Ligand-Engaged Urokinase-Type Plasminogen Activator Receptor (uPAR) and Activation of the CD11b/CD18 (Mac1) Integrin Inhibit Late Events of HIV Expression in Monocytic Cells

Short title: uPA/uPAR and MAC-1 inhibit late HIV expression

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Abstract

Urokinase-type plasminogen activator (uPA) signaling via its receptor uPAR inhibits late events in HIV-1 replication in acutely infected primary monocyte-derived macrophages (MDM) and promonocytic U937 cells. Here we show that U937-derived chronically infected U1 cells stimulated with phorbol myristate acetate (PMA) express integrins, uPA and soluble uPAR at levels similar to those of MDM. UPA inhibited HIV expression in U1 cells incubated with either PMA or tumor necrosis factor-α (TNF-α), but not with other HIV-inductive cytokines or lipopolysaccharide. Of interest, only PMA and TNF-α, but not other HIV-inductive stimuli, induced surface expression of the αM chain CD11b in U1 cells constitutively expressing CD18, the β2 chain of the Mac-1 integrin. Like uPA, fibrinogen, a Mac-1 ligand, and M25, a peptide homologous to a portion of the β-propeller region of CD11b preventing its association with uPAR, inhibited HIV virion release in PMA-stimulated U1 cells. Both uPAR siRNA and soluble anti-β1/-β2 mAbs abolished the anti-HIV effects of uPA, whereas CD11b siRNA reversed the anti-HIV effect of M25, but not that induced by uPA. Thus, either uPA/uPAR interaction, Mac-1 activation or prevention of its association with uPAR triggers a signaling pathway leading to the inefficient release of HIV from monocytic cells.
Introduction

Urokinase-type plasminogen activator (uPA), a serine protease that activates plasminogen to plasmin\(^1\), is synthesized as an inactive precursor (pro-uPA) that undergoes a rapid proteolytic activation. uPA binds to a specific glycosyl-phosphatidyl-inositol (GPI)-anchored receptor, uPAR, localized at the cell surface.\(^2\) Both uPA and uPAR are expressed by inflammatory cells including neutrophils, monocytes, macrophages, and activated T lymphocytes\(^2\) in which they play important roles in cell activation, adhesion and migration.\(^3,4\) In addition to localize the enzymatic activity of uPA on the leading edge of migrating cells, uPAR mediates signaling by uPA.\(^5\) Binding of uPA to uPAR induce migration, adhesion and proliferation of different cell types independently of the catalytic activity of uPA.\(^6,7\) As a GPI-receptor lacking an intracellular domain, uPAR requires the interaction with transduction-competent receptors, such as the G-protein coupled receptor formyl peptide receptor-like-1 (FPRL1)\(^5\), the gp130 signal transducing chain of the IL-6 receptor family\(^8\), or integrins such as \(\alpha_5\beta_1\) in epidermal cancer cells and CD11b-CD18 (Mac-1) in monocytes-macrophages.\(^5,6,9,10\)

High serum and cerebrospinal spinal fluid levels of soluble uPAR (suPAR) were correlated to the severity of HIV-1 disease independently of CD4\(^+\) T cell counts or viremia levels.\(^11-14\) Furthermore, uPA expression was observed in the brain of HIV\(^+\) individuals staining negative for both HIV-1 p24 Gag antigen and uPAR\(^15\), suggesting a potential role of uPA as negative regulator of HIV-1 expression. In vitro uPA inhibits HIV-1 replication in lymphoid histocultures, primary monocyte-derived macrophages (MDM), promonocytic U937 cells acutely infected with HIV and chronically infected promonocytic U1 cells stimulated with the differentiating agent phorbol 12, myristate, 13-acetate (PMA) or tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)).\(^16,17\) In particular, uPA was shown to promote the sequestration of HIV particles in cytoplasmic vacuoles, likely belonging to multivescicular bodies (MVB)\(^18-20\), an effect that was fully accounted by its signaling-competent amino-terminal fragment (ATF).\(^5\) More recently, we showed that vitronectin (VN)-dependent cell adhesion is crucial for uPA-mediated inhibition of virus replication in MDM and in PMA-
stimulated U1 cells. A better definition of this signaling pathways and of its determinants may be relevant for understanding the dynamics of tissue seeding by infected leukocytes that may affect their ability or efficiency to establish HIV reservoirs in sanctuary sites and cause organ/tissue specific pathology, such as HIV-associated dementia, interstitial lung disease, nephropathy, enteropathy and wasting syndrome.

In the present study we investigated which among the known uPAR-associated signaling-competent receptors mediate its inhibitory signal on HIV-1 expression in monocytic cells. Our findings indicate that such inhibitory effect is mediated by β1 and/or β2 integrins, but does not require the expression of CD11b. In addition, we also demonstrate that stimulation of the CD11b/CD18 (Mac-1) integrin by fibrinogen (FNG) or prevention of the association between CD11b and uPAR fully mimicked uPA/uPAR dependent inhibition of late events in virus expression reinforcing the hypothesis of a common pathway controlling the late phase of HIV assembly and release from infected monocytic cells.
Materials and Methods

Reagents. LPS-free (<2x10^5 EU/IU, corresponding to <10^10 EU/mg) human pro-uPA (MW: 52 kDa) was provided by Dr. Jack Henkin (Abbot Laboratories, Illinois, USA), whereas the ATF peptide was purchased from American Diagnostica (Stamford, CT). Pro-uPA and ATF were used at 10 nM. Fibrinogen (FNG), phosphatidylinositol-specific phospholipase C (PIPLC) from Bacillus (B.) cereus, crystal violet, goat anti-mouse FITC-antibody (Ab), rabbit anti-goat FITC Ab and phorbol-12, myristate-13, acetate (PMA) were purchased from Sigma Chemical Corp. (St. Louis, MS). Interleukin-6 (IL-6) and interferon-γ (IFN-γ) were purchased from R&D Systems (Minneapolis, MN). PMA, IL-6 and IFN-γ were resuspended as recommended by the manufacturers and used at final concentrations of 6, 10 and 50 ng/ml, respectively, based on previous studies. PIPLC was resuspended at 20 U/ml in culture medium and used at the final concentration of 10 U/ml. M25 (PRYQHIGLVAMFRQNTG) and its scrambled peptide (scM25, HQIPGAYRGVNQRFTML) were purchased from PRIMM (Milan, Italy) and dissolved at 20 mM in DMSO and used at the indicated concentrations. The list of Ab used and their sources is detailed in the “Supplementary Material”. All experiments were performed in 96-well microtiter plates (Falcon, BD Biosciences, Bedford, MA) unless otherwise specified.

