BRIEF REPORT

Association of Cytokines With Exosomes in the Plasma of HIV-1–Seropositive Individuals

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Human immunodeficiency virus (HIV)-infected and viremic individuals exhibit elevated levels of plasma cytokines. Here we show that most cytokines are not in free form but appear associated with exosomes that are distinct from virions. Purified exosomes were analyzed to determine the levels of 21 cytokines and chemokines and compared with exosome-depleted plasma. Most cytokines were markedly enriched in exosomes from HIV-positive individuals relative to negative controls and to plasma. Moreover, exposure of naive peripheral blood mononuclear cells to exosomes purified from HIV-positive patients induced CD38 expression on naive and central memory $CD4^+$ and $CD8^+$ T cells, probably contributing to inflammation and viral propagation via bystander cell activation.

Keywords. exosomes; HIV; cytokines; chemokines; immune activation.

Chronic immune activation is one of the strongest predictors of human immunodeficiency virus (HIV) disease progression, associated with chronic levels of detectable cytokines and elevated expression of activation markers on the surface of T lymphocytes [1–3]. Despite extensive studies highlighting direct and indirect viral induction of chronic immune activation, the mechanisms underlying the chronically activated immune state during HIV infection are not fully elucidated. Exosomes are small membrane delimited vesicles released both constitutively and on stimulation from a variety of cell types [4]. They are found in a number of biological fluids and are known to carry a variety of proteins and nucleic acid molecules [4]. Although they were originally thought to be little more than reservoirs for cellular debris, their role in regulating biological functions as well as disease is increasingly appreciated [4]. In this study, exosomes isolated from the plasma of HIV-infected individuals were analyzed for cytokine and chemokine content compared with exosomes from uninfected controls and assessed for immunomodulatory potential on bystander $CD4^+$ and $CD8^+$ T cells.

EXPERIMENTAL METHODS

Human Subjects

Samples were obtained from the Hope Clinic of Emory University (HIV-1 seronegative [n = 15]) and from the Infectious Disease Program of Grady Health System (antiretroviral naive HIV-1 seropositive [n = 10]). Plasma viral loads of the HIV-infected volunteers ranged from 1423 to 536 436 HIV-1 RNA copies/mL, with an average viral load of 205 957 HIV RNA copies/mL. All participants gave written informed consent, and the study was approved by the institutional review boards of Emory University and Morehouse School of Medicine.

Isolation of Exosomes From Human Plasma

Data from our laboratory and others have shown Nef to be secreted from infected cells in exosomes (exNef), and it is present in the plasma of infected individuals at nanogram levels [5–8]. Optiprep (Sigma-Aldrich) velocity gradients were found to be efficient in purifying exosomes from infected human plasma [9] (Figure 1*D*). Exosomes from plasma of infected donors, including exNef, were found to segregate in the low-density/upper fractions of the iodixanol gradient, whereas virus particles segregated in the high-density/lower fractions. Subsequently, virion particles were identified by p24 analysis of gradient fractions, and exosomes were identified using multiple protein markers AChE, CD9, CD63, and CD45. The very upper low-density fractions were collected for exosomes, because they were found to have no p24 contamination. Plasma collected from whole blood in ethylenediaminetetraacetic acid tubes was subjected to differential centrifugation at 30 000 *g* for 30 min and at 100 000 *g* for 2 hours. The 100 000 *g* pellet was resuspended in 1 mL of ×1 phosphate-buffered saline, loaded onto Optiprep velocity gradients, and subjected to flotation centrifugation at 250 000 *g* for 2 hours. Eleven fractions were collected from each gradient and assayed for acetylcholinesterase (AChE) activity or

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CD63, markers for exosomes, and p24, a marker for viral particles (Figure 1*D*). Fractions with peak exosome content (fractions 2 and 3) were pooled, diluted 1:3 with phosphate-buffered saline, centrifuged for 2 hours at 400 000 *g*, and stored at 4°C.

