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Construction of Nef-positive doxycycline-dependent HIV-1 variants using bicistronic expression elements

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ABSTRACT

Conditionally replicating HIV-1 variants that can be switched on and off at will are attractive tools for HIV research. We previously developed a genetically modified HIV-1 variant that replicates exclusively when doxycycline (dox) is administered. The nef gene in this HIV-rtTA variant was replaced with the gene encoding the dox-dependent rtTA transcriptional activator. Because loss of Nef expression compromises virus replication in primary cells and precludes studies on Nef function, we tested different approaches to restore Nef production in HIV-rtTA. Strategies that involved translation via an EMCV or synthetic internal ribosome entry site (IRES) failed because these elements were incompatible with efficient virus replication. Fusion protein approaches with the FMDV 2A peptide and human ubiquitin were successful and resulted in genetically-stable Nef-expressing HIV-rtTA strains that replicate more efficiently in primary T-cells and human immune system (HIS) mice than Nef-deficient variants, thus confirming the positive effect of Nef on in vivo virus replication.

Introduction

The Nef protein encoded by the primate lentiviruses - human immunodeficiency virus type 1 and 2 (HIV-1, HIV-2) and simian immunodeficiency virus (SIV) - is considered to be an accessory protein, because it is dispensable for viral replication in T cell lines but contributes to efficient replication and disease progression in HIV-1 infected individuals and SIV-infected macaques. Nef interacts with various cellular proteins and a multitude of Nef activities have been described, including down-modulation of surface expression of CD4 and MHC-I in infected cells and counteracting cellular restriction factors (Basmaciogullari and Pizzato, 2014; Fackler et al., 2006; Kirchhoff et al., 2008; Rosa et al., 2015; Usami et al., 2015).

Inactivation of Nef results in attenuated HIV and SIV variants that yield a reduced viral load upon infection (Gibbs et al., 1994; Kestler et al., 1991; Watkins et al., 2013; Zou et al., 2012). Because attenuated viruses can be very effective in inducing protective immunity against pathogenic viruses (e.g. smallpox, polio and measles virus), Nef-deleted HIV and SIV variants were considered as prophylactic vaccine candidate. Vaccination of macaques with such SIV variants does indeed provide robust protection against a challenge with pathogenic strains (reviewed in (Desrosiers, 1998; Johnson, 1999; Mills et al., 2000)). However, the attenuated vaccine virus persists and can revert to virulence, which caused disease in a minority of the vaccinated animals (Baba et al., 1995, 1999; Chakrabarti et al., 2003; Whatmore et al., 1995). Also some of the long-term survivors of the Sydney Blood Bank Cohort infected with a natural Nef-deficient HIV-1 variant did eventually progress to AIDS (Zaunders et al., 2011). Furthermore, an HIVΔ3 variant with deletions in the nef, vpr and LTR sequences regained substantial replication capacity in long-term cell culture infections by acquisition of compensatory changes elsewhere in the viral genome (Berkhout et al., 1999). These results highlight the genetic instability and evolutionary capacity of attenuated HIV and SIV strains, which poses a serious safety risk for the application of live-attenuated HIV vaccines in humans.

We and others previously presented a Nef-deleted HIV-1 variant that replicates in the presence of doxycycline (dox) but not in the absence of this antibiotic (Berkhout et al., 2002; Das et al., 2002, 2004a; Smith et al., 2001; Verhoef et al., 2001). In this HIV-rtTA variant, the Tat-TAR regulatory mechanism that normally controls viral gene expression was inactivated by mutation of both the Tat gene and the TAR RNA structure, and functionally replaced by the

