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Vpu-mediated tetherin antagonism of ongoing HIV-1 infection in CD4$^+$ T-cells is not directly related to the extent of tetherin cell surface downmodulation

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ABSTRACT

Tetherin is a host cell restriction factor that acts against HIV-1 and other enveloped viruses. The antiviral activity of tetherin is antagonized by the HIV-1 protein Vpu, that downregulates tetherin from the cell surface. Here, we report the specific detection of cell surface tetherin levels in primary activated CD4$^+$ T-cells and in CD4$^+$ T-cell lines. Differences were observed regarding tetherin cell surface expression, Vpu-mediated tetherin downmodulation and promotion of virus release. However, Vpu expression in all T-cell lines resulted in a 2-fold increase in numbers of infected cells after three days. This implies a Vpu-mediated effect in ongoing infection and possibly in cell-to-cell viral spread that is independent of the extent of Vpu-mediated tetherin cell surface downmodulation. Endogenous cell surface tetherin levels in T-cell lines were also down-modulated following infection with Vpu-deleted virus, suggesting an additional Vpu-independent mechanism of tetherin cell surface downmodulation following HIV-1 infection in T-cell lines.

Introduction

Tetherin (BST-2/CD317/HM1.24) is a host cell restriction factor that contributes to cellular defense against infection by HIV-1 and other enveloped viruses; tetherin-mediated restriction is interferon responsive (Jouvenet et al., 2009; Neil et al., 2008; Van Damme et al., 2008). In HIV-1 infections, the viral protein Vpu antagonizes tetherin-mediated restriction and promotes virus release (Neil et al., 2008; Van Damme et al., 2008). The antiviral action of tetherin is due to its presence in the membrane of budding viral particles, tethering nascent viral particles to the cell surface and to each other (Kupzig et al., 2003; Neil et al., 2008; Perez-Caballero et al., 2009; Van Damme et al., 2008). At the cell surface, tetherin localizes to lipid rafts (Goffinet et al., 2009; Kupzig et al., 2003; Rollason et al., 2009, 2007), which, during the HIV-1 life cycle are the focus of viral assembly, budding, as well as entry; lipid rafts are involved in both cell-free virus spread and direct cell-to-cell spread (reviewed in (Waheed and Freed, 2009)). Direct cell-to-cell spread is reported to increase the efficiency of HIV-1 transmission by 100–18,000 times compared to cell-free spread and is considered to be the predominant mode of HIV-1 spread in T-cell lines and in secondary lymphoid tissue (Chen et al., 2007; Dimitrov et al., 1993; Gummuluru et al., 2000; Hübner et al., 2009; Sourisseau et al., 2007) (reviewed in (Mothes et al., 2010; Sattentau, 2008)). In addition to restricting virus release and subsequently cell-free viral spread, we and others have shown that tetherin also inhibits direct cell-to-cell transmission in T-cells (Casartelli et al., 2010; Kuhl et al., 2010b). Others have reported that HIV-1 might overcome tetherin-mediated restriction of direct cell-to-cell viral spread (Jolly et al., 2010).

The capacity of tetherin to restrict virus release is commonly attributed to its cell surface expression. Vpu activity in counteracting tetherin-mediated restriction is believed to result from Vpu-mediated tetherin cell surface down-regulation, which either results from tetherin degradation or from its sequestration in intracellular compartments (Dubé et al., 2010; Dube et al., 2009; Goffinet et al., 2009; Iwabu et al., 2009; Mangeat et al., 2009; Perez-Caballero et al., 2009; Van Damme et al., 2008). Most of these data were obtained using the HeLa epithelial cell line, which expresses high endogenous levels of tetherin or with the 293T human embryonic kidney cell line, which naturally lacks tetherin expression and must be transfected with tetherin-expressing plasmids.

The specific detection of tetherin expression at the cell surface has been reported for only a few cell lines (HeLa, MT-4, COS-7) and for primary B-cells, plasmacytoid dendritic cells (pDCs) and monocyte derived macrophages (MDMs) (Blasius et al., 2006; Mitchell et al.,

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2009; Miyagi et al., 2009; Rong et al., 2009; Sato et al., 2009; Van Damme et al., 2008; Vidal-Laliena et al., 2005). Interferon-α (IFNα) increased cell surface expression in these cells and also induced detectable cell surface expression in 293T cells (Van Damme et al., 2008). In peripheral blood mononuclear cells (PBMCs), total cellular tetherin expression had previously only been shown after IFNα-treatment by Western blot of cell lysates (Miyagi et al., 2009).

It was recently reported that endogenous tetherin is differentially modified at the post-translational level compared to tetherin that is derived from an exogenous source (Andrew et al., 2010). Cell-line specific differences have been reported for expression patterns of other host cell restriction factors, such as APOBEC3G, which also confers resistance to HIV-1 infections and which is antagonized by the viral accessory protein Vif (reviewed in (Henriet et al., 2009; Niewiadomska and Yu, 2009)). APOBEC3G is expressed and restricts viral replication in CEM-CCRF cells but not in a derivative cell line CEM-SS (Foley et al., 1965; Sheehy et al., 2002).

Few studies have investigated the relationship between cell surface tetherin expression and virus release in infected T-cell lines (Miyagi et al., 2009; Rong et al., 2009; Sato et al., 2009). While virus release might be attributed to expression levels of cell surface tetherin in MT-4 (Harada et al., 1985; Sato et al., 2009) and stably transduced Sup-T1 cells (Rong et al., 2009), other work that used the H9 T-cell line and the CEMx174 T/B-cell fusion cell line reported tetherin-mediated restriction that was independent of tetherin cell surface levels, suggesting the possibility of cell-type specific differences in the effect of tetherin on virus release (Miyagi et al., 2009). To address this, we determined tetherin cell surface expression in relation to virus release and infection rates. Here, we report specific detection of cell surface tetherin expression in primary activated $CD4^+$ T-cells and in multiple T-cell lines. Strong differences in regard to tetherin cell surface expression, Vpu-mediated tetherin downmodulation, and promotion of virus release were observed among them. We show that the influence of Vpu on multiple-round infections was equivalent in all T-cell lines, and that twice as many cells were infected at 72 h post infection (p.i.) in the case of *vpu*-containing compared to Δ*vpu* infections. This implies a tetherin-mediated effect on cell-to-cell spread that is not directly related to its cell surface expression. In addition, we report a Vpu-independent downregulation of endogenous tetherin following infection of $CD4^+$ T-cell lines.

