Exosomes Decrease in vitro Infectivity of HIV-1 Preparations: Implication for CD4+T Lymphocyte Depletion in vivo

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1. Introduction
1.1 Nature and role of exosomes
Since their identification by Johnstone et al. (Johnstone et al., 1987), exosomes have gained importance in understanding many biological processes. Exosomes are vesicles expelled by cells into the extracellular milieu. They originate from internal endocytic compartments called multivesicular bodies (MVB) and are released following fusion of MVB with the plasma membrane (Stoorvogel et al., 2002). Numerous cell types, including tumour, foetal, epithelial and haematopoietic cells share the characteristics of releasing exosomes upon activation by cytokines (Abu samra et al., 2005; Ahn and Johnstone, 1993; Altieri, Khan, and Tomasi, 2004; Peche et al., 2006; Segura, Amigorena, and Thery, 2005; Taylor, Akyol, and Gercel-Taylor, 2006; van Niel and Heyman, 2002). Initially associated with the elimination of obsolete proteins during reticulocyte maturation, exosomes are now known to play several roles in intercellular communication (for reviews, (Chaput and Thery, 2010) and (Record et al., 2011)). Based on the presence of various molecules within the vesicle membrane or lumen, it has been proposed that exosomes are particularly involved in regulation of the immune response, for example tolerance induction (Admyre et al., 2006; Frangsmyr et al., 2005; Kapsogeorgou et al., 2005; Karlsson et al., 2001; Kim, Morse, and Choi, 2006; Larregina et al., 2004; Mallegol, van Niel, and Heyman, 2005; Ostman, Taube, and Telemo, 2005; Peche et al., 2003; Peche et al., 2006; Quah and O’Neill, 2005a; Segura, Amigorena, and Thery, 2005; Taylor, Akyol, and Gercel-Taylor, 2006; Van Niel et al., 2003), antigen presentation (Andre et al., 2004; Chaput et al., 2004; Clayton et al., 2003; Kleijmeer et al., 2001; Peche et al., 2003; Raposo et al., 1996; Thery et al., 2002), cancer immunotherapy (Amigorena, 2000; Andre et al., 2001; Mignot et al., 2006; Quah and O’Neill, 2000; Zitvogel et al., 1998), control of receptor expression (Ahn and Johnstone, 1993; Hawari et al., 2004; Levine, 2004), mechanisms involved in cell death (Abusamra et al., 2005; Farsad, 2002; Iero et al., 2008; Lenassi et al., 2010; Zhang et al., 2006) and control of inflammation (Abusamra et al., 2005; Kim et al., 2006; Levine, 2004). Exosomes may also contain functional miRNA (Pegtel et al., 2010) or deliver bioactive lipids (Esser et al., 2010; Subra et al., 2010). Depending on the function and on
the activation state of the secreting cells, exosomes thus regulate multiple pathways in neighbouring cells in autocrine, paracrine and juxtacrine fashion.
The mechanism by which molecules are sorted in exosomes involves a recycling process that is influenced by molecular lateral mobility within lipid domains (de Gassart et al., 2003; de Gassart et al., 2004). MHC-II, co-stimulatory molecules, enzymes (Alonso et al., 2007; Baynes et al., 1991) and heat-shock proteins (HSP) (Lancaster and Febbraio, 2005) are among the proteins associated with exosomes (Segura, Amigorena, and Thery, 2005; Segura et al., 2005; Skokos et al., 2001).

Exosomes are similar to retroviruses not only in terms of size but also the molecules they incorporate and their ability to activate immune cells. Exosomes are slightly smaller and more heterogeneous in size (30-100 nm) than HIV-1 particles (100 nm). The most obvious similarity between these two types of particles is the presence of molecules of host origin. For example, incorporation of MHC-I and MHC-II by virions and by exosomes has been described (Cantin, Fortin, and Tremblay, 1996; Cantin, Martin, and Tremblay, 2001; Gansuvd et al., 2003; Raposo et al., 2002; Vincent-Schneider et al., 2002). In addition, several cell-surface molecules such as LFA-1 integrins (CD11a, CD18), co-stimulatory molecules (CD28, CD54) and complement-neutralizing molecules (CD55, CD59) are associated with both particles (Cantin, Methot, and Tremblay, 2005; Nguyen et al., 2003; Thery et al., 2001; Thery et al., 1999). Finally, the buoyant density of exosomes ranges from 1.13 to 1.21 g/l, while that of HIV-1 particles ranges from 1.16 to 1.18 g/l (Thery et al., 2001; Wang et al., 1999). Similar protein and lipid composition as well as buoyant densities render the separation of exosomes from virions quite difficult using standard techniques such as density-gradient centrifugation. These problems prompted us to use an Optiprep™-based velocity gradient method (Cantin et al., 2008), which has allowed us to show clearly that exosomes can be separated completely from viruses, based on detection of exosome marker (acetylcholinesterase) and HIV-1 marker (capsid protein p24).