Exosome Characterization

Purified exosomes were assayed by Western analysis for exosomal markers CD9, CD45, and CD63, and immunolabeled with anti-CD63 and examined via electron microscopy to confirm the quality of the purified exosome preparations. Purified exosomes and whole plasma were analyzed for proinflammatory cytokine and chemokine expression using a human cytokine/chemokine 21-plex magnetic bead kit (Affymetrix; Table 1). Data were acquired using a luminex-200 system and analyzed using Bio-Plex Manger software, version 6.0 (Figure 1*B* and 1*C*; Table 1).

Assay for Immunomodulatory Potential

A total of 3.0×10^6 peripheral blood mononuclear cells (PBMCs) were cultured at 37°C in Roswell Park Memorial Institute 1640 medium supplemented with 20% heat-inactivated exosome-depleted fetal bovine serum and exposed to either pooled exosomes isolated from the plasma of 3 HIV-1–seropositive or HIV-1–seronegative individuals, while parallel untreated cultures and cultures treated with concanavalin A (ConA; 5 μg/mL) served as negative and positive controls, respectively. At 48 hours after exposure, naive ($CD45RA^+/CD62L^+$), central (TCM; $CD45RA^-/CD62L^+$), and effector (TEM; $CD45RA^-/CD62L^-$) memory $CD4^+$ and $CD8^+$ T-cells were analyzed for CD38 expression with flow cytometry. Exosome preparations were normalized by total protein and added at a concentration of 1 μg/mL. Statistical analysis was performed, and graphs were generated using SigmaPlot 10 or GraphPad Prism 6.0 software. All tests were set at a P level of $< .05$.

Antibodies

The following antibodies were used in this study: rabbit polyclonal anti-CD63 (Santa Cruz), rabbit polyclonal anti-CD9 (Santa Cruz), rabbit polyclonal anti-CD45 (Abcam), rabbit polyclonal anti-p24 (ImmunoDiagnostic), murine monoclonal anti-AChE (EMD Millipore), and goat anti-mouse or anti-rabbit immunoglobulin G (IgG) (H+L) labeled with horseradish peroxidase (Thermo Fisher Scientific). The following fluorochrome-conjugated monoclonal antibodies were used for flow cytometry analyses: Alexa Fluor 700–labeled anti-CD3 (UCHT1; BD Bioscience), allophycocyanin (APC)/cyanine 7 (Cy7)–labeled anti-CD4 (OKT4; Biolegend), peridinin chlorophyll protein complex-labeled anti-CD4 (RPA-T4; BD Bioscience), V450-labeled anti-CD8 (RPA-T8; BD Bioscience), biotin-labeled anti-CD45RA (HI100; BD Bioscience), phycoerythrin (PE)/Cy7–labeled anti-CD62L (DREG-56; Biolegend), PE/cyanine 5 (Cy5)–labeled anti-CD38 (HIT2; Biolegend), APC/CY7-labeled anti-HLADR (L243; Biolegend), PE-Texas Red–labeled anti-streptavidin (BD Bioscience), PE/Cy5-labeled mouse IgG1K isotype control (MOPC-21; Biolegend), and APC-Cy7–labeled mouse IgG2aK isotype control (MOPC-173; Biolegend).

RESULTS

Exosomes purified from the plasma of HIV-1–seropositive and seronegative individuals were characterized for AChE activity, the presence of CD45, CD9, and CD63 (Figure 1*A*–*C*), and the absence of HIV p24 (Figure 1*D*) to ascertain their purity. They were then compared for levels of 21 cytokines/chemokines by multiplex assay (Table 1). In the exosomes isolated from HIV-positive individuals (n = 10; Table 1), all 21 cytokines/chemokines were detected. In addition, their levels were also found to be significantly elevated (Figure 1*E*) relative to both their corresponding plasma levels and when compared with exosomes isolated from HIV-negative controls (n = 15; Table 1, Figure 1*E*). Only interleukin 1α, interferon α, and CXCL10 were elevated in the plasma of HIV-1–viremic individuals compared with controls (Figure 1*F*; Table 1).

To test the potential clinical relevance of exosome cytokine content, PBMCs from uninfected human donors were exposed to pooled exosomes isolated from 3 HIV-positive individuals or HIV-negative controls for 48 hours and assessed for induction of CD38 and HLA-DR, markers for activation, on $CD4^+$ and $CD8^+$ T cells via flow cytometry. PBMCs exposed to ConA and untreated PBMCs were used as positive and negative controls, respectively. A significant increase in cell surface expression of CD38 was observed on both naive and central memory $CD4^+$ and $CD8^+$ T cells exposed to HIV positive exosomes compared with cells exposed to HIV-negative exosomes and untreated controls (Figure 1*G*), but, interestingly, HLA-DR was not significantly up-regulated by coculture with exosomes (data not shown).