Results

Variation of tetherin cell surface expression in T-cell lines

We first assessed the cell surface expression of endogenous tetherin by flow cytometry in CEM-CCRF, CEM-SS, and H9 cells, in addition to Sup-T1 cells stably transduced with human tetherin (Kuhl et al., 2010b; Rong et al., 2009). We were able to specifically detect and assess cell surface expression of tetherin in all of these cell lines (Fig. 1A). Cell surface expression of tetherin varied between the cell lines; relative mean expression levels were 9.1 in H9 cells, 23.4 in CEM-CCRF cells and 44.5 in CEM-SS cells. In the case of the tetherin-inducible Sup-T1 cell line, we employed a doxycycline titration method to specifically induce cell surface tetherin levels that resemble levels detected on T-cell lines (Figs. 1A and B). Induction with 5 ng/ml doxycycline resulted in a relative mean cell surface expression of tetherin of 8.3, which was similar to that obtained in H9 cells.

We also assessed cell size and cell granularity/complexity by flow cytometric analysis of forward scatter (FSC) and side scatter (SSC), respectively. Side scatter patterns differed between cell types due to differences in cell granule content which in combination with FSC is a commonly used characteristic for identification of cell populations. All cell lines tested here were of similar size (FSC) and granularity/complexity (Figs. 1C and D).

Cell line specific differences of vpu-mediated tetherin downmodulation

Next, we assessed the capacity of Vpu to downregulate cell surface expression of tetherin following HIV-1 infection in the various cell lines. Cells were infected to a level of ~10%, as assessed by flow cytometric detection of eGFP expression at 48 h p.i., with single-round infections, *i.e. env*-deleted, *wt* (*vpu*-positive), or Δ*vpu* (*vpu*-deleted) virus, pseudotyped for entry with the Vesicular stomatitis virus protein G (VSV-G) envelope. Tetherin cell surface expression in infected (eGFP positive) and uninfected (eGFP negative) cells was determined by flow cytometry. In the stably transduced Sup-T1 cell line infected with *wt* virus, tetherin cell surface levels were significantly downregulated compared to uninfected controls (~40%). Uninfected H9 cells showed similar tetherin cell surface expression as did uninfected Sup-T1 cells induced with 5 ng/ml doxycycline (Figs. 2A and B). However, infection of H9 cells with *wt* virus resulted in only a modest tetherin down-regulation (~27%) compared to uninfected cells (Fig. 2A). CEM-SS cells exhibited significantly higher tetherin cell surface levels than did the parental CEM-CCRF cell line (Figs. 2C and D). In both these cell lines, cell surface tetherin was downregulated in *wt* infection but to different extents (CEM-SS: ~78%; CEM-CCRF: ~45%).

Vpu-independent tetherin modulation

In CEM-SS cells, cell surface tetherin was also downregulated after infection by Δ*vpu* virus, compared to uninfected controls (~47%) (Fig. 2D); similar trends were observed with CEM-CCRF (~33%) and H9 cells (~13%) (Figs. 2A and C). This effect was not observed in the transduced Sup-T1 cell line (induced with 5 ng/ml doxycycline); such cells, when infected with Δ*vpu* virus, showed a slight, but statistically insignificant upregulation of tetherin (~15%) (Fig. 2B).

Cell line specific effect of Vpu-mediated tetherin modulation on virus release

To assess the effect of Vpu on virus release we infected the cell lines with equal amounts of *wt* or Δ*vpu* virus, based on CA p24 levels, and measured virus release into the supernatant at 24, 48 and 72 h p.i. using a quantitative reverse transcription-based assay (Fig. 3). Virus release at baseline (24 h p.i.) was similar in all infections. Starting at 48 h p.i. Vpu mediated increased levels of virus release in all cell lines, though the effect differed among them. At 72 h p.i., the extent of the Vpu effect on virus release (comparing *wt* and Δ*vpu*) ranged from ~60% in H9 cells to ~550% in CEM-SS cells (CEM-CCRF: ~170%; induced Sup-T1: ~300%) (Fig. 3). In order to compare the direct effect of Vpu on tetherin cell surface downmodulation and virus release, we normalized the Vpu-mediated increase of virus release to the extent of Vpu-mediated tetherin cell surface downregulation (Table 1). A greater ratio implies a stronger correlation of virus release and changes in tetherin cell surface expression. The similarity of the ratios between CEM-CCRF cells (10.2) and derivative CEM-SS cells (10.5) shows that tetherin-mediated restriction of virus release was strongly affected by tetherin cell surface expression levels in both instances. H9 cells showed a low ratio (3.5), indicating a lower correlation between tetherin-mediated restriction of virus release and cell surface expression, while inducible Sup-T1 cells showed an intermediate level of correlation (6.1).

Similar influence of Vpu in ongoing infection

Using flow cytometry detection of virus-derived eGFP expression at 72 h p.i., we assessed cell line susceptibility to *wt* virus. Infections with equal amounts of *wt* virus, based on CA p24 levels, resulted in infection rates ranging from ~1% in H9 cells to ~40% in inducible Sup-T1 cells (CEM-SS: ~3%; CEM-CCRF: ~20%) (Fig. 4). The presence of Vpu (comparing *wt* and Δ*vpu* virus) increased infection rates at 72 h p.i. to similar levels in all cell lines, ranging from a 107% increase in CEM-CCRF

