous log-phase growth with replenishment of medium alone or medium supplemented with exosomes. At each measurement time point during culture, cells were counted, and aliquots containing 10^3 cells from three independent experiments were pelleted and stored at -70°C . Frozen cell pellets were assayed for intracellular HIV-1 RNA copy number using a Nuclisens HIV-1 RNA quantification assay (BioMérieux, Durham, NC).

Confocal microscopy of exosome-cell interaction. TZM-bl or HeLa Tat cells were labeled, according to the manufacturer's instructions, with PKH-67 (Sigma, Dekalb, MO), a lipophilic dye that binds cell membrane lipids by its long aliphatic tail. Cells were plated on 35-mm uncoated dishes (type P35G-0-10C;

Mattek Corp., Ashland, MA) at 75% confluence in 10% RPMI medium. Freshly harvested exosomes were quantified for protein concentration by Bradford's assay (Bio-Rad Laboratories). Exosomes were then resuspended in HBSS to a total volume of 1 ml. A 1:250 dilution of the Cy5 dye (GE LifeSciences, Buckinghamshire, United Kingdom) was made, and 10 μl of the diluted dye was added to 30 μg of exosome protein. The incubation of dye with exosomes was performed at 4°C in the dark for 1 h. Following incubation, the exosomes were washed three times with HBSS and resuspended to a concentration of 1 to 1.5 $\mu\text{g}/\mu\text{l}$. Thirty micrograms of labeled exosomes was added to each dish of labeled cells, and incubation was carried out for 10 min or for 12 h at 37°C in the dark. Fixation was done at the required time points by 2% paraformaldehyde. Dishes containing fixed cells and exosomes were viewed under an Olympus Fluoview 1000 instrument. Images were analyzed using MetaMorph software.

CAT assay. U3A and U3AR cells were grown to 90% confluence. Cells in designated wells were exposed to medium only or medium supplemented with exosomes. At 12 to 16 h after incubation, cells were trypsinized, replated, and then transfected with HIV LTR-chloramphenicol acetyltransferase (CAT) (38). Transfections were performed using Lipofectamine Plus (Invitrogen, Carlsbad CA), according to the manufacturer's instructions. At 36 h posttransfection, cells were treated with 20 ng/ml PMA for 8 h. Cells were lysed, and the amount of CAT protein was measured using a CAT ELISA (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions.

RESULTS

HIV-1 suppressive activity of CD8⁺ T-cell membrane and exosomes. As a source of HIV-1 suppression activity, we utilized the CD8⁺ T-cell line, TG, which was cloned by HVS transformation of PBMC of an HIV-1-infected subject (7). The utility of HVS-transformed CD8⁺ T-cell lines in mechanistic studies of noncytotoxic HIV-1 suppression has been widely reported (5, 8, 17, 23, 32). The noncytotoxic HIV-1 suppression activity of these CD8⁺ T-cell lines has been shown to be dependent on the parental primary CD8⁺ T-cell clone activity and not the HVS transformation process itself (5, 8, 32). To quantify noncytotoxic HIV-1 suppression activity, we utilized a previously described quantitative acute infection assay that measures with a relatively high degree of precision the inhibition of HIV-1 replication in acutely infected primary CD4⁺ T cells (7, 8). The TG cell line readily displayed potent dose-dependent nontoxic HIV-1 suppression against the heterologous CD4⁺ T cells infected with primary HIV-1 isolate 33015 (Fig. 1A). We purified an organelle-free cell membrane fraction from the CD8⁺ T-cell line and observed that cell membrane alone suppressed HIV-1 replication in a dose-dependent nontoxic manner (Fig. 1B). A time course analysis demonstrated that purified cell membrane from TG cells suppressed HIV-1 replication with the same kinetics as TG cell-mediated inhibition (Fig. 1C), suggesting that there may be a common antiviral mechanism underlying both sources of noncytotoxic HIV-1 suppression.

In order to understand how the observed membrane-mediated HIV-1 suppression activity was linked to the secreted form of the same antiviral activity, we explored the possibility that a vesicular form of this activity might be secreted into culture supernatant by the CD8⁺ T-cell line. We hypothesized that if HIV-1 suppression activity specifically trafficked through the endocytic pathway, then a membrane-localized form of the antiviral activity would be found in the form of exosomes secreted by the cells.

Exosomes are typically isolated from cell-conditioned culture supernatant by isolation of membrane vesicles pelleted at a relative centrifugal force (RCF) greater than $10,000 \times g$ (14, 41). We therefore harvested extracellular membrane fractions

FIG. 1. Noncytolytic suppression of HIV-1 by secreted membrane fractions from a CD8⁺ T-cell line. Suppression of HIV-1 replication in an acute infectious assay was quantified for the HVS-transformed CD8⁺ T-cell line, TG (A), and membrane prepared from TG cells (B). (C) TG cells (filled squares) and TG membrane (open squares) suppressed HIV-1 replication with the same time kinetics. (D) HIV-1 suppression activity was assayed for secreted membrane pellets harvested first at 15,000 × g RCF followed by pellets depleted at 60,000 × g by serial centrifugation of cell-free culture supernatant (filled bars). (E) Harvested pellets were treated with 0.1 M sodium carbonate, pH 11.5, to deplete peripheral proteins from the vesicles, followed by neutralization, pelleting, resuspension in medium at original volume, and assaying for HIV-1 suppression activity (open bars). (F) The 15,000 × g vesicle pellet was fractionated by sucrose density gradient. Vesicle fractions corresponding to 1.0 to 1.14 g/ml and 1.14 to 1.21 g/ml were isolated, purified, and assayed for HIV-1 suppression activity. Results are given as means ± standard deviations (*n* = 3).