DISCUSSION

In this report we describe evidence of active and selective enrichment of most screened cytokine and chemokines in plasma exosomes of HIV-seropositive individuals. The levels of these factors were significantly increased compared with both the soluble plasma levels, and the levels in exosomes in the plasma of HIV-seronegative individuals. We used AChE normalization to get a real comparison of cytokine levels between the exosomal vs the soluble plasma fraction, confirming that the increase of cytokine/chemokines levels in exosomes is real and significant. Finally, we found that the exosomes from HIV-seropositive individuals was biologically active inducing increases in the activation marker, CD38, on the surface of naive and central memory $CD4^+$ and $CD8^+$ lymphocytes. There was no comparable effect on the same cell subtypes due to exosomes from HIV-seronegative

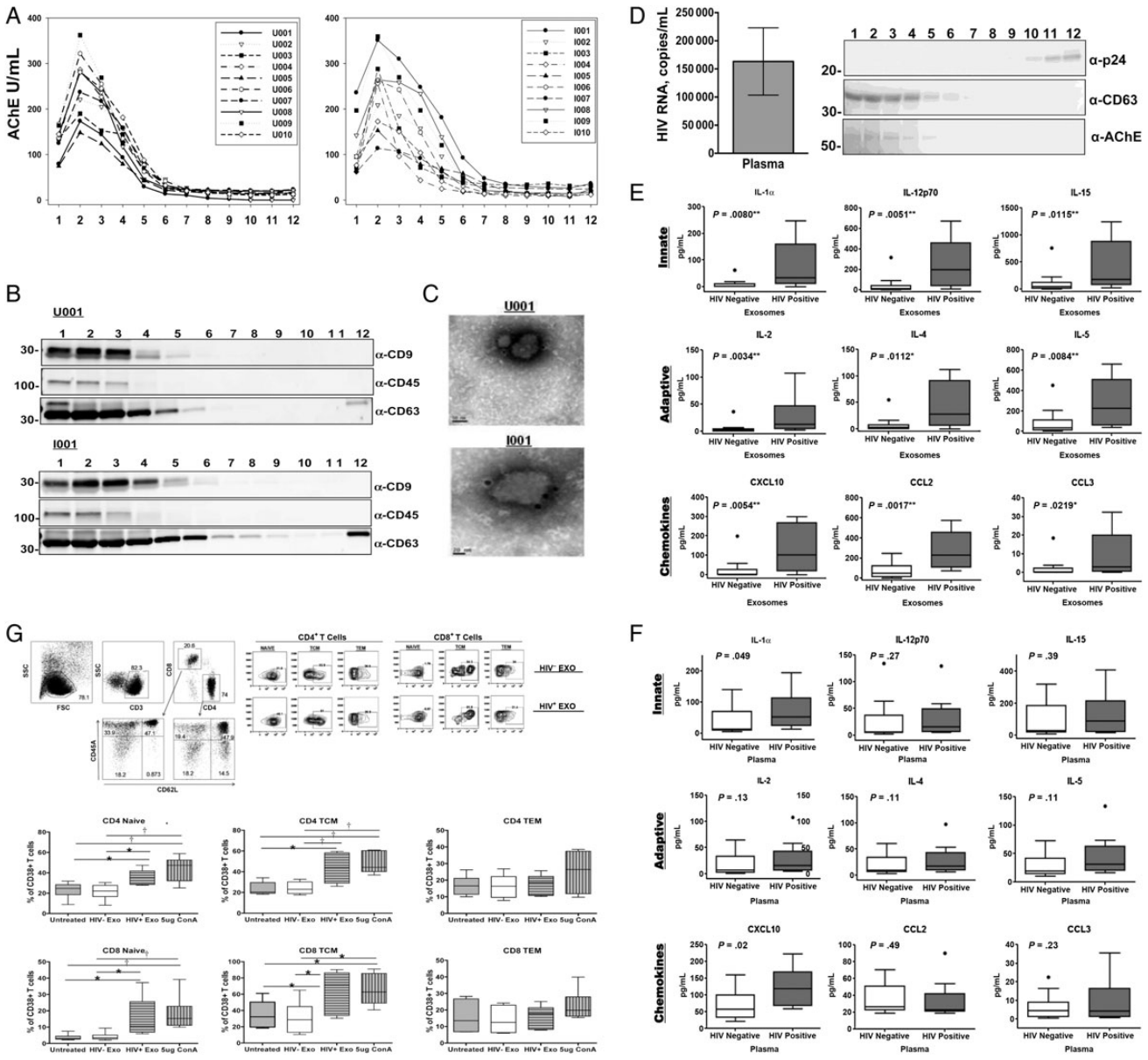

Figure 1. Exosomes are efficiently purified from human plasma, analyzed for cytokine content, and examined for immunomodulatory potential. Plasma collected from whole blood in ethylenediaminetetraacetic acid tubes was subjected to differential centrifugation at 30 000 *g* for 30 minutes and 100 000 *g* for 2 hours. The 100 000 *g* pellet was resuspended in 1 mL of ×1 phosphate-buffered saline, loaded onto Optiprep velocity gradients, and subjected to flotation centrifugation at 250 000 *g* for 2 hours. *A–C,* Fractions from individuals seropositive (n = 10) or seronegative (n = 10) for human immunodeficiency virus (HIV) were subjected to an enzymatic assay for acetylcholinesterase (AChE), a marker for exosomes (*A*) and Western blot analysis for exosomal markers CD9, CD45, and CD63 (*B*) and were immunolabeled with anti-CD63 and examined with electron microscopy to confirm preparation of purified exosomes (*C*). *D*, Plasma viral loads of HIV-infected volunteers ranged from 1423 to 536 436 HIV-1 RNA copies/mL, with an average viral load of 205 957 HIV RNA copies/mL. Representative fractions from an HIV-1–seropositive