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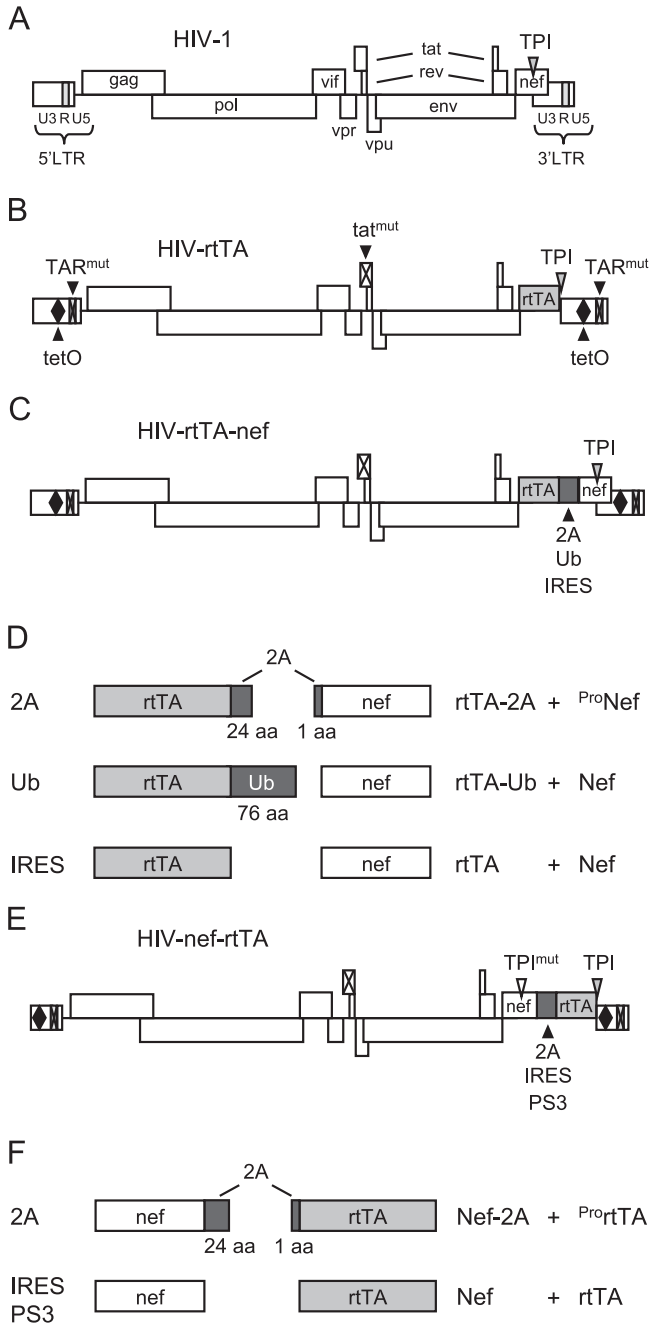

Fig. 1. *HIV-rtTA constructs.* (A) Schematic of the HIV-1 proviral DNA genome with the LTR region subdivided in U3, R and U5 domains. The nef gene overlies the TPI region, which consists of the T-rich sequence, polypurine tract (ppt), and attachment/integration sequence (att/int). (B) In HIV-rtTA, the Tat-TAR axis of transcription regulation has been inactivated by a Tyr-26-Ala substitution in Tat (Tat^{mut}) and multiple nucleotide substitutions in the bulge and loop of TAR (TAR^{mut}). Transcription and replication of the virus was made dox-dependent by the introduction of tetO elements in the U3 promoter region and replacing the nef gene by the rtTA gene. Furthermore, a 198-nt fragment of the nef/U3 sequence downstream of the TPI region was deleted to reduce the genome size. (C) In the HIV-rtTA-nef variants, the wild-type nef, TPI and U3 sequences were restored. The rtTA and nef genes were separated by the 25-amino acid 2A sequence, the 76-amino acid Ub sequence or the 585-nt EMCV IRES element (indicated with IRES). (D) Nef and rtTA protein products resulting from the rtTA-2A-nef, rtTA-Ub-nef and rtTA-IRES-nef configuration. (E) In the HIV-nef-rtTA variants, the nef gene with a mutated TPI sequence (TPI^{mut}) was introduced downstream of the env gene and the U3 region was further truncated by deleting all nef/U3 sequences downstream of the TPI (from the EcoRV site at U3 position 35) and upstream of the tetO sites. The nef and rtTA genes were separated by the 25-amino acid 2A sequence, the EMCV IRES element or the 50-nt PS3 IRES element (indicated with PS3). (F) Nef and rtTA protein products resulting from the nef-2A-rtTA, nef-IRES-rtTA and nef-PS3-rtTA configuration.

