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Brief Communication

Immune evasion activities of accessory proteins Vpu, Nef and Vif are conserved in acute and chronic HIV-1 infection

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ABSTRACT

Heterosexual HIV-1 transmission has been identified as a genetic bottleneck and a single transmitted/founder (T/F) variant with reduced sensitivity to type I interferon initiates productive infection in most cases. We hypothesized that particularly active accessory protein(s) may confer T/F viruses with a selective advantage in establishing HIV infection. Thus, we tested *vpu*, *vif* and *nef* alleles from six T/F and six chronic (CC) viruses in assays for 9 immune evasion activities involving the counteraction of interferon-stimulated genes and modulation of ligands known to activate innate immune cells. All functions were highly conserved with no significant differences between T/F and CC viruses, suggesting that these accessory protein functions are important throughout the course of infection.

Introduction

Studies have defined a genetic 'bottleneck' at mucosal HIV-1 transmission with a single inferred variant initiating peripheral viremia in most cases (Baalwa et al., 2013; Fenton-May et al., 2013; Haaland et al., 2009; Ochsenbauer et al., 2012; Parrish et al., 2013; Salazar-Gonzalez et al., 2009). In particular it has been noted that transmitted founder (T/F) Envelope proteins are R5 tropic with both increased sensitivity to broadly neutralizing antibodies (Wilen et al., 2011) and reduced ability to interact with maraviroc-bound CCR5 compared to viruses isolated from chronic infection (Parker et al., 2013). Furthermore, preferential transmission of ancestral as opposed to contemporary strains in donor individuals has been reported (Redd et al., 2012), suggesting specific viral determinants for sexual transmission that differ from determinants for propagation of chronic infection. Intriguingly, it has been reported that T/F viruses are less restricted by interferon (IFN) in primary T cells than chronic controls (Fenton-May et al., 2013; Parrish et al., 2013).

Lentiviral accessory proteins have evolved the ability to mitigate the detrimental effects of an IFN-induced antiviral immune response by interfering with various host immunity factors (Harris et al., 2012; Moll et al., 2010; Shah et al., 2010). For example, HIV-1 Vpu and Vif counteract the IFN-inducible restriction factors tetherin/BST-2/CD317 (Neil et al., 2008; Van Damme et al., 2008) and APOBEC3D/F/G, respectively (Bishop et al., 2004; Hultquist et al., 2011; Sheehy et al., 2002; Zheng et al., 2004). Vpu also prevents expression of IFN and IFN-stimulated genes by inhibiting the activation of NF-κB (Bour et al., 2001; Sauter et al., 2015), and Nef is known to modulate a range of host immune receptors including CD4 and MHC molecules (Kirchhoff, 2010).

We hypothesized that accessory gene(s) from T/F viruses confer advantages to establishment and early propagation of HIV-1 infection either through counteraction of IFN-stimulated genes or modulation of ligands known to activate NK, NKT and other immune cells. We therefore directly compared functional activities of the accessory proteins Vpu, Vif and Nef from six T/F and six CC viruses (see Table 1 for patient characteristics and Supplementary Figs. 1–3 for multiple sequence alignments).

Results

Counteraction of tetherin/BST-2/CD317 and NF-κB activation by Vpu

Vpu proteins from T/F and CC viruses were tested for their ability to increase virion release by counteracting tetherin/BST-2/CD317. Tetherin serves as a physical tether between viral and cellular membranes and

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Table 1
Origins and characteristics of viruses used in this study.

Virus	Country of origin	Gender	Transmission route	Fiebig stage	Viral load at isolation	Cd4 count at isolation	Coreceptor tropsim	TF/CC	HIV-1 subtype
ZM246F	Zambia	F	Heterosexual	II	10,013,800	NA	R5	TF	C
ZM247F	Zambia	F	Heterosexual	II	10,823,500	NA	R5	TF	C
ZM249M	Zambia	M	Heterosexual	IV	>2,000,000	NA	R5	TF	C
CH040	USA	M	MSM	II	2,197,248	NA	R5	TF	B
WITO	USA	M	Heterosexual	II	325, 064	NA	R5	TF	B
CH077	USA	M	MSM	II/III	394, 649	NA	R5/X4	TF	B
CH432	Malawi	M	Heterosexual	NA	40,570	261	R5	CC	C
CH457	Tanzania	F	Heterosexual	NA	234,671	450	R5	CC	C
CH534	South Africa	F	Heterosexual	NA	NA	NA	NA	CC	C
RHGA	USA	M	MSM	NA	50,000	571	R5	CC	B
WARO	USA	F	MSM	NA	16,758	598	R5	CC	B
STCOr1	USA	M	MSM	NA	67,964	796	R5/X4	CC	B

Key: MSM: men who have sex with men; TF – transmitted/founder virus; CC- Chronic virus; NA – not available; Feibig stage as described previously (Fiebig et al., 2003)

inhibits the egress of budding virions from infected cells. All Vpus were expressed (Fig. 1A) and enabled efficient virus release from tetherin expressing HEK293T cells. Anti-tetherin activity was similar for Vpus from T/F and CC viruses ($p=0.51$, Fig. 1B), consistent with a previous study demonstrating conservation of tetherin antagonism over extended periods in chronically infected patients (Pickering et al., 2014).