Quantification of uPA and suPAR secretion. Both molecules were measured in culture supernatants of U1 cells and MDM. uPA concentrations were measured by a commercial kit (IMUBIND uPA ELISA kit no. 894, American Diagnostica, Greenwich, CT) with a lower detection limit of 10 pg/ml. suPAR and the uPA-suPAR complex were determined using an in-house sandwich ELISA. In this assay, suPAR is captured using a mAb as capture Ab and the associated uPA detected using a polyclonal Ab directed against uPA. This assay does not detect suPAR and uPA alone but displays a linear dose–response to complexes between suPAR and uPA, as previously described.14,15

Chronically HIV infected U1 cell line. The promonocytic U1 cell line contains two copies of integrated X4 HIV-1_LAI/IIIB provirus per cell, and it is characterized by a constitutive state of
relative viral latency.\textsuperscript{24} High levels of virus expression are rapidly induced by stimulation of U1 cells with either PMA or cytokines.\textsuperscript{25,26} U1 cells were stimulated at the concentration of 2x10^5 cells/ml in RPMI 1640 containing 10% of heat-inactivated foetal bovine serum (FBS), and were firstly incubated with either uPA or peptides 30 min before the addition of PMA or HIV-inductive stimuli.

**U1 cell adhesion assay.** U1 cells were resuspended in culture medium containing 10% FBS and were left either unstimulated or were stimulated and seeded in triplicates into 96 well tissue culture plate. At the indicated time points, cells were washed with warm culture medium, fixed for 15 min with 3% paraphormaldehyde in PBS, stained for 10 min with 0.5% crystal violet (in 20% methanol/80% water), washed 3 times and lysed with 100 µl of 1% SDS in water. The results were obtained by measuring the absorbance at 570 nm.

**Acute CCR5-dependent (R5) infection of primary human monocyte-derived macrophages (MDM).** Circulating monocytes were isolated from Ficoll-Hypaque purified peripheral blood mononuclear cells (PBMC) of several independent healthy HIV-1 seronegative donors by isoosmotic Percoll gradient.\textsuperscript{27} Their purity was approximately 90% based on morphology and CD14 staining (data not shown). Monocytes were allowed to differentiate for 7 days in culture in D-MEM enriched with 10% FBS and 10% human AB serum before infection.\textsuperscript{17} Thus, human MDM were treated for 30 min with uPA or ATF and then infected with the R5 laboratory-adapted HIV-1\textsubscript{BaL} strain at the multiplicity of infection (m.o.i.) of 0.1.\textsuperscript{17} MDM cultures were carried on for 3-4 weeks after infection and supernatants were collected every 3-4 days and stored at -20 °C for determination of their reverse transcriptase (RT) activity content. Collected culture supernatants were replenished with fresh culture medium, added of uPA or ATF in treated cultures.

**Acute X4 HIV-1 infection of the human promonocytic U937 cell line.** U937 cells were resuspended in RPMI 1640 plus 10% FBS and infected with the laboratory-adapted X4 HIV-1\textsubscript{LAI/HXB} strain at the m.o.i. of 1 after 30 min pre-incubation with FNG. Cell cultures were carried on for 3-4 weeks after infection and supernatants were collected every 2-3 days and stored at -20 °C for
determination of their RT activity content. Collected culture supernatants were replenished with fresh culture medium, added of FNG in treated cultures.

**HIV-1 quantification by RT activity assay.** HIV-1 expression was monitored by determination of Mg\(^{++}\)-dependent RT activity in culture supernatants, reflecting the production of new progeny virions.\(^{28}\) Release of cell-associated virions by five consecutive cycles of cell freezing and thawing was performed as previously described.\(^{16}\)

**Cytofluorimetric analysis.** U1 cells were washed twice in 2% FBS/PBS and stained by using the indicated Ab at 1 µg/10\(^5\) cells unless otherwise specified. After 30 min of incubation on funding ice U1 cells were centrifuged twice in 2% FBS/PBS at 1,500 rpm for 5 min at +4 °C; the cells were then incubated with 1 µg of the secondary anti-mouse-FITC or anti-rabbit-FITC Ab. The cells were spun again after 30 min of incubation on funding ice and resuspended in 2% formaldehyde/ PBS. Fifteen-thousand cells were acquired using a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometry apparatus and analyzed by the CellQuest software (Becton Dickinson).

**Cell proliferation.** Cell proliferation was assessed by the uptake of [\(^3\)H]-thymidine. One µCi of [\(^3\)H]-thymidine was added to 4×10\(^4\) cells in 200 µl medium and incubated 16 h at 37 °C, 5% CO\(_2\). Cells were harvested and the β emission was counted in a 1450 β-counter (1450 Microbeta Plus, Wallac, Sweden).