individual were assayed for exosomal markers and HIV-1 viral particles via Western blot analysis to confirm purification of exosomes from HIV-1 viral particles. Purified exosomes and whole plasma from individuals seropositive (n = 10) or seronegative (n = 15) for HIV-1 were analyzed for proinflammatory cytokine and chemokine expression using a 21-plex multiplex array. *E*, All 21 proinflammatory cytokines and chemokines measured (interleukin 1α [IL-1α], interleukin 2 [IL-2], interleukin 2Rα [IL-2Rα], interleukin 4 [IL-4], interleukin 5 [IL-5], interleukin 7 [IL-7], interleukin 9 [IL-9], interleukin 12p70 [IL-12p70], interleukin 15 [IL-15], interleukin 16 [IL-16], CD40L, granulocyte colony-stimulating factor (G-CSF), interferon [IFN] β and α2, CXCL10, CCL2, CCL3, CCL4, soluble Fas ligand [sFasL], soluble intracellular adhesion molecule 1 [sICAM], and tumor necrosis factor [TNF] α) were associated with and significantly elevated in the exosomes of HIV-1–seropositive individuals compared with seronegative controls (a selection of representative cytokines comparing HIV-1–seropositive and seronegative controls is displayed). *F*, Alternatively, IL-1α, IFN-α2, and CXCL10 were significantly elevated in the corresponding plasma of HIV-1–seropositive individuals compared with seronegative controls. Error bars represent mean and standard error of the mean (SEM) from independent donors. Difference between groups were tested for statistical significance with the Mann–Whitney *U* test. $^{*}P < .05$; $^{\dagger}P < .01$; $^{\ddagger}P < .001$. *G*, CD38 expression was increased on the surface of naive and central memory $CD4^+$ and $CD8^+$ T-cells. A total of 3.0×10^6 peripheral blood mononuclear cells (PBMCs) from 6 HIV-1–seronegative individuals were exposed to either pool exosomes isolated from the plasma of 3

Table 1. Purified Exosomes and Whole Plasma From HIV-1–Seropositive (n = 10) and HIV-1–Seronegative (n = 15) Individuals Analyzed for Proinflammatory Cytokine and Chemokine Expression Using a 21-Plex Multiplex Array[a]