The relationship between exosome biogenesis and retrovirus assembly has not yet been described in satisfactory detail. Although considerable evidence points to the takeover of the intracellular machinery responsible for MVB biogenesis (located at the cytoplasmic membrane) in the case of HIV-1 budding from CD4+ T lymphocytes (CD4TL), virions are found in endosomes of macrophages and dendritic cells (DCs), suggesting an internal budding process (Booth et al., 2006; Gould, Hildreth, and Booth, 2004; Morita and Sundquist, 2004; Nguyen et al., 2003). Comparative studies of exosomes and HIV-1 particle production pathways (Nguyen et al., 2003) based on observations of similar viral budding (Derse et al., 1987) and uptake by cells (Izquierdo-Useros et al., 2010) indicate that retroviruses evolved by exploiting the exosome release pathway.

The highly varied exosome composition and content suggest crucial roles for these vesicles in intercellular communication (Thery, Zitvogel, and Amigorena, 2002), transport of genetic material (mRNA or microRNA) (Valadi et al., 2007) and exchange of proteins (Andre et al., 2004; Thery, Zitvogel, and Amigorena, 2002), or in inflammation by carrying bioactive lipids (Esser et al.; Subra et al., 2010). Numerous studies indicate more efficient T cell activation by exosomes released from mature (mDCs) than from immature DCs (iDCs) (Admyre et al., 2006; Chaput and Thery, 2010; Segura, Amigorena, and Thery, 2005). In other studies, an inhibitory role for exosomes in the immune response has been described and particularly in the induction of T cell death via either FasL or galactin-9 by tumour-derived exosomes (Abusamra et al., 2005; Alonso et al., 2007; Chaput and Thery, 2010; Klibi et al., 2009; Ren et al.; Xie et al., 2010) (Andreola et al., 2002; Monleon et al., 2001). Compelling evidence for a
role of the HIV-1 protein Nef (released in association with exosomes) in inducing apoptosis of bystander CD4TL has been published recently (Lenassi et al., 2010). All of these data have led us to examine the involvement of exosomes in CD4TL depletion during HIV-1 infection.

1.2 Rapid depletion of CD4TL during primary infection

HIV-1-caused disease is characterized by a state of chronic immune activation due to sustained inflammation and immune hyperactivation that persists even under antiretroviral therapy (HAART) (Imami et al., 2001). Several observations from non-pathogenic simian immunodeficiency virus (SIV) infection, HIV-1 infected “elite controllers”, “elite suppressors” or long-term non-progressors reveal a good correlation between low level of activation of the immune system and absence of clinical signs of AIDS (Bailey et al., 2008; Fontaine et al., 2011; Milush et al., 2007; Shacklett, 2010; Silvestri et al., 2003). In contrast, strongly increased immune activation characterized by dysregulated neutrophil and macrophage functions (Roilides et al., 1990; Torre et al., 2002), polyclonal B cell activation (Aberg et al., 2005), increased T cell turnover (Aberg et al., 2005), increased numbers of T cells with an activated phenotype (Aberg et al., 2005) and increased levels of pro-inflammatory molecules are hallmarks of disease progression in pathogenic infections by primate (HIV/SIV) lentiviruses (Ascher and Sheppard, 1988), (Giorgi et al., 1999; Liu et al., 1997). More significant is that a major rapid loss of mucosal CD4TL occurs in the gut-associated lymphoid tissues quite early in HIV-1 infection (Brenchley, Price, and Douek, 2006; Brenchley et al., 2004; Mehandru et al., 2004). At this stage, both mucosal lymph node destruction (which initiates immune dysfunction) and loss of integrity of the gut epithelium allow microbial products to cross the intestinal barrier. This translocation phenomenon produces high levels of circulating bacterial lipopolysaccharides (Brenchley, Price, and Douek, 2006) and thus contributes to the maintenance of the inflammatory state and systemic immune activation observed in chronic HIV-1-infected patients (Brenchley, Price, and Douek, 2006; Marchetti et al., 2008). These studies all point to early events in HIV-1 infection as decisive determinants of the irreversible damage inflicted on immune cells. It is well established that dendritic cells (DCs) are involved early in HIV-1 transmission (Granelli-Piperno et al., 1998; Manel et al., 2010; Tsunetsugu-Yokota et al., 1997). It is also known that CD4TL, more particularly the Th17 mucosal subset (Cecchinato and Franchini, 2010; Cecchinato et al., 2008; Elhed and Unutmaz, 2010; Favre et al., 2009; Milush et al., 2011; Paiardini, 2010) are dysregulated (Elbim et al., 2009; Hofman et al., 1999; Okada, Takei, and Tashiro, 1997; Okada, Takei, and Tashiro, 1998; Pitrik et al., 1996; Roilides et al., 1993; Roilides et al., 1990; Szalc et al., 1992; Thorsen, Busch-Sorensen, and Sondergaard, 1989) in pathogenic HIV-1/SIV infection (Brenchley et al., 2008; Elbim et al., 2009; Elbim et al., 2008; Favre et al., 2009).