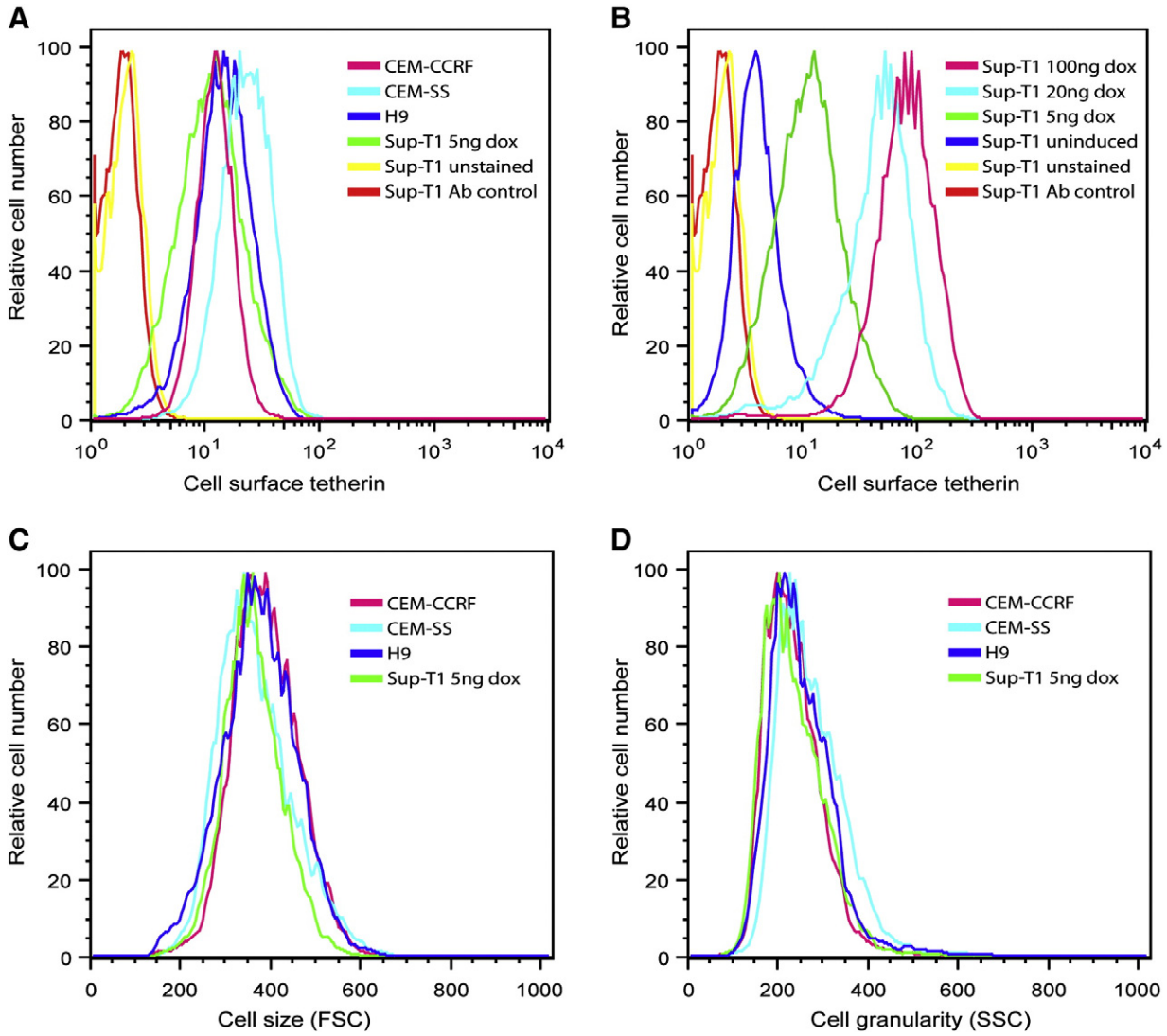

Fig. 1. Tetherin cell surface levels in T-Cell lines. Representative overlay of tetherin cell surface expression levels (A and B), cell size (C) and cell granularity (D) Cell surface tetherin levels assessed by flow cytometry detection of PerCP-levels in (A) H9 (*blue*), CEM-CCRF (*rose*), CEM-SS (*turquoise*) and Sup-T1 cells, induced with 5 ng/ml doxycycline (*green*), and (B) in Sup-T1 cells induced with 5 ng/ml (*green*), 20 ng/ml (*turquoise*) and 100 ng/ml (*rose*) doxycycline, and in uninduced Sup-T1 cells (*blue*); (A and B) Controls are unstained, induced Sup-T1 cells (100 ng/ml doxycycline, *yellow*) and induced Sup-T1 cells (100 ng/ml doxycycline, *red*) stained with the secondary antibody only. Cell size (C) and granularity (D) of H9 (*blue*), CEM-CCRF (*rose*), CEM-SS (*turquoise*) and Sup-T1 cells, induced with 5 ng/ml doxycycline (*green*), were analyzed according to flow cytometry detection of forward scatter (FSC) and side scatter (SSC), respectively.

cells to a 127% increase in H9 cells (CEM-SS: 122%; inducible Sup-T1: 126%)(Fig. 4).

Vpu and tetherin expression in infected primary CD4$^+$ T-cells

In order to account for the importance of the described role of Vpu/tetherin in T-cell lines, we next assessed this interrelationship in primary activated CD4$^+$ T-cells. In brief, PBMCs were negatively selected for CD4$^+$ T-cells and activated using phytohemagglutinin A (PHA) and interleukin 2 (IL-2). We were able to specifically detect endogenous tetherin cell surface expression in activated primary CD4$^+$ T-cells (Fig. 5A). Tetherin cell surface expression was elevated by 55% upon induction with 50 U/ml IFNα compared to untreated controls with mean expression levels rising from 19 (absence of IFNα) to 29.4 (50 U/ml IFNα) (Fig. 5B). Higher concentrations of IFNα did not result in further significant elevation of tetherin cell surface levels (250 U/ml IFN: 29.3; 500 U/ml IFN: 30.7) (Fig. 5B).

To assess the effect of Vpu on tetherin downmodulation and on ongoing infections in primary CD4$^+$ T-cells, cells were infected with equal amounts of *wt* and Δ*vpu* virus, and cell surface tetherin levels were measured using flow cytometry. Cells were also treated with IFNα (250 U/ml) at 24 h p.i., a time point which allowed integration to occur. In the absence of IFNα, Vpu downmodulated tetherin cell surface levels in infected (eGFP positive) cells by 27% compared to the uninfected (eGFP negative) population (Fig. 5C); in IFNα-treated populations, *wt* infected cells showed similarly reduced tetherin expression levels (25% lower than in uninfected cells) (Fig. 5D). Infections with Δ*vpu* virus in IFN-treated and untreated cell populations resulted in a slight, but statistically insignificant, upregulation of tetherin cell surface levels compared to uninfected (eGFP negative) cell populations (untreated: 5%; IFN-treated (250 U/ml): 15%) (Figs. 5C and D). As with our analysis of T-cell lines, we normalized Vpu-mediated virus release to the ability of Vpu to downmodulate tetherin cell surface levels. IFNα-treated and untreated primary CD4$^+$ T-cells showed similar ratios of 1.8 (no IFNα) and 2 (250 U/ml IFNα) and therefore, compared to T-cell lines, might have lower tetherin cell surface activity on virus release (Table 1).