by serial ultracentrifugation of cell-free culture supernatants from the antiviral CD8⁺ T-cell line and assayed their HIV-1 suppression activity. We observed peak anti-HIV activity in vesicle fractions pelleted from TG cell culture supernatant at 15,000 × g (15,000 × g fraction) with residual HIV-1 suppression activity pelleting in the 60,000 × g fraction (Fig. 1D). We treated the HIV-1-suppressing 15,000 × g extracellular membrane fractions with 0.1 M sodium carbonate, a high-alkali (pH 11.5) treatment that dissociates peripheral membrane proteins and disrupts the circular integrity of vesicles into linear bilayer sheets (15, 50). Sodium carbonate treatment did not deplete the antiviral activity from the extracellular vesicle fractions (Fig. 1E), indicating a relatively strong membrane association of the activity.

Since potent HIV-1 suppression activity in the 15,000 × g fraction was consistent with the presence of exosomes, we subjected this sample to further fractionation using a two-layer discontinuous sucrose-density gradient with ultracentrifugation at 100,000 × g. After fractionation of the sample, two distinct membrane fractions representing sucrose densities of 1.0 to 1.14 g/ml and 1.14 to 1.21 g/ml were extracted, purified by recentrifugation in HBSS, standardized to equivalent pro-

tein concentrations, and assayed for HIV-1-suppressive activity. Peak HIV-1 suppression was consistently observed in the 1.14- to 1.21-g/ml sucrose density membrane fractions (Fig. 1F), a sucrose density previously reported for exosomes from other cell types (13, 40, 41, 43).

Characterization of the HIV-1-suppressing extracellular membrane fraction as exosomes. Transmission electron microscopy of the sucrose gradient-purified 15,000 × g HIV-1-suppressing membrane fraction demonstrated the presence of 30- to 100-nm vesicles morphologically identified as exosomes (Fig. 2A). To confirm the endosomal origin of these purified vesicles and their identity as exosomes, we employed a previously described immunomagnetic exosome bead capture technique (9) to probe the antigenic content of the HIV-1-suppressing nanovesicles. Previous characterizations of exosomes from other cell types describe a specific enrichment of MHC class II molecules and proteins of the tetraspanin family such as CD9, CD63, and CD81 (14, 40). A specific lysosomal targeting signal contained in CD63 (39) further delineates the intracellular origin of exosomes and their secretion via the endocytic pathway (13, 14, 18, 33, 40, 41, 49). The bead capture technique utilizes paramagnetic polystyrene beads coated with

FIG. 2. Characterization of the sucrose density gradient-purified HIV-1-suppressing secreted vesicle fraction. (A) TEM of the 1.14- to 1.21-g/ml sucrose gradient-purified 15,000 $\times$ g vesicle fraction (bar, 100 nm). (B) Cryo-TEM section of an immunomagnetic bead after incubation with the HIV-1-suppressing nanovesicle fraction. The box depicts an expanded view; arrows point to vesicles with exosome morphology; the dashed line delineates the bead surface (bar, 100 nm). Flow cytometry analysis was performed on exosome-saturated beads (C) and TG cells (D) (y axis, percentage of maximum; x axis, log-scale relative fluorescence). Unshaded areas represent the antibody isotype; shaded areas indicate specific fluorescence by the antibody labeled above each graph. (E) CD63 levels expressed as a percentage of CD9 on exosome and cell surfaces. (F) CD63 levels expressed as a percentage of CD81 on exosome and cell surfaces.

antibodies specific for MHC class II molecules. Saturation of the 4.5- μ m-diameter bead surface with MHC class II-positive vesicles allows for direct probing of vesicle antigenic content by flow cytometry. We incubated purified HIV-1-suppressing nanovesicles with the anti-MHC class II-coated paramagnetic beads. After incubation, the beads were prepared for TEM analysis and flow cytometry analysis to confirm their capture and probe their antigenic content. TEM of cryo-TEM sections of the immunomagnetic beads after incubation with the HIV-1-suppressing exosome vesicles demonstrated saturation of bead

surfaces with the exosome-sized particles (Fig. 2B). Aliquots of the vesicle-coated beads were subsequently analyzed by flow cytometry using monoclonal antibodies specific for particular exosome- and T-cell-specific markers. We readily detected the specific presence of exosome markers MHC class II, CD9, CD63, and CD81 on the HIV-1-suppressing nanovesicles (Fig. 2C), confirming their identity as exosomes. Interestingly, the antiviral exosomes contained the CD3 T-cell marker but did not display the CD8 molecule. We therefore probed the cell surface expression of these same antigens in the TG CD8⁺

FIG. 3. Exosome-mediated nontoxic suppression of HIV-1 replication. (A) Suppression of HIV-1 replication by cell membranes and exosomes harvested from cultured primary CD8⁺ T cells from two therapy-naïve HIV-1-infected patients. Filled bars represent data from patient 1 and open bars represent data from patient 2. (B) MTT assays were performed to quantify relative cell viability of HIV-1-infected CD4⁺ T cells cultured in medium only and in medium supplemented with HIV-1-suppressing exosomes. (C) Exosome-mediated suppression of CCR5-tropic HIV-1 isolates 33015, IN/93/999, and ME1 and CXCR4-tropic HIV-1 isolates 33074, IIIB, and ME46. Results are given as means $\pm$ standard deviations ($n = 3$).

