Cell activation and HIV-1 replication in unstimulated CD4+ T lymphocytes ingesting exosomes from cells expressing defective HIV-1

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Abstract

Background: A relevant burden of defective HIV-1 genomes populates PBMCs from HIV-1 infected patients, especially during HAART treatment. These viral genomes, although unable to codify for infectious viral particles, can express viral proteins which may affect functions of host cells as well as bystander ones. Cells expressing defective HIV-1 have a lifespan longer than that of cells producing infectious particles. Hence, their interaction with other cell types, including resting lymphocytes, is expected to occur frequently in tissues where HIV actively replicates. We investigated the effects of the expression of a prototype of functionally defective HIV-1 on bystander, unstimulated CD4+ T lymphocytes.

Results: We observed that unstimulated human primary CD4+ T lymphocytes were activated and became permissive for HIV-1 replication when co-cultivated with cells expressing a functionally defective HIV-1 (F12/Hut-78 cells). This effect depended on the presence in F12/Hut-78 supernatants of nanovesicles we identified as exosomes. By inspecting the underlying mechanism, we found that ADAM17, i.e., a disintegrin and metalloprotease converting pro-TNF-α in its mature form, associated with exosomes from F12/Hut-78 cells, and played a key role in the HIV-1 replication in unstimulated CD4+ T lymphocytes. In fact, the treatment with an inhibitor of ADAM17 abolished both activation and HIV-1 replication in unstimulated CD4+ T lymphocytes. TNF-α appeared to be the downstream effector of ADAM17 since the treatment of unstimulated lymphocytes with antibodies against TNF-α or its receptors blocked the HIV-1 replication. Finally, we found that the expression of NefF12 in exosome-producing cells was sufficient to induce the susceptibility to HIV-1 infection in unstimulated CD4+ T lymphocytes.

Conclusions: Exosomes from cells expressing a functionally defective mutant can induce cell activation and HIV-1 susceptibility in unstimulated CD4+ T lymphocytes. This evidence highlights the relevance for AIDS pathogenesis of the expression of viral products from defective HIV-1 genomes.

Keywords: Defective HIV-1, Exosomes, ADAM17, CD4+ T lymphocytes, Nef

Background

The high frequency of mutations occurring in HIV-1 retrotranscription can lead to the production of defective HIV-1 genomes. They can persist in peripheral blood mononuclear cells (PBMCs) of HIV-1 infected patients since host cells are expected to have a lifetime longer than that of cells infected by replication-competent quasispecies. First evidences about the significant presence in vivo of defective HIV-1 genomes came from the observation that the number of PBMCs containing HIV-1 DNA greatly exceeds that of cells expressing infectious HIV-1 [1,2]. Later, 46% of HIV-1 genomes detected in PBMCs from 10 infected patients was found deleted, while PBMCs from 3 patients harbored only deleted or rearranged HIV-1 genomes [3]. Sequence analysis of the HIV-1 RT gene in PBMCs and rectal tissue of highly active anti-retroviral therapy (HAART)-treated patients revealed a great number of stop codons in all samples analyzed [4]. More recently, the analysis of 213 proviral clones from treated patients demonstrated the presence of 88.3% of genomes with identifiable defects [5].
Of major importance, mutations do not necessarily hamper the expression of defective HIV-1 genomes. Accordingly, defects in basically all HIV-1 genes except tat were identified in genomes of HIV-1 isolated from plasma of HAART-treated patients [6-10]. At least part of these mutated viral genomes are expected to integrate in host cell DNA thereby expressing defective HIV-1. Thus, the presence in HIV-1 infected patients, especially those treated by HAART, of defective but transcriptionally active HIV-1 genomes can be relevant, and investigating their role in the development of the disease would be of interest. We looked at the effects of the expression of a prototype of functionally defective HIV-1 (i.e., F12/HIV-1) [11] on bystander unstimulated CD4+ T lymphocytes. This system can mirror the events occurring in vivo upon interaction of resting lymphocytes with cells harboring defective HIV-1 genomes expressing either fully or partially functional viral products. The Hut-78 cells chronically infected with the non-producer F12/HIV-1 strain (referred to as F12/Hut-78 cells) were obtained by cloning cells infected by supernatants of PBMCs from an HIV-1 infected patient [11]. Cells expressing such HIV-1 mutant do not release infectious viral particles, meanwhile expressing a complete viral protein pattern comprising a truncated Vpr, an uncleaved Env gp160, and a mutated Nef (Table 1) [12]. In the present study, we provide evidence that exosomes released by F12/Hut-78 cells can influence the cell activation state of bystander, unstimulated CD4+ T lymphocytes.

Exosomes are nanovesicles released by all cell types. They are lipid bilayer vesicles of 50–100 nanometers which form intracellularly upon inward invagination of endosome membranes [16]. This event leads to the formation of intraluminal vesicles which then become part of multivesicular bodies. Subsequently, they can undergo lysosomal degradation or, alternatively, be released into extracellular space upon fusion of multivesicular bodies with plasma membrane. Exosomes can be released also through direct extrusion of plasma membrane [17]. Current protocols of purification and marker analysis cannot discriminate between endosome-produced nanoparticles and vesicles with similar size but originating from cell membranes. For the sake of clarity, cell-produced nanoparticles are here defined exosomes irrespective to their origin.

It is now accepted that exosomes are part of the intercellular communication network while they were originally thought to secrete only waste cell material [18]. Exosomes incorporate messenger RNAs, microRNAs, and proteins which are functional in target cells [19]. Exosomes from HIV-1 infected cells incorporate Gag [20] and Nef HIV-1 proteins [21,22]. HIV-1 Gag molecules associate with exosomes by virtue of their higher-order oligomerization, while Nef is incorporated in exosomes upon anchoring into lipid raft microdomains through its N-terminal myristoylation and a stretch of basic amino acid residing in its alpha-helix 1.

Here, we demonstrate that the cell activation induced by exosomes from F12/Hut-78 cells in unstimulated human primary CD4+ T lymphocytes couples with HIV-1 replication. These effects relied on the expression of NefF12.

