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Construction of doxycycline-dependent mini-HIV-1 variants for the development of a virotherapy against leukemias

Rienk E Jeeninga¹, Barbara Jan¹, Henk van den Berg² and Ben Berkhout*¹

Address: ¹Laboratory of Experimental Virology, Department of Medical Microbiology Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands and ²Department of Paediatric Oncology, Emma Children Hospital, Academic Medical Center of the University of Amsterdam, Amsterdam, The Netherlands

Email: Rienk E Jeeninga - r.jeeninga@amc.uva.nl; Barbara Jan - janbarbara@hotmail.com; Henk van den Berg - h.vandenberg@amc.uva.nl; Ben Berkhout* - b.berkhout@amc.uva.nl

* Corresponding author

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Abstract

T-cell acute lymphoblastic leukemia (T-ALL) is a high-risk type of blood-cell cancer. We describe the improvement of a candidate therapeutic virus for virotherapy of leukemic cells. Virotherapy is based on the exclusive replication of a virus in leukemic cells, leading to the selective removal of these malignant cells. To improve the safety of such a virus, we constructed an HIV-1 variant that replicates exclusively in the presence of the nontoxic effector doxycycline (dox). This was achieved by replacement of the viral TAR-Tat system for transcriptional activation by the Escherichia coli-derived Tet system for inducible gene expression. This HIV-rtTA virus replicates in a strictly dox-dependent manner. In this virus, additional deletions and/or inactivating mutations were introduced in the genes for accessory proteins. These proteins are essential for virus replication in untransformed cells, but dispensable in leukemic T cells. These minimized HIV-rtTA variants contain up to 7 deletions/inactivating mutations (TAR, Tat, vif, vpR, vpU, nef and U3) and replicate efficiently in the leukemic SupT1 T cell line, but do not replicate in normal peripheral blood mononuclear cells. These virus variants are also able to efficiently remove leukemic cells from a mixed culture with untransformed cells. The therapeutic viruses use CD4 and CXCR4 for cell entry and could potentially be used against CXCR4 expressing malignancies such as T-lymphoblastic leukemia/lymphoma, NK leukemia and some myeloid leukemias.

Background

Virotherapy has been proposed as a novel therapeutic means against certain cancers and is currently being evaluated in clinical trials [1-3]. This novel strategy is based on the selective replication of viruses in specific target cells to efficiently remove these cells from the patient. Initial successes have been reported in the treatment of head and neck cancers using an engineered adenovirus [4-7], but doubts remain about the absolute restriction of virus replication in cancer cells [8]. In an ideal setting, the thera-

peutic virus should replicate exclusively in malignant cells. A large number of target cells will enable a fast spreading viral infection at the start of therapy. Consequently, the number of target cells will rapidly decline and result in a concurrent reduction of the virus population. It may be necessary to modify therapeutic viruses to increase their replication specificity and/or to modulate their cytopathogenicity. For instance, cytotoxic genes may be incorporated into the viral genome or virus spread may be improved by inclusion of genes encoding fusogenic pro-

teins [9]. Experiments have thus far focused on virotherapy of solid tumors. Therapeutic viruses have been described based on adenovirus [10,11], herpes simplex virus [12], Newcastle disease virus, poliovirus, vesicular stomatitis virus, measles virus and reovirus [1-3]. No therapeutic viruses have been described that replicate in lymphoid-leukemic cells.

We explored the possibility to use HIV-1 derived viruses, which specifically target T-lymphocytes, as therapeutic virus for leukemia and recently reported the proof of principle with a minimal HIV-1 variant [13]. Our approach was based on the observation that several accessory proteins are not needed for HIV-1 replication in transformed T-cell lines, yet are important for virus replication in primary cells. A minimized derivative of HIV-1 with five gene deletions (vif, vpR, vpU, nef and U3) was demonstrated to replicate in several leukemic T cell lines, but not in normal peripheral blood mononuclear cells (PBMC).