T-cell line. In contrast to expression in exosomes, cell surface expression of both CD3 and CD8 was detected (Fig. 2D), indicating a distinct membrane origin of the antiviral nanovesicles. From cell surface and exosome flow cytometry data, we calculated CD63 expression relative to tetraspanins CD9 (Fig. 2E) and CD81 (Fig. 2F). CD63 content appeared elevated on exosomes in comparison to the cell surface, a result consistent with exosome biogenesis from internal endocytic membrane compartments (14).

Characterization of CD8⁺ T-cell exosome-mediated HIV-1 suppression. To confirm that the HIV-1-suppressive effect from TG membrane and exosomes was not due to the HVS transformation of this cloned CD8⁺ T-cell line, we examined primary CD8⁺ T cells obtained from two therapy-naïve HIV-1-infected subjects. Although it was not possible to obtain large numbers of PBMC from these subjects, we were able to isolate and expand primary CD8⁺ T cells from these individuals to sufficient numbers for low-yield cell membrane and exosome preparations. The membrane and exosome preparations were performed as described for TG cells but omitting sucrose gradient fractionation in the last step due to the sample losses inherent in the multistep purification process. Nonetheless, we found that both cell membrane and exosome fractions from the primary CD8⁺ T cells of these two therapy-naïve HIV-1-positive individuals could suppress HIV-1 replication (Fig. 3A). This result demonstrated that exosome-mediated HIV-1 suppression in the TG CD8⁺ T-cell line was not the result of the HVS transformation process, a result consistent with previous characterizations of CAF from these HVS-transformed CD8⁺ T-cell lines. In order to obtain high yields of anti-HIV exosomes, we utilized the TG CD8⁺ T-cell line since the cell cultures could be expanded indefinitely to harvest high yields of secreted exosomes maintaining consistent anti-HIV activity for further characterization studies. The anti-HIV activity of these CD8⁺ cell secreted exosomes was found to have no toxic effect on infected CD4⁺ T-cell targets as determined by MTT-based cell viability assays (Fig. 3B), consistent with the nontoxic nature of the CD8⁺ T-cell effector function.

A major hallmark of nontoxic HIV-1 suppression by CD8⁺ T cells is the inhibition of both CCR5- and CXCR4-tropic virus replication (5, 16, 24). We assayed exosome-mediated antiviral activity against a panel of CCR5- and CXCR4-tropic isolates in infected primary CD4⁺ T cells. The panel of isolates included CCR5-tropic HIV-1 isolates 33015, IN/93/999, and ME1 and CXCR4-tropic HIV-1 isolates 33074, IIIB, and ME46. In quantitative acute infectious assays, we found that purified TG exosomes suppressed HIV-1 replication regardless of coreceptor use (Fig. 3C).

Exosomes mediate HIV-1 suppression through a protein moiety. We investigated the biophysical nature of the antiviral activity of the HIV-1-suppressing exosomes. To confirm that a protein factor was involved in mediating the antiviral action, exosomes were subjected to a 6-h protease treatment with a combination of trypsin and chymotrypsinogen A. Untreated and protease-treated exosomes were pelleted by centrifugation, washed, resuspended in cell culture medium, and assayed for HIV-1-suppressive activity. We observed that protease treatment inactivated the antiviral action of exosomes (Fig. 4A), suggesting that the antiviral factor was a protein moiety possessing a membrane orientation consistent with extracellular surface presentation of its antiviral domain. We next examined whether the putative protein factor's tight membrane association regulated its antiviral action by depleting the lipid content of the exosomes. The lipid and protein fractions of the vesicles were separated by treatment with 2:1 chloroform-methanol as previously described (49). The antiviral activity was specifically observed in the precipitated protein fraction ($63\% \pm 5\%$), with no significant activity found in the chloroform-extracted lipid fraction ($12\% \pm 13\%$) (Fig. 4B). To further confirm the nonrequirement of lipid, we performed an alternative experiment using cold acetone to remove exosome lipid from the protein fraction. Exosomes were dissolved in cold acetone, followed by precipitation of the protein content by ultracentrifugation. The protein pellet was vacuum dried and resuspended in medium, followed by recentrifugation to remove insoluble particulates. The antiviral activity was again

FIG. 4. Biochemical analysis of HIV-1-suppressing exosomes. (A) Protease inactivation of exosome-mediated antiviral activity. The asterisk indicates a statistically significant difference at a P value of <0.05 (Student's t test). (B) Chloroform-methanol treatment of HIV-1-suppressing exosomes followed by separation and purification of precipitated exosome protein and lipid fractions. The asterisk indicates a statistically significant difference at a P value of <0.01 (Student's t test). (C) Cold acetone delipidation of exosomes with recovery of antiviral activity in the lipid-free protein fraction. Results are given as means $\pm$ standard deviations ($n = 3$).

recovered in the delipidated protein fraction (Fig. 4C). The results of the exosome delipidation experiments were consistent with the membrane-bound protein moiety exerting its antiviral activity independently of its apparent lipid tethering.

Exosomes mediate repression of HIV-1 LTR promoter activity in acute and chronic models of infection. We explored whether the broad anti-HIV activity displayed by exosomes was elicited by transcriptional repression of the HIV-1 LTR promoter. Toward this aim, we adapted an LTR gene reporter assay that mimics an acute HIV-1 infection model. Our adaptation of this assay utilized the HeLa-derived TZM-bl cell line containing a stably integrated HIV-1 LTR promoter fused to a β -galactosidase gene. The TZM-bl cell line has been further engineered to express CD4 and CCR5 molecules to support productive HIV-1 infection. Expression of the β -galactosidase reporter can be activated in the TZM-bl cell line by HIV-1 infection, Tat activation alone, or exogenous stimulation with an LTR-responsive mitogen such as PMA. Previous studies

demonstrating CAF-mediated suppression of the LTR promoter in HeLa cells had determined that the extent of LTR promoter inhibition was dependent on the amount of time target cells were exposed to CAF (5). We assayed whether the same phenomenon was true for the HIV-1-suppressing exosomes by incubating TZM-bl cells with the purified exosomes for variable lengths of time, followed by acute infection with the CCR5-tropic HIV-1 isolate 33015. We observed that maximum exosome-mediated suppression of LTR-activated β -galactosidase expression occurred after a 16-h incubation (Fig. 5A), a result consistent with previous characterizations of CAF (5). We additionally found that the percentage of LTR promoter suppression correlated with time on a logarithmic scale (correlation factor r , 0.99), further demonstrating a kinetic relationship between length of exosome exposure and extent of LTR repression.