### Results

**Unstimulated CD4+ T lymphocytes become susceptible to HIV-1 infection when co-cultivated with cells expressing a defective HIV-1 strain**

We looked at possible effects of the expression of defective HIV-1 on bystander unstimulated CD4+ T lymphocytes by

### Table 1 Proteome of F12/HIV-1*  

| Viral gene | Amino acid substitutions | Viral protein product |
|------------|--------------------------|-----------------------|
| gag        | p17: 1                   | Inefficient cleavage of the p55 precursor [12,13] |
|            | p24: 6                   |                                      |
|            | p15: 3                   |                                      |
| pol        | Pro: 3                   | Apparently intact [12] |
|            | RT: 10                   |                                      |
|            | Endo: 2                  |                                      |
| env        | gp120: 4                 | No cleavage of the gp160 precursor [11,12] |
|            | gp41: 2                  |                                      |
| nef        | 3                        | Defects in trafficking and signaling functions [13,14] |
| tat        | 0                        | Functional [12] |
| rev        | 0                        | Functional [12] |
| vif        | 14                       | Functionally defective [12,15] |
| vpr        | 3                        | Premature stop codon at aa 76 [12] |
| vpu        | 0                        | Apparently intact [12] |

*The F12/HIV-1 amino acid sequence was compared to a consensus sequence obtained with multiple alignments of 5 type B HIV-1 isolates. No nucleotide substitutions have been found in the LTRs, TAR, and RRE HIV-1 regulatory sequences.
setting up trans-well co-cultures of F12/Hut-78 cells with unstimulated CD4+ T lymphocytes isolated from healthy donors. F12/Hut-78 cells or, as control, parental uninfected Hut-78 cells were put in the upper chamber of a 0.4 μm trans-well plate where unstimulated CD4+ T lymphocytes were seeded in the bottom chamber. After overnight cocultivation, unstimulated CD4+ T lymphocytes were recovered from trans-well plates and infected with HIV-1 in the presence or not of azidothymidine (AZT). Three days later, HIV-1 replication in unstimulated cells was evaluated by FACS analysis for the presence of intracytoplasmic HIV-1 Gag-related products. HIV-1 replicated in unstimulated CD4+ T lymphocytes from co-cultures with F12/Hut-78 cells but not parental ones (Figure 1A). The HIV-1 replication did not occur also in CD4+ T lymphocytes from co-cultures carried out in the presence of AZT (Figure 1A), thus ensuring that what we detected was consequence of authentic HIV-1 infection.

Considering that HIV-1 replication in lymphocytes needs cell activation, we hypothesized that the expression of the defective HIV-1 generates a signal leading to lymphocyte activation. To identify the nature of such a signal, we repeated the co-culture experiments in the presence of a number of drugs or antibodies. Among the drugs we tested, HIV-1 replication in bystander cells was successfully blocked by 1 μM of both GW4869 and Spiroepoxide (Figure 1B), i.e., two structurally unrelated inhibitors of neutral sphingomyelinase blocking exosome release [23-28]. This result prompted us to focus on exosomes as possible effectors of the induction of HIV-1 susceptibility in unstimulated CD4+ T lymphocytes.

Characterization of exosomes from F12/Hut-78 cells
Exosomes from F12/Hut-78 cells were isolated by differential centrifugations and then purified by 6-18% gradients of iodixanol. As control, the same procedure was applied to supernatants from D10/Hut-78 cells, i.e., a cell line chronically infected with a producer HIV-1 strain [11]. Gradient fractions were assayed for the activity of acetylcholinesterase (AchE, a marker of exosomes) [29], and the presence of HIV-1 Gag products (Figure 2A). As previously described [30], 6-18% iodixanol gradients concentrated exosomes in fractions 5–8. Here, Gag products also were detectable (Figure 2A), likely consequence of their association with exosomes [20]. Conversely, in denser fractions, where viral particles are expected to float, Gag products remained undetectable in gradients loaded with nanovesicles from F12/Hut-78 cells, while they massively accumulated in gradients with nanovesicles from D10/Hut-78 cells. Therefore, supernatants from F12/Hut-78 cells appeared to be free of viral particles. Hence, still possible residual viral particles were expected to only minimally contaminate the exosome preparations from F12/Hut-78 cells obtained through differential centrifugations.

Nanovesicles from F12/Hut-78 cells were formally identified as exosomes by FACS analysis demonstrating the presence of both monosialotetrahexosylganglioside (GM1) [31,32], using FITC-conjugated cholera toxin B (CTX-B), and CD63 (Figure 2B). In addition, western blot analysis revealed that, consistently with what previously described for exosomes from Gag- [20] or Nef-expressing cells [21,22], both HIV-1 CAp24 and Nef proteins associate with exosomes from F12/Hut-78 cells (Figure 2C).

Primary unstimulated CD4+ T lymphocytes release both IL-2 and TNF-α in response to the treatment with exosomes from F12/Hut-78 cells
HIV-1 replication in quiescent CD4+ T lymphocytes requires cell activation which associates with the release of interleukin (IL)-2 and tumor necrosis factor (TNF)α. We asked whether unstimulated CD4+ T lymphocytes release IL-2 and TNF-α in response to the treatment with exosomes from F12/Hut-78 cells. Human unstimulated CD4+ T lymphocytes were challenged with equal amounts of exosomes derived from F12/Hut-78 cells or, as control, uninfected Hut-78 cells. Exosomes were quantified in terms of the activity of AchE as detailed in the Material and Methods section. We assessed the number of IL-2 producing cells by ELISPOT assay 48 hours after exosome challenge, and the TNF-α release by ELISA on supernatants harvested at different hours after challenge. We found that 60 μU of exosomes from F12/Hut-78 cells applied on 10^5 unstimulated CD4+ T lymphocytes cells generated a number of cells producing IL-2 significantly increased compared to untreated cells or cells treated with exosomes from parental Hut-78 cells (Figure 3A). Consistently, higher levels of TNF-α were detected when unstimulated CD4+ T lymphocytes were treated with scaled amounts (i.e., from 15 to 60 μU/10^5 cells) of exosomes from F12/Hut-78 cells than from parental ones (Figure 3B). The TNF-α release peaked at 6 hours post challenge, and returned to control levels 16 hours after challenge (data not shown). No TNF-α was detected in the exosome preparations we used for the challenges (not shown).

These data implied that the treatment of unstimulated CD4+ T lymphocytes with exosomes from F12/Hut-78 cells leads to cell activation.

The treatment with exosomes from F12/Hut-78 cells renders unstimulated CD4+ T lymphocytes susceptible to HIV-1 replication
Next, we investigated whether the cellular activation induced by exosomes released from F12/Hut-78 cells couples with HIV-1 replication. As previously described [33], timing of quiescent CD4+ T lymphocyte activation strongly influences viral replication. Thus, exosomes were added to unstimulated CD4+ T lymphocytes either: i) six hours
before infection with the T-tropic NL4-3 HIV-1 strain; ii) together with HIV-1, and iii) six hours after HIV-1 infection. HIV-1 replication was assayed by FACS analysis 3 days after infection by FACS analysis for the intracellular expression of HIV-1 Gag-related products. We measured 0.4 to 0.9% of HIV-1 Gag positive cells in unstimulated cells from different donors challenged with HIV-1 alone. Conversely, when exosome challenge preceded HIV-1 infection, a strong increase in the number of HIV-1 Gag expressing cells was observed (Figure 4A). The increase appeared slightly but reproducibly less striking when cells were simultaneously treated with exosomes and HIV-1, whereas negligible effects were observed when HIV-1 infection preceded exosome challenge (Figure 4A). On the basis of these results, the subsequent experiments were carried out by infecting cells with HIV-1 6 hours after exosome treatment.