A rapid decrease thus occurs in the numbers of both infected and uninfected CD4TL within the very first weeks of infection. CD4TL are known to play a pivotal role in orchestrating the immune response as well as in the development, maturation and maintenance of cytotoxic T cells (Matloubian, Concepcion, and Ahmed, 1994; Zajac et al., 1998), the development of a humoral response and B cell antibody class switching (Tsuji et al., 1994), control of the bactericidal activity of macrophages and induction of HIV-1-specific CD4 and CD8 T cell responses. In fact, HIV-1 infection and the specific immune response to it depend largely on CD4TL functionnality and depletion of these cells during primary infection constitutes major interference, perhaps explaining the long-term inability of the host immune response to control the infection. Different mechanisms have been proposed to explain the significant
depletion of CD4TL in the gut-associated lymphatic tissues. Among these, direct infection of CD4TL by the virus (Arnoult et al., 2003), cytotoxic activity of CD8 T cells against infected cells (Sewell et al., 2000) and cytopathic effects on bystander cells or abortive infection (Doitsh et al., 2010) are the most plausible. However, additional factors, including HIV-1 proteins such as Vpr, Tat, Nef, VpU, proteases and gp120 (Varbanov, Espert, and Biard-Piechaczyk, 2006; Wan and Chen, 2010), mechanisms such as activation-induced cell death (AICD) mediated by Fas, TNF and TRAIL/APO2 (Lichtner et al., 2004) or dysregulation of cytokine/chemokine production (Saelens et al., 2004) can contribute to CD4TL death. Moreover, the detection of Nef in exosomes and the known involvement of this viral protein in apoptosis add support to the potential role of exosomes in bystander cell viability (Lenassi et al., 2010). We therefore propose another mechanism involving the release, from HIV-1-loaded iDCs, of exosomes that can induce functional defects in CD4TL and contribute to their elimination.

1.3 The role of dendritic cells in HIV-1 primary infection

The weakening of the immune system begins soon after the virus enters the body, which it does principally via the mucosal tissues. Following transmission of HIV-1, the virus crosses the mucosal barrier and is met by DCs, which are among the first cells to encounter the virus (Hladik and McElrath, 2008). A major immune system cell type involved in capturing and internalizing HIV-1 is the iDCs, which then migrates principally to the lymph nodes of the gastrointestinal tract, a site of HIV-1 replication during acute infection. Despite the progress that has been made in understanding iDC/HIV-1 interactions as well as virion sequestration and transmission to CD4TL, several fundamental questions surrounding the near total depletion of memory CD4TL observed during the acute infection (Brenchley et al., 2004; Guadalupe et al., 2003; Li et al., 2005; Mattapallil et al., 2005; Mehandru et al., 2004) remain unanswered. It is well known that both cells play a pivotal role in the dissemination of HIV-1, in the establishment of infection and also in anti-HIV-1 immunity.

It is now well established that HIV-1 entry into CD4TL is mediated by cellular chemokine receptors such as CCR5 or CXCR4. However, we, along with others, have found that additional factors, such as the DC-SIGN and DCIR lectins can mediate virus attachment to DCs and its subsequent endocytosis (Cambi et al., 2009; Geijtenbeek et al., 2000; Lambert et al., 2008; Permanyer, Ballana, and Este, 2010). Indeed, several recent studies have shown virions concentrated in late endocytic compartments also called multivesicular bodies (MVBs) or MHC class II compartments in mature DCs (Garcia et al., 2005; Izquierdo-Useros et al., 2009; Kwon et al., 2002), where they are sheltered both from the action of antiviral drugs and the immune response. Mature DCs are capable of stockng viral particles and migrating via the lymphatic network to lymph nodes and thus constitute reservoirs of virions. At this stage, it is thought that mDCs can transmit virions to T cells through two sequential routes: an early route known as trans-infection, via passive transport through late endosomes, or a later route called cis-infection following productive infection (Turville et al., 2004). Fusion of late endosomes with the DC plasma membrane releases large numbers of virions into intercellular space called the virological synapse, which can infect nearby target cells (Moir, Chun, and Fauci, 2010; Piguet and Steinman, 2007). Endosomes contain the intraluminal vesicles that become exosomes when delivered into this space at the same time as the viral particles contained in HIV-1-loaded DCs. Exosomes and virions thus pass via the late endosome across the cell to be exchanged with other cells (Izquierdo-Useros et al., 2010).
After their migration to the lymph nodes, iDCs likely transfer HIV-1 to CD4TL with great efficiency and simultaneously release exosomes. The ability of exosomes to activate CD4TL, thereby enhancing HIV-1 replication, or to induce T cell apoptosis directly, could contribute...
to the massive depletion of CD4TL. Indeed, our preliminary observations show that HIV-1 increases exosome release from iDCs. Some studies have shown that exosomes can activate T cells and consequently are involved in the regulation of the immune response (Admyre et al., 2006; Segura et al., 2005; Thery et al., 2002). Furthermore, the Fas-ligand on exosomes can also induce apoptosis of both CD4 and CD8 T cells (Abusamra et al., 2005; Alonso et al., 2007; Alonso et al., 2005; Segura, Amigorena, and Thery, 2005). It should be noted that the capacity of exosomes to regulate the viability of CD4 T cell sub-populations has not yet been fully investigated in the context of HIV-1 infection. In order to answer this important fundamental question, new methods are needed for separating mixtures of exosomes and HIV-1 to purity.