To confirm that exosomes induced transcriptional repression at the HIV-1 promoter, we tested whether different meth-

FIG. 5. Suppression of HIV-1 at the level of transcription in acute and chronic models of infection. (A) Time-dependent suppression of LTR gene reporter expression by exosomes in TZM-bl cells. (B) Exosome-mediated suppression of LTR gene reporter expression after inducing the LTR with either an HIV-1 isolate, transfection with a plasmid expressing viral Tat protein, or mitogen stimulation with PMA. (C) Real-time RT-PCR measurement of LTR-activated β -galactosidase mRNA in TZM-bl cells in the absence or presence of exosomes. Values for β -galactosidase mRNA measurements was normalized by coquantification of 18S rRNA. (D) Intracellular HIV-1 RNA measurements in chronically infected 8E5 cells cultured in the presence (filled circles) or absence (open circles) of HIV-1-suppressive exosomes. Results are given as means $\pm$ standard deviations ($n = 3$).

ods of LTR promoter activation in the TZM-bl cell line resulted in gene reporter suppression after exosome exposure. TZM-bl cells were exposed to the HIV-1-suppressing exosomes for 18 h followed by either infection with HIV-1, transfection with a plasmid expressing the viral Tat protein, or replenishment of medium supplemented with 100 ng/ml PMA. In accordance with our observations of virus-induced LTR activation, we observed that the purified CD8⁺ T-cell-secreted exosomes also potently suppressed Tat- and PMA-activated LTR promoter-driven β -galactosidase expression in the TZM-bl cell line (Fig. 5B). The suppression of the PMA-activated LTR was especially significant since it demonstrated that the exosomes induced a transcriptional inhibition of the viral promoter independent of any HIV-1 protein expression, another hallmark of the noncytotoxic HIV-1-suppressing activity of CD8⁺ T cells (11, 12, 42). To further confirm that LTR gene reporter expression was inhibited at the level of transcription, real-time RT-PCR analysis of β -galactosidase mRNA was performed on TZM-bl cells cultured in the absence and presence of antiviral exosomes. RT-PCR values for β -galactosidase mRNA were normalized relative to RT-PCR values of 18S rRNA expression. The results of this experiment confirmed that the observed reductions in β -galactosidase protein expression in TZM-bl cells were the result of a specific decrease in LTR-activated reporter transcripts (Fig. 5C).

Since the TZM-bl LTR gene reporter assay essentially mimicked an acute infection model, we sought to determine if the CD8⁺ T-cell-secreted exosomes were capable of suppressing HIV-1 transcription in a chronic model of infection. Toward this aim, we utilized the chronically infected 8E5 cell line as target cells to measure HIV-1 transcriptional repression. The CEM-derived 8E5 cells contain a single full-length integrated copy of a mutant CXCR4-tropic HIV-1_{LAV} genome with a null mutation in its reverse transcriptase. The resulting virions from this T-cell line are thus rendered noninfectious. Since no cell-to-cell transmission of virus occurs, any suppression of HIV-1 in the 8E5 cell line will be specifically directed at a postintegration step in the virus life cycle. We cultured 8E5 cells in the absence or presence of the purified antiviral exosomes over a 25-day time period. The level of HIV-1 RNA copies per 1,000 cells was measured every 5 days, and cells were replenished with medium alone or medium supplemented with exosomes at each time point, along with cell concentration adjustments to maintain consistent log-phase growth of the 8E5 cells. Although a transient spike in HIV-1 RNA at day 5 was observed for 8E5 cells cultured in the presence of the exosomes (Fig. 5D), beyond 5 days of culture, we observed a precipitous and consistent decline in intracellular HIV-1 transcripts in 8E5 cells cultured in the presence of exosomes compared to medium controls (Fig. 5D, days 5 to 25). A 100-fold decrease in HIV-1 transcripts was observed at day 25 for 8E5 cells cultured in the presence of exosomes while 8E5 cells cultured in medium alone maintained consistently high HIV-1 mRNA copy numbers throughout the time interval. Combined with HIV-1 promoter suppression in the acute LTR gene reporter model, potent exosome-mediated suppression of HIV-1 RNA production in chronically infected 8E5 cells demonstrated that the purified CD8⁺ cell-secreted exosomes specifically suppress HIV-1 replication by inducing a transcriptional block on the LTR promoter.

FIG. 6. Confocal imaging of exosome-cell interaction. Exosomes were labeled with Cy5 (red), and cell membranes of TZM-bl cells were labeled with PKH-7 (green). (A) Exosome interaction with the cell periphery at 10 min of coinubation. (B) Exosome interaction with the cell periphery after 12 h of coinubation. Images shown are the focal plane at a 5- to 7- μ m depth inside stationary cells.