Vpu promoted virus release in both IFNα-treated and untreated activated cells. In untreated populations, Vpu promoted virus release by ~45%, comparing *wt* to Δ*vpu* infections, as assessed by reverse

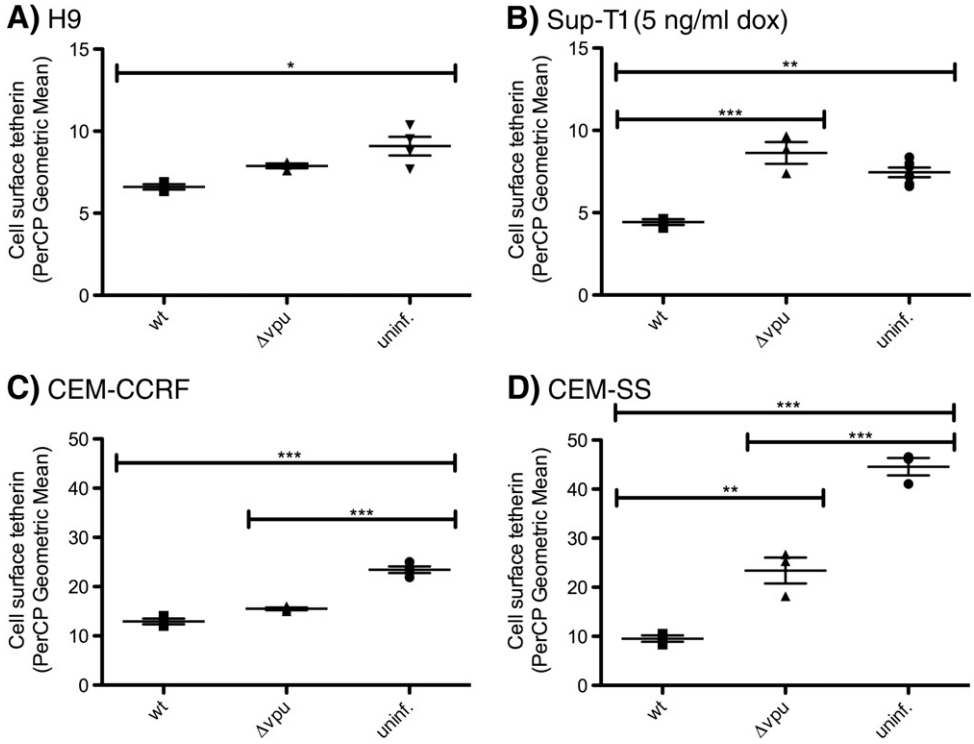

Fig. 2. Tetherin cell surface modulation in infected T-cell lines. Geometric means of cell surface expression of tetherin in T-cell lines H9 (A), Sup-T1 (B; induced by 5 ng/ml doxycycline), CEM-CCRF (C) and CEM-SS (D). Cells were infected at a rate of 10% with VSV-G pseudotyped *wt* and Δ*vpu* BR-NL43-IRES-eGFP. At 48 h p.i., cells were gated into uninfected and infected populations based on their virus-derived eGFP expression profile, and cell surface levels of tetherin were assessed. Data are derived from a minimum of three independent experiments. Error bars represent the SEM. Statistical significance between uninfected cells, cells infected with *wt* or Δ*vpu* virus, as assessed by One-Way ANOVA, coupled with Bonferroni's post-test, is indicated at the top of the graph.

transcriptase activity in culture supernatants at 72 h p.i. (Fig. 5E). This Vpu-mediated effect was elevated to ~70% in IFNα-treated populations. Treatment with IFNα decreased virus release by ~27% and ~50% in *wt* and Δ*vpu* infections, respectively.

IFN-α treatment reduced the susceptibility of $CD4^+$ T-cell populations to productive infection, as assessed by flow cytometry detection of eGFP at 72 h p.i.; infection rates were reduced from ~7% to ~2% (~70% reduction) in *wt* infections and from ~1.6% to ~0.4%

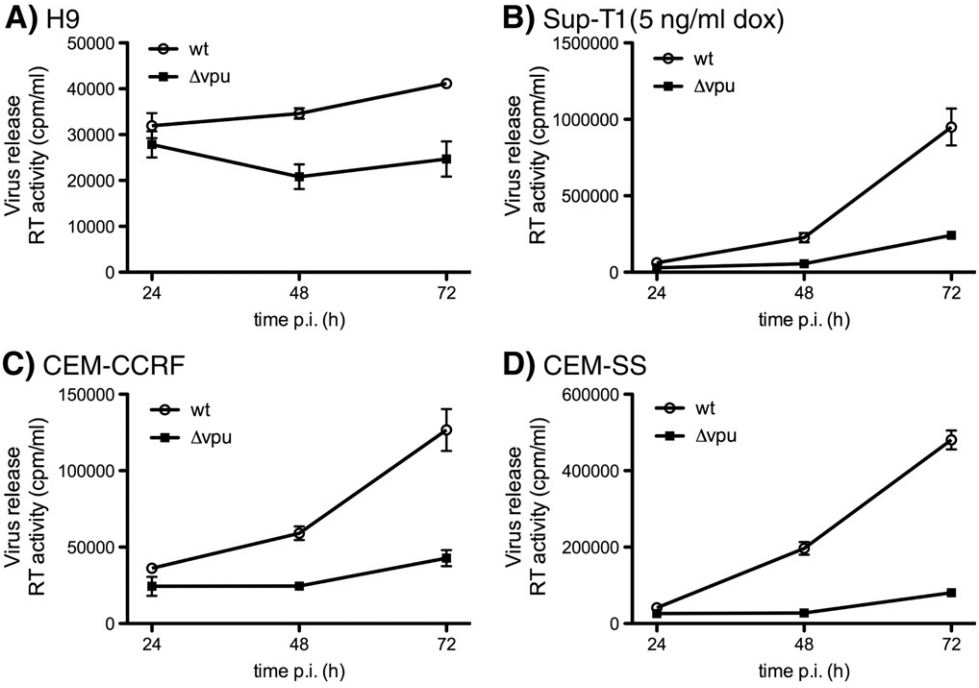

Fig. 3. Varying impact of Vpu on virus release. The T-cell lines H9 (A), Sup-T1 (induced, B), CEM-CCRF (C) and CEM-SS (D) were infected with equal amounts of *wt* or Δ*vpu* virus (600 ng p24 per 10^6 cells) by spinoculation. Synchronized infections were also carried out. Virus release was assessed at 24 h, 48 h and 72 h p.i. by reverse transcriptase assay. Data from three independent experiments are presented; error bars represent SEM. Statistical analysis was assessed using Student's *t*-test.

