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Dendritic Cells Pulsed with HIV-1 Release Exosomes that Promote Apoptosis in CD4+ T Lymphocytes

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Abstract

Loss of mucosal CD4TL (CD4+ T lymphocyte) cells is a salient characteristic of infection by the human immunodeficiency virus-1 (HIV-1). While several mechanisms promoting T cell apoptosis have been proposed, they fail to explain fully the observed T cell loss. Dendritic cells (DCs) are thought to play a pivotal role in the spread of HIV-1 throughout the organism, both establishing and maintaining the infection. DCs capture virions, enclose them in late endocytic compartments and subsequently deliver them to target cells. Internalized viral particles are found in cellular compartments that also contain nanovesicles known as exosomes. These vesicles and virions are released together by DCs. Whether or not exosomes are benign in HIV-1 pathogenesis is unknown. We therefore examined the effect of exosomes derived from HIV-1-infected DCs on CD4TL viability and HIV infectivity in CD4TL. DCs exposed to HIV-1 release more exosomes into the extra-cellular media than do control cells. By processing culture supernatant of infected DCs to separate HIV-1 from exosomes, we showed that the latter produce a pro-apoptotic profile in CD4TL. The purified HIV-1 fraction allows greater viability of CD4TL but is more infectious than the exosome-containing fraction. Altogether, our results suggest that exosomes derived from HIV-1-infected DCs can bring about apoptosis of CD4TL and might thereby limit HIV infectivity in the infectious synapse. Exosome release appears to be an important immune modulator mechanism while appearing paradoxically to contribute to the T-cell depletion observed following HIV infection.

Keywords: Microvesicles; Velocity gradient; Acute HIV-1 infection; T-cell apoptosis; HIV-1 purification; Exosomes

Abbreviations: DCs: Dendritic cells; CD4TL: CD4+ T lymphocytes; HIV-1: Human immunodeficiency virus-1; HAART: Highly active antiretroviral therapy; SIV: Simian immunodeficiency virus

Introduction

In North America, where antiretroviral treatments are readily accessible, AIDS-related death is in rapid decline. However, antiretroviral treatments have debilitating side effects, and drug resistance is a major problem. Moreover, the pandemic keeps spreading, especially in Africa and Southeast Asia. No effective HIV vaccine has yet been developed, and current antiretroviral agents fail to eradicate the virus from the body. Failure to develop a preventive vaccine is at least in part the result of incomplete understanding of the pathogenic mechanisms of the disease. HIV-1 disease is characterized by a state of chronic immune activation due to sustained inflammation and immune hyperactivation, observed even under conditions of highly active antiretroviral therapy (HAART) [1]. Numerous observations of non-pathogenic simian immunodeficiency virus (SIV) infection and of HIV-1-infected patients who spontaneously control the disease ("elite controllers" or long-term non-progressors) indicate that a good correlation exists between a low level of activation of the immune system and the lack of clinical signs of AIDS [2-6]. In contrast, strongly increased immune activation characterized by dysregulated neutrophil and macrophage functions [7,8], polyclonal B cell activation, [9] increased T cell turnover [9], increased numbers of T cells with an activated phenotype [9] and increased levels of pro-inflammatory molecules are all indicators of disease progression in primate lentivirus (HIV/SIV) pathogenic infections [10-12]. These studies and several others point to early events in HIV-1 infection as highly determinant in the irreversible damage inflicted on immune cells [13-17]. It is well established that dendritic cells (DCs) are involved early in HIV-1 transmission [18-20]. DCs are the first immune cells to come into contact with HIV-1. They internalize the virus and migrate to secondary lymphoid organs, where they communicate with cells of the innate and adaptive immune systems [18,21]. Physical contact between HIV-1-loaded DC and CD4TL appears sufficient for internalization of virions by the latter [22], leading to virion production in both cell types [23-27]. CD4 T lymphocytes (CD4TL), in particular Th17 residing in mucosal lymphoid tissues, are major sites for HIV-1 replication and are selectively depleted in pathogenic models of the disease [28-30]. Their numbers are dramatically reduced in HIV-1-infected subjects [30]. CD4TL apoptosis is an indicator of HIV-1 pathogenesis in both early and late phases of AIDS. CD4TL play an important role in the immune response to HIV-1. Weakening of the immune system by rapid sustained depletion of mucosal CD4TL [23-27] with ensuing irreversible damage [23,27,31] begins very soon after HIV enters the body. The observation of rapid depletion of CD4TL in gut-associated lymphoid tissues has reoriented AIDS research [23-27]. Destruction of mucosal lymphoid tissue and loss of gut epithelial integrity allow microbial products to cross the intestinal barrier, resulting in high

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levels of circulating bacterial lipopolysaccharides [31] and maintaining the chronic state of inflammation and systemic immune activation observed in patients with chronic HIV-1-infection [31,32]. The mechanisms proposed to explain the drastic depletion of CD4TL in gut-associated lymphoid tissues include direct infection of CD4TL by the virus [33], cytotoxic activity of $CD8^+$ T cells against infected cells [34] and cytopathic effects on bystander cells, due to accumulation of abortive HIV-1 reverse transcripts [35]. Other secreted factors and vesicles similar to virions may be involved in this process. More recently, extra-cellular particles called *exosomes* have been identified as plausible intercellular mediators [36]. Measuring from 30 to 100 nm in diameter, exosomes are microvesicles originating from endocytic compartments called multivesicular bodies (MVB) and are released into the extra-cellular milieu following fusion of MVB with the plasma membrane [37]. Exosome release upon cell activation by cytokines is a characteristic of numerous cell types, including tumor, fetal, epithelial and hematopoietic cells [38-44]. Roles for exosomes in intercellular communication, possibly related to various proteins and other macromolecules they bear on their membrane or in their lumen, have been described in the context of immune response regulation, notably in tolerance induction [41-43,45-55], antigen presentation [53,56-61], cancer immunotherapy [62-66], control of receptor expression [39,67,68], cell death mechanisms [38,69-72] and control of inflammation [38,67,73]. Exosomes can also contain functional miRNA [74] and can deliver bioactive lipids [75, 76]. Depending on the function and the activation state of the secreting cells, exosomes could thus regulate multiple pathways in neighboring cells in a paracrine fashion. Delimited by a lipid bilayer, they display a characteristic flotation density ranging between 1.13-1.19 g/ml on sucrose gradients. On velocity gradients, exosomes are recovered in the fractions containing 9.6 to 12% iodixanol [77]. Common exosome membrane proteins include tetraspanins, LAMP-2, actin, acetylcholinesterase (AChE), Tsg101 and heat shock proteins.