Similar to what we observed for TNF-α release, the extents of HIV-1 replication in CD4+ T lymphocytes correlated with the exosome input (Figure 4B). Notably, the treatment with 10 μM AZT led to a strong reduction of HIV-1 Gag-expressing CD4+ T lymphocytes (Figure 4C). This result formally excluded that possible carry-over from
exosome- and/or virus-associated CAp24 interfered with the results we obtained by FACS analysis, meanwhile indicating that HIV-1 was indeed expressed in unstimulated CD4+ T lymphocytes challenged with exosomes from F12/Hut-78 cells. 

Next, we were interested in establishing whether HIV-1 expressing CD4+ T lymphocytes released infectious virus. To this end, CD4+ T cells were challenged with exosomes from Hut-78 or F12/Hut-78 cells and then with HIV-1 in the presence or not of AZT. Three days later, CD4+ T cell cultures were washed, and co-cultures with Rev-CEM reporter cells, i.e., a cell line expressing GFP in the presence of both HIV-1 Tat and Rev products [34], were set up. After additional 3 days, the percentages of GFP+ cells were evaluated by FACS (Figure 4D). No significant increase in the percentage of GFP+ cells was observed in Rev-CEM from co-cultures with CD4+ T lymphocytes treated with control exosomes, whereas a sharp increase was detectable when the lymphocytes were treated with exosomes from F12/Hut-78 cells. Such an increase was no more detectable when challenged CD4+ T lymphocytes were cultured in the presence of AZT until the addition of the reporter cells. These results indicated that unstimulated CD4+ T lymphocytes release infectious virus when treated with exosomes from F12/Hut-78 cells. In addition, the lack of infected Rev-CEM cells in the co-cultures with CD4+ T lymphocytes challenged with control exosomes indicated that HIV-1 replicating in Rev-CEM cells did not originate from the viral input used to infect the unstimulated CD4+ T lymphocytes.

We concluded that the exosome-dependent activation of CD4+ T lymphocytes rendered them susceptible to productive HIV-1 infection.

**ADAM17 is involved in both activation and HIV-1 replication in unstimulated CD4+ T lymphocytes treated with exosomes from F12/Hut-78 cells**

Next, we investigated the mechanism underlying the cell activation in unstimulated CD4+ T lymphocytes targeted.
by exosomes from F12/Hut-78 cells. It has been recently reported that the expression of HIV-1 Nef leads to incorporation of active ADAM17 into exosomes. Upon ingestion of these exosomes, unstimulated CD4+ T lymphocytes release TNF-α as consequence of the cleavage of pro-TNF-α driven by exosome-associated ADAM17 [35]. ADAM17 belongs to the family of ADAM (a disintegrin and metalloprotease) enzymes [36]. It is a multi-domain, transmembrane, Zn2+-dependent proteinase whose inactive form presents a pro-domain which can be cleaved by furin in trans-Golgi network. The most characterized function of active ADAM17 is the cleavage of pro-TNF-α to its active form. We asked whether exosome-associated ADAM17 was involved in the CD4+ T lymphocyte activation we observed. To this aim, we first assayed the presence of active ADAM17 in exosomes from F12/Hut-78 cells. Western blot analysis highlighted the presence of active ADAM17 in F12/Hut-78 cells but not in parental Hut-78 cells (Figure 5A). Consistently, active ADAM17 was found in exosomes from F12/Hut-78 cells, or mock-challenged (Nil). As control, cells were also treated with 2 μg/mL of PHA. After extensive washings, the cells were seeded in complete medium, and supernatants were harvested 6 hours later. TNF-α contents were determined by ELISA. Shown are the mean concentrations ± SD as calculated from three independent experiments. Nd: not detectable. *p < 0.05.

Figure 3 Unstimulated CD4+ T lymphocytes release IL-2 and TNF-α when treated with exosomes from F12/Hut-78 cells. A. Detection of IL-2-producing cells. 2 × 10⁵ unstimulated CD4+ T lymphocytes were challenged with 60 μU of exosomes from either Hut-78 or F12/Hut-78 cells, or mock-challenged (Nil), and then incubated for 48 hours in ELISPOT microwells previously coated with an anti-IL-2 monoclonal antibody. Shown are the mean number ± SD of IL-2 spot forming units (SFU)/10⁶ cells calculated from five independent experiments. *p < 0.05. B. Detection of TNF-α on supernatants. 10⁵ unstimulated CD4+ T lymphocytes were challenged with increasing amounts (i.e., from 15 to 60 μU) of exosomes from Hut-78 or F12/Hut-78 cells, or mock-challenged (Nil). As control, cells were also treated with 2 μg/mL of PHA. After extensive washings, the cells were seeded in complete medium, and supernatants were harvested 6 hours later. TNF-α contents were determined by ELISA. Shown are the mean concentrations ± SD as calculated from five independent experiments. *p < 0.05.
Figure 4 (See legend on next page.)
the viral replication in unstimulated CD4+ T lymphocytes challenged with exosomes from F12/Hut-78 cells and then infected with HIV-1. Alternatively, exosomes and HIV-1 were added to cells at the same time, or cells were first infected and then challenged with the exosomes. As control, cells were infected with or without PHA. Three days later, the cells were scored for the expression of HIV-1 Gag by FACS analysis. In the upper panels, shown are the dot-plots from a representative experiment. Percentages of HIV-1 Gag positive cells are indicated. In the bottom panel, shown are the fold increases of the percentages of HIV-1 CAp24-positive cells compared to cultures treated with HIV-1 alone. Shown are the mean of fold increases + SD as calculated from five independent experiments with duplicates. *p < 0.05. B. Dose–response effect of exosomes. Shown are the mean of fold increases + SD as calculated from three independent experiments with triplicates. *p < 0.05. C. Effects of AZT. Cultures were run in the absence (Nil) or in the presence of 10 μM AZT. Shown are the mean of fold increases + SD as calculated from three independent experiments with duplicates. *p < 0.05. D. Detection of infectious HIV-1. 10^5 CD4+ T lymphocytes were challenged with 60 μL of exosomes from either Hut-78 or F12/Hut-78 cells, and then infected with HIV-1 with or without AZT. Three days later, co-cultures with Rev-CEM cells were undertaken. After additional 3 days, GFP positive Rev-CEM cells were scored by FACS. Shown are the mean of fold increases of GFP+ Rev-CEM cells from exosome-treated cells compared to co-cultures with lymphocytes treated with HIV-1 alone, as calculated from two independent experiments with duplicates.

Figure 5 Active ADAM17 associates with exosomes from F12/Hut-78 cells. Western blot analysis for the expression of ADAM17 in both cells (A) and exosomes (B) from Hut-78 and F12/Hut-78 cells. On the left of blots for ADAM17, arrows identify both inactive and active ADAM17 forms. Signals from cellular ADAM17 were normalized with β-actin signals, while both anti-ICAM-1 and anti-CD63 monoclonal antibodies served to normalize ADAM17 signals from exosomes. On the right of each panel, molecular weight markers are given in kDa. Results are representative of five independent experiments.
increased HIV-1 replication in a dose-dependent manner (Figure 7A).