Table 1
Cell line-specific correlations of Vpu-mediated tetherin cell surface downmodulation and virus release, based on comparison of *wt* infection to Δ*vpu* infection.

Cell type	Δ Cell surface tetherin (ΔCST)	Δ Virus release (ΔVR)	Tetherin cell surface activity (ΔVR/ΔCST)
H9	17%	60%	3.5
Sup-T1 (5 ng/ml dox)	49%	300%	6.12
CEM-CCRF	16%	168%	10.5
CEM-SS	54%	553%	10.2
Primary $CD4^+$ (no IFNα)	26%	45%	1.8
Primary $CD4^+$ (250 U/ml IFNα)	35%	70%	2

(~75% reduction) in Δ*vpu* infected populations (Fig. 5F). Vpu mediated an increase in infection rate by ~400% in IFNα-treated cells and ~330% in untreated populations, based on equal levels of CAp24 input.

Discussion

Here, we have investigated the roles of tetherin-mediated restriction and Vpu-mediated antagonism in the context of ongoing infection in primary $CD4^+$ T-cells and in the $CD4^+$ T-cell lines H9, CEM-CCRF, CEM-SS and the tetherin-inducible Sup-T1 cell line. CEM-SS is a direct derivative cell line of CEM-CCRF; however, these two cell lines show different susceptibility to HIV-1 infection and were shown to differ in their expression profile of host cell restriction factor APOBEC3G (Sheehy et al., 2002). It is not known whether these cell lines also exhibit differences in regard to tetherin. Additionally, CEM-CCRF cells were used for development of the T-cell/B-cell fusion cell line CEMx174 (Foley et al., 1965; Salter et al., 1985); tetherin in CEMx174 and H9 cells was recently proposed to restrict virus release independent of its cell surface expression (Miyagi et al., 2009). Induced tetherin expression of stably transduced Sup-T1 cells, was recently shown to restrict direct cell-to-cell spread of HIV-1 in the absence of Vpu (Kuhl et al., 2010b).

In our studies, endogenous tetherin cell surface expression in all T-cell lines differed, with endogenous cell surface tetherin in Sup-T1 cells being at the limit of detection by flow cytometry. Expression levels among H9, CEM-CCRF and CEM-SS cells varied ~4-fold (Fig. 1A). Using a doxycycline titration method in tetherin-inducible Sup-T1 cells, we were able to mimic these endogenous cell surface levels (Fig. 1B). The similar size and granularity/complexity observed among the cells confirms their overall similarity and implies that differences in tetherin detection are due to genuine differences in tetherin expression levels at the cell surface and not due to differences in size and/or cell complexity between the cell lines tested here (Figs. 1C and D).

We have also been able to specifically detect tetherin cell surface expression in primary activated $CD4^+$ T-cells by flow cytometry (Fig. 5A). Mean cell surface tetherin levels of activated $CD4^+$ T-cells were upregulated by 50–60% by treatment with IFNα. This effect seemed to be saturable (Figs. 5A and B), as previously observed in MDMs (Miyagi et al., 2009). To our knowledge this is the first report of specific detection and quantification of tetherin cell surface expression in primary $CD4^+$ T-cells. A previous study failed to specifically detect cell surface tetherin expression in activated (CD3/CD28) and/or

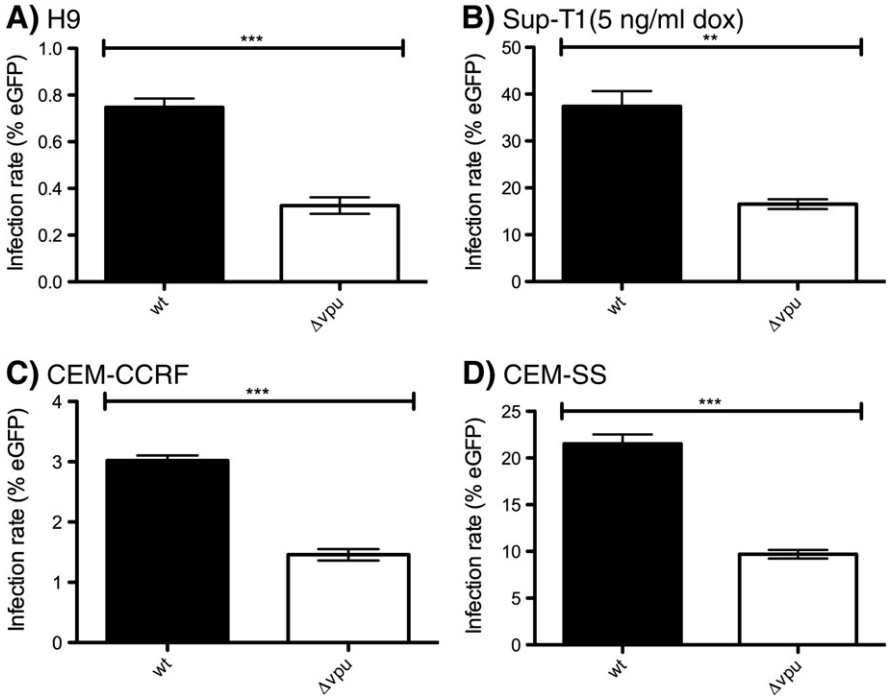

Fig. 4. Vpu promotes viral spread in various T-cell lines at similar levels. The T-cell lines H9 (A), CEM-CCRF (C), CEM-SS (D) and the induced cell line Sup-T1 (B) were infected with equal amounts of *wt* or Δ*vpu* BR-NL43-IRES-eGFP virus (600 ng p24 per 10^6 cells) by spinoculation. Infection rates were determined at 72 h p.i. by assessing virus-derived eGFP expression. Data are derived from three independent experiments; error bars represent SEM.