The relationship between exosome biogenesis and retrovirus assembly is not yet certain [78]. Although much evidence points to usurpation of the plasma-membrane-associated MVB biogenesis machinery by HIV-1 budding from CD4TL, virions are also found in endosomes of macrophages and DCs, suggesting instead an internal budding process [79-82]. Comparative study of exosomes and HIV-1 particles [80] and observation of similar viral budding [83] and uptake by cells [84] suggest that retroviruses evolved by exploiting the exosome release pathway. Exosome release by DCs has been studied extensively [85-88], but not in the case of HIV-1-loaded DCs. In some studies, an inhibitory effect on the immune response has been described for exosomes, most notably T cell death induced by tumor-derived exosomes bearing either FasL or galectin-9 [38,89-95]. More recently, a research group has emphasized the role of HIV-1 protein Nef released with exosomes in inducing the apoptosis of bystander CD4TL [72]. These reports have led us to investigate the involvement of exosomes in CD4TL depletion during HIV- 1 infection in order to determine what role they might play in HIV-1 pathogenesis. The aim of the present study was to evaluate the role of exosomes in CD4TL viability using an in vitro cell model of HIV-1 infection. We focused on vesicles released from DCs pulsed with HIV-1 and thus showed that exosomes appear to be involved in CD4TL death. Our results suggest a significant role for exosomes in pathogenic developments following HIV-1 infection.

Methods

Reagents

Optiprep™ (60% iodixanol) was obtained from Sigma Aldrich (Oakville, ON, Canada). Protein A/G PLUS-Agarose was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). GM-CSF was purchased from Genscript (Paletta Court Burlington, CA). Human recombinant IL-2 (rhIL-2) was obtained through the AIDS Repository Reagent Program (Germantown, MD). IL-4 and FITC-VAD-FMK (Apostat) were from R&D Systems (Minneapolis, MN). LPS-EB Ultrapur and Primocin were purchased from Invivogen (San Diego, CA). RPMI 1640, high-glucose DMEM, lymphocyte separation medium, fetal bovine serum (FBS), penicillin G, streptomycin and glutamine were purchased from WISENT Inc. (St Bruno, QC, Canada). Phytohemagglutinin-L (PHA-L), acetylthiocholine 5,5-dithio-bis(2-nitrobenzoic acid) and 2,2'-dithiodipyridine (AT-2) were purchased from Sigma-Aldrich (St. Louis, MO).

Antibodies: Anti-HLA-DR-producing hybridomas (L243, 2.06) and anti-acetylcholinesterase producing clone AE-1 were obtained from the American Type Culture Collection (Manassas, VA). When necessary, antibodies were purified using mAbTrap protein affinity columns according to the manufacturer's instructions (Pharmacia Technology AB, Uppsala, Sweden). Anti-HLA-DRα (DA6.147), anti-Icam-1 (G-5), anti-actin (I-19), anti-MHC-I (F-3) and anti-Lamp-2 (H4B4) were purchased from Santa Cruz Biotechnologies® Inc. (Santa Cruz, CA). Isotype control IgG1 was purchased from Sigma-Aldrich (St. Louis, MO). Anti-goat-HRP or anti-mouse-HRP secondary antibodies were obtained from Jackson Immunoresearch (Baltimore, MA). For the enzyme-linked immunosorbent assay (ELISA) of HIV-p24, coating was done using anti-p24 antibody (hybridoma 183-H12), and detection was done using biotin-conjugated p24 antibody (hybridoma 31-90-25), both antibodies being generous gifts from Dr. Michel Tremblay (Université Laval, QC, Canada). Streptavidin-PolyHRP40 was obtained from Fitzgerald Industries (Burlington, CA).

Cells: The human embryonic kidney cell line 293T (HEK293T) was obtained from the ATCC and maintained in DMEM complete medium. Human DCs and primary CD4TL were isolated from peripheral blood mononuclear cells (PBMCs) obtained from anonymous healthy volunteer donors. DCs were generated from $CD14^+$ monocytes as described previously [96]. Briefly, PBMCs were prepared by centrifugation on lymphocyte separation medium. $CD14^+$ cells were then isolated using a selection kit according to the manufacturer's instructions (Stemsep Human $CD14^+$ Selection Kit, STEMCELL Technologies, Vancouver, BC, Canada) with an autoMACS® (Miltenyl Biotech). $CD14^+$ cells were cultured in 6-well plates at a concentration of 10^6 cells/ml. To generate DCs, purified monocytes were cultured in complete culture medium that was supplemented every two days with GM-CSF (1,000 U/ml) and IL-4 (200 U/ml) for 6–7 days. When necessary, DC maturation was induced on the fifth day by culturing for 48 h with the above-mentioned cytokines supplemented with interferon-γ (IFN-γ) (1,000 U/ml) and LPS (100 ng/ml). Immature DCs express HLA-DR, CD86, DC-SIGN and low levels of CD14, while mature DCs express CD83 and high levels of ICAM-1, HLA-DR and CD86, but lower levels of DC-SIGN and CD14. Expression of CD3 and CD19 was measured to assess contaminating T and B cells respectively. CD4TL were isolated using a negative selection kit according to the