Obvious safety concerns remain for the development of therapeutic viruses based on the human pathogen HIV-1. One of the major concerns is the high mutation and recombination rate of HIV-1 that allows the generation of escape variants over time. For instance, virus evolution frequently leads to the appearance of drug-resistant mutants in patients on antiviral therapy. It could be argued that repair of gene deletions would be impossible, but one cannot exclude alternative viral strategies to improve its fitness or replication capacity. Such an indirect escape strategy has been reported for a HIV-1 vaccine candidate with three gene deletions [14]. Gradual improvement of viral fitness has also been reported for persons infected with a nef-deleted virus variant, coinciding with AIDS disease progression in some of these patients [15]. We therefore designed a method to gain full control over viral replication. For this, we combined the minimal HIV-1 strategy with that of the HIV-rtTA virus [16], a vaccine candidate that was engineered to replicate exclusively in the presence of the nontoxic effector dox. The latter was achieved by replacement of the viral TAR-Tat system for transcriptional activation by the *Escherichia coli*-derived Tet system for inducible gene expression [17]. HIV-rtTA lacks several protein coding genes and non-coding structural elements and replicates in a strictly dox-dependent manner, and has been proposed as a safe form of an attenuated vaccine strain because its replication can be turned on and off at will.

We designed two molecular clones based on HIV-rtTA. rtTA Δ 6^A carries four deletions (vif, vpR, nef and U3) and two genome regions with inactivating mutations (TAR, vpU). rtTA Δ 6^B has five deletions (vif, vpR, vpU, nef and U3) and inactivating mutations in TAR. The efficacy of these therapeutic viruses was tested by replication studies

in the leukemic T-cell line SupT1 and PBMC. Both viruses replicate efficiently and in a dox-dependent manner in SupT1 cells, resulting in rapid cell killing. In contrast, these viruses are unable to replicate in PBMC. Furthermore, the rtTA Δ 6^A and rtTA Δ 6^B viruses were able to selectively infect and remove the SupT1 cells from a mixed culture with PBMC.

Results

Design of dox-inducible mini-HIV variants

We recently reported the development of a mini-HIV-1 variant for virotherapy of T-ALL [13]. This minimized HIV-1 derivative carries five deletions (vif, vpR, vpU, nef and U3). The deleted genes/motifs contribute to virus replication in untransformed cells, but are dispensable for replication in leukemic T-cells. To obtain control over virus replication, we now combined the mini-HIV approach with the dox-dependent HIV-rtTA concept [16,18]. The nef gene in HIV-rtTA is replaced by the gene encoding the rtTA protein (Fig. 1). In the presence of dox, the transcriptional activator rtTA protein binds to tetO binding sites that were introduced in the U3 domain of the LTR promoter (Fig. 1, black box). Tat-mediated transcriptional activation is abrogated by an inactivating mutation in the tat gene (Tyr26Ala)[19,20] and multiple inactivating mutations in the TAR hairpin (indicated by crosses in Fig. 1) [16]. HIV-rtTA also carries a deletion of a large upstream part of the U3 domain [21] and thus represents a Δ 4 HIV-rtTA genome (Fig. 1, Δ TAR, tat, nef, U3). HIV-rtTA was further minimized by deletion of genes encoding the accessory proteins Vif and VpR. Additionally, the vpU gene was inactivated in rtTA Δ 6^A (Fig. 1) by mutation of the startcodon (AUG to AUA) and in rtTA Δ 6^B by gene deletion. Due to the vpU-cloning procedure, the wild type tat gene was restored. These minimized rtTA Δ 6^A and rtTA Δ 6^B variants express the basic set of HIV-1 proteins (gag, pol, env), the essential Rev and Tat proteins, but lack the accessory proteins Vif, VpR, VpU and Nef. The RNA genome of rtTA Δ 6^B is 8,872 nt compared to 9,229 nt for full length HIV-1 LAI and 9,607 nt for the parental HIV-rtTA virus.