To further strengthen the role of TNF-α, experiments with neutralizing antibodies against the TNF-α receptors (TNFR)-1 and 2, either alone or in combination, were performed. According to what we observed with anti-TNF-α antibodies, the treatment with anti-TNFR1 and –TNFR2 antibodies alone or in combination inhibited the HIV-1 replication in unstimulated CD4+ T lymphocytes challenged with exosomes from F12/Hut-78 cells (Figure 7B). This result suggests that the activation of both TNFR1- and

Figure 6 The inhibition of ADAM17 activity impairs both TNF-α release and HIV-1 replication in unstimulated CD4+ T lymphocytes treated with exosomes from F12/Hut-78 cells. 
A. Effect of TAPI-2 on TNF-α release. 10^5 unstimulated CD4+ T lymphocytes were challenged with 60 µU of exosomes from either Hut-78 or F12/Hut-78 cells, or, as control, treated with 2 µg/mL of PHA. After extensive washings, the cells were cultured for 6 hours in the presence or not (Nil) of the indicated concentrations of TAPI-2. Thereafter, supernatants were harvested and titrated for the presence of TNF-α. Shown are the mean + SD of TNF-α concentrations as calculated from three independent experiments with duplicates. *p < 0.05. 
B. Effect of TAPI-2 on HIV-1 replication. 10^5 unstimulated CD4+ T lymphocytes were challenged with 60 µU of exosomes from either Hut-78 or F12/Hut-78 cells, and then infected by HIV-1. As control, unstimulated cells were challenged with HIV-1 alone, or in the presence of 2 µg/mL of PHA. The cells were then left in culture for 3 days in the presence or not (Nil) of the indicated concentrations of TAPI-2. Finally, the cells were analyzed for HIV-1 expression by FACS analysis. The fold increases of the percentages of HIV-1 CAp24-positive cells compared to cultures treated with HIV-1 alone are presented. Shown are the mean of fold increases + SD as calculated from three independent experiments with duplicates. *p < 0.05.

Figure 7 TNF-α drives the HIV-1 replication in unstimulated CD4+ T lymphocytes treated with exosomes from F12/Hut-78 cells. 
A. Neutralization of soluble TNF-α. 10^5 unstimulated CD4+ T lymphocytes were challenged with 60 µU of exosomes from either Hut-78 or F12/Hut-78 cells, and then infected with HIV-1. After washings, the cells were cultured in the presence of anti-TNF-α neutralizing antibodies or with unrelated IgGs. As control, unstimulated CD4+ T lymphocytes were treated with the indicated doses of recombinant TNF-α, infected by HIV-1, and then cultured in the presence of anti-TNF-α neutralizing antibodies or IgGs. Three days after challenges, the cells were analyzed for HIV-1 expression by FACS analysis. The fold increases of the percentages of HIV-1 CAp24-positive cells compared to cultures treated with HIV-1 alone are presented. Shown are the mean of fold increases + SD as calculated from three independent experiments with duplicates. *p < 0.05. 
B. Effects of the block of TNFR1 and TNFR2. The same procedure described for panel A was applied, except than unstimulated lymphocytes were incubated with neutralizing anti-TNFR1 or –TNFR2 antibodies either alone or in combination, or irrelevant isotype IgGs for 1 hour at 4°C before exosome and HIV-1 challenges. Three days later, the cells were analyzed for HIV-1 expression by FACS analysis. The fold increases of the percentages of HIV-1 CAp24-positive cells compared to lymphocytes treated with HIV-1 alone are presented. Shown are the mean of fold increases as calculated from two independent experiments with duplicates.
TNFR2-related intracellular signals is required for the HIV-1 replication in unstimulated CD4+ T lymphocytes.

Overall, these results supported the data we obtained with TAPI-2, and are consistent with the idea that TNF-α was the downstream effector of exosome-associated ADAM17.

**Unstimulated CD4+ T lymphocytes replicate HIV-1 when targeted by exosomes released by cells expressing NefF12**

Active ADAM17 was previously reported to be uploaded in exosomes as consequence of the ectopic expression of wild-type Nef in producer cells [35]. The upload of active ADAM17 in exosomes from F12/Hut-78 cells was suggestive of a key role of NefF12 in the induction of HIV-1 susceptibility in CD4+ T lymphocytes. To investigate this point, 293 T cells were transiently transfected with vectors expressing NefF12 or, as control, NefG2A, i.e., a mutant which is not expected to assemble the Nef-associated kinase complex required for ADAM17 uploading in exosomes [38]. This phenotype is the consequence of the lack of the N-terminal myristoylation site which dramatically decreases the efficiency of Nef association with cell membranes. We noticed that NefF12, differently to NefG2A, was incorporated into exosomes (Figure 8A). Unstimulated CD4+ T lymphocytes were challenged with equal amounts of exosomes derived from cells expressing either NefF12 or NefG2A. As control, cells were treated with exosomes derived from either mock-transfected cells or F12/Hut-78 cells. Six hours later, the cells were infected with HIV-1, and after additional 3 days, analyzed for HIV-1 expression. HIV-1 replicated at similar extents in cell cultures treated with exosomes from F12/Hut-78 cells or NefF12-expressing cells, while no HIV-1 replication was detectable in cells treated with control or NefG2A exosomes (Figure 8B).

These results indicate that the expression of NefF12 in exosome-producing cells is sufficient to render unstimulated CD4+ T lymphocytes susceptible to HIV-1 infection.

**Unstimulated CD4+ T lymphocytes become susceptible to HIV-1 infection when co-cultivated with activated CD4+ T lymphocytes infected by a NefF12-expressing, non-producer HIV-1**

Finally, we were interested in establishing whether NefF12 expressed in both viral and cellular contexts different from F12/Hut-78 cells maintains its key role in inducing HIV-1 susceptibility on bystander CD4+ T lymphocytes. To this end, PHA-activated primary CD4+ T lymphocytes were infected by a NL4-3 HIV-1 strain where wt Nef was replaced by NefF12 [39], and which was pseudotyped with the G protein from vesicular stomatitis virus (VSV-G). As for F12/Hut-78 cells, cells expressing this HIV-1 strain do not release viral particles, in the presence however of the expression of a complete viral protein pattern [39]. Both mock and infected cell cultures were put in the upper chamber of trans-well plates where unstimulated CD4+ T lymphocytes were seeded in the lower one, in the presence or not of the exosome inhibitors GW4869 and Spiroepoxide. After an overnight incubation, unstimulated CD4+ T lymphocytes were recovered from trans-well plates and infected with HIV-1. Three days later, HIV-1 replication in unstimulated cells was evaluated by FACS analysis. We noticed that HIV-1 replicated in unstimulated CD4+ T lymphocytes from co-cultures with cells infected with the NL4-3/NefF12 HIV-1 strain but not mock infected cells (Figure 9). The viral replication was no more detectable when the co-cultures were carried out in the presence of the exosome inhibitors (Figure 9).