HIV-1 is a retrovirus that causes a slow but sustained depletion of CD4TL during the chronic stage of infection, leading to progressive failure of the immune system. The principal immune cell type that captures and internalizes HIV-1 is the iDC. HIV-1-loaded iDCs migrate to the lymph nodes of the gastrointestinal tract, a major site of HIV-1 replication during acute infection. Given that CD4TL play a pivotal role in the orchestration of the immune response, the rapid and sustained disappearance of mucosal CD4TL (within the first 15 days) compromises the development of both the cellular and humoral responses to HIV-1 infection. Exosomes release by DCs or CD4TL can contribute to elimination of CD4TL as well as other cell deregulations characterizing this crucial phase.

2. Protocol to study the role of exosomes in CD4TL viability in the context of HIV-1 infection

2.1 Cell purification

Experiments were performed using human primary cells, DCs and CD4TL. Cells were isolated from peripheral blood mononuclear cells (PBMCs) obtained from anonymous and healthy volunteer donors. PBMCs were prepared by centrifugation on a lymphocyte separation medium from Wisent Inc. (St Bruno, QC, Canada). CD14+ cells were then isolated using a monocyte positive selection kit according to the manufacturer’s instructions (Stemsep Human CD14 positive selection Kit, STEMCell Technologies, Vancouver, BC, Canada) using an AutoMacs (Miltenyl Biotech, Auburn, CA, USA) and a previously established procedure (Bounou et al., 2004; Gilbert et al., 2007a; Gilbert et al., 2007b). CD14+ cells were cultured in six-well plates at a concentration of 1 X 10^6 cells/ml. To generate iDCs, purified monocytes were cultured in complete culture medium supplemented every two days with granulocyte-macrophage colony-stimulating factor (1,000 U/ml) from Genscript (Cedarlane Laboratories, Burlington, ON, Canada) and IL-4 (200 U/ml) from R&D Systems (Minneapolis, MN, USA) for 6 to 7 days. Expression of CD3 and CD19 was measured to assess contamination with T and B cells respectively. Expression of HLA-DR, CD86, DC-SIGN, CD83 and CD14 was monitored to verify the immature phenotypes of DCs. In the immature state, DCs express a high level of DC-SIGN and low level of CD83, whereas mature DCs express CD83 and high levels of ICAM-1, HLA-DR and CD86. 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 phytohemagglutinin-L (1 µg/ml) to obtain mitogen-stimulated cells and maintained at a density of 2 x 10^6 cells/ml in RPMI supplemented with IL-2 (30 U/ml) obtained through the AIDS Repository Reagent Program.
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(Germantown, MD, USA). Experiments were performed with cell preparations that were devoid of contamination (i.e. DC purity > 95%; CD4TL purity > 98%). In all culture media, bovine exosomes from foetal bovine serum (FBS) were eliminated by O/N ultracentrifugation at 100,000xg. Complete RPMI 1640 culture medium contains FBS, penicillin G, streptomycin, glutamine from Wisent and primocin and plasmocin from Invivogen (San Diego, CA, USA).

2.2 Virus production and purification
Virions were produced by transient transfection in human embryonic kidney 293T cells (HEK293T) as previously described (Cantin et al., 1997). Plasmids used include pJR-CSF (R5-tropic), pNLAD8 (R5-tropic), pNL4-3balenv (R5-tropic) and pNL4-3 (X4-tropic). The pNL4-3balenv vector (provided by R. Pomerantz, Thomas Jefferson University, Philadelphia, PA, USA) 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 (Dornadula et al., 1999). Other plasmids were obtained from the AIDS Repository Reagent Program (Germantown, MD, USA). Several viral preparations were obtained from primary cells. Peripheral blood from healthy donors was centrifuged on lymphocyte separation medium (Wisent) to obtain PBMCs. NL4-3Balenv virions were propagated by acute infection of PBMCs (1.5x10⁷ cells/ml) with virus (500 ng/ml) for six days.