**Small interference RNA (siRNA) transient transfection.** Three pairs of validated stealth siRNA (25 nt) for each target antigen were purchased from Invitrogen LTD (Paisley, GB) and resuspended at the final concentration of 20 µM in DEPC-treated water, as detailed in the “Supplementary Material”. Typically, 2×10\(^6\) cells were transfected with 1 µM siRNA, then resuspended at 1×10\(^6\) cells/ml and finally diluted 4 h later at 2×10\(^5\) cells/ml before incubation with uPA and stimuli. Cells transiently transfected with irrelevant stealth siRNA duplex containing 45%-55% GC (Mock siRNA, Invitrogen) was used as negative control.
**Statistical analysis.** Results are reported as mean values + SD, and the analysis of variance ($p$ value) performed by a two-tailed student $t$ test for paired observations (unless otherwise specified). The observed differences were considered significant if the $p$ value obtained was below 0.05.
Results

The anti-HIV activity of uPA depends on its ATF-receptor binding domain and by the cell surface expression of uPAR. Exogenously added pro-uPA is immediately cleaved in vitro into its active form uPA by the plasmin present in the cell culture serum-enriched medium. The anti-HIV effect of uPA in stimulated U1 cells was already shown to be dependent upon its receptor-interacting component ATF and not by its catalytic activity. This finding was here confirmed in that only uPA peptides maintaining an ATF growth factor-like domain, including the so-called “Omega loop”, but not the enzymatically competent low molecular weight fragment, inhibited HIV expression in chronically infected U1 cells stimulated with either PMA (Figure 1A) or TNF-α (data not shown).

In order to demonstrate the essential role of the GPI-anchored protein uPAR in uPA-mediated HIV inhibitory signals, U1 cells were incubated with PIPLC from B. cereus that specifically removes GPI anchors. PIPLC indeed reduced the expression of uPAR on U1 cell surface 2-24 h after incubation, as measured by cytofluorimetric analysis (Figure 1B). Concomitantly, PIPLC promoted the release of soluble uPAR (suPAR) in the culture supernatant, from 33 (Table 1) to 220 pM (not shown), while it did not modulate the cell surface expression of non GPI-anchored receptors including integrins, as measured by FACS analysis (data not shown). PIPLC did not interfere with HIV expression of either unstimulated or PMA-stimulated U1 cells (Figure 1C) suggesting that neither uPAR per se nor endogenous uPA played a significant role in HIV-1 expression from U1 cells. In contrast, PIPLC abrogated the anti-HIV activity of exogenous uPA (Figure 1C) without any cytotoxic or cytostatic effects, as tested by [³H]-Thymidine uptake (data not shown).

We next determined the concentrations of uPA, suPAR and of their complex in culture supernatants of both unstimulated and stimulated U1 cells (Table 1). Most of the uPA released from stimulated U1 cells was indeed complexed with suPAR (Table 1), as previously reported in vivo.

Addition of suPAR per se did not alter HIV expression from either unstimulated or PMA

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stimulated U1 cells (Figure 1D). Furthermore, suPAR did not interfere with HIV-1 replication in acutely infected U937 cells (data not shown, n=3) reinforcing the concept that its role in virus production is strictly related to mediate uPA-dependent cell signaling. In contrast, a 20:1 molar excess of suPAR vs. uPA abrogated the anti-HIV activity of uPA (Figure 1D), whereas, viceversa, a 5:1 excess of uPA vs. suPAR overcame the inhibitory effect of suPAR (data not shown).

**ATF inhibits HIV replication in primary human monocyte-derived macrophages (MDM).**

We have previously reported that uPA inhibits virus replication in primary MDM\(^\text{17}\) and we here report that exogenously added ATF exerts an anti-HIV activity in *in vitro* infected MDM established from several donors (Figure 2A).

Both uninfected and infected MDM released uPA, uPA and uPA-suPAR complex about 3-fold more uPA and 6-fold more suPAR than U1 cells on a per cell basis (Table 1). However, as observed in U1 cells, most soluble uPA was complexed by suPAR, with a suPAR/uPA ratio always >20 (Table 1). There was not a significant correlation between the levels of released uPA and suPAR in infected MDM (Figure 2B), or between the levels of released uPA or suPAR and peak HIV replication in MDM, although a positive trend between higher levels of (complexed) uPA and virus production was noted (Figure 2C).

**PMA and TNF-\(\alpha\), but not other HIV-inductive stimuli, upregulate CD11b expression in U1 cells.** Unstimulated U1 cells showed constitutive cell surface expression of uPAR and of CD18, but negligible presence of CD11b (Table 2 and Figure 3A). PMA stimulation upregulated the levels of cell surface uPAR (reaching a plateau approximately 20 h after stimulation, as previously reported in both U937\(^\text{33}\) and MCF-7 cells\(^\text{34}\)) and induced the expression of CD11b, thus allowing the formation of Mac-1 (Figure 3A, top panels). Superimposable results, although inducing lower levels of CD11b expression, were observed by TNF-\(\alpha\) stimulation (Figure 3A, middle panels). In contrast, IL-6 strongly upregulated both HIV-1 and uPAR expression, but failed to induce detectable levels of CD11b (Figure 3A, lower panels). Of interest, uPA inhibited HIV expression in U1 cells stimulated with both PMA and TNF-\(\alpha\), but not with IL-6 (Figure 3B). U1 cells were also stimulated
with IFN-γ or GM-CSF plus lipopolysaccharide (LPS)\textsuperscript{35-37}, which upregulated virus expression to a comparable level, in the presence or absence of uPA.\textsuperscript{26} However, as for IL-6, they did not induce expression of CD11b (data not shown) whereas, concomitantly, uPA failed to inhibit HIV production induced by these two additional stimuli.

Among other uPA co-receptors and integrin chains expressed by U1 cells neither FPRL1, nor α\textsubscript{5}β\textsubscript{1}, α\textsubscript{6} and α\textsubscript{X} integrin chains were demonstrated to be involved in uPA-mediated inhibition of HIV expression (see “Supplementary results and Figures S1 and S2”).