Interaction of exosomes with the cell surface. The presence of an HIV-1 suppression factor on the extracellular surface of the exosomes as well as a time-dependent induction for LTR promoter repression suggested the involvement of an exosome-triggered signaling mechanism. We therefore sought to determine whether the exosomes directly interacted with the surface of target cells in their induction of HIV-1 suppression. We performed confocal microscopy using TZM-bl cells labeled

FIG. 7. STAT1-dependence of HIV-1 LTR promoter suppression by TG exosomes. U3A (STAT1 deficient) and U3AR (STAT1 rescued) cell lines were each treated either with medium only or medium supplemented with TG exosomes for 12 h prior to transfection with a wild-type LTR-CAT gene reporter plasmid. After an 8-h LTR induction by PMA, cells were harvested, and intracellular CAT protein expression was measured by ELISA. The asterisk indicates a statistically significant difference at a P value of <0.01 (Student's t test). The percent suppression of LTR gene reporter expression was determined by the level of exosome-treated CAT expression versus medium-only controls in triplicate experiments. Results are given as means $\pm$ standard deviations ($n = 3$).

with the lipophilic dye PKH67 to stain internal and external cell membranes. Exosomes were labeled with Cy5, a dye that covalently binds to the primary amine group of proteins. The Cy5-labeled exosomes were incubated with PKH67-labeled cells at 37°C in the dark for various times, and three-dimensional confocal microscopy was performed following fixation. After only 10 min of incubation, exosomes were found to concentrate at the cell surface, limiting their localization only to the periphery of TZM-bl cells (Fig. 6A). After 12 h of incubation, a time by which near-maximal HIV-suppressive activity was detected (Fig. 5A), the Cy5-labeled exosomes were still limited to the periphery of TZM-bl cells (Fig. 6B). No evidence for internalization of exosome membrane was found at 12 h, indicating the involvement of exosomes only at cell surfaces during their induction of HIV-1 LTR promoter repression.

Dependence of exosome-mediated HIV-1-suppressive activity on intracellular STAT1 activity. An earlier study on CAF-mediated HIV-1 transcriptional suppression had determined that the antiviral activity was dependent on STAT1 signaling (5). The previous investigators showed that HIV-1 suppression activity could not be induced in cells deficient in the STAT1 protein expression. We sought to determine if the secreted CD8⁺ T-cell exosomes also shared this particular hallmark of CAF in inducing transcriptional repression of the HIV-1 LTR promoter. For this purpose, we utilized two fibrosarcoma cell lines, the STAT1-deficient U3A cell line and the constitutively STAT1-expressing U3AR cell line, both derived from the parental 2fTGH fibrosarcoma cell line. U3A and U3AR cells were incubated in medium only or medium supplemented with HIV-1-suppressing exosomes for 10 h. After incubation, all cells were liposome-transfected with an HIV-1 LTR-CAT gene reporter plasmid followed by mitogen induction of the LTR with PMA. After an 8-h PMA stimulation, intracellular CAT protein concentration was measured by ELISA. We observed no HIV-1 suppression in STAT1-deficient U3A cells but found

61% HIV-1 suppression in STAT1-expressing U3AR cells (Fig. 7).

DISCUSSION

The data presented in this report describe for the first time the existence of a membrane protein factor mediating noncytotoxic HIV-1 suppression that is expressed by CD8⁺ T cells and secreted via exosomes. HIV-1 suppression activity was observed in both primary CD8⁺ T cells and our transformed CD8⁺ T-cell line, TG, indicating that the antiviral properties displayed by exosomes were not the result of the HVS transformation process. The antiviral exosomes possessed many of the hallmarks defining noncytotoxic HIV-1 suppression activity by CD8⁺ T cells including nontoxicity to target cells, suppression of both CCR5- and CXCR4-tropic HIV-1 isolates, and transcriptional suppression of the HIV-1 LTR promoter. This transcriptional repression was observed in models mimicking both acute and chronic HIV-1 infection, as evidenced by suppression of LTR-activated transcripts in both TZM-bl and 8E5 cells. In the acute model, we found that the CD8⁺ T-cell-secreted exosomes could suppress PMA-induced HIV-1 LTR transcription in the absence of *tat* or any viral protein expression, a result that is consistent with previous characterizations of the noncytotoxic antiviral activity in culture supernatant of CD8⁺ T cells (5, 12).

Our results demonstrate that, unlike β -chemokines, the antiviral activity of exosomes was not mediated at the level of entry but through induction of an unknown signal that represses the lentiviral LTR promoter. Our biochemical characterization of the suppressive activity in exosomes indicated that HIV-1 suppression is mediated by a protein moiety present at the extracellular surface of exosomes. Whether the exosome-localized factor is a single molecule or a set of multiple molecules triggering intracellular transcriptional repression of HIV-1 remains to be determined. What we did observe from our confocal analysis was that exosomes did not internalize and remained at the target cell periphery even after 12 h of exposure. The lack of internalization of exosomes and the orientation of the antiviral activity to the extracellular face of the nanovesicles suggest that HIV-1 transcriptional repression is triggered by one or more ligand-receptor interactions that occur upon membrane contact of exosomes with the target cell surface.

That a signaling mechanism may be occurring is consistent with our observation that exosome-mediated transcriptional repression of the LTR is dependent on STAT1, a component of the interferon-mediated signaling pathway (5). Our time course experiment of LTR repression in TZM-bl cells indicated that at least a 12- to 16-h exposure to exosomes was required in for maximum LTR transcriptional suppression to be reached, a result in agreement with previous analysis of CAF-mediated HIV-1 LTR transcription (5). This delayed induction of LTR promoter repression, which was also observed in chronically infected 8E5 cells, suggests a possible involvement of secondary gene expression in the intracellular suppression of HIV-1 transcription, something that is known to occur through the STAT1-mediated interferon pathway (5). Interestingly, interferons have been shown not to be responsible for the elusive CAF activity (26). It is not clear what the putative

target cell receptors that facilitate transcriptional repression of HIV-1 are. A hint that the putative receptor(s) may be somewhat ubiquitously expressed can be gleaned from our observation that HIV-1 LTR promoter repression occurred in cell lines as divergent as fibrosarcoma, HeLa, and T cells. Further study may reveal what the exact involvement of STAT1 is and the resulting signal cascade that facilitates intracellular repression of the HIV-1 promoter.