These results add significance to what we previously observed with 293 T transfected cells in terms of the key role of NefF12 expression in the HIV-1 replication in bystander CD4+ T lymphocytes.

**Discussion**

Formal demonstration of the presence of grossly defective HIV-1 genomes in PBMCs of AIDS patients was achieved in mid ’90s [3]. Physical mapping of defective genomes indicated that the frequency of deletions is proportional to their proximity to the central part of the viral genome [3]. Subsequent studies demonstrated the presence of genetically damaged sequences in transcriptionally active proviral HIV-1 [40]. Moreover, HAART treatment further selects for mutated/deleted viral genomes in both PBMCs and rectal tissues [4,41]. The identification of an in vitro cell system comprehensively reproducing the events which in vivo lead to the formation of defective HIV-1 DNA is still an unmet challenge. The functionally defective F12/HIV-1 genome [42] could recapitulate some features of different defective HIV-1 species which in vivo remain transcriptionally active.

Our investigations started from the observation that unstimulated CD4+ T lymphocytes became susceptible to HIV-1 infection upon trans-well co-culture with F12/Hut-78 cells. This experimental setting was useful to avoid misinterpretations due to possible effects induced by cell-to-cell contact. However, it is fairly conceivable that it mirrors events occurring in free co-cultures and, more important, in tissues most relevant for HIV replication, e.g., gut mucosa and lymph nodes.

Unstimulated CD4+ T lymphocytes challenged by exosomes from F12/Hut-78 cells released both IL-2 and TNF-α. The fact that no IL-2 production was detectable until 36–48 hours after exosome challenge (data not shown) suggested that its release was consequence of the cellular activation induced by an autocrine/paracrine stimulus of TNF-α. The prompt release of TNF-α appears to be the key event leading to the activation of
unstimulated CD4+ T lymphocytes challenged by exosomes from F12/Hut-78 cells.

It was reported that HIV-1 does not replicate in quiescent CD4+ T lymphocytes due to its inability to complete retrotranscription and integration steps [33,43,44]. On the other hand, TNF-α has manifold effects on HIV infection: for instance, it activates resting CD4+ T lymphocytes meanwhile stimulating TAK, i.e. a cell kinase required for the Tat trans-activation [45]. It can also re-activate latent HIV-1 through the action of its downstream effectors [46]. Our evidences that unstimulated CD4+ T lymphocytes became susceptible to HIV-1 infection when the exosome-mediated stimulus was applied before but not after HIV-1 infection are highly reminiscent of previously published data on resting CD4+ T lymphocytes stimulated with anti-CD3/CD28 antibodies before or after HIV-1 challenge [33]. In this experimental setting, a great increase of both fully retrotranscribed and integrated products was found in pre-stimulated compared to post- and non-stimulated cells. On the basis of these literature data, we hypothesize that the TNF-α released upon exosome challenge helps the viral genome to complete early replication events. Upon provirus integration, the activation of the downstream effectors of both TNF-α and IL-2 would contribute to foster HIV-1 transcription.

We found active ADAM17 in F12/Hut-78 cells and exosomes released by them, but not in uninfected cells.

Figure 8 HIV-1 replicates in unstimulated CD4+ T lymphocytes treated with exosomes from NefF12-expressing cells. A. Detection of Nef in cells expressing either NefF12 or NefG2A, and in exosomes purified from the respective supernatants. Shown is the anti-Nef western blot analysis of both cells and exosomes from either 293 T cells transiently transfected with vectors expressing NefF12 or NefG2A, or mock-transfected cells (Ctrl). Signals from cellular Nef were normalized with β-actin detection, and anti-ICAM-1 analysis served to normalize exosome signals. On the left of Nef-specific panels, arrows indicate the specific signals. On the right, molecular markers are given in kDa. Results are representative of two independent experiments. B. HIV-1 replication in unstimulated CD4+ T lymphocytes treated with exosomes from cells expressing either NefF12 or NefG2A. 10⁵ unstimulated CD4+ T lymphocytes were challenged with 60 μU of exosomes from either F12/Hut-78 cells, 293 T cells transfected with NefF12- or NefG2A-expressing vectors, or mock-transfected cells (Ctrl), and then infected with 50 ng of HIV-1. As control, unstimulated cells were challenged with HIV-1 alone. Three days later, the cells were analyzed by FACS for HIV-1 expression. The fold increases of the percentages of HIV-1 CAp24-positive cells compared to cultures treated with HIV-1 alone are reported. Shown are the mean of fold increases + SD as calculated from three independent experiments with duplicates. *p < 0.05.
Despite the cells accumulate much higher amounts of inactive ADAM17 compared to the active form, only the latter seemed to be uploaded in exosomes. The mechanism of selective upload of active ADAM17 deserves further investigations.

We cannot formally exclude that NefF12 delivered in target cells by exosomes could contribute to the cell activation/HIV-1 replication in unstimulated lymphocytes. However, the fact that NefF12 allele lacks many Nef signaling functions [13,14] runs against this hypothesis. Most important, the domain critical for ADAM17 activation and uploading into exosomes (i.e., the N-terminal 11–40 region) [35,38] is conversely well conserved in NefF12 [12].

Our results allow to propose a model where cells expressing defective HIV-1 genomes release ADAM17-loaded exosomes which, when ingested by bystander quiescent CD4+ T lymphocytes, can induce the cleavage of pre-stored pro-TNF-α. The mature cytokine may act in an autocrine/paracrine way by activating intracellular signals supporting the progression of the life cycle of infectious HIV-1. Thus, our results suggest that defective HIV-1 genomes which are transcriptionally active could play a relevant role in
supporting the spread of replication-competent HIV. Furthermore, they strengthen previous evidences about the key role of TNF-α in HIV pathogenesis [47]. This mechanism would be of particular importance in HAART-treated patients where defective HIV-1 genomes accumulate in the presence of the block of the replication of infectious virus. In this scenario, the activation of resting CD4+ T lymphocytes induced by ADAM17-uploaded exosomes may foster the spread of replication-competent latent HIV, which was estimated to represent 11.7% of genomes [5], as well as the emergence of drug-resistant HIV quasispecies and, when the therapy is interrupted, infectious HIV. In addition, a sustained production of exosomes inducing the release of active TNF-α could contribute to the overall immune activation still present in successfully HAART-treated patients.

Conclusions
Exosomes released by cells expressing a defective HIV-1 genome activate bystander, resting CD4+ T lymphocytes and render them susceptible to HIV-1 infection. Exosome-associated ADAM17 plays a key role in both cell activation and HIV-1 replication observed in CD4+ T lymphocytes, and TNF-α is its downstream effector. Overall, our data reveal a so far neglected effect of defective but transcriptionally active HIV-1 genomes on bystander lymphocytes which could play a relevant role in the spread of HIV-1 in infected persons.