The Mac-1 ligand FNG inhibits late events of HIV expression in U1 cells and in acutely infected U937 cells. We next evaluated whether known Mac-1 ligands could also affect HIV expression in either PMA or TNF-α stimulated U1 cells in the absence of uPA. U1 cells were incubated with FNG, known to bind to both Mac-1\textsuperscript{38} and the VN receptor α\textsubscript{V}β\textsubscript{3} (that, however, was not expressed by U1 cells, as shown in Table 2)\textsuperscript{17}; the cells were then stimulated by either PMA or HIV-inductive cytokines. Incubation of U1 cells with FNG in the absence of other stimuli did not activate HIV expression (data not shown); however, FNG inhibited HIV expression in U1 cells stimulated with PMA (Figure 4A), but not with IL-6 (data not shown). In analogy to uPA\textsuperscript{16}, FNG inhibition of HIV expression in PMA-stimulated U1 cells was reversed by cell disruption (Figure 4B). Furthermore, FNG inhibited the acute replication of X4 HIV-1 in U937 cells (Figure 4C). As observed with U1 cells, U937 cell disruption at time points before and after the peak of HIV replication restored the levels of RT activity to those of control cells (Figure 4D).

Preventing association of CD11b/CD18 from uPAR in the absence of receptor ligands inhibits HIV-1 expression. In order to investigate the role of Mac-1 interaction with uPAR in terms of inhibition of HIV expression, we tested the potential effect of M2, a peptide known to prevent or disrupt the integrin association to uPAR.\textsuperscript{39} Surprisingly enough, M25 alone, but not its control scrambled peptide, inhibited HIV expression in PMA-stimulated U1 cells in the absence of uPA in a concentration-dependent fashion, reaching more than 90% of inhibition at 80 µM (Figure 5A). Furthermore, M25 inhibited the residual levels of RT activity in the presence of uPA (Figure 5A);
identical results were obtained in U1 cells stimulated with TNF-α (data not shown). M25 anti-HIV mechanism resembled that induced by either uPA or FNG in that it was fully reverted by cell disruption (Figure 5B), suggesting that prevention of the association between Mac-1 and uPAR triggers an inhibitory pathway of HIV-1 particle release.

The anti-HIV effect triggered by uPA/uPAR interaction is independent of CD11b. Experiments were next carried out after transfection of U1 cells with siRNA directed to either uPAR or CD11b. As shown earlier, the constitutive levels of expression of uPAR in U1 cells were enhanced by 24 h stimulation with PMA (Figure 6A, upper left panel) while CD11b, which was not expressed by unstimulated cells, was clearly induced by PMA (Figure 6A, upper right panel). Transfection of their respective siRNA partially reduced the expression of uPAR (Figure 6A, lower left panel) while it abrogated PMA-induced CD11b expression (Figure 6A, lower right panel); these suppressive effects were stable for at least 3 days after cell stimulation (data not shown).

Mock siRNA did not alter the inhibitory effects of either uPA or M25 on HIV production, while CD11b siRNA abolished the anti-HIV activity of M25 but did not affect the inhibitory capacity of uPA (Figure 6B, left and right panels, respectively). In contrast, the reduced expression of uPAR abolished the anti-HIV activity of both uPA and M25 peptide (Figure 6B, middle panel).

Given the dependence of uPA anti-HIV effect on VN-mediated cell adhesion, we next investigated the potential interference of these siRNA on U1 cell adhesion to the plastic substrate in the presence to either uPA or M25. As expected, M25 did not affect plastic adhesion of both unstimulated (not shown) and PMA-stimulated U1 cells (Figure 6C). Transfection of CD11b siRNA did not alter the ability of uPA to induce U1 cell adhesion, whereas this effect was lost in U1 cells transfected with uPAR siRNA (Figure 6C).

Cell adhesion-dependent uPA inhibition of HIV expression involves integrin β chains. We then investigated the potential contribution of β₁ and β₂ integrin chains. Different anti-integrin blocking Ab were either coated to the plastic of tissue culture microwells, thereby inducing integrin-dependent cell adhesion. In addition, we also tested the same Ab in suspension in order to
Plates coated with Ab recognizing antigens not expressed by unstimulated U1 cells, such as CD3 and CD11b (Table 2), failed to induce cell adhesion (Figure 7, upper panel). In contrast, Ab recognizing $\beta_1$, but not $\beta_2$, integrin chains induced cell adhesion in both unstimulated and uPA-stimulated cells (Figure 7, upper panel). PMA stimulation enhanced the levels of cell adhesion in plates coated with anti-CD11a, anti-CD11b and anti-$\beta_1$, but not anti-$\beta_2$, integrin chains (Figure 7, upper panel). Consistently, uPA increased the levels of cell adhesion in PMA-stimulated cells in plates coated with Ab directed against CD11a, CD11b, $\beta_1$, but not $\beta_2$ integrin chains. As expected, when Ab were added in suspension, either no effect or reduced levels of adhesion were observed in respect to what observed in their homologous Ab-coated plates (Figure 7, upper panel).

None of the Ab tested modify the levels of HIV expression in unstimulated cells or in cells incubated with uPA alone (Figure 7, lower panel). When U1 cells were stimulated with PMA no effects were observed in the presence of all tested Ab except in plates coated with anti-CD11b that inhibited virus production, while no effects were observed when the Ab were diluted in cell culture supernatants. UPA maintained its anti-HIV activity in all tested conditions except in the presence of soluble anti-$\beta_1$ and anti-$\beta_2$ integrin chains that abolished its antiviral effect, without affecting uPA-mediated enhancement of PMA-induced cell adhesion.