Tetherin does not only restrict virion release but also acts as an innate sensor activating NF-κB-dependent expression of type I IFN and IFN-stimulated genes (Galao et al., 2012). We therefore investigated whether T/F Vpus are more efficient in blocking tetherin signaling than Vpus from CC viruses. A dual luciferase reporter assay in HEK293T cells revealed that all Vpus efficiently inhibited the activation of NF-κB with no significant differences between the T/F and CC groups ($p=0.81$, Fig. 1C). Since Vpu has also been reported to block activation of NF-κB independently of tetherin (Bour et al., 2001; Sauter et al., 2015), we repeated the experiment using a constitutively active mutant of IKKβ instead of tetherin as the inducer. Again, T/F and CC Vpus suppressed NF-κB activation to similar degrees ($p=0.31$, Fig. 1D).

Cell surface downregulation of CD4, NTB-A and CD1d by Vpu

Besides counteracting tetherin and inhibiting NF-κB activation, Vpu also decreases cell surface expression of CD4, NTB-A and CD1d. While downmodulation of CD4 prevents superinfection and enables the release of fully infectious progeny virions from infected cells (Kimura et al., 1994; Lama et al., 1999; Wildum et al., 2006), downmodulation of NTB-A and CD1d has been suggested to protect HIV-1 infected cells from NK or NKT cell mediated killing, respectively (Moll et al., 2010; Shah et al., 2010). Using flow cytometry, we monitored expression of these receptors on the surface of HEK293T cells transfected with Vpu or a vector control. Vpu reduced surface levels of all three receptors as expected, without significant differences between T/F and CC viruses (CD4: $p=0.84$, NTB-A: $p=0.61$, CD1d: $p=0.34$, Fig. 1E–J).

Counteraction of APOBEC3F/G by Vif

The accessory protein Vif induces the ubiquitination and subsequent degradation of the restriction factors APOBEC3DF/G/H to prevent their incorporation into viral particles (Bishop et al., 2004; Hultquist et al., 2011; Sheehy et al., 2002). APOBEC3F/G are cytidine deaminases, inhibiting HIV-1 replication through G to A hypermutation of viral cDNA as well as direct inhibition of reverse transcription itself (Gillick et al., 2013). We analyzed the activity of T/F and CC Vif proteins against human APOBEC3F and APOBEC3G by co-transfecting HEK293T cells with plasmids encoding *vif*-deficient HIV-1 along with APOBEC3F or APOBEC3G and *vif* alleles/empty plasmid. As Vif expression/ stability varied between alleles tested, the amount of Vif expression plasmid was modified in order to achieve similar amounts of Vif protein in the producer cells (Fig. 2A). Under these conditions the infectivity of supernatant viruses was determined, revealing that Vifs from T/F and CC viruses have equivalent activity against APOBEC3F and APOBEC3G (Fig. 2B and C).

Cell surface downregulation of CD4, CD28 and MHC class I by Nef

The accessory protein Nef downregulates various cell surface proteins including CD4, MHC class I and CD28 to escape immune surveillance (Garcia and Miller, 1991; Schwartz et al., 1996; Swigut et al., 2001) and may thus also be involved in the reduced sensitivity of T/F viruses to IFN. CD4 and CD28 downregulation were analyzed by transient transfection of HEK293T cells with vectors expressing Nef and either CD4 or CD28. All Nef proteins were efficiently expressed (Fig. 3A) and reduced surface expression of both receptors with no significant differences between T/F and CC viruses (CD4: $p=0.71$, CD28: $p=0.76$, Fig. 3B–E). To explore MHC class I downregulation, a HeLa cell line expressing HLA-B*27 was transfected with different *nef* alleles and MHC class I cell surface expression was analyzed by FACS 48 h post-transfection. Nef proteins of both groups of viruses downmodulated MHC class I to a similar extent ($p=0.22$, Fig. 3F and G).

Nef-mediated enhancement of virion infectivity

It has been shown that HIV-1 virion infectivity is reduced in the absence of a functional *nef* gene (Aiken and Trono, 1995; Chowers et al., 1994). We thus tested the effect of Nef proteins on virion infectivity by transfecting HEK293T cells with *nef* alleles and *nef*-deficient HIV-1 NL4-3. Supernatants harvested two days post-transfection were used to infect indicator TZM-bl cells with normalized amounts of p24. Despite variation between individual Nef proteins in their ability to enhance virion infectivity, there was no statistical difference when T/F and CC Nefs were compared ($p=0.77$, Fig. 3H).

Discussion

In summary, our results show that established functions of the accessory proteins Vpu, Vif and Nef are conserved across T/F and CC viruses. Specifically, our results from transient expression studies suggest that the increased IFN resistance of newly transmitted HIV-1 strains is not the result of a particularly efficient Vpu-mediated inhibition of NF-κB activation or counteraction of the restriction factors tetherin and APOBEC3F/G. An important limitation is that viral proteins were not expressed from a provirus in our experiments, and therefore it is possible that *in vivo* expression may differ.