manufacturer's instructions (Stemsep Human CD4 T cell Enrichment Kit, STEMCell Technologies). In some experiments, these cells were activated with 1 µg/ml PHA-L (to obtain mitogen-stimulated cells) or not (quiescent cells) and maintained in complete culture medium supplemented with IL-2 (30 U/ml) at a density of 2 x 10^6 cells/ml. Experiments were performed with nearly pure cell preparations (i.e. DC > 95%; $CD14^+$ T > 98%). In all culture media, bovine exosomes derived from FBS were eliminated by overnight ultracentrifugation of serum at 100,000xg.

Production of Virus Stock: Virions were produced by transient transfection in human embryonic kidney 293T cells as described previously [97]. The plasmids used include pJR-CSF (R5-tropic), pNLAD8 (R5-tropic), pNL4-3Bal*env* (R5-tropic) and pNL4-3 (X4-tropic). The pNL4-3Bal*env* vector (provided by R. Pomerantz, Thomas Jefferson University, Philadelphia, PA) was generated by replacing the *env* gene of the T-tropic HIV-1 strain NL4-3 with that of the macrophage-tropic HIV-1 Bal strain, thus resulting in an infectious molecular clone with R5-tropic properties [98]. Other plasmids were obtained from the AIDS Repository Reagent Program (Germantown, MD). Several virion preparations were obtained from primary cells. Briefly, peripheral blood was obtained from healthy donors and PBMCs were prepared by centrifugation on a Ficoll-Hypaque density gradient. NL4-3Bal*env* virions were produced by acute infection of PBMCs for 6 d (1.5 x 10^7 PBMC per ml with 500 ng of virion per ml). On day 6, the virus containing supernatants were filtered through 0.22-µm cellulose acetate membrane, purified by ultracentrifugation and normalized for virion content using a sensitive in-house double-antibody sandwich ELISA specific for the viral protein $p24^{gag}$ [99].

Immunodepletion of Exosomes in Highly Purified Viral Preparations: One hundred µl of ultracentrifuged cell supernatant were diluted in 400 µl of PBS and incubated with 20 µL of protein A/G beads pre-coated with 20 µg of anti-acetylcholine or with isotype control antibody for 1 h at room temperature (RT) using a rotating platform. The immune complexes were then recovered by centrifugation and the supernatant containing the exosome-depleted viral preparation was subjected to a second round of immunodepletion under the same conditions. The final supernatant and beads were kept for determination of $p24^{gag}$ content and acetylcholinesterase activity.

Acetylcholinesterase Activity Assay: Acetylcholinesterase activity was measured using a previously described procedure [77]. Briefly, 30 µl of standard or sample were added to equal volumes of acetylthiocholine (in PSB, pH 8) and 5,5-dithio-*bis*(2-nitrobenzoic acid) (in PBS, pH 7) to obtain final concentrations of 1.25 mM and 0.1 mM respectively in a final volume of 200 µl and held at RT. Changes in absorbance were monitored at 450 nm for 10 min with a microplate spectrophotometer (ELX808, BIO-TEK Instruments, Winooski, VT).

Loading DCs with HIV-1 and Purification of HIV-1 Particles by Velocity Gradient: Purified DCs were contacted with HIV-1 (10 ng $p24^{gag}/10^6$ cells) for 48 h at 37°C in complete RPMI 1640 containing GM-CSF and IL-4, and the supernatants were harvested and subjected to sequential centrifugation before Optiprep separation. Virions and exosomes were first concentrated by ultracentrifugation using an Optima L-90K Beckman Coulter apparatus (Fullerton, CA) for 45 min at 100,000x*g* (31,500 rpm) in a 70 Ti rotor. The pellet containing virions and exosomes was re-suspended in 500 µl of PBS and then centrifuged through a 6-18% Optiprep velocity gradient as described previously [77,100]. The virus preparations were centrifuged using the Optima L-90K apparatus for 75 min at 250,000x*g* in a NVT65 rotor. Gradient fractions were collected from the top. The different fractions were then analyzed using a sensitive in-house double-antibody sandwich ELISA specific for $p24^{gag}$ and the acetylcholinesterase activity assay [77,100].

ELISA against HLA-DR: Measurement of HLA-DR-containing microvesicles (i.e. exosomes) was done using an in-house enzymatic assay. Briefly, flat-bottom 96-well plates were initially coated with anti-HLA-DR (50 µg/ml, from hybridoma 2.06) in carbonate buffer (pH 9). After removal of excess antibody by three washes with 0.1% Tween 20 in PBS, non-specific sites were blocked with 1% BSA in PBS plus 0.1% Tween 20 for 1 h at RT. The plates were washed again and wells were filled with sample (100 µl) followed by holding for 1h at RT. The plate was then washed three times and a second biotinylated anti-HLA-DR antibody (0.5 µg/ml, from L-243) in blocking solution was added followed again by 1 h at RT. The plates were then washed three times and streptavidin-peroxidase conjugate (streptavidin-HRP-40; Fitzgerald Industries, Wicklow, IRL) was added for 30 min at RT. Plates were washed again and detection was performed by adding 3,3′,5,5′-tetramethylbenzidine sulfate substrate followed by 1M H3PO4 to terminate the reaction. HLA-DR purified from Raji CD4 cells was used as standard curve.