Thus, the cell adhesion dependent antiviral signal triggered by uPA binding to uPAR requires downstream activation of $\beta$ integrin chains.
Discussion

In the present study we have investigated the role of potential signaling partners mediating uPA/uPAR dependent inhibition of late events in HIV-1 replication in monocytic cells such as primary MDM and promonocytic U1 and U937 cells lines. UPA anti-HIV activity in U1 cells was restricted to PMA and TNF-α stimulation, but was ineffective when these cells were stimulated with other cytokines or LPS. This restricted pattern of uPA-mediated anti-HIV effect was paralleled by the upregulation of CD11b on the surface of U1 cells pairing with constitutively expressed the β2 chain (CD18) to form Mac-1 (also known as Complement Receptor-3, CR3) known to provide signaling competence to the uPA/uPAR complex in myelomonocytic cells. Independently of uPA/uPAR, FNG binding to Mac-1 inhibited late events in HIV production in both stimulated U1 cells and acutely infected U937 cells. Furthermore, prevention of uPAR association with CD11b by the M25 peptide showed similar HIV-inhibitory effects in PMA-stimulated U1 cells. SiRNA targeting uPAR reversed the inhibitory effect of both uPA and M25, whereas siRNA-mediated downregulation of CD11b abolished M25 but did not affect uPA-dependent antiviral effect. Soluble Ab directed against β1 and/or β2 integrin chains, but not against the α chains CD11a-b-c, prevented uPA interference with HIV expression. These findings suggest a model whereby a similar, if not identical, anti-HIV signal interfering with late events in the virus life cycle is triggered by uPA binding to uPAR, but also by uPAR-independent ligation of Mac-1 or by preventing the interaction between CD11b and uPAR.

As observed in cancer, the uPA/uPAR system plays a relevant role in HIV disease. Soluble levels of suPAR represent a potent predictor of HIV disease progression independently of the levels of peripheral CD4+ T cell counts and viremia. These observations were extended and confirmed in patients with HIV-associated dementia. Cultivation and infection of both primary MDM and promonocytic cell lines, such as U1 and U937 cell lines, revealed that both uPA and suPAR levels are expressed in vitro at similar levels than observed in vivo. Furthermore, in vitro released uPA
was mostly complexed with suPAR (Table 1), as described in vivo.¹⁵ This observation likely accounts for the lack of evidence of an autocrine/paracrine role of endogenous uPA on HIV replication in our model systems. Since free uPA inhibits acute HIV-1 replication in primary MDM and U937 cells as well as virus expression in chronically infected U1 cells¹⁶, its sequestration by suPAR to form a biologically inactive uPA-suPAR complex may indeed prevent the anti-HIV effects of uPA therefore providing a potential explanation to the CD4 and viremia independent prognostic value of increased suPAR concentrations for HIV disease progression.

Furthermore, HIV-1 infection of primary MDM did not alter levels of expression of both uPA and suPAR (Table 1). This observation may account for the lack of correlation between circulating levels of uPA and suPAR and patients’ viremia.¹¹ In contrast, suPAR levels were found to be strictly correlated with the state of immune activation of HIV-infected individuals, as determined by the serum levels of TNF-α⁴³, sTNFrII¹², CCL2, CCL4 and CCL5⁴⁵ as well as with the lipid and glucose metabolism⁴³,⁴⁶, as likely reflected in vitro by a trend of association among the levels of HIV-1 replication in acutely infected MDM, suPAR and uPA-suPAR complexes (Figure 2).

UPA-dependent inhibition of virus expression in U1 cells was restricted to PMA and TNF-α stimulation, while other HIV-inductive stimuli (including IL-6, IFN-γ and GM-CSF plus LPS) were unaffected. Of interest, both PMA and TNF-α upregulate HIV expression via activation of NF-kB⁴⁷ whereas the other stimuli act by activation of an ERK-1/-2 AP-1 dependent pathway upregulating virus transcription.²⁶,⁴⁸ Since the inhibitory effect of uPA occurs at a post-transcriptional/post-translational level resulting in the accumulation of virions in intracellular vacuoles¹⁶,⁴⁹, the selectivity of the anti-HIV effect of uPA was unlikely related to interference with virus transcription, as previously described.¹⁶,¹⁷,⁴⁹

We have previously demonstrated that the catalytic component of uPA was dispensable for its anti-HIV effects, unlike its signaling component ATF¹⁶,⁴⁹, as here confirmed in stimulated U1
cells with smaller ATF peptides and extended to the infection of primary MDM. Removal of uPAR by either cleavage of its GPI anchor or reduction of its expression by means of siRNA resulted in the abolition of the antiviral effect of uPA. Since uPAR is a GPI-anchored receptor signal transduction is usually mediated by other receptors and, in monocytic cells, either by FPRL1\(^{50}\) or by integrins such as CD11b/CD18 (Mac-1).\(^{51}\) Indeed, both FPRL1 and some integrins, including \(\alpha_5\beta_5\), \(\alpha_5\beta_1\) and other integrin chains, were constitutively expressed by U1 cells and were not affected by HIV-inductive stimuli. However, no evidence of interference with uPA-dependent anti-HIV effects were obtained by incubating U1 cells with agonists of either FPRL1 or of these integrins.