Tet-On system for inducible gene expression (Fig. 1A and B)(Baron and Bujard, 2000). The rtTA gene encoding a man-made transcriptional activator was inserted in place of the nef gene and tet-operator (tetO) DNA binding sites were inserted into the LTR promoter. Since the rtTA protein can only bind tetO and activate transcription in the presence of dox, HIV-rtTA replicates exclusively when dox is administered. The HIV-rtTA variant has been improved significantly by virus evolution (Das et al., 2004b; Marzio et al., 2001; Marzio et al., 2002; Zhou et al., 2006a, 2006b, 2006c) and we have demonstrated dox-dependent replication in vitro in T cell lines, ex vivo in human lymphoid tissue (Kiselyeva et al., 2004) and in vivo in humanized mice (Legrand et al., 2012).

In principle, the conditional replication feature may improve the safety of a live-attenuated virus vaccine because replication can be temporarily activated by dox to the extent needed for induction of the immune system. Subsequent dox-withdrawal will stop viral gene expression and replication, which should prevent evolution of the vaccine virus toward a pathogenic variant. Application of a conditionally replicating HIV-rtTA as a vaccine in humans will require additional safety measures. Safety could be increased by the inclusion of an additional drug-dependent control mechanism (Das et al., 2005) and by deletion of the integrase (IN) function. Because IN-deficient HIV-1 variants do not replicate in most cell lines and primary cells (Nakajima et al., 2001), an alternative replication mechanism, like the simian virus 40 replication signals, should be integrated (Lu et al., 2004). An efficiently replicating, drug-controlled and non-integrating HIV-1 variant has however not yet been developed.

Nevertheless, the dox-controlled HIV-rtTA and a similar SIV variant (Das et al., 2007, 2008) can be used in vaccination studies in laboratory animals to learn - for example - whether continuous low-level replication, which is typical for a regular live-attenuated virus, is required for protection and what protective correlates are induced by an effective vaccine (Manoussaka et al., 2013). Furthermore, these viruses can be used in cell culture and animal experiments to study HIV-1 and SIV biology. Because the nef gene was replaced with the rtTA gene, HIV-rtTA will however replicate suboptimally in primary cells and in vivo. HIV-rtTA did indeed demonstrate an attenuated phenotype when tested in a humanized mouse model of hematopoiesis that harbors all major components of the human immune system (HIS) (Legrand et al., 2012). Although infection of dox-fed HIS mice resulted in the establishment of a productive infection, HIV-rtTA replication was delayed compared to the parental X4-tropic HIV-1 LAI strain and was not accompanied by $CD4^+$ T-cell depletion (Legrand et al., 2012). In vaccination studies, this attenuated phenotype is likely to restrict the induction of protective immune responses (Lohman et al., 1994; Wyand et al., 1996). The vaccine response may also be suboptimal as no response against Nef will be induced. We therefore considered different approaches to construct a Nef-positive HIV-rtTA variant. This study resulted in HIV-rtTA-nef variants that express all HIV-1 proteins, which can be used for future vaccination studies in the humanized mouse model (An et al., 2007). These virus variants can also be used as a research tool for studying the Nef protein function.

Results

Design of Nef-positive HIV-rtTA variants

The nef gene is positioned at the 3′ end of the HIV-1 genome and partially overlaps the U3 sequence of the 3′ LTR (Fig. 1A). Moreover, the nef gene overlies the T-rich sequence (T) and the polypurine tract (PPT), which are important motifs for reverse transcription of the RNA genome into a DNA copy, and the