SDS-PAGE and Western blot analysis: Twenty-five µl of the 100 µl of exosome preparation were added to an equal volume of 2X sample buffer at 100°C and held at 100°C for 7 min as described [101]. Electrophoresis was then done on 7.5-20% SDS-polyacrylamide gradient gel followed by transfer to Immobilon PVDF membrane (Millipore Corporation, Bedford, MA). Immunoblotting was done overnight at 4°C using antibody diluted 200-fold in non-fat skim milk powder (5% in Tris buffer saline with Tween 20 (TBST)) or 10,000-fold in gelatin (2% in TBST) for actin and revealed using the Luminata Forte horseradish peroxidase substrate (Millipore Corporation, Bedford, MA) for exosome detection, or Western Lightning® Plus ECL (Perkin Elmer, Montreal, Canada) for detection in cells. HRP-conjugated secondary anti-mouse or antigoat antibodies (Jackson Immunoresearch (Baltimore, MA)) were used at a dilution of 1/20,000.

Immunocapture of exosomes: Exosomes diluted in 500µl of PBS, were captured directly with 50 µl of protein A/G beads pre-coated with anti-AChE (40 µg of antibody per ml of final solution) or isotype control antibody (IgG1). Exosomes were then eluted from the beads using 50 µl of isotonic 0.2 M glycine solution in 0.1 M KH2PO4 (pH 3) for 2 min. The pH was then brought to neutrality by adding 100 µl of 0.1 M K2HPO4 (pH 8.8).

Infectivity Measurements Using Spliced Tat Levels: Expression levels of TAT splice transcripts were determined using a Rotor-Gene system (Corbett Life Science). Total RNA (from 10^6 cells) was isolated using an RNA extraction kit (GE). After elution, RNA quantity and quality were measured in terms of absorbance at 260 and 280 nm. RNA was the reverse-transcribed using Superscript® III Reverse Transcriptase, followed by qRT-PCR quantification of transcripts using TaqMan Universal PCR Master Mix system (Applied Biosystems) with primers designed for the TAT splice form (TAT splice-F [GAAGCATC-CAGGAAGTCAGC], TAT splice-R [CTATTCCTTCGGGCCTGTC], 18S-F [TAGAGGGACAAGTGGCGTTC] and 18S-R [CGCTGAGC-CAGTCAGTGT]). Normalization with 18S rRNA levels was performed to express the final values. A standard curve was derived for each gene of interest using serial dilutions of RNA pooled from all samples. The probe sequences are: for TAT splice 5′-(d carboxyfluorescein (FAM))-TATCAAAGCAACCCACCCACCTCC-(BHQ-1)-3′ and for

18S 5′-(d FAM)-AACAGGTCTGTGATGCCCTT-(BHQ-1)-3′. Two µl of cDNA were used in each reaction.

Primers and probes were used at 5 µM and 2 µM respectively. These control experiments were performed in the Gene Quantification Core Laboratory of the Centre de Génomique de Québec (CRCHUQ, Québec, QC, Canada).

Apoptosis Studies and Flow Cytometric Analyses: Five µl of cell-permeant FITC-conjugated pan-caspase inhibitor FITC-VAD-FMK were added to a final volume of 400 µl of medium containing 5 x 10^5 cells followed by 30 min at 37°C. Cells were then washed and re-suspended in PBS containing 2 mM EDTA and 0.5% BSA and fixed with 2% *p*-formaldehyde (final concentration) for subsequent analysis by flow cytometry (FACSCalibur™ platform, Becton-Dickinson). Data were acquired using BD CellQuest Pro software and analyses were done using Cyflogic software. Fifty µl of exosome suspension from 4 x 10^6 cells were added to the CD4TL culture for the indicated time.

Statistical analysis: Means were compared using either Student's *t*-test or single-factor ANOVA followed by Dunnett's multiple comparison when more than two means were considered [102]. *P* values < 0.05 were considered statistically significant. Bonferroni correction was applied to minimize the probability of type-I errors in Figure 2. For all figures, an asterisk (*) denotes a *p*value < 0.05, whereas two asterisks (**) denotes a *p*-value < 0.01 and three asterisks (***) denotes a *p*-value < 0.001. Calculations were done using GraphPad Prism software.

Results

Obtaining exosome-free viral preparations

Exosome secretion by DCs has been studied extensively during the past few years [87,89]. However, the release and function of exosomes from DCs in the context of HIV-1 infection remains poorly documented, due mainly to the difficulty of separating microvesicles from HIV-1 particles [84,103]. We therefore began by developing a method of producing exosome-free preparations of HIV-1. Cultured HEK-293T cells were transfected with a plasmid expressing the R5 molecular clone NL4-3Bal*env*. The final pellet from the sequential centrifugation of the culture supernatant, containing both exosomes and virions, was re-suspended and subjected to immunocapture using protein A/G beads pre-coated with an anti-acetylcholinesterase (AE-1),

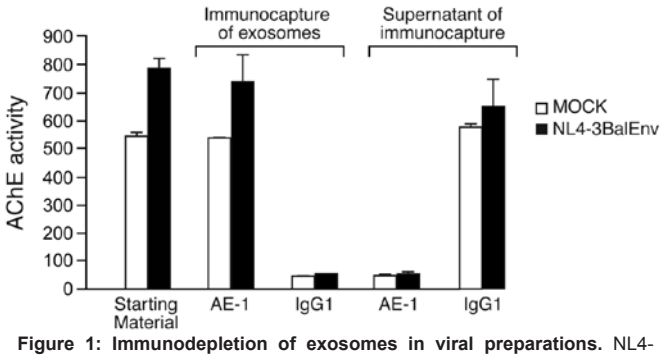

Figure 1: Immunodepletion of exosomes in viral preparations. NL4-3Bal*env* virions were produced by transfection of HEK-293T cells. Culture supernatant was centrifuged and the pellet was re-suspended and incubated with protein A/G beads pre-coated with anti-AChE (AE-1) or with isotype control (IgG1) antibody. AChE activities associated with the A/G beads (immunocaptured exosomes), in the bead supernatant and in the original re-suspended centrifugal pellet were measured.