2.3 Separation of exosomes and virions by velocity gradient
Exosomes, microvesicles and virions contained in cell-free supernatants were passed through a 0.22 µm filter or centrifuged, 10 min at 10,000xg to eliminate micro-particles. Filtered virus and/or exosomes from 293T cells or PBMCs were concentrated by ultracentrifugation in an Optima L-90K Beckman Coulter centrifuge (Fullerton, CA) for 45 min at 31,500 rpm (100,000xg) in a 70 Ti rotor. The pellet containing virions and microvesicles/exosomes was re-suspended in 500 µl of PBS. HIV-1 viral particles were then centrifuged through a 6-18% Optiprep™ (60% iodixanol) separation gradient from Sigma Aldrich® (Winston Park, CA, USA) as previously described (Dettenhofer and Yu, 1999). The densities of each gradient fraction were below 1.13 g/ml, as shown in Figure 2. The virus preparations were then centrifuged using the Optima L-90K centrifuge for 75 min at 52,000 rpm (250,000xg) in a NVT65 rotor. Gradient fractions were collected from the top. Optiprep™ separation medium possesses intrinsic properties such as neutral pH and physiological osmolarity (Dettenhofer and Yu, 1999; Ford, Graham, and Rickwood, 1994; Van Veldhoven, Baumgart, and Mannaerts, 1996) that make it highly suitable for efficient separation of viral entities from cellular debris and microvesicles (Dettenhofer and Yu, 1999; Hermens et al., 1999; Moller-Larsen and Christensen, 1998; Zolotukhin et al., 1999). This technique, identical to the velocity gradient adapted by Dettenhofer (Dettenhofer and Yu, 1999) for HIV-1 separation, is also very efficient for separating microvesicle contaminants from HIV-1 preparations. The results depicted in Figure 3A (black rectangles) express the separation efficiency in terms of quantity of viral p24⁰⁰⁰⁰⁰ protein, which is concentrated in fraction 15.6. Electron microscopy, performed at the Armand Frappier Institute Microscopy Laboratory using standard protocol (Alain et al., 1987; Hammond et al., 1981), confirmed that fractions 15.6 and 16.8 contain concentrated and homogenous viral particles (10¹¹ per ml). Virion contents were normalized by means of an in-house sensitive double-antibody
sandwich enzyme-linked immunosorbent assay (ELISA) specific for the viral p24 protein (Bounou, Leclerc, and Tremblay, 2002).

The distribution of fractions containing exosomes along the gradient was evaluated by measuring acetylcholinesterase (AChE) activity, which is a commonly used specific exosome marker (Fig. 3A, open rectangles) (Gastpar et al., 2005; Rieu et al., 2000). This enzyme activity was measured following a procedure previously described (Cantin et al., 2008). Briefly, 30 µl of standard or sample were mixed with PBS pH 8 containing acetylthiocholine and PBS pH 7 containing 5,5-dithio-bis-(2-nitrobenzoic acid) to obtain final reagent concentrations of respectively 1.25 mM and 0.1 mM in a volume of 200 µl and held at room temperature. Changes in absorption at 450 nm were monitored for 10 min with a plate reader spectrophotometer (ELX808, BIO-TEK instruments, Winooski, VT, USA).

Glycosylphosphatidylinositol-anchored AChE as well as other GPI proteins are localized in the MVB and are part of the exosome membrane (Gastpar et al., 2005; Johnstone et al., 1987). The results illustrated in Figure 3 show that AChE activity is concentrated in fractions 8.4 to 12 and microscopic observations showed that 10^8 particles/ml are present in these fractions. To confirm the efficiency of this velocity gradient method for separating HIV-1 from exosomes, we also checked for the presence of infectious particles in each gradient fraction (i.e. infectivity on TZM-bl cells (Cantin et al., 2008) or measure of spliced TAT on CD4TL). Infectivity assays using the TZM-bl indicator cell line showed that fractions 14.4 to 16.8 contained fully infectious virus while fractions 8.4 to 12 contained insignificant amounts. These results thus clearly indicate that our procedures can isolate exosomes and HIV-1 particles differentially and independently. We are satisfied that fractions 8.4 to 12 of the Optiprep™ gradients contained exosomes exclusively, while fractions 14.4 to 16.8 contained infectious viruses.