Unstimulated U1 cells express abundant levels of the integrin \(\beta\) chain CD18, but not of the \(\alpha\) chains CD11b or CD11c. Only PMA and TNF-\(\alpha\) upregulated the expression of CD11b and CD11c, as reported\(^{52}\), suggesting that uPA anti-HIV effect could be potentially mediated by either Mac-1 (CD11b/CD18) or p150/95 (CD11c/CD18). However, prevention of CD11c expression by means of siRNA did not affect HIV expression in PMA-stimulated cells (data not shown), while different CD11b ligands, including FNG and coated anti-CD11b mAb triggered an HIV-inhibitory signal in PMA-stimulated U1 cells. Like uPA, we demonstrated that FNG interfered with the late stage of virus production both in U1 and acutely infected U937 cells. Before our observation, Mac-1 was shown to enhance CD4/CCR5-dependent HIV entry in monocytes, macrophages and dendritic cells\(^{53-55}\), whereas, conversely, several Mac-1 ligands, including FNG, have shown inhibitory effect on the enhancement of opsonized HIV infection.\(^{53-55}\) Of interest, another Mac-1 ligand, soluble CD16 (sCD16), was reported to be significantly decreased in HIV disease progression.\(^{53,56}\) In addition, intercellular adhesion molecule-1 (ICAM-1), a ligand of Mac-1, is incorporated into the virion envelope.\(^{57}\) A confounding aspect of the role of Mac-1 in HIV replication is the observation that ligation of the \(\beta_2\) integrin chain CD18 by immobilized Ab has been shown to increase HIV transcriptional activation in THP1 monocytic cells.\(^{58,59}\) Similar results were described in primary monocytes and U937 cells in the presence of FNG\(^{52,54}\), in U1 cells co-cultivated with HUVEC\(^{60}\),
and in chronically infected promyelocytic OM10.1 cells in which an autocrine release of TNF-α was involved in the upregulation of virus expression. However, the adhesion of LPS-stimulated THP1 cells to tissue culture plastic has been reported to downregulate the release of HIV virions in both stimulated THP1 cell lines and primary MDM. In agreement with these earlier observations, we have previously reported the abolition of uPA inhibitory effects and upregulation of virus production was observed in both acutely infected MDM and PMA-stimulated U1 cells cultivated in the absence of VN or in non-adherent conditions obtained in Teflon-coated plates.

Quite surprisingly, incubation of U1 cells with M25, a peptide derived from the β propeller region of CD11b known to disrupt its association with uPAR, also inhibited late events in HIV expression in PMA-stimulated U1 cell. Since CD11b is not expressed in unstimulated U1 cells, M25 likely prevents its association with uPAR following PMA stimulation. Thus, expression of Mac-1 uncoupled from uPAR leads to an inhibitory effect on HIV expression independently of the exogenous addition of either uPA or integrin ligands other than those present in the culture medium (such as heat-inactivated C3). In this regard, disruption of uPAR-integrin interaction has been previously shown to prevent the association between β integrin and src kinases, thereby affecting adhesion-dependent signal transduction. This effect has been linked to the ability of uPAR to stabilize complexes of caveolin, integrins and src kinases ultimately responsible for adhesion-dependent signalling.

Interference with either uPAR or CD11b expression by siRNA resulted in different biological effects. UPAR targeting siRNA, as well as PIPLC treatment, only partially decreased the levels of uPAR expression in PMA-stimulated U1 cells, yet it abolished uPA and M25 anti-HIV effects. Conversely, almost complete prevention of CD11b expression by siRNA resulted in the expected loss of M25 anti-HIV effect, but was irrelevant for uPA-dependent effects on HIV expression and cell adhesion. These results suggest that uPAR may functionally replace CD11b in terms of triggering a β chain-dependent anti-HIV signal, as previously suggested.
uPA may promote an indirect activation of β₁ and β₂ integrin chains following the recruitment of VN to form a uPA/uPAR/VN supercomplex competent in mediating cell adhesion and mechano-transduction. In support of this interpretation, soluble anti-β₁ and anti-β₂ Ab did not prevent uPA induced cell adhesion, as recently reported, while they fully prevented its anti-HIV effect. Overall these results suggest that an interplay between integrins and uPAR may occur in lipid rafts known to play a fundamental role in both viral entry and for the exit of new progeny virions from infected cells.

In addition to uPA/uPAR and Mac-1 and its ligands, other extracellular stimuli, including IFN-γ and CCL2/MCP-1, have been previously shown to inhibit virion release by favouring intracellular virion accumulation. In this regard, assembly and release of HIV virions in multivesicular bodies (MVB) belonging to the hexosomal pathway is a crucial aspect characterizing the infection of macrophages. Although the relevance of intracellular virion accumulation in macrophages has been recently questioned, different monocytic cells, including U937, HL-60, THP1 and MonoMac cell lines, primary monocytes or macrophages derived from CD34⁺ bone marrow precursors, show accumulation and release of HIV virions in intracellular compartments for several weeks in addition to their generation at the plasma membrane. Even more importantly, these morphogenetic features of virion assembly in sub-cellular vacuolar compartments have been documented \textit{in vivo} in the brain of individuals with HIV encephalitis.

In conclusion, our results suggest a general model whereby interacting, yet distinct ligands can trigger a signaling pathway resulting in the inhibition of HIV spreading by interfering with late stage virion assembly and release in macrophages. At least three converging signals leading to this common final pathway can be postulated based on the present results: 1. ligation of uPAR by its natural ligand uPA via its ATF domain; 2. ligand binding and cross-linking of Mac-1; 3. prevention of uPAR interaction with CD11b resulting in the assembly of Mac-1 dissociated from uPAR. As for other examples of biological redundancy, these observations suggest that control of HIV assembly,
maturation and virion release is a crucial check-point for efficient viral spreading from macrophages to other susceptible cell types.
Acknowledgments

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Authorship

Massimo Alfano designed and performed experiments, analyzed and interpreted the results and contributed to the drafting of the manuscript; Samanta A. Mariani and Chiara Elia performed experiments and interpreted the results; Ruggero Pardi and Francesco Blasi designed experiments, contributed to interpretation of the results and drafting of the manuscript; Guido Poli designed experiments, analyzed and interpreted the results and contributed to the drafting the manuscript.