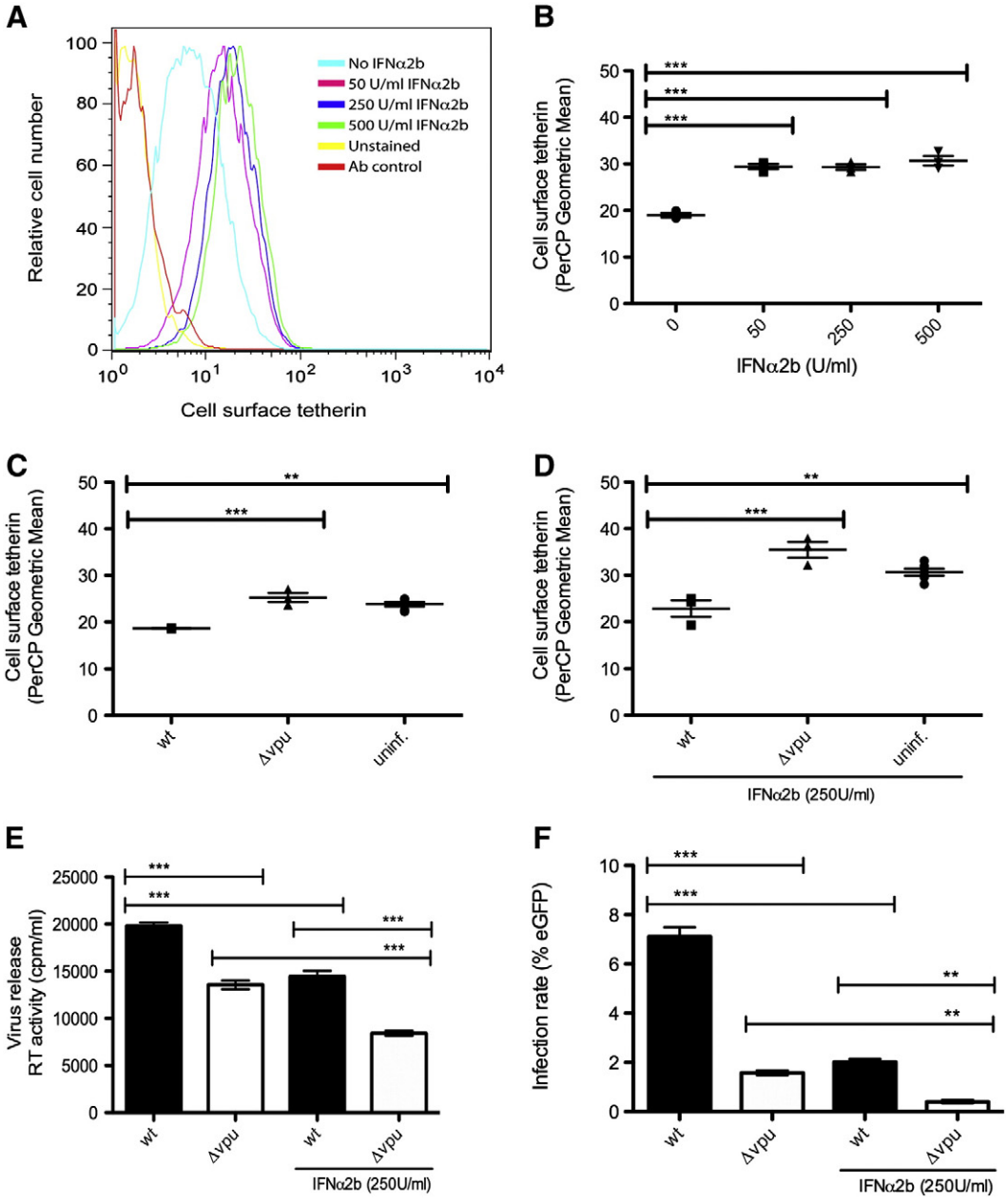

Fig. 5. Relationship between tetherin and Vpu in primary CD4$^+$ T-cells. (A and B) Representative overlay (A) and quantification of geometric means (B) of tetherin cell surface expression levels. Cell surface tetherin levels assessed by flow cytometry detection of PerCP-levels in PHA/IL-2 activated CD4$^+$ T-cells (*turquoise*) and activated cells treated with 50 U/ml (*purple*), 250 U/ml (*green*) and 500 U/ml (*blue*) IFNα2b; (A) Controls are IFN-treated (500 U/ml IFNα2b) cells, unstained (*yellow*) or stained with the secondary antibody only (*red*). (C–F) Primary CD4$^+$ T-cells were infected with equal amounts of *wt* or Δ*vpu* BR-NL43-IRES-eGFP virus (600 ng p24 per 10^6 cells) by spinoculation. At 72 h p.i. flow cytometry was utilized to assess infection rates by detection of virus derived eGFP expression (F) and tetherin cell surface expression in infected (eGFP positive) and uninfected (eGFP negative) populations via Per-CP detection (E). (D–F) Primary CD4$^+$ T-cells were stimulated with 250 U/ml IFNα2b at 24 h p.i. (B–F). Data are derived from a minimum of three independent experiments. Error bars represent the SEM. Statistical significance of differences, as assessed by One-Way ANOVA, coupled with Bonferroni's post-test, is indicated at the top of the graph.

IFNα-treated PBMCs, although the detection of tetherin upregulation of levels of cellular tetherin using Western blot techniques on cell lysates has been reported (Miyagi et al., 2009). The specific detection of tetherin cell surface expression, and its elevation by IFNα, as detected by flow cytometry in activated CD4$^+$ T-cells that were separated from PBMCs, suggests that cell surface expression in other types of PBMC populations might occur but be below the level of detection of common assays. IFNα might also induce overall cellular tetherin levels in PBMCs, as tetherin was detected in Western blots of IFN-treated PBMCs (Miyagi et al., 2009).

Furthermore, the cell surface presence of tetherin in the absence of IFNα supports the notion that tetherin be characterized as an intrinsic restriction factor in activated primary CD4$^+$ T-cells that is elevated upon IFNα induction. It is unknown whether activation of T-cells induces tetherin expression.

Tetherin cell surface expression was downregulated following *wt* infection in all cell lines and in primary CD4$^+$ T-cells, though cell specific differences of up to 4-fold were observed. The capacity of Vpu to promote tetherin cell surface expression in primary CD4$^+$ T-cells was similar in IFNα-treated and untreated populations and was in the middle of the range of Vpu activity in T-cell lines (Figs. 2 and 5C and D).