![Graph showing density of gradient fractions](image)

**Fig. 2. Density of gradient fractions**

The particularity of velocity gradient separation resides in its capacity to isolate particles of similar density. The density range of 6 to 18 % of Optiprep™ is 1.03 to 1.13 g/l.
2.4 Rapid purification of exosomes
A quick alternative procedure for eliminating exosomes prior to contacting the cells with HIV-1 preparation consisted of immuno-depleting AChE-bearing vesicles directly from 100,000 x g pellet (starting material) as shown in Figure 3B. Briefly, ultracentrifuged cells supernatants were diluted in PBS and incubated with protein A/G beads pre-coated with anti-AChE (AE-1 from ATCC) or with isotypic control antibodies (IgG1). The beads (exosomes) and final supernatants (infectious viruses without exosomes) were kept for viral Titer determination (data not shown) and AChE assay (Fig. 3B). The results show that 95% of the AChE activity was recovered using the anti-AChE beads after the first round of precipitation. Exosomes can be eluted from the beads in two minutes using 50 µl of 0.2M glycine in 0.1M KH₂PO₄ pH 3. Acidic pH was then neutralized by adding 100 µl of 0.1M K₂HPO₄ (pH 8.8). It should be noted that this treatment does not affect AChE activity.

2.5 Analysis of protein contained in each gradient fraction
Several proteins including LFA-1 are known to be present on both particles and were quantified in exosome/HIV-1 preparations by means of an in-house enzymatic sandwich-type immunoassay. Plates were initially coated with anti-LFA-1 (MEM-25) (50 µg/ml) in carbonate buffer. After three washes with PBS/0.1% Tween 20, the non-specific sites were blocked with PBS/0.1% Tween 20/1% BSA for 1 hr at RT. The plates were washed and 75 µl of each gradient fraction mixed with 0.5% Triton X-100 to cause lysis were added and the plates were then held overnight at 4°C. The plates were then washed three times and biotinylated anti-LFA-1 (TSI/22) (0.5 µg/ml) was added in blocking solution for 1 hr at RT. After three more washings, streptavidin-peroxidase conjugate was added for 30 min at RT. The plates were washed and the detection was performed by the addition of the TMB-S substrate followed by the addition of H₃PO₄. Absorbance at 450 nm was read to quantify LFA-1 in each fraction. Figure 3C shows that LFA-1 is present on both vesicles (exosomes and fully infectious virions) and also in uncharacterized particles from fractions 13.2 and 14.4.

Both methods (Optiprep™ gradient and immunodepletion) were used to purify exosomes. These methods were employed to eliminate exosomes from all viral or mock preparations before contact with cells and results depicted in Figure 3D show that depletion of exosomes from the HIV-1 preparation increased cell viability.

2.6 Electron microscopy procedure and examination of negative-stain specimens
The protocol described by Hammond et al. and Alain et al was performed with each analytical fraction (Alain et al., 1987; Hammond et al., 1981). Briefly, microvesicle preparation was fixed with an equal volume of 2% paraformaldehyde and 50-200 µL in a micro-ultracentrifuge tube with a formvar-coated electron microscopy grid at the bottom were concentrated for 5 minutes at 120,000 x g (20 psig) using an Airfuge ultracentrifuge (Beckman, Palo Alto, CA, USA). The grids were dried on bibulous paper and stained for 1 min with a drop of 3% phosphotungstic acid (pH 6.0). The concentration, shape and overall appearance of the microvesicles were examined with a Hitachi 7100 (Hitachi, Japan) transmission electron microscope (see Fig. 3A).

2.7 Virus infection assays
Indicator cell line TZM-bl, which carries a stably integrated luciferase reporter gene under the control of the HIV-1 regulatory element known as the long terminal repeat (Thibault et
Fig. 3. Exosome and virus purification

The upper panel illustrates the purification protocols based on Optiprep™ velocity gradient and on immunocapture. Panel A shows velocity gradient purification of NL4-3balenv virions produced on HEK293T cells by transient transfection. Gradients were overlaid with 100,000xg centrifugal pellet. Viral protein p24gag (black rectangles) was measured by ELISA. AChE activity (x10⁶ OD/min, open rectangles) was evaluated in each fraction by colorimetry. Each fraction was examined by electron microscopy for particle quality and quantity. Panel B represents the results obtained by immunocapture with anti-AChE. Panel C shows the presence of LFA-1 in each gradient fraction. Finally, panel D shows that depletion of exosomes increased cell viability. These results show that we are able to separate exosomes from HIV-1 particles.
al., 2007; Wei et al., 2002; Zhao et al., 2005), was used to quantify infectious viral particles after exosomes depletion. Each well contained 1.5 x 10^4 cells plus 100 µl of fraction in a final volume of 200 µl. Plates were incubated for 48 hrs. All experimental points were done in triplicate. Luciferase activity was determined following a modified version of a known protocol (Barbeau et al., 1997; Berube et al., 1996). Briefly, cell-free supernatant (100 µl) withdrawn from each well and mixed with solution containing 25 mM Tris phosphate pH 7.8, 2 mM dithiothreitol, 1% Triton X-100 and 10% glycerol (25 µl) to cause vesicle lysis was held at room temperature for 30 min. A 20-µl aliquot of this mixture was then mixed with 100 µl of luciferase assay buffer (20 mM Tricine, 1.07 mM (MgCO_3)_4 Mg(OH)_2·5 H_2O, 2.67 mM MgSO_4, 0.1 mM EDTA, 270 µM coenzyme A, 470 µM luciferin, 530 µM ATP and 33.3 mM dithiothreitol) and the luciferase reaction was monitored on a Dynex MLX microplate luminometer for 20s/well after a 2-5s delay.