	Exosome Cytokine Concentration, Median (IQR), pg/mL			Plasma Cytokine Concentration, Median (IQR), pg/mL		
Cytokine	HIV Seronegative	HIV Seropositive	*P* Value	HIV Seronegative	HIV Seropositive	*P* Value
IL-1α	0.333 (0.0–11.37)	33.22 (10.03–158.7)	.008[b]	14.60 (9.250–71.35)	52.52 (24.70–114.5)	.05[c]
IL-2	1.838 (0.0–4.382)	12.99 (4.424–47.22)	.003[b]	7.040 (2.370–33.0)	15.85 (6.028–43.23)	.13
IL-2Rα	21.49 (5.782–49.86)	172.1 (46.21–373.9)	.006[b]	83.67 (48.75–124.7)	83.75 (72.25–108.8)	.60
IL-4	2.930 (0.0–7.782)	27.86 (6.355–91.18)	.01[c]	9.270 (6.050–34.0)	17.27 (8.965–42.99)	.11
IL-5	34.19 (9.028–112.9)	227.4 (57.89–509.3)	.008[b]	19.81 (14.50–42.16)	31.44 (20.02–63.13)	.11
IL-7	26.85 (5.273–82.68)	217.9 (48.84–619.1)	.01[c]	9.530 (7.620–50.10)	29.85 (7.458–84.45)	.31
IL-9	71.79 (14.68–308.6)	686.3 (240.0–1529)	.005[b]	37.85 (30.81–122.1)	68.24 (40.02–140.2)	.11
IL-12p70	13.79 (2.812–41.35)	197.9 (39.47–459.5)	.005[b]	6.200 (4.520–37.07)	16.02 (5.955–49.67)	.27
IL-15	43.53 (6.533–121.0)	173.8 (75.65–881.8)	.01[c]	29.88 (20.21–185.1)	90.27 (22.11–214.2)	.39
IL-16	38.18 (5.550–72.10)	142.6 (74.56–442.1)	.01[b]	335.3 (146.1–1405)	253.7 (151.0–450.5)	.33
TNF-α	2.211 (0.555–6.778)	36.70 (6.687–95.20)	.002[b]	24.81 (17.06–116.6)	66.30 (17.90–180.3)	.42
IFN-α2	0.0 (0.0–15.93)	32.35 (14.76–122.6)	.005[b]	18.02 (8.140–70.48)	55.96 (34.91–146.8)	.01[c]
IFN-β	0.0 (0.0–0.9324)	44.14 (0.0–316.6)	.005[b]	14.16 (5.400–55.91)	7.885 (0.0–90.70)	.49
CXCL10	0.0 (0.0–27.15)	102.2 (17.91–268.9)	.005[b]	57.82 (33.15–100.8)	118.7 (67.38–168.5)	.02[c]
CCL2	49.90 (15.38–124.0)	232.6 (106.9–457.7)	.002[b]	26.80 (22.47–51.15)	22.85 (21.13–42.18)	.60
CCL3	0.0 (0.0–2.248)	2.928 (0.465–20.0)	.02[c]	4.470 (1.220–8.940)	4.355 (1.225–16.42)	.49
CCL4	0.0 (0.0–0.0)	11.76 (0.0–137.3)	.02[c]	24.81 (17.06–116.6)	66.30 (17.90–180.3)	.42
CD40L	1.877 (0.740–10.04)	31.89 (6.800–114.5)	.01[b]	187.2 (100.2–634.4)	217.6 (123.1–342.5)	.80
G-CSF	8.170 (0.0–58.81)	103.4 (20.90–435.4)	.02[c]	9.760 (5.220–71.12)	25.24 (8.748–74.59)	.21
sICAM	86.16 (51.02–167.5)	446.4 (145.4–1363)	.01[b]	2433 (985.9–4633)	1489 (1288–1976)	.56
sFasL	1.998 (0.348–6.099)	12.55 (6.914–52.34)	.006[b]	5.370 (4.390–14.85)	8.415 (5.600–16.31)	.26

Abbreviations: G-CSF, granulocyte colony-stimulating factor; HIV, human immunodeficiency virus; IFN, interferon; IL-1α, interleukin 1α; IL-2, interleukin 2; IL-2Rα, interleukin 2Rα; IL-4, interleukin 4; IL-5, interleukin 5; IL-7, interleukin 7; IL-9, interleukin 9; IL-12p70, interleukin 12p70; IL-15, interleukin 15; IL-16, interleukin 16; IQR, interquartile range; sFasL, soluble Fas ligand; sICAM, soluble intracellular adhesion molecule 1; TNF, tumor necrosis factor.