Our evidence of an exosome-bound antiviral factor expressed by CD8⁺ T cells opens a new avenue in the search for candidate molecules comprising the elusive CAF, one beyond the realm of cytokines and chemokines. Our findings that secreted CD8⁺ T-cell exosomes can suppress HIV-1 suggest the existence of a more elaborate membrane-mediated mechanism underlying the noncytotoxic antiviral activity than previous lymphokine secretion models have envisioned (10, 26, 52). This includes the possibility that exosomes could be facilitating the colocalization of multiple membrane factors that work in tandem to trigger a specific intracellular pathway suppressing HIV-1 transcription. Exosome biogenesis is an elaborate and active process involving complex membrane and cytoskeletal reorganization in the luminal membranes of late endosomes (41). Previous characterizations of exosomes from a number of mammalian cell types have demonstrated a consistent pattern of proteins that are specifically included in the membrane and cargo of the nanovesicles (14, 40, 41, 43, 49). Our immunophenotyping of the CD8⁺ T-cell line TG-secreted antiviral exosomes is consistent with these previous exosome characterizations, showing a high enrichment of MHC class II molecules and tetraspanin proteins (14). Consequently, the localization of an LTR-suppressive factor to exosomes suggests a potential regulation of this antiviral activity by the same tetraspanin scaffolds mediating MHC class II trafficking and function.

Our findings, consequently, raise the question of how intracellular membrane trafficking inside CD8⁺ T cells might determine the cellular presentation of noncytotoxic HIV-1 suppression activity. Cellular microdomains consisting of tetraspanin scaffolds (19, 28, 48, 51), something exosome membranes are highly enriched for (13, 40, 41), have been shown to be critical mediators of intracellular trafficking and lateral membrane protein translocation in immune cells (28, 39, 48, 51). Our finding of reduced appearance of lysosomal marker CD63 at the CD8⁺ cell surface compared to exosomes (Fig. 2E and F) is in line with the molecule's known function in shuttling internal endosomal membrane components to lysosomes (39). This raises the question of whether our observed membrane-bound HIV-1-suppressing activity might also traffic to lysosomes within CD8⁺ T cells. With broad lipid and protein degradation occurring in these compartments, potential lysosomal targeting of the putative antiviral factor might result in its removal from a membrane anchor producing a soluble isoform eliciting the same HIV-1 suppression activity. Indeed, our biophysical analysis did demonstrate that the antiviral action of the putative membrane factor was elicited independently of its lipid tethering. A more thorough characterization of exosome versus cell surface membrane factors, in conjunction with further confocal imaging studies, may provide valuable clues to understanding the cell contact dependence previously observed for the CD8⁺ T-cell effector function. An exhaustive proteomic delineation of exosome proteins will likely prove

challenging as exosomes possess biochemical complexities on par with general cell membrane compartments (41). Nonetheless, further efforts toward identifying the membrane factor(s) responsible for the antiretroviral activity will allow for a much needed mechanistic understanding of this effector function of CD8⁺ T cells.

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REFERENCES

1. Barker, E., K. N. Bossart, S. H. Fujimura, and J. A. Levy. 1999. The role of CD80 and CD86 in enhancing CD8⁺ cell suppression of HIV replication. *Cell Immunol.* **196**:95–103.
2. Blackburn, D. J., C. P. Locher, B. Ramachandran, S. W. Barnett, K. K. Murthy, K. D. Carey, K. M. Brasky, and J. A. Levy. 1997. CD8⁺ cells from HIV-2-infected baboons control HIV replication. *AIDS* **11**:737–746.
3. Blackburn, D. J., C. E. Mackewicz, E. Barker, T. K. Hunt, B. Herndier, A. T. Haase, and J. A. Levy. 1996. Suppression of HIV replication by lymphoid tissue CD8⁺ cells correlates with the clinical state of HIV-infected individuals. *Proc. Natl. Acad. Sci. USA* **93**:13125–13130.
4. Chang, T. L., F. Francois, A. Mosoian, and M. E. Klotman. 2003. CAF-mediated human immunodeficiency virus (HIV) type 1 transcriptional inhibition is distinct from alpha-defensin-1 HIV inhibition. *J. Virol.* **77**:6777–6784.
5. Chang, T. L., A. Mosoian, R. Pine, M. E. Klotman, and J. P. Moore. 2002. A soluble factor(s) secreted from CD8⁺ T lymphocytes inhibits human immunodeficiency virus type 1 replication through STAT1 activation. *J. Virol.* **76**:569–581.
6. Chen, C. H., K. J. Weinhold, J. A. Bartlett, D. P. Bolognesi, and M. L. Greenberg. 1993. CD8⁺ T lymphocyte-mediated inhibition of HIV-1 long terminal repeat transcription: a novel antiviral mechanism. *AIDS Res. Hum. Retrovir.* **9**:1079–1086.
7. Chen, Y., D. Dampf, M. Chen, K. Kulka, D. J. Volsky, K. Saha, and P. Gupta. 2000. Dependence of CD8⁺ T-cell-mediated suppression of HIV type 1 on viral phenotypes and mediation of phenotype-dependent suppression by viral envelope gene and not by beta-chemokines. *AIDS Res. Hum. Retrovir.* **16**:117–124.
8. Chen, Y., C. Rinaldo, and P. Gupta. 1997. A semiquantitative assay for CD8⁺ T-cell-mediated suppression of human immunodeficiency virus type 1 infection. *Clin. Diagn. Lab. Immunol.* **4**:4–10.
9. Clayton, A., J. Court, H. Navabi, M. Adams, M. D. Mason, J. A. Hobot, G. R. Newman, and B. Jasani. 2001. Analysis of antigen presenting cell derived exosomes, based on immuno-magnetic isolation and flow cytometry. *J. Immunol. Methods* **247**:163–174.
10. Cocchi, F., A. L. DeVico, A. Garzino-Demo, S. K. Arya, R. C. Gallo, and P. Lusso. 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* **270**:1811–1815.
11. Copeland, K. F., P. J. McKay, and K. L. Rosenthal. 1995. Suppression of activation of the human immunodeficiency virus long terminal repeat by CD8⁺ T cells is not lentivirus specific. *AIDS Res. Hum. Retrovir.* **11**:1321–1326.
12. Copeland, K. F., P. J. McKay, and K. L. Rosenthal. 1996. Suppression of the human immunodeficiency virus long terminal repeat by CD8⁺ T cells is dependent on the NFAT-1 element. *AIDS Res. Hum. Retrovir.* **12**:143–148.
13. Denzer, K., M. J. Kleijmeer, H. F. Heijnen, W. Stoorvogel, and H. J. Geuze. 2000. Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. *J. Cell Sci.* **113**:3365–3374.
14. Escola, J. M., M. J. Kleijmeer, W. Stoorvogel, J. M. Griffith, O. Yoshie, and H. J. Geuze. 1998. Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* **273**:20121–20127.
15. Fujiki, Y., S. Fowler, H. Shio, A. L. Hubbard, and P. B. Lazarow. 1982.