3. Results

3.1 Loading of DCs or CD4TL with HIV-1 induces the release of large amounts of exosomes into the extracellular medium

Exosomes contain several molecules that can either eliminate or activate CD4TL (Quah and O'Neill, 2005b; Segura et al., 2005; Thery et al., 2002). Moreover, exosome-like vesicles found in plasma induce apoptosis in a FasL-like manner (Ren et al., 2010). Although exosomes
release by DCs have been studied extensively (Chaput et al., 2006; Izquierdo-Useros et al., 2009; Thery et al., 2001; Thery et al., 1999), the release mechanism and the nature of the exosomes produced by HIV-1-loaded cells (DCs or CD4TL) have not been thoroughly investigated. To begin to answer this question, the experimental set up proposed in Figure 4 and methods presented in Figure 3 were used.

DCs and CD4TL pulsed with NL4-3balenv and washed several times were cultured for respectively 2 or 5 days. Exosomes and virions were isolated initially by differential centrifugation and exosome levels were determined by measuring exosomal AChE activity (Cantin et al., 2008). These results, presented in Figure 5, confirmed higher levels of exosomes secreted by iDCs and CD4TL pulsed with purified HIV-1 (1.4 fold and 1.9 respectively, Fig. 5A, B). Using Optiprep™ velocity gradients to separate exosomes and HIV-1, we processed the pellet obtained following sedimentation centrifugation. As

![AChE activity in 100 000 g pellet](image)

**A) DCs**

![Optiprep gradient](image)

**C) DCs**

**D) CD4TL**

Fig. 5. Exosomes released by DCs and CD4TL after pulsing with HIV-1

I DCs (A, C) or CD4TL (B, D) were incubated for 2h with exosome-free NL4-3balenv HIV-1 virus or mock preparation and cultured for an additional 72h. Cell-free supernatants were obtained by centrifugation and exosomes in the pellets were quantified by measuring AChE activity (x10^6 DO/min). Exosomes were then separated from HIV-1 on an Optiprep gradient and the exosome content (based on AchE activity) of each fraction was determined. Data are representative of five independent donors. These results show that HIV-1 induced exosome release (in fractions 9.6 through 12) by both cell types (mean increases of 1.4-fold for DCs and 1.9-fold for CD4TL, based on at least 5 independent experiments).
expected, exosomes were concentrated in iodixanol fractions 8.4-12.0% on the Optiprep™ gradient (Fig. 5C, D). Large amounts of exosomes produced by HIV-1-loaded cells accumulated in fractions starting at 9.6% iodixanol (in comparison to the control condition, open bar). The velocity method thus allows efficient separation of exosomes which accumulate in iodixanol fractions 8.4 to 12% as illustrated in Figure 3. Immature DCs release exosomes and are highly relevant to HIV-1 primary infection since they are involved in the capture of HIV-1 in mucosal tissues and play a crucial role in the subsequent transmission of the virus to CD4TL in the lymph nodes (Gilbert et al., 2007a; Gilbert et al., 2007b; Turville et al., 2004) as illustrated in Figure 1.

3.2 Impact of exosome depletion on CD4TL p24 production and infectivity

The large increase in exosome release by HIV-1-pulsed cells, combined with the results showing that exosomes from these cells induced apoptosis, is particularly relevant in the context of HIV-1 infection for several reasons. These results could explain in part the severe depletion of mucosal CD4TL, a cell type very susceptible to HIV-1. In addition, these cells play a pivotal role in orchestrating immune response and their decline during the early phase of infection undoubtedly delays the specific response to HIV-1. Furthermore, most laboratory preparations of HIV-1 contain exosomes, which may explain in vitro observations such as cytokine release, apoptosis, atypical gene expression, infectivity and so on. Since it became clear that exosomes play a major role in several aspects of the immune response to HIV-1, we sought to evaluate their impact on HIV-1 p24 production and infectivity. NL4-3balenv produced by transfection of 293T cells was made free of exosomes by immunocapture with anti-AE-1. Activated CD4TL were pre-incubated for 2h at 37°C with either exosome-free or exosome-containing HIV-1 preparation washed and then incubated for up to 5 days. ELISA was used to determine viral protein p24 in culture supernatants. Figure 6A shows that infection in the presence of exosomes is transient and less efficient.