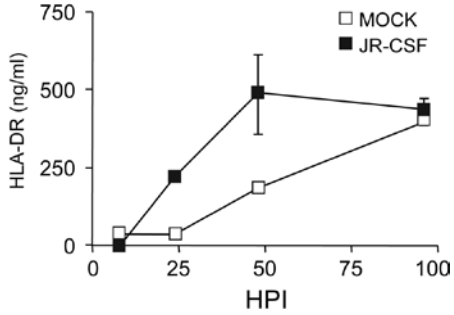

Figure 2: Release of exosomes from dendritic cells after HIV-1 capture. DCs were incubated for 1h with mock viral suspension or with HIV-1 clone JR-CSF and then cultured for an additional 24h, 48h or 96h. Exosomes and HIV-1 virions were separated by differential centrifugation of the cell-free supernatants and the amount of exosomes in the pellet was determined by means of an ELISA targeting the exosome marker HLA-DR. Data represent the mean ± SEM of triplicate samples for one donor and are representative of three independent experiments. Sequential Bonferroni correction indicated that p < 0.01.

or with an isotype control (IgG1) antibody [77]. As shown in terms of AChE activity, a single round of immunocapture allowed quantitative recovery of the exosomes (Figure 1) while virions remained in the suspension. Protein A/G beads coated with the isotype control antibody did not remove exosomes from the suspension. These results also show that NL4-3Bal*env*-transfected HEK-293T cells secreted large amounts of exosomes compared to mock transfected cells. This increased production of exosomes was confirmed using both R5 and X4 tropic strains. Immunocapture via AChE was therefore effective for the selective removal of exosomes from suspensions containing HIV-1.

Dendritic cells release exosomes after contact with HIV-1

In order to study the effect of HIV-1 on DC secretions, we first examined *in vitro* exosome production by DCs following exposure to HIV-1. It should be noted that only 5–10% of DCs capture HIV-1 and that the vast majority of the virions thus taken up are degraded by lysosomes. DCs were contacted for 1h with R5 molecular clone JR-CSF (obtained by transfecting HEK-293T cells and purifying the supernatant using the above-mentioned immunocapture technique), washed several times and then cultured for 24h, 48h or 96h. Figure 2 shows the release of exosomes from DCs into the culture supernatant beginning at 24h of culture. Since the replication of HIV-1 in DCs during the first 48h does not reach levels that are detectable by the usual methods [56,103,104] and since HEK-293T cells and hence the virions they produce lack HLADR, the HLA-DR-bearing particles detected were DC-derived exosomes. The release of exosomes from DCs was also increased following contact with X4 viruses such as NL4-3 and R5 NL4-3Bal*env* (data not shown).

Efficient separation of exosomes from virions

We next considered the effect of HIV produced in primary cells on exosome release from DCs, using NL4-3Bal*env* obtained from PBMC culture supernatant made free of exosomes by immunocapture as described above. Exosomes and virions released by PBMCs were separated by differential centrifugation and exosome levels were determined by measuring AChE activity. Increased exosome release (1.4 fold) following exposure to HIV-1 was again confirmed (Figure 3a). Considering that only a small percentage of DC is able to internalize virions and that the observed increase is very reproducible

and corroborates the results described in Figure 2, we consider this increase as significant. The pellets obtained by centrifugation (Figure 3a) were fractionated on an Optiprep velocity gradient. Based on measured AChE activity, large amounts of exosomes were found at 9.6–12.0% iodixanol (Figure 3b) compared to the control condition (mock exposure). The virions accumulated in fractions 13.2–18% iodixanol, as revealed by HIV-1 capsid protein detection (Figure 3c). Capsid protein corresponded to infectious virus, since these fractions were able to infect autologous CD4TL, as measured by spliced TAT RNA by real-time PCR five days post-infection (Figure 3d).

Characterization of exosomes released from HIV-1-loaded DCs

Exosome release from HIV-1-infected DCs under physiological conditions of inflammation and their effects on CD4TL was investigated. To simulate inflammation, we incubated DCs with LPS (at a concentration corresponding to steady-state levels observed in chronic HIV-1 infection) and IFN-γ (added as a supplementary agent [105]) during contact with HIV-1, thus inducing DC maturation. Exosomes were collected from DC culture medium by differential centrifugation 48h after contact with HIV-1 and/or LPS/IFN-γ (Figure 4a) and characterized by Western blot analyses. The presence of Lamp-2, ICAM-1, HLA-DR, MHC class I and actin was thus observed, while only lamp-2 was detectable under the control condition (Figure 4a). It is noteworthy that all of these markers are common characteristics of exosomes. While the HIV-1 membrane too can carry all of these proteins except for HLA-DR (which is not present in the HEK-293T cells used to produce the virions), de novo synthesis of HIV-1 does not occur in DCs prior to 48h after infection. To confirm that the particles obtained were exosomes only, we measured AChE activity (Figure 4b). These results confirm that NL4-3Bal*env* (as do R5 HIV-1 clones such as NLAD8, data not shown) increases the accumulation of exosomes in the extra-cellular medium. LPS/IFN-γ treatment increased exosome release independently of the presence of HIV-1 and this effect was not cooperative with HIV-1.