Methods
Cell cultures and isolation
Hut-78, D10/Hut-78, F12/Hut-78 [11], and Rev-CEM cells [34] were grown in RPMI medium plus 10% heat-inactivated fetal calf serum (FCS). Human embryonic kidney 293 T cells were grown in Dulbecco’s modified Eagle’s medium plus 10% heat-inactivated fetal calf serum (FCS). CD4+ T lymphocytes were isolated from PBMCs of healthy donors by negative selection using an immunomagnetic-based kit (Miltenyi), and cultivated in RPMI medium plus 10% FCS. The cell cultures were checked for their purity through FACS analysis for CD4, CD8, and CD14 markers. Cell preparations having more than 3% of CD8+ cells and/or 1% of CD14+ cells were discarded. For activation, 2 μg/mL of phytohemagglutinin (PHA) were added to CD4+ T lymphocyte cultures. TAPI-2 was purchased from Santa Cruz Biotechnology. Recombinant human TNF-α was from R&D Systems. AZT was obtained from NIH AIDS Research and Reference Reagent Program. For anti-TNF-α neutralization experiments, 1 μg of either anti-TNF-α neutralizing antibodies (polyclonal rabbit antibodies, Fitzgerald Industries) or normal rabbit IgGs was added to CD4+ T lymphocyte immediately after exosome and HIV-1 challenges. The same amounts of antibodies were then re-added after 24 hours of culture. For TNFR-blocking experiments, anti-TNFRI clone #16085 and anti-TNFRII clone #22210 neutralizing monoclonal antibodies (both from R&D Systems) were used. CD4+ T lymphocytes were incubated for 1 hour at 4°C with 1 μg of the antibodies either alone or in combination or, as control, with the same amount of isotype control IgGs, and then challenged with exosomes and HIV-1. Antibodies were then re-added 24 hours later.

Molecular clones, transfections, and HIV-1 infections
Preparations of T-tropic HIV-1 were obtained from the supernatants of 293 T cells 48 hours after transfection with the pNL4-3 HIV-1 molecular clone. The VSV-G pseudotyped NL4-3/NefF12 HIV-1 strain [39] was obtained by co-transfecting the HIV-1 molecular clone with a pcDNA3.1 (Invitrogen)-based vector expressing the VSV-G in a 5:1 molar ratio. Both NefF12 and NefG2A alleles were cloned in the pcDNA3.1 vector after PCR amplification from the HIV-1 molecular clones expressing the respective Nef mutant [12,48]. Transfections were performed using Lipofectamine 2000 (Invitrogen). Supernatants were clarified and concentrated by ultracentrifugation on a 20% sucrose cushion as previously described [49]. This method ensured that exosomes from transfected cells were excluded from the vesicle pellet [50]. Virus preparations were titrated in terms of HIV-1 CAp24 content using quantitative enzyme-linked immunosorbent assay (ELISA, Innogenetic). Infections with HIV-1 were carried out by spinoculation at 400 × g for 30 min at room temperature (r.t.) using 500 ng CAp24 equivalent of HIV-1 for 10⁶ cells.

Trans-well co-cultures
Trans-well co-cultures were carried out in 12-well plates using Cell Culture Insert Falcon Membrane (25 mm diameter, 0.4 μm pore size, Becton Dickinson). They were set up by putting 10⁶ F12/Hut-78 or parental Hut-78 cells in the upper chamber, while 2 × 10⁶ unstimulated CD4+ T lymphocytes were seeded in the bottom chamber. The co-cultures were then run overnight in the presence or not of 1 μM of the inhibitors of exosome release GW4869 (Sigma) and Spiroepoxide (Santa Cruz). Then, CD4+ T lymphocytes were recovered and infected with HIV-1, washed, and left in culture 3 days in the presence or not of 10 μM AZT. Afterwards, lymphocytes were analyzed by FACS for the HIV-1 expression through the detection of intracytoplasmic HIV-1 CAp24.

Nanovesicle purification and challenge
Cell culture supernatants containing exosome-depleted FCS were processed following already described methods for exosome purification. In detail, supernatants were centrifuged at 500 × g for 10 min and filtered with 0.22 μM pore size. Then, the supernatants underwent differential
centrifugations consisting in a first ultracentrifugation at 10,000 × g for 30 min. Supernatants were then harvested and ultracentrifuged at 70,000 × g for 1 h. The pelleted vesicles were resuspended in 1 × PBS, and ultracentrifuged again at 70,000 × g for 1 h. Afterwards, the pellet was resuspended in 200 to 400 μL of 1 × PBS and, in some cases, subjected to discontinuous iodixanol (Axis-Shield) gradient. It was performed essentially as described [51]. Briefly, concentrated vesicles were ultracentrifuged at 200,000 × g for 1.5 hours at 4°C in an SW41 Ti rotor (Beckman) through a 6 to 18% iodixanol density gradient formed by layering iodixanol in 1.2% increments. Then, 0.7 ml fractions were collected starting from the top. In some instances, half of each fraction was diluted with 2 volumes of 0.9% sodium chloride and ultracentrifuged for 30 min at 95,000 rpm in a TL-100 tabletop ultracentrifuge. Finally, the beads were washed, resuspended in 200 to 400 μL of Tris–HCl pH 7.4 10 mM, NaCl 100 mM, EDTA 1 mM, 0.1% Triton X-100.

**AchE activity assay**

The vesicle-associated AchE activity was evaluated through the Amplex Red kit (Molecular Probes) following the manufacturer’s recommendations. The AchE activity was measured as mU/mL, where 1 mU is defined as the amount of enzyme which hydrolyzes 1 pmole of acetylcholine to choline and acetate per minute at pH 8.0 at 37°C.

**FACS analysis of cells and nanovesicles**

For the detection of intracytoplasmic HIV-1 CAp24, cells were treated with trypsin for 15 min at 37°C. Then, they were labeled using the KC57-RD anti-CAp24 monoclonal antibody (Coulter) upon permeabilization with Cytofix/Cytoperm solutions (BD Pharmingen) as previously described [52]. Double staining of nanovesicles was performed by incubating them with 5 μl of surfactant-free white aldehyde/sulfate latex beads overnight at r.t. on a rotating plate. The binding of exosomes with the beads was necessary for both antibody labeling and FACS analysis. Afterwards, nanovesicle-bead complexes were washed and incubated at 37°C for 2 hours with FITC-conjugated CTX-B. Then, the samples were washed and incubated with PE-conjugated anti-CD63 monoclonal antibody (BD Pharmingen) 1 h at 37°C. Finally, the beads were washed, resuspended in 1 × PBS-2% v/v formaldehyde, and FACS analyzed.