Interestingly, cell surface expression of tetherin was downregulated following infection with Δ*vpu* virus in H9, CEM-CCRF and CEM-SS cells (Fig. 2). This effect was neither observed in the transduced Sup-T1 cell

line (induced with 5 ng/ml doxycycline (Fig. 2B) or 100 ng/ml doxycycline (Kuhl et al., 2010b)) or in primary CD4$^+$ T-cells (Figs. 5C and D), nor in HeLa or transfected 293T cells (Perez-Caballero et al., 2009; Van Damme et al., 2008), and might therefore be limited to endogenous tetherin in T-cell lines. Vpu-independent partial downregulation of endogenous tetherin in T-cell lines might be mediated by direct antagonism of tetherin *via* another viral protein or be due to more systemic effects of viral infection. As HIV-1 viral proteins, other than Vpu, have not been shown to interact with tetherin, a more systemic effect of viral infection seems likely. This effect might regulate tetherin expression at the transcriptional or post-transcriptional level.

HIV-1 was recently proposed to disrupt important signaling and transcription pathways of the immune system (Doehle et al., 2009; Mogensen et al., 2010). DNA constructs for expression of exogenous tetherin employ viral promoters for transcriptional regulation (*e.g.* CMV promoter), which differ from the promoter region responsible for transcription of the endogenous tetherin gene (Ohtomo et al., 1999). Systemic regulation at the transcription level might therefore only affect endogenous tetherin expression. A recent report on the differential processing of endogenous and exogenous tetherin suggests a systemic effect at a post-translational level (Andrew et al., 2010). In Kaposi's sarcoma-associated herpes virus (KSHV) infection, tetherin is proposed to be antagonized only at the post-translational level by viral ubiquitin ligase K5 (Mansouri et al., 2009). The slight, but statistically insignificant upregulation of tetherin in induced Sup-T1 cells (Fig. 2B) and in primary CD4$^+$ T-cells (Figs. 5C and D) has been previously described (Kuhl et al., 2010b) and was also observed in CEMx174 T/B-cells (Andrew et al., 2010).

We and others have previously shown that *wt* and Δ*vpu* virus are equally infectious in initial cell-free infections and that tetherin and Vpu do not affect the kinetics of the initial round of HIV-1 infection (up to 48 h p.i.) (Kuhl et al., 2010b; Miyagi et al., 2009; Sato et al., 2009; Vendrame et al., 2009). The cell lines tested here showed differential susceptibility towards HIV-1 infection (Fig. 4). As expected, virus release was influenced by differences in infection rates; however, virus release was increased in all cell lines by the presence of Vpu (comparing *wt* and Δ*vpu* virus). The Vpu-mediated effect showed cell line specific variability of up to 10-fold (Fig. 3, Table 1). In primary CD4$^+$ T-cells, Vpu also promoted virus release, an effect that was increased in the IFNα-treated population; IFNα decreased virus release more strongly in Δ*vpu* infected populations than in *wt* infected populations (Fig. 5E, Table 1). Due to the systemic induction of antiviral interferon-stimulated genes (ISGs) (reviewed in Sadler and Williams, 2008), a systemic reduction of virus release (*wt* and Δ*vpu* virus) is not surprising. However, as tetherin is IFNα-inducible and is antagonized by Vpu in HIV-1 infections (Figs. 5A and B, (Neil et al., 2008; Van Damme et al., 2008)), it seems reasonable to argue that the pronounced effect of Vpu on virus release in the IFNα-treated population, when compared to untreated populations, is due to Vpu-mediated tetherin antagonism.

The Vpu-independent effect of cell surface tetherin downmodulation following HIV-1 infection in T-cell lines (Fig. 2), leads to the question whether the Vpu-mediated increase in virus release is directly related to its tetherin cell surface downmodulation activity, as suggested previously (Perez-Caballero et al., 2009; Van Damme et al., 2008). Normalization of the Vpu-mediated increase of virus release by the extent of Vpu-mediated tetherin cell surface downregulation allows such comparison (Table 1). CEM-CCRF cells and their derivative CEM-SS cells showed equally strong correlations of Vpu-mediated tetherin downmodulation and virus release. This similarity also implies that, despite their differences in cell surface expression, tetherin exerts antiviral function in both CEM-CCRF and CEM-SS cells in a similar manner, in contrast to observed differences in regard to APOBEC3G expression (Sheehy et al., 2002). The strong correlation of virus release and modulation of cell surface tetherin levels in CEM-CCRF and CEM-SS cells indicates that cell surface independent tetherin activity in T/B-cell CEMx174 fusion cells might be derived from the B-cell fusion partner rather than from CEM-CCRF cells (Miyagi et al., 2009). The low dependency of virus release on Vpu-mediated tetherin downmodulation in H9 cells is supportive of previous work that suggested an antiviral activity of tetherin independent of its cell surface expression (Miyagi et al., 2009). The low ratios in primary CD4$^+$ T-cells further support the existence of additional, cell surface independent, tetherin-mediated antiviral activities. The intermediate correlation of induced Sup-T1 cells substantiates the use of inducible cell lines as a research tool in regard to tetherin-mediated effects (Kuhl et al., 2010a, 2010b; Rong et al., 2009).

Although there appears to be an inverse correlation between cell surface tetherin and virus release, the effect of Vpu on viral spread at 72 h p.i. was found to be not directly comparable to the extent of Vpu-mediated cell surface downregulation in T-cell lines. For example, Vpu mediates a 2.1–2.3-fold increase of viral spread in all T-cell lines examined (Fig. 4). We have previously shown that initial infection (up to 48 h p.i.) is not affected by Vpu or tetherin (Kuhl et al., 2010b), after which direct cell-to-cell transmission is predominant in T-cell cultures (Chen et al., 2007; Gummuluru et al., 2000; Kuhl et al., 2010b; Sourisseau et al., 2007). Therefore, the Vpu-mediated increase in viral spread is likely to be affected predominantly by Vpu-mediated effects on direct cell-to-cell transmission. In the context of cell-to-cell spread, Vpu might impose a fitness cost to the virus in the absence of tetherin; however, in the presence of tetherin, Vpu mediates an increase in viral spread through its tetherin-antagonizing activity (Kuhl et al., 2010b). Thus, in the context of cell-to-cell transmission of HIV-1 in T-cells, neither tetherin-mediated restriction nor Vpu-mediated antagonism appears to be directly related to particular tetherin cell surface expression levels. This provides further support for tetherin-mediated restriction of cell-to-cell spread (Casartelli et al., 2010; Kuhl et al., 2010b). We have previously reported a ~2-fold Vpu-mediated increase of cell-to-cell spread in Sup-T1 cells induced to ~10-fold higher tetherin cell surface expression (induced by 100 ng/ml doxycycline, Fig. 1B) (Kuhl et al., 2010b) and others reported similar effects of Vpu/tetherin in various cell types, *i.e.* IFN-treated T-cell cultures and primary T-lymphocytes, as well as 293T cells transfected with tetherin (Casartelli et al., 2010; Vendrame et al., 2009).