Non-infectious particles released from HIV-1-infected DCs participate in CD4TL apoptosis

Given that CD4TL depletion begins in the early stages of a new infection, we examined the apoptotic effect on CD4TL of vesicles obtained in the 100,000x*g* pellet of culture supernatant of HIV-1-infected DCs stimulated or not with LPS/IFN-γ. We sought to determine in particular the impact of the quantity of vesicles on inducing apoptosis. Virions in the culture supernatant were inactivated with AT-2 [106] and resting or activated CD4TL were incubated with the isolated exosomes and with inactivated viral particles. Apoptosis was measured using the fluorescent pan-caspase inhibitor peptide FITC-VAD-FMK. Figure 5 shows greater susceptibility of CD4TL to apoptosis in the presence of exosomes obtained from infected DCs than in their absence. This effect is seen in both resting cells (panel a) and activated cells (panel b). We note with interest that neither particles recovered from DCs stimulated with LPS/IFN-γ alone nor HIV-1 itself induced the observed effects on CD4TL in spite of the significant increase in their release (Figure 4b), suggesting that the exosomes produced under these conditions are of a different nature, independent of the amount of vesicles. The non-infectious vesicles derived from HIV-1-loaded DCs also increased apopotosis in Th17-polarized CD4 (data not shown). Furthermore, the particles had no effect on CD4TL proliferation measured in the presence of 5-(or -6)-carboxyfluorescein diacetate succinimidyl ester or 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide treatment under any of these conditions (data not shown). Altogether, our results suggest that exosomes produced by DCs after contact with HIV-1 can

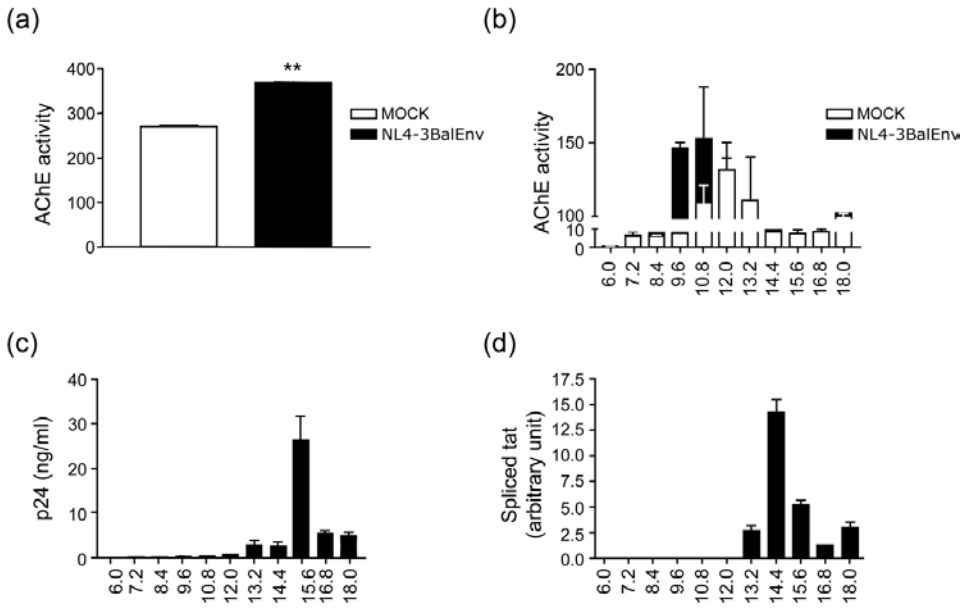

Figure 3: DCs infected with HIV-1 release exosomes and virions having distinctive markers and sedimentation velocities. DCs were incubated for 1h with mock viral suspension (open bars) or with exosome depleted suspension of NL4-3Bal*env* produced on PBMCs (black bars) and then cultured for an additional 48h. Cell-free supernatants were subjected to differential centrifugations to pellet exosomes and HIV-1. Exosomes were then separated from virions using Optiprep™ gradient separation. Exosomes in the 100,000x*g* pellet (a) and in each individual Optiprep fraction (b) were quantified by measuring AChE activity. The virions were quantified using an anti-p24^{gag} ELISA (c). Data correspond to the mean ± SEM of triplicate samples from one donor and these results are representative of three independent donors. ** $P < 0.01$. The infectivity of viruses present in each gradient fraction was evaluated by means of real-time PCR targeting the spliced TAT gene in autologous T CD4$^+$ lymphocytes (d).

affect the viability of activated or resting T cells independently of the amount of exosomes produced.

Exosomes from HIV-1-loaded DCs promote CD4TL apoptosis

These results prompted us to examine in further details the role of exosomes released from HIV-1-loaded DCs in causing CD4TL death. DCs were contacted with NL4-3Bal*env* and exosomes and virions released into the culture medium were collected. Exosomes were immunocaptured using anti-AChE-coupled beads with IgG1-coupled beads as an isotype control. Exosomes were eluted from the beads and added to autologous CD4TL. Apoptosis was measured using FITC-VAD-FMK staining after 24h of culture. As expected, exosomes increased apoptosis in resting T cells (1.6-fold increase) whereas isotype control had little effect (Figure 6). These data support the assertion that purified exosomes from HIV-1-loaded DCs can promote T cell death.

HIV-1 preparations depleted of exosomes infect greater numbers of target cells

Since exosomes produced by HIV-1-pulsed DCs induced apoptosis in CD4TL, we hypothesized that depletion of exosomes from HIV-1 suspensions might actually increase the infectivity of the virus (i.e. the total number of cells infected and producing new virions as a result). CD4TL were therefore infected with virus suspension made from culture supernatant of infected HEK-293T cells and removing the exosomes (Figure 7a, AE-1) or not (Figure 7a, IgG1). We found that CD4TL infection with purified virus was more efficient at producing spliced TAT RNA, implying greater infectivity. Furthermore, the viable CD4TL count was higher when the cells were incubated with the purified virus (Figure 7b, AE-1), indicating better survival of CD4TL in the absence of exosomes. As was observed above with exosomes released from infected DCs (Figures 5 and 6), CD4TL exposed to virus concurrently with exosomes obtained from HEK-293T cells are highly susceptible to apoptosis.