**Western blot assay**

Western blot analysis on cell lysates was performed by washing cells twice with 1 × PBS (pH 7.4) and lysing them for 20 min on ice with lysis buffer (20 mM HEPES pH 7.9, 50 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.5% nonionic detergent IGEPAL CA-630, 0.5 mM dithiothreitol, 20 mM sodium molybdate, 10 mM sodium orthovanadate, 100 mM sodium fluoride, 10 μg/mL leupeptin, 0.5 mM phenylmethylsulfonyl fluoride). Whole cell lysates were centrifuged at 6,000 × g for 10 min at 4°C. The protein concentration of cell extracts was determined by the Lowry protein quantitation assay. Aliquots of cell extracts containing 30 to 50 μg of total proteins were resolved by 8-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred by electrophoretic transfer onto a 0.45 μm pore size nitrocellulose membrane (Amersham) overnight using a Bio-Rad Trans-Blot. For western blot analysis of exosomes, they were lysed and analyzed as described for cell lysates. For immunoassays, membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Triton X-100 for 1 h at room temperature, then incubated overnight at 4°C with specific antibodies diluted in PBS containing 0.1% Triton X-100. The antibodies used in immunoblots were: polyclonal rabbit anti-HIV-1 Gag Cap24 #4250 (NIH AIDS Research and Reference Program), sheep polyclonal anti-Nef ARP444 (a generous gift from Dr. Mark Harris), rabbit polyclonal anti-ADAM17 from Cell Signaling, monoclonal anti-ICAM-1 15.2 from Santa Cruz Biotech., monoclonal anti-CD63 from R&D Systems, and monoclonal anti-β-actin AC-74 (Sigma). Immune complexes were detected with horse-radish peroxidase conjugated goat anti-rabbit or goat anti-mouse antibodies (GE Healthcare) and enhanced chemiluminescence reaction (Euroclone).

**IL-2 and TNF-α detection**

The release from CD4+ T lymphocytes of IL-2 and TNF-α was detected by ELISpot and ELISA, respectively. For IL-2, cells were treated with exosomes and then cultivated for 48–72 hours in ELISpot microwells (Millipore) previously coated with a monoclonal antibody against human IL-2 (Mabtech). Afterwards, the cells were removed, and a biotinylated antibody against human IL-2 was added, followed by the addition of a streptavidin-alkaline phosphatase. The plate was finally developed using BCIP/NBT substrate (Sigma). Spot-forming cells were analyzed and counted using an ELISpot reader (Amplicond Bioline A-EL-VIS GmbH). The measurement of TNF-α was performed through ELISA kits from Immunological Sciences following the manufacturer’s recommendations.

**Statistical analysis**

When appropriate, data are presented as mean ± standard deviation (SD). In some instances, the paired Student’s t-Test was used and confirmed using the non-parametric Wilcoxon rank sum test. P < 0.05 was considered significant.

**Competing interests**

The authors declare that they have no competing interests.
Authors’ contributions
CA carried out the most part of cell and infection experiments. CC carried out ELISA, ELSI-SPOT, FACS, and western blot analyses. SCC performed and purified exosomes, and carried out Aché-based quantification assays of exosomes. FM performed blocking experiments with anti-TNFα antibodies and virus titrations using Rev-CM cells. MF supervised the research and wrote the manuscript. All authors read and approved the final manuscript.

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References
1. Cao Y, Qin L, Zhang L, Safrit J, Ho DD: Virologic and immunologic characterization of long-term survivors of human immunodeficiency virus type 1 infection. N Engl J Med 1995, 332:201–208.
2. Daar ES, Chernyavsky T, Zhao JQ, Chen IS, Zack JA: Sequential determination of viral load and phenotype in human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses 1995, 11:85–9.
3. Sanchez G, Xu X, Chermann JC, Hirsch I: Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected individuals. J Virol 1997, 71:2233–2240.
4. Fourni S, Lambert-Niclot S, Soule M, Cilet J, Valantin MA, Curr J, Curr J, Mury B, Carcelain G, Katlama C, Calvez V, Marcellin AG: HIV-1 genome is often defective in PBMCs and rectal tissues after long-term HAART as a result of APOBEC3 editing and correlates with the size of reservoirs. J Antimicrob Chemother 2012, 67:2323–2336.
5. Ho Y-C, Shan L, Hommane NW, Wang T, Laskey SB, Rosenblum DI, Lai J, Blankson JN, Siliciano RD, Siliciano RF: Rapamycin-dependent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. Cell 2013, 155:540–551.
6. Saurya S, Lichtenstein Z, Karpas A: Characterization of pol, vif, vpr, and vpu genes of HIV type 1 in AIDS patients with high viral load and stable CD4+ T cells on combination therapy. AIDS Res Hum Retroviruses 2002, 18:1151–1155.
7. Saurya S, Lichtenstein Z, Karpas A: Characterization of nef gene of HIV type 1 in highly active antiretroviral therapy treated AIDS patients with discordance between viral load and CD4+ T cell counts. AIDS Res Hum Retroviruses 2002, 18:983–987.
8. Saurya S, Lichtenstein Z, Karpas A: Characterization of gag gene of plasma HIV type 1 in combination therapy-treated AIDS patients with high viral load and stable CD4+ T cell counts. AIDS Res Hum Retroviruses 2003, 19:73–76.
9. Saurya S, Lichtenstein Z, Karpas A: Defective rev response element (IRE) and rev gene in HAART treated AIDS patients with discordance between viral load and CD4+ T cell counts. J Clin Virol 2005, 33:324–327.
10. Saurya S, Lichtenstein Z, Karpas A: Deletions in env gene of HIV-1 in AIDS patients treated with highly active antiretroviral therapy (HAART). J Med Virol 2003, 71:167–172.
11. Federico M, Tinari A, Bartoli S, Credi C, Marzola FS, Moglia M, Verani P, Rosi GB: Biologic and molecular characterization of producer and nonproducer clones from HUT-78 cells infected with a patient HIV isolate. AIDS Res Hum Retroviruses 1989, 5:385–396.
12. D’Alopo P, Olivetta E, Bona R, Nappi F, Pedacchia D, Pugliese K, Ferrari G, Verani P, Federico M: Gag, vif and nef genes contribute to the homologous viral interference induced by a nonproducer human immunodeficiency virus type 1 (HIV-1) variant: identification of novel HIV-1-inhibiting viral protein mutants. J Virol 1998, 72:4308–4319.
13. D’Alopo P, Santarcangelo AC, Arol S, Baur A, Federico M: Genetic and functional analysis of the human immunodeficiency virus (HIV) type 1-inhibiting F12-HIV nef allele. J Gen Virol 2001, 82:2735–2745.
14. Fackler DT, D’Alopo P, Baur AS, Federico M, Peterlin BM: Nef from human immunodeficiency virus type 1(12) inhibits viral production and infectivity. J Virol 2001, 75:6601–6608.
15. Vallant G, Lupr R, Federico M, Mavilio F, Bovieneta C: T lymphocytes transduced with a lentiviral vector expressing F12-αf are protected from HIV-1 infection in an APOBEC3G-independent manner. Mol Ther 2005, 12:976–970.
16. Sygry B, Stazio T, Pastori M, Pal Z, Mijas P, Aradi B, Laszlo V, Pallinger E, Fiao E, Kittel A, Nagy G, Falus A, Buzas B: Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci 2011, 68:2667–2688.
17. Booth AM, Fang Y, Fallon JK, Yang JM, Hillhreth JEK, Gould SJ: Exosomes and HIV Gag bud from endosome-like domains of the T cell plasma membrane. J Cell Bio 2006, 172:293–335.
18. Mathivanan S, Liu H, Simpson RJ: Exosomes: extracellular organelles important in intercellular communication. Proteomics 2010, 13:1907–1920.
19. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT, Carter BS, Kirchevsky AM, Breakefield XO: Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol 2008, 10:1470–1476.
20. Fang Y, Wu N, Gan X, Yan WH, Morrell JC, Gould SJ: Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. PLoS Biol 2007, 5:1267–1283.
21. Lenassi M, Cagnone G, Liu MF, Vaupotic T, Bartholomeeusen K, Cheng YF, Krogan NJ, Plemenitas A, Peterlin BM: HIV Nef is secreted in exosomes and triggers apoptosis in bystander CD4+ T cells. Traffic 2010, 11:110–122.
22. Munetani C, Cavallone LE, Kratzel K, Tinari A, De Milito A, Fais S, D’Alopo P, Federico M, Vullo V, Formina A, Mesri EA, Superti F, Baur AS: Massive secretion by T cells is caused by HIV Nef in infected cells and by Nef transfer to bystander cells. Cell Host Microbe 2009, 6:218–230.
23. Chairoungdou A, Smith DL, Pochard P, Hull M: Caplan MI: Exosome release of beta-catenin: a novel mechanism that antagonizes Wnt signaling. J Cell Biol 2010, 190:1079–1091.
24. Kogure T, Liu WL, Yan RK, Bricaci C, Patel T: Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth. Hepatology 2011, 54:1237–1248.
25. Kosaka N, Iuchi H, Yoshikawa Y, Takeshita F, Matsuji Y, Ochya T: Secretory mechanisms and intercellular transfer of microRNAs in living cells. J Biol Chem 2010, 285:17442–17452.
26. Kosaka N, Iuchi H, Yoshikawa Y, Hagiwara K, Takeshita F, Ochya T: Competitive interactions of cancer cells and normal cells via secretory microRNAs. J Biol Chem 2012, 287:1407–1425.
27. Trajkovic K, Hsu C, Chiantia S, Rajendran L, Wenzel D, Wieland F, Schwille P, Bruegger B, Simons M: Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 2008, 319:1244–1247.
28. Yuwana K, Sun H, Mutsukaze S, Igarashi Y: Sphingolipid-modulated exosome secretion promotes clearance of amyloid-beta by microglia. J Biol Chem 2012, 287:10907–10919.
Schuler G, Federico M, Baur AS: HIV Nef, paullin, and Pak1/2 regulate activation and secretion of TACE/ADAM10 proteases. Mol Cell 2013, 49:668–679.