- Polypeptide and phospholipid composition of the membrane of rat liver peroxisomes: comparison with endoplasmic reticulum and mitochondrial membranes. *J. Cell Biol.* **93**:103–110.
16. Geiben-Lynn, R., N. Brown, B. D. Walker, and A. D. Luster. 2002. Purification of a modified form of bovine antithrombin III as an HIV-1 CD8⁺ T-cell antiviral factor. *J. Biol. Chem.* **277**:42352–42357.
 17. Greenberg, M. L., S. F. Lacey, C. H. Chen, D. P. Bolognesi, and K. J. Weinhold. 1997. Noncytolytic CD8 T cell-mediated suppression of HIV replication. *Springer Semin. Immunopathol.* **18**:355–369.
 18. Heijnen, H. F., A. E. Schiel, R. Fijnheer, H. J. Geuze, and J. J. Sixma. 1999. Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* **94**:3791–3799.
 19. Hemler, M. E. 2005. Tetraspanin functions and associated microdomains. *Nat. Rev. Mol. Cell Biol.* **6**:801–811.
 20. Hsueh, F. W., C. M. Walker, D. J. Blackburn, and J. A. Levy. 1994. Suppression of HIV replication by CD8⁺ cell clones derived from HIV-infected and uninfected individuals. *Cell Immunol.* **159**:271–279.
 21. Kannagi, M., L. V. Chalifoux, C. I. Lord, and N. L. Letvin. 1988. Suppression of simian immunodeficiency virus replication in vitro by CD8⁺ lymphocytes. *J. Immunol.* **140**:2237–2242.
 22. Kao, S. Y., A. F. Calman, P. A. Luciw, and B. M. Peterlin. 1987. Antitermination of transcription within the long terminal repeat of HIV-1 by *tat* gene product. *Nature* **330**:489–493.
 23. Lacey, S. F., K. J. Weinhold, C. H. Chen, C. McDaniel, C. Oei, and M. L. Greenberg. 1998. Herpesvirus saimiri transformation of HIV type 1 suppressive CD8⁺ lymphocytes from an HIV type 1-infected asymptomatic individual. *AIDS Res. Hum. Retrovir.* **14**:521–531.
 24. Le Borgne, S., M. Fevrier, C. Callebaut, S. P. Lee, and Y. Riviere. 2000. CD8⁺-cell antiviral factor activity is not restricted to human immunodeficiency virus (HIV)-specific T cells and can block HIV replication after initiation of reverse transcription. *J. Virol.* **74**:4456–4464.
 25. Leith, J. G., K. F. Copeland, P. J. McKay, C. D. Richards, and K. L. Rosenthal. 1997. CD8⁺ T-cell-mediated suppression of HIV-1 long terminal repeat-driven gene expression is not modulated by the CC chemokines RANTES, macrophage inflammatory protein (MIP)-1 alpha and MIP-1 beta. *AIDS* **11**:575–580.
 26. Levy, J. A. 2003. The search for the CD8⁺ cell anti-HIV factor (CAF). *Trends Immunol.* **24**:628–632.
 27. Levy, J. A., C. E. Mackewicz, and E. Barker. 1996. Controlling HIV pathogenesis: the role of the noncytotoxic anti-HIV response of CD8⁺ T cells. *Immunol. Today* **17**:217–224.
 28. Levy, S., and T. Shoham. 2005. The tetraspanin web modulates immune-signalling complexes. *Nat. Rev. Immunol.* **5**:136–148.
 29. Mackewicz, C., and J. A. Levy. 1992. CD8⁺ cell anti-HIV activity: nonlytic suppression of virus replication. *AIDS Res. Hum. Retrovir.* **8**:1039–1050.
 30. Mackewicz, C. E., D. J. Blackburn, and J. A. Levy. 1995. CD8⁺ T cells suppress human immunodeficiency virus replication by inhibiting viral transcription. *Proc. Natl. Acad. Sci. USA* **92**:2308–2312.
 31. Mackewicz, C. E., M. R. Garovoy, and J. A. Levy. 1998. HLA compatibility requirements for CD8⁺-T-cell-mediated suppression of human immunodeficiency virus replication. *J. Virol.* **72**:10165–10170.
 32. Mackewicz, C. E., R. Orque, J. Jung, and J. A. Levy. 1997. Derivation of herpesvirus saimiri-transformed CD8⁺ T cell lines with noncytotoxic anti-HIV activity. *Clin. Immunol. Immunopathol.* **82**:274–281.
 33. Morelli, A. E., A. T. Larregina, W. J. Shufesky, M. L. Sullivan, D. B. Stolz, G. D. Papworth, A. F. Zahorchak, A. J. Logar, Z. Wang, S. C. Watkins, L. D. Faló, Jr., and A. W. Thomson. 2004. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* **104**:3257–3266.