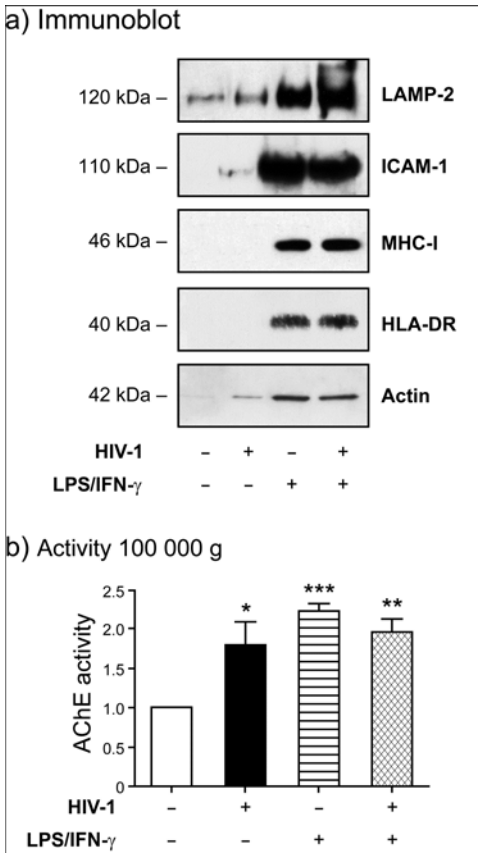

Figure 4: Properties of exosomes released from HIV-1-infected DCs. DCs were cultured in the presence of LPS/IFNγ and exosome-free suspension of NL4-3Bal*env* for 48h. Differential centrifugation of the cell-free supernatants yielded a pellet containing exosomes and HIV virions, which were analyzed by Western blotting for the indicated exosome markers (a). Results shown are representative of five experiments. AChE activity carried by exosomes is also shown (b). Data are expressed as the ratio of AChE activity relative to the mock condition and represent the mean ± SEM of three experiments.

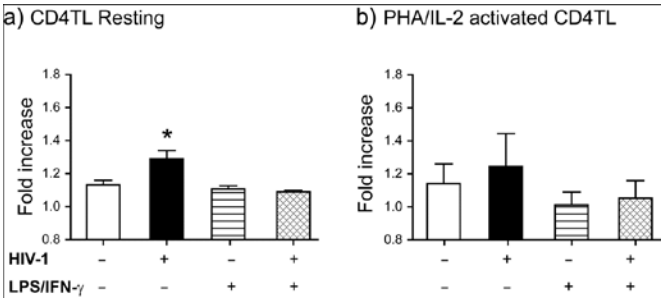

Figure 5: Effect of released particles on CD4TL death. Resting CD4TL (panel a) or PHAL/IL-2-activated CD4TL (panel b) were incubated for 5d with exosomes and attenuated HIV-1 obtained from the 100,000x*g* pellet from culture of NL4-3Bal*env*-infected DCs. The fluorescent pan-caspase inhibitor (FITC-VAD-FMK) was added 30 min before flow cytometry analysis. Results are expressed as the relative increase compared to the control condition without exosome. Data represent the mean ± SEM from five independent experiments and the asterisk denotes statistically significant data ($P < 0.05$).

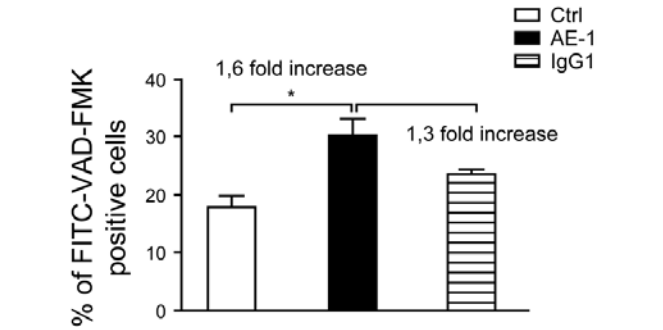

Figure 6: Exosomes released from DCs infected with HIV-1 increase apoptosis of CD4TL. DCs were cultured in the presence of NL4-3Bal*env* for 48h. Exosomes in the cell-free supernatant were isolated from virions by immunocapture using protein A/G beads pre-coated with anti-AChE (AE-1) or with an isotype (IgG1) antibody (control). The suspension eluted from the beads (AE-1) was added to a culture of resting autologous CD4TL. FITC-VAD-FMK was added 30 min before flow cytometry analysis. Data are expressed as a percentage of positive cells for FITC-VAD-FMK and represent the mean ± SEM from four independent experiments.