36. Gooz M: ADAM-17: the enzyme that does it all. Crit Rev Biochem Mol Biol 2010, 45:146–169.

37. Moss ML, Rasmussen FH: Fluorescent substrates for the proteinases ADAM17, ADAM10, ADAM8, and ADAM12 useful for high-throughput inhibitor screening. Anal Biochem 2007, 366:144–148.

38. Wolf D, Witte V, Clark P, Blume K, Lichtenheld MG, Baur AS: HIV Nef enhances Tat-mediated viral transcription through a hnRNP-K-nucleated signaling complex. Cell Host Microbe 2008, 4:398–408.

39. Olivetta E, Pugliese K, Bona R, D’Albora P, Ferrantelli F, Santarcangelo AC, Matta G, Verani P, Federico M: Cis expression of the F12 human immunodeficiency virus (HIV) nef allele transforms the highly productive NL4-3 HIV type 1 to a replication-defective strain: involvement of both Env gp41 and CD4 intracytoplasmic tails. J Virol 2000, 74:483–492.

40. Janini M, Rogers M, Brix DR, McCutchan FE: Human immunodeficiency virus type 1 DNA sequences genetically damaged by hypermutation are often abundant in patient peripheral blood mononuclear cells and may be generated during near-simultaneous infection and activation of CD4 (+) T cells. J Virol 2001, 75:7973–7986.

41. Fourati S, Lambert-Niclot S, Soulie C, Wirden M, Malet I, Valantin MA, Tubiana R, Simon A, Katlama C, Carcelain G, Calvez V, Marcelin AG: Differential impact of APOBEC3-driven mutagenesis on HIV evolution in diverse anatomical compartments. AIDS 2014, 28:487–491.

42. Carlini F, Nicolini A, d’Albora P, Federico M, Verani P: The non-producer phenotype of the human immunodeficiency virus type 1 provirus F12/HIV-1 is the result of multiple genetic variations. J Gen Virol 1996, 77:2009–2013.

43. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip AM, Chen ISY: HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 1990, 62:213–222.

44. Zack JA, Haislip AM, Krogstad P, Chen ISY: Incompletely reverse-transcribed human immunodeficiency virus type 1 genomes in quiescent cells can function as intermediates in the retroviral life cycle. J Virol 1992, 66:1717–1725.

45. Ghose R, Liou LY, Herrmann CH, Rice AP: Induction of TAK (cyclin T1/P-TEFb) in purified resting CD4 (+) T lymphocytes by combination of cytokines. J Virol 2001, 75:11336–11343.

46. McNamara LA, Ganesh JA, Collins KL: Latent HIV-1 infection occurs in multiple subsets of hematopoietic progenitor cells and is reversed by NF-kappa B activation. J Virol 2012, 86:9337–9350.

47. Herber G, Khan KA: Is HIV infection a TNF receptor signaling-driven disease? Trends Immunol 2008, 29:61–67.

48. Chowles MY, Spina CA, Kwoh TJ, Fitch NJS, Richman DD, Guatelli JC: Optimal infectivity in-vitro of human immunodeficiency virus type 1 requires an intact nef gene. J Virol 1994, 68:2906–2914.

49. Federico M, Peracino Z, Olivetta E, Fiorucci G, Muratori C, Micheli A, Romeo G, Affrato E: HIV-1 Nef activates STAT1 in human monocytes/macrophages through the release of soluble factors. Blood 2001, 98:2752–2761.

50. Park IW, He JX: HIV-1 is budded from CD4+ T lymphocytes independently of exosomes. Virology 2010, 72:34.

51. Dettenhofer M, Yu XF: Highly purified human immunodeficiency virus type 1 reveals a virtual absence of vif in virions. J Virol 1999, 73:1460–1467.

52. Muratori C, Setigui A, Ruggiero E, Falchi M, Bacigalupo I, Palladino C, Toschi E, Federico M: Macrophages transmit human immunodeficiency virus type 1 products to CD4-negative cells: Involvement of matrix metalloproteinase 9. J Virol 2007, 81:9078–9087.

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