In primary CD4$^+$ T-cells, Vpu mediated an ~3 fold increase of infection rates when comparing *wt* and Δ*vpu* infected populations (Fig. 5F). As with virus release, the effect was pronounced (~4 fold) in IFNα-treated populations and might be attributable to Vpu-mediated tetherin antagonism. Interferon-α treatment reduced infection rates more than virus release, which indicates a restriction of ongoing infections, with IFNα-mediated restriction acting at both late and early infection stages in the viral life cycle (Vendrame et al., 2009).

Here, we report T-cell line differences in the relationship between tetherin cell surface levels and virus release. Further, Vpu antagonism of tetherin-mediated restriction in ongoing infection in T-cell lines is independent of the level of tetherin cell surface downmodulation. These results, combined with reports of source-dependent tetherin modifications (Andrew et al., 2010) and cell type specific functions unrelated to antiviral function (Cao et al., 2009; Rollason et al., 2009), suggest that single round infection experiments in cells other than T-cells (HeLa or 293T) might not always be representative of tetherin-mediated restriction/function in HIV-1 infections. To our knowledge, this is the first report of specific detection of tetherin cell surface expression and its Vpu-mediated downmodulation in primary activated CD4$^+$ T-cells.

Material and methods

Cell lines

H9, CEM-CCRF and CEM-SS cells were maintained in RPMI-1640 culture medium (Gibco) supplemented with 10% FBS. Transduced Sup-T1 cells were maintained in RPMI-1640 supplemented with 10% tetracycline-free bovine serum albumin (BSA), 2 μg/ml puromycin

(Sigma), and 1 mg/ml G418 (Sigma). Tetherin expression in Sup-T1 cells was induced using doxycycline at concentrations of 100, 20 and 5 ng/ml (Sigma). CEM-CCRF cells were obtained from American Type Culture Collection (ATCC) (Foley et al., 1965); H9 and CEM-SS cells were obtained from the NIH AIDS Research and Reference Reagent Program (Mann et al., 1989; Nara and Fischinger, 1988); Sup-T1 cells stably transduced with the human tetherin gene were previously described (Rong et al., 2009).

Primary CD4$^+$ T-cells

Primary CD4$^+$ T-cells were derived from peripheral mononuclear cells (PBMCs). First, PBMCs were isolated from whole blood of pooled donors by the Ficoll–Hypaque method. CD4$^+$ T-cells were then separated by negative selection using an Untouched Human CD4 T Cells kit (Invitrogen). Cells were cultured in RPMI-1640 culture medium (Gibco) supplemented with 10% FBS and 20 U/ml human interleukin-2 (IL-2), and stimulated with 10 μg/ml phytohemagglutinin A (PHA) for 72 h. For IFNα-treatment of activated cells, culture medium was supplemented with 50 U/ml, 250 U/ml or 500 U/ml Interferon-α-2b (Invitrogen) (Rong et al., 2009).

Viruses

Site-directed mutagenesis, using the QuickChange XLII Site-Directed Mutagenesis Kit (Stratagene), was used to introduce nucleotide changes into the coding regions of *vpu* and/or *env*, resulting in two stop codons at the beginning of Vpu and/or Env coding regions of the viral clone pBR43-IRES-eGFP (NIH AIDS Research and Reference Reagent Program), expressing enhanced green fluorescent protein (eGFP) from an internal ribosomal entry site downstream of *nef*. Virus was produced in 293T cells using Lipofectamine2000 (Invitrogen) as a transfection reagent; virus was pseudotyped with Vesicular stomatitis virus protein G (VSV-G). Virus was collected after 48 h, filtered (0.45 μm), and viral capsid/p24 protein (CA p24) content was quantified by a Vironostika HIV-1 Ag kit (bioMérieux).

HIV-1 infections

For single round infections, cells populations were infected with VSV-G pseudotyped *wt* or Δ*vpu* virus to 10% infection rates, as determined by flow cytometric detection of virus-derived eGFP expression at 48 h p.i., in order to minimize superinfection events. For studies of ongoing infection, cells were infected with 600 ng CA p24 per 10^6 cells by spinoculation (1500×*g*, at 37 °C for 2 h), followed by incubation for 1 h at 37 °C, after which virus was removed by centrifugation and cell washing.

Flow cytometry

Infection rates were determined by studying virus-derived eGFP expression. Infection rates in single round infections were determined at 48 h p.i.; infection rates of ongoing infections was determined at 72 h p.i. Levels of cell surface tetherin in infected and uninfected cells were assessed by flow cytometry for PerCP (peridinin chlorophyll protein) at 72 h p.i. Staining for cell surface tetherin was performed using a primary rabbit anti-human-tetherin polyclonal antibody (1:3000) (NIH AIDS Research and Reference Reagent Program (Miyagi et al., 2009)), followed by a PerCP-labeled secondary goat anti-rabbit antibody (1:250) (Santa Cruz Biotechnology). Cells were stained at 4 °C for 30 min and fixed in 4% paraformaldehyde for 25 min. Uninfected and infected cells were distinguished by virus-derived eGFP expression. Flow cytometry analysis was performed on a minimum of 30,000 cells using a FACSCalibur instrument (Becton Dickinson) and FlowJo 7.5 software (Tree Star).

Virus release

Virus release into the supernatant was analyzed at 24, 48 and 72 h p.i. using a quantitative reverse transcription-based assay (Oliveira et al., 2009).

Statistical analysis

Data from at least three independent experiments were analyzed utilizing GraphPad PRISM 5 software. Differences were analyzed for statistical significance using a one-way analysis of variance (ANOVA) with Bonferroni's post-test for groups and Student's *t*-test for pairs of data.

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