[a] All 21 proinflammatory cytokines and chemokines measured were associated with and significantly elevated in the exosomes of HIV-1–seropositive individuals compared with seronegative controls. Alternatively, IL-1α, IFN-α2, and CXCL10 were significantly elevated in the corresponding plasma of HIV-1–seropositive individuals compared with seronegative controls. The difference between groups was tested for statistical significance with the Mann–Whitney *U* test.

[b] $P < .01$.

[c] $P < .05$.

individuals. Although HLA-DR was up-regulated with ConA stimulation, expression of this activation marker surprisingly was not significantly up-regulated during coculture with the HIV-seropositive exosomes (data not shown). Although both activation markers are often associated, they also have been shown to undergo distinct regulation in HIV-infected patients [10].

These data suggest a potentially interesting mechanism by which HIV infection could indirectly recruit novel targets through activation induction of naive $CD4^+$ T cells and contribute to the continuous chronic viral replication in vivo. In addition, generalized activation of naive and memory $CD8^+$ T cells could contribute to dysfunction of this cell pool as a whole, resulting in poor responses to HIV and other pathogens, exhaustion of antigen-specific T cells, and induction of chronic immune activation.

Interestingly, this mechanism has remarkable similarity to a host mechanism leading to immune privilege during pregnancy. Multiple roles of extracellular vesicles have been examined in the complex process of a successful pregnancy. Furthermore, their involvement in the pathology of preeclampsia has been examined, where elevated circulating extracellular vesicles have been implicated in contributing to exacerbated maternal systemic innate immune cell activation and vascular dysfunction [11]. This same process has also been found to be

Figure 1 continued. HIV-1–seropositive (HIV^+ Exo) or HIV-1–seronegative (HIV^- Exo) individuals, left untreated, or treated with 5 μg/mL of concanavalin A (ConA) as a positive control. 48 hours after exposure, Naive ($CD45RA+/CD62L^+$), central (TCM; $CD45RA^-/CD62L^+$) and effector (TEM; $CD45RA^-/CD62L^-$) memory $CD4^+$ and $CD8^+$ T cells were analyzed for CD38 expression using flow cytometry. Exosomes were normalized by total protein and added at a concentration of 1 μg/mL. Error bars represent mean and SEM values from 6 independent donors. Differences between groups were tested for statistical significance with 1-way analysis of variance. $^*P < .05$; $^\dagger P < .01$; $^\ddagger P < .001$.

hijacked during carcinogenesis, leading to immunomodulation/inflammation and ultimately allowing tumor growth [12]. It is clear from the literature in this area that knowledge of the role played by these extracellular vesicles would allow harnessing their ability for immunotherapy [13].

One of the characteristics of chronic immune activation is chronically elevated cytokine levels [3]. While cytokine levels in the plasma of HIV-seropositive individuals have been the subject of intense investigation, the results presented here demonstrate that a significant amount of cytokines/chemokines is not released in free form but is associated with and seemingly enriched within exosomes. Given the increased stability of molecules carried by exosomes [14], it is plausible that association of cytokines and chemokines within exosomes would imply their increased half-life, as well as their wider distribution to specific target cells distal from the producer cells. Although there are several reports by our group and others of increased exosome release from HIV-infected cells and Nef-transduced cell lines [6–8, 15], the origin of cytokine-laden exosomes in HIV-infected individuals remains to be fully elucidated. Consequently, further investigations will be needed to ascertain the respective contribution of infection, HIV proteins, and host cell responses in the release of cytokine-laden exosomes into the circulation. In summary, our results suggest a potentially important mechanism that may contribute to chronic viral replication and chronic immune activation during HIV infection, leading to exciting avenues for future inquiry.

Notes

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