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All authors declare no conflict of interests.
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Table 1. Concentrations of uPA, suPAR and of the uPA-suPAR complex in culture supernatants of U1 cells and of primary MDM acutely infected or not with HIV-1.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Stimulus/infection</th>
<th>RT Activity (cpm/µl)</th>
<th>suPAR (pM)</th>
<th>uPA (pM)</th>
<th>suPAR/uPA ratio</th>
<th>suPAR-uPA complex (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U1</td>
<td>Nil</td>
<td>&lt;100</td>
<td>33±0</td>
<td>2.9±0.6</td>
<td>11.38</td>
<td>2.9±0.5</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>1,657±221*</td>
<td>49±1**</td>
<td>2.5±1</td>
<td>19.6^</td>
<td>2.4±0.4</td>
</tr>
<tr>
<td></td>
<td>TNF-α</td>
<td>2,021±310*</td>
<td>34±1</td>
<td>4.6±1</td>
<td>7.4***</td>
<td>4.5±0.3***</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>2,421±212*</td>
<td>33±1</td>
<td>2.7±0.6</td>
<td>12.2</td>
<td>2.6±0.2</td>
</tr>
<tr>
<td>MDM</td>
<td>Control#</td>
<td>--</td>
<td>217±102</td>
<td>8±2</td>
<td>27.2</td>
<td>7±1</td>
</tr>
<tr>
<td></td>
<td>HIV</td>
<td>6,967±882*</td>
<td>182±93</td>
<td>8±2</td>
<td>22.8</td>
<td>7±1</td>
</tr>
</tbody>
</table>

The levels of expression of the analytes were measured 48 h after stimulation of U1 cells and at the peak of viral replication in MDM. Since MDM were seeded at twice the cell concentration of U1 cells, the results shown have been normalized dividing by a factor of 2. The results are expressed as mean±SD of the concentrations determined in 3 independent experiments with U1 cells and from MDM cultures established from 5 independent donors (see Figure 2). #Control: uninfected MDM. Significantly higher levels of HIV production were observed in PMA or cytokine-stimulated U1 cells vs. unstimulated cells (Nil) (*p=10^-5), as well as in infected vs. uninfected MDM (*p=10^-5). Furthermore, increased levels of suPAR were observed after U1 cell stimulation with PMA, but not with cytokines (**p=10^-3 vs. Nil) as well as of the suPAR/uPA ratio in PMA and TNF-α stimulated U1 cells (^p=0.0045 and ***p=0.01, respectively) and of the suPAR/uPA concentrations in cells stimulated with TNF-α (**p=0.01).
Table 2. Expression of integrin chains and cell surface receptors in U1 cells.

<table>
<thead>
<tr>
<th>Integrin chain</th>
<th>Nil</th>
<th>PMA</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>αIIb (CD41)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>αL (CD11a)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>αE (CD103)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>αM (CD11b)</td>
<td>Neg</td>
<td>+*</td>
<td>Neg</td>
</tr>
<tr>
<td>αX (CD11c)</td>
<td>Neg</td>
<td>+*</td>
<td>Neg</td>
</tr>
<tr>
<td>αV (CD51)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>αVβ3 (VN-R)†</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>αVβ5 (FN-R, VN-R)‡</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α5β1 (FN-R)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α1 (VLA1)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>α2 (CD49b)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>α3 (CD49c)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>α4 (CD49d)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α5 (CD49e)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α6 (CD49f)</td>
<td>Neg</td>
<td>+*</td>
<td>Neg</td>
</tr>
<tr>
<td>β1 (CD29)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>β2 (CD18)</td>
<td>+</td>
<td>++*°</td>
<td>+</td>
</tr>
<tr>
<td>β3 (CD61)</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
</tr>
<tr>
<td>β4 (CD104)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

Receptors

<table>
<thead>
<tr>
<th></th>
<th>Nil</th>
<th>PMA</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>uPAR</td>
<td>+</td>
<td>++*</td>
<td>++*</td>
</tr>
<tr>
<td>FPRL1</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Cell surface expression of the indicated molecules was evaluated by cytofluorimetric analysis after 24 h of stimulation (n=4 for all antigens, except uPAR and CD11b that were tested in 9 independent experiments). An identical pattern of expression was observed up to 72 h post stimulation. Neg: negative cells; +: positive cells; °++: cells testing positive with a geometric mean fluorescence intensity >2-fold that of unstimulated cells; *p<0.05 vs. Nil. VN-R: vitronectin receptor; FN-R: fibronectin receptor. Upregulation of CD11b and CD11c in U937 cells stimulated with PMA was previously reported.63
Figure legends

**Figure 1.** UPA inhibits HIV expression in chronically infected U1 cells via binding to uPAR with its ATF domain. **A.** U1 cells were preincubated with different concentrations of either uPA or peptides representing the low-molecular weight (LMW), ATF, the growth factor domain (GFD) or the Omega loop components of uPA. Cells were then stimulated with PMA and followed for virus expression in culture supernatants. All peptides significantly inhibited (p<10^{-5}) virus expression in the same range of concentrations except for LMW that was ineffective at all tested concentrations. **B.** U1 cells were analyzed for uPAR expression 2 and 24 h after incubation with PIPLC (10 U/ml). Down modulation of uPAR expression was clearly detectable at both time points. Geometric mean fluorescence intensities (MFI): isotype (MFI: 4), unstimulated cells – Nil - (MFI: 31), PIPLC (MFI: 14). **C.** U1 cells were incubated with PIPLC for 2 h and then stimulated with PMA in the presence or absence of uPA (10 nM). The RT activity levels were measured in the culture supernatants at the peak of virus expression (day 4 post-stimulation). PIPLC abolished the inhibitory effect of uPA on virus expression without affecting the inductive effect of PMA (*p<10^{-5}). **D.** U1 cells were stimulated with PMA in the presence or absence of suPAR and uPA and RT activity was determined in the culture supernatants after 3 days of culture(*p<10^{-5}); a similar pattern of virus expression was observed even at days 2 and 4 of culture (data not shown). The error bars indicate the standard deviation (SD) of duplicate samples. All the described experiments have been performed in duplicate wells and repeated 3 times and provide identical findings.