Discussion

HIV-1 is a retrovirus that causes a slow but sustained depletion of CD4TL, a major characteristic of AIDS, which leads to progressive failure of the whole immune system. Our study provides relevant insight into several aspects of HIV-1 transmission by DCs, and more particularly the role of exosomes in the elimination of CD4TL. Characterization of exosomes produced by DCs after HIV-1 capture is therefore of

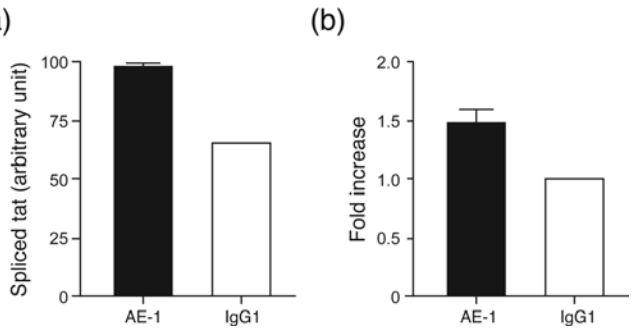

Figure 7: Depleting exosomes from HIV-1 suspensions increases CD4TL survival. CD4TL cultures were incubated for 2h with NL4-3Bal*env* viral suspensions with (*filled* bar) or without exosomes (*open* bar) then washed and cultured for four days. CD4TL RNA was reverse transcribed, purified and spliced TAT was quantified. Surviving cells were counted (panel c). Results are expressed as increases relative to the IgG1 control (n = 4).

the utmost importance in the control of HIV-1 transmission and for understanding the pathogenesis of the disease. Residing in mucosal tissues, DCs capture and internalize HIV-1 virions, thus hiding them and helping them escape immune surveillance during their migration to secondary lymphoid organs, where they are transmitted to CD4TL and replicate. It is crucial to understand the process of CD4TL depletion, especially in the mucosae, because these cells are known to play a pivotal role in mounting the immune response. Since exosomes provide a mechanism of communication between cells, they could signal DCs to orchestrate the immune response as well as participate indirectly in the immunodepletion observed in the acute phase of HIV-1. The pathways of exosome biogenesis and viral assembly converge in a common intracellular compartment and both particles are released jointly from this compartment during the transinfection process [86]. Accumulation of exosomes into the extra-cellular milieu in the context of HIV-1 infection has received much less attention. Our study provides evidence that the exosomes released by HIV-1-pulsed DCs can affect the viability of neighboring cells such as CD4TL. Our preliminary proteomic analyses (data not shown) performed on exosomes derived from HIV-1-loaded DCs (Figure 3) revealed the presence of apoptotic proteins such as apoptotic peptidase activating factor 1 (Apaf-1) and death-associated protein 3 (Dap-3), which are both involved in pro-apoptotic pathways [107,108]. However, how and to what extent factors such as Apaf-1, Dap-3 or viral proteins included in exosomes can initiate apoptosis remains to be elucidated. This hypothesis is supported by recent work showing exosome secretion after HIV-1 infection of T lymphocytes [72]. The authors demonstrated that the viral protein Nef is enclosed in exosomes, thus conferring the capacity to trigger apoptosis in uninfected bystander T cells [72].

Among the numerous important functions attributed to exosomes, immune modulation is by far the most studied [89]. Moreover, their involvement in pathogen spreading has been suggested in the case of HIV-1 [84]. Our results show that DCs infected in vitro with HIV-1 release large amounts of exosomes, and time course analysis shows that maximal production of exosomes is observed between 24h and 48h (Figure 2), which corresponds to the time taken by HIV-1-loaded DCs to migrate from the mucosa to the lymph nodes, as previously shown in a simian model [109]. Several groups have proposed a model in which viral particles are endocytosed into an intracellular compartment of DCs and then transmitted to CD4TL, without any need for productive infection, in a process known as trans-infection [104]. Furthermore, the model proposed by Wiley and coworkers shows that close association with exosomes increased HIV-1 infectivity [103]. Similarities in both protein and lipid composition and buoyant density render the separation of exosomes and virions quite difficult using conventional techniques such as sucrose density gradient separations. Using the Optiprep velocity gradient method [77], we clearly showed that exosomes could be separated from HIV enough to make virions undetectable (using the usual means) in the exosome fraction. Our data also suggest that exosomes do not promote HIV-1 infectivity, but might in fact limit the extent of CD4TL infection by triggering apoptosis, which is certainly a double-edged sword.

Regarding the current interest in the role of microbial translocation in HIV pathogenesis, we showed increased secretion (two-to-three-fold) of exosomes from LPS/IFNγ-treated DCs compared to immature DCs (Figure 4b), this treatment reproducing an inflammatory environment and allowing DC maturation. Maturation has been described as a partial loss of the intracellular multivesicular compartments, which become elongated tubular structures. However, the remaining MVBs still produce exosomes in these cells [87,110,111]. Moreover, even if receptor bound antigens are efficiently internalized by mature DCs [112], it is generally assumed that maturation reduces the capacity of DCs for endocytosis [113-115], a necessary step prior to exosome accumulation and release into the extra-cellular medium. In this study, we showed that it is possible to increase exosome secretion by DCs by pulsing the cells with HIV virions. Other studies have shown that such increases can also be observed when cells are stimulated via specific receptors associated with immune function [87]. We therefore suggest that like cytokines, exosomes could act as extra-cellular messengers of DCs in the regulation of the immune system. Moreover, the fact that mature DCs release more exosomes than do immature DCs suggests that exosomes could have a specific role in the inflammatory reaction. In summary, our results highlight the potential role of exosomes secreted by DCs in affecting neighboring bystander T cells in the context of HIV-1 infection. Since helper T cells play diverse and important roles in the immune response, it is not surprising that they influence the state of immunity against a variety of pathogens. This work may lead to better understanding of the failure of immune cell function during primary HIV-1 infection. We think that exosomes play a key role in this failure.

Competing Interests

All authors have no potential conflicts of interest with the submitted material.

Authors' Contribution

C.S. performed research, analyzed data and wrote the first draft. S.S. performed experiments. A.B. and A.A.L. performed preliminary experiments. S.M. performed experiments for preliminary proteomic analysis. D.G. performed a preliminary proteomic experiment and assisted in editing the manuscript. C. Gilbert designed, supervised the study, analysed the data and wrote the final version of the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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