 34. Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55–63.
 35. Ohashi, T., M. Kubo, H. Kato, A. Iwamoto, H. Takahashi, M. Fujii, and M. Kannagi. 1999. Role of class I major histocompatibility complex-restricted and -unrestricted suppression of human immunodeficiency virus type 1 replication by CD8⁺ T lymphocytes. *J. Gen. Virol.* **80**:209–216.
 36. Pan, B. T., K. Teng, C. Wu, M. Adam, and R. M. Johnstone. 1985. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J. Cell Biol.* **101**:942–948.
 37. Raposo, G., H. W. Nijman, W. Stoorvogel, R. Liejendekker, C. V. Harding, C. J. Melief, and H. J. Geuze. 1996. B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* **183**:1161–1172.
 38. Southgate, C. D., and M. R. Green. 1991. The HIV-1 Tat protein activates transcription from an upstream DNA-binding site: implications for Tat function. *Genes Dev.* **5**:2496–2507.
 39. Takino, T., H. Miyamori, N. Kawaguchi, T. Uekita, M. Seiki, and H. Sato. 2003. Tetraspanin CD63 promotes targeting and lysosomal proteolysis of membrane-type 1 matrix metalloproteinase. *Biochem. Biophys. Res. Commun.* **304**:160–166.
 40. Thery, C., M. Boussac, P. Veron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, and S. Amigorena. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J. Immunol.* **166**:7309–7318.
 41. Thery, C., L. Zitvogel, and S. Amigorena. 2002. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* **2**:569–579.
 42. Tomaras, G. D., S. F. Lacey, C. B. McDaniel, G. Ferrari, K. J. Weinhold, and M. L. Greenberg. 2000. CD8⁺ T cell-mediated suppressive activity inhibits HIV-1 after virus entry with kinetics indicating effects on virus gene expression. *Proc. Natl. Acad. Sci. USA* **97**:3503–3508.
 43. Valadi, H., K. Ekstrom, A. Bossios, M. Sjostrand, J. J. Lee, and J. O. Lotvall. 2007. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* **9**:654–659.
 44. van Niel, G., G. Raposo, C. Candalh, M. Boussac, R. Hershberg, N. Cerf-Bensussan, and M. Heyman. 2001. Intestinal epithelial cells secrete exosome-like vesicles. *Gastroenterology* **121**:337–349.
 45. Walker, C. M., A. L. Erickson, F. C. Hsueh, and J. A. Levy. 1991. Inhibition of human immunodeficiency virus replication in acutely infected CD4⁺ cells by CD8⁺ cells involves a noncytotoxic mechanism. *J. Virol.* **65**:5921–5927.
 46. Walker, C. M., and J. A. Levy. 1989. A diffusible lymphokine produced by CD8⁺ T lymphocytes suppresses HIV replication. *Immunology* **66**:628–630.
 47. Walker, C. M., G. A. Thomson-Honniebier, F. C. Hsueh, A. L. Erickson, L. Z. Pan, and J. A. Levy. 1991. CD8⁺ T cells from HIV-1-infected individuals inhibit acute infection by human and primate immunodeficiency viruses. *Cell Immunol.* **137**:420–428.
 48. Wright, M. D., G. W. Moseley, and A. B. van Spruiel. 2004. Tetraspanin microdomains in immune cell signalling and malignant disease. *Tissue Antigens* **64**:533–542.
 49. Wubbolts, R., R. S. Leckie, P. T. Veenhuizen, G. Schwarzmann, W. Mobius, J. Hoernschemeyer, J. W. Slot, H. J. Geuze, and W. Stoorvogel. 2003. Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J. Biol. Chem.* **278**:10963–10972.
 50. Yates, J. R., III, A. Gilchrist, K. E. Howell, and J. J. Bergeron. 2005. Proteomics of organelles and large cellular structures. *Nat. Rev. Mol. Cell Biol.* **6**:702–714.
 51. Yunta, M., and P. A. Lazo. 2003. Tetraspanin proteins as organisers of membrane microdomains and signalling complexes. *Cell Signal.* **15**:559–564.
 52. Zhang, L., W. Yu, T. He, J. Yu, R. E. Caffrey, E. A. Dalmasso, S. Fu, T. Pham, J. Mei, J. J. Ho, W. Zhang, P. Lopez, and D. D. Ho. 2002. Contribution of human alpha-defensin 1, 2, and 3 to the anti-HIV-1 activity of CD8 antiviral factor. *Science* **298**:995–1000.