**Figure 2.** ATF inhibits acute R5 HIV-1 replication in MDM. **A.** The anti-HIV activity of uPA and ATF were tested on primary MDM infected with an R5 HIV-1. The compounds were supplemented to the cell cultures at the time of infection and every 3 days after infection. The results of a single experiment representative of 5 independently performed with cells of different donors are shown. The error bars indicate the SD of triplicate samples, whereas asterisks at the indicated time points indicate statistical significance (p=10^{-3}) among Nil and uPA or ATF treated cells; no significant differences were observed in the inhibition levels induced by uPA or ATF. **B.**
Culture supernatants of uninfected MDM and *in vitro* infected MDM (A) were analyzed for the levels of uPA and suPAR at the day corresponding to the peak of viral replication (RT Activity) in 5 independent experiments performed with cells from different donors. Differences in the levels of suPAR, uPA, the uPA-suPAR complex, and the suPAR/uPA ratio between uninfected and infected cells tested not significant. R²: linear regression; p was calculated by Spearman rank test.

**Figure 3. Induction of CD11b expression in U1 cells and uPA-mediated inhibition of HIV-1 expression.** A. U1 cells were gated based on their light scatter and analyzed for both uPAR (R4 mAb) and CD11b (αM44 mAb) expression 20 h after stimulation with PMA, TNF-α or IL-6; the integrin β₂ chain CD18 is constitutively expressed on U1 cells (Table 1). All stimuli upregulated the levels of uPAR on the cell surface, but only PMA and, to a lesser extent, TNF-α, promoted the expression of CD11b on U1 cells. B. uPA inhibits HIV expression in U1 cells stimulated with PMA and TNF-a, but not with IL-6, IFN-γ or GM-CSF plus LPS. The results were obtained form a single experiment representative or 5 or more independently performed. The error bars indicate the SD of duplicate samples.

**Figure 4. FNG inhibits HIV expression in PMA-stimulated U1 cells and in U937 cells acutely infected with an X4 HIV-1.** A. U1 cells were incubated with the indicated concentrations of FNG and then stimulated with PMA. B. Cell disruption rescues the RT activity levels of U1 cells stimulated with PMA in the presence of FNG, as detected after 4 days of culture (arrowhead, panel A). C. U937 cells were incubated with FNG (100 µg/ml) and then infected with the X4 strain HIV-1LAI/IIIB; FNG was supplemented to the cell cultures every 72 h, when 50% of culture supernatants were replaced with culture medium. D. Cell disruption rescued the RT activity levels of acutely infected U937 cells in the presence of FNG at the indicated days post-infection (*p<10⁻⁵). The results shown were derived from one experiment representative of 3 (panels A and B) and 2 (panels C and D) independently performed.

**Figure 5. M25 inhibits HIV expression in PMA-stimulated U1 cells both in the absence and presence of uPA.** A. U1 cells were incubated with different concentrations (from 5 to 80 µM)
of the M25 peptide or of its scrambled form (M25scr) in the presence or absence of uPA (10 nM) and were then stimulated with PMA. A concentration-dependent inhibition of HIV expression was observed in the presence of M25, but not of its control peptide, both in the presence and absence of uPA. B. Cell disruption rescued HIV expression from the inhibitory effect of M25 (40 µM) or uPA (10 nM) after 5 days of culture (*p<10^{-5}, **p<10^{-4}). The results shown were derived from one experiment representative of 6 (A) and 3 (B) independently performed.

Figure 6. CD11b is required for the anti-HIV activity of M25 but not of uPA. A. Expression of both uPAR and CD11b in unstimulated and PMA-stimulated U1 cells before (upper panels) and after (lower panels) transfection with their respective siRNA and control mock siRNA. The uPAR siRNA showed only a partial inhibitory effect on its target whereas the CD11b siRNA abolished the expression of the integrin chain. B. CD11b siRNA abolished the M25 inhibitory effects on HIV expression but did not affect uPA dependent inhibition, whereas decreased uPAR expression abrogated the inhibitory effects of both agents. C. Lack of CD11b expression did not interfere with uPA/uPAR dependent adhesion of PMA-stimulated U1 cells to the plastic substrate that was otherwise inhibited by uPAR siRNA (*; p<10^{-6}). No effect of M25 on the adhesion of PMA-stimulated U1 cells was observed. The results shown were derived from one experiment representative of 4 independently performed.

Figure 7. Differential effect of soluble and coated Ab directed against α and β integrin chains on U1 cell adhesion and HIV-1 expression. Microtiter wells were coated with different Ab (1 µg/well each) before seeding and stimulation of U1 cells (2x10^5 cells/ml). In parallel, 2x10^5 cells/ml U1 cells were resuspended in medium enriched of the indicated Ab (10 µg/ml) before seeding and stimulation with PMA in the presence or absence of uPA. The same concentration of Ab was supplemented after 24 h of cell culture. Cell adhesion (upper panel) and virus expression (lower panel) were measured 48 h after cell seeding. The results shown were derived from one experiment representative of 3 independently performed. Significantly enhanced cell adhesion was observed in all PMA-stimulated cells vs. unstimulated cells (Nil) (*p=10^{-3}). Furthermore, uPA
increased PMA-stimulated cell adhesion (**p=10^{-6} vs. PMA alone). Significantly increased levels of cell adhesion were observed also when otherwise unstimulated U1 cells were incubated with anti-CD29 mAb coated to the plastic surface (\textasciitilde p=10^{-4} vs. Nil). Virus expression was significantly inhibited by uPA in all conditions (^p=10^{-5} vs. PMA), but not in uPA-treated, PMA-stimulated cells incubated with either soluble anti-CD29 or soluble anti-CD18 Ab vs. BSA-treated cells (*p=10^{-3}). Finally, significantly lower levels of HIV production were observed in PMA-stimulated U1 cells incubated in the presence of coated anti-CD11b mAb (**p=0.004).
Figure 1

A.

B.

C.

D.
Figure 2

A.

B.

C.

RT Activity (cpm/µL) x 10^4
Figure 3

A.

B.
Figure 4

A. 

B. 

C. 

D.
Figure 5

A.

B.
Ligand-engaged urokinase-type plasminogen activator receptor (uPAR) and activation of the CD11b/CD18 (Mac1) integrin inhibit late events of HIV expression in monocytic cells

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