![Figure 6A](image-url) Fig. 6. Impact of exosomes on p24 production and HIV-1 infectivity

Panel A) P24 production was evaluated in the supernatants of CD4TL cultured for up to 5 days after pulsing with NL4-3balenv preparation either free of exosomes (Balenv AE-1) or not (Balenv IgG1). Panel B) TZM-bl cells were incubated with several dilutions of HIV-1 preparation either immunodepleted (Balenv AE-1) or not (Balenv IgG1) and maintained in culture for 48 hrs before lysis. Results are representative of two independent experiments.
than in their absence (solid bar). To evaluate virion infectivity, indicator cell line TZM-bl was incubated with several dilutions of HIV-1 preparation either exosome-depleted or not. Panel B of Figure 6 shows that the exosome-depleted preparation is more infectious than the non-depleted preparation. All these results provide additional evidence that exosomes derived from HIV-1-pulsed cells influence cell viability (figure 3D) and indirectly p24 production and infectivity. They suggest that the presence of exosomes in culture supernatants of HIV-1-stimulated cells should be considered in all laboratory experiments with HIV-1.

4. Conclusion

Exosome biogenesis and the HIV-1 virion assembly pathway converge in a common intracellular compartment. Moreover, both types of vesicle can be released during the trans-infection process in DCs (Izquierdo-Useros et al., 2009). However, exosome secretion in the context of HIV-1 infection has not been properly investigated, due primarily to lack of effective methods of separating the two types of vesicles. Their separation using antibodies directed against specific membrane antigens is often suboptimal since exosomes and HIV-1 display approximately the same antigen expression pattern in addition to several other surface molecules. This is why flow cytometry, ELISA or bead capture techniques based on specific markers are not sufficiently discriminating for the separation of exosomes, extraneous micro-particles and HIV-1. Alternatively, immunocapture with anti-CD45 (Chertova et al., 2006; Trubey et al., 2003), used to separate only micro-particles derived from leucocyte plasma membranes, does not eliminate exosomes originating from the endosomal membrane and cannot be used to separate exosomes from HIV-1. We have shown that AChE appears essentially excluded from the HIV-1 fraction, since the major portion of its activity is recovered in the early Optiprep™ fractions (8.4 to 12), in which no virus is detected (Cantin et al., 2008). Based on this observation, depletion of 100,000xg pellets with protein-A/G-bound anti-AChE on agarose beads appears to provide excellent means of rapidly purifying virions or capturing exosomes (Cantin et al., 2008). Using these methods, we have observed that HIV-1 contact with DCs or CD4TL enhances extracellular exosome release and that these exosomes can affect the viability of nearby cells such as CD4TL. These results are in agreement with observations concerning the pro-apoptotic role of Nef accessory proteins. Using Optiprep™ gradients, recent work has shown that the viral protein Nef is enclosed in exosomes, conferring to it the capacity to trigger apoptosis of uninfected bystander T cells (Lenassi et al., 2010).

In summary, the results of the present study show that relatively simple methods of purifying both exosomes and HIV-1 contained in the same cell supernatant are now available. Achieving very highly purified exosomes from HIV-1 preparations is a definite advantage in studying the respective roles of both vesicles as well as the links between them. These methods could also provide the opportunity for specific isolation of exosomes secreted by a variety of cell types and could prove useful in experiments that require highly purified exosome preparations to study their roles in various biological processes. Indeed, these purification steps are crucial in studies involving mixtures of exosomes and HIV-1 (or for that matter, other retroviruses) as starting material. We may anticipate that these methods will constitute a significant contribution to the use of exosomes for vaccination or gene therapy. In addition, we strongly believe that an improved and standardized method of exosome purification should lead to more comparable results among different
laboratories and lessen discrepancies such as those seen among several studies in recent years as well as facilitate the interpretation of new results to be published in this subject area.

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Like any other book on the subject of HIV/AIDS, this book is not a substitute or exhausting the subject in question. It aims at complementing what is already in circulation and adds value to clarification of certain concepts to create more room for reasoning and being part of the solution to this global pandemic. It is further expected to complement a wide range of studies done on this subject, and provide a platform for the more updated information on this subject. It is the hope of the authors that the book will provide the readers with more knowledge and skills to do more to reduce HIV transmission and improve the quality of life of those that are infected or affected by HIV/AIDS.

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