Replication characteristics of the mini-rtTA viruses

Viral gene expression and production of virus particles was tested by transfection of the mini-rtTA plasmids in C33A cells. These cells lack the CD4 receptor and are thus not susceptible for multiple rounds of HIV-1 replication. We measured no difference in virus production of the mini-rtTA viruses compared with the original HIV-rtTA construct (Fig. 2). All constructs are fully dependent on dox for gene expression. These results demonstrate that none of the deleted/mutated genes/motifs play an important role in viral gene expression (transcription, splicing, and translation) and the assembly of new virions. Virus production of all dox-dependent rtTA viruses is somewhat

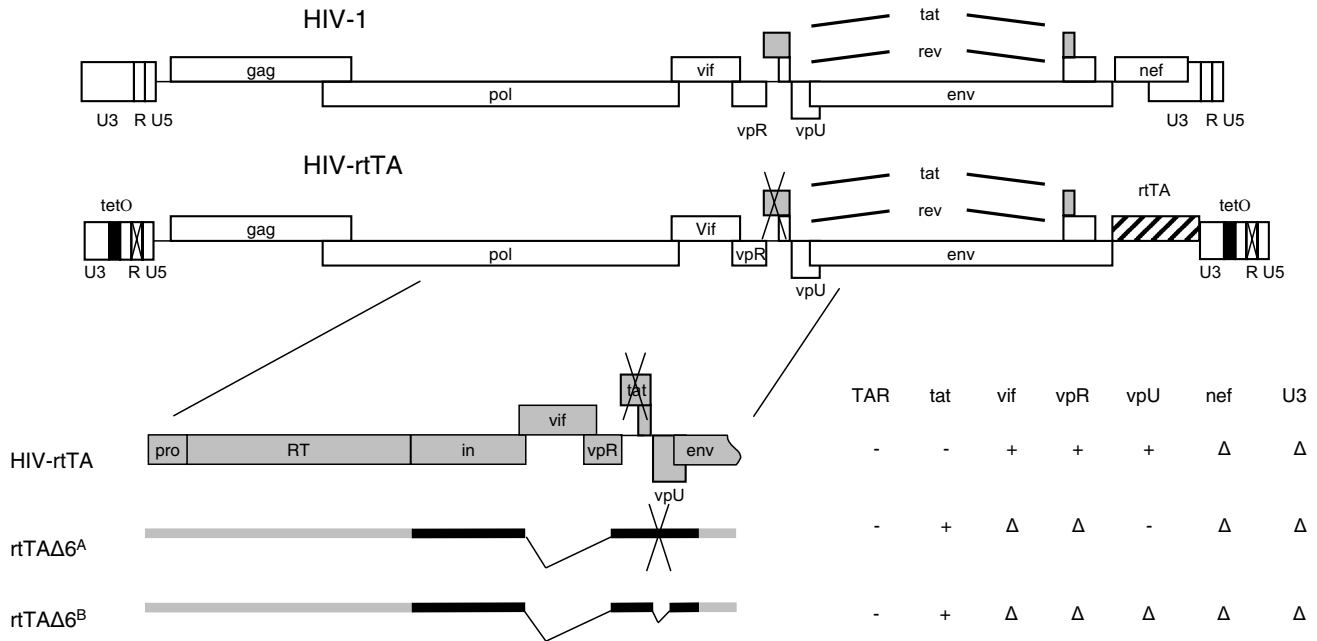


Figure 1

Overview of the minimal HIV-rtTA molecular clones. Schematic overview of the different molecular clones used in this study. The position of the various deletions and a summary of the inactivated (-) or deleted (Δ) viral genes/motifs is provided. See the Materials and Methods section for details on the construction. See also Fig. 7 for further details.

lower than that of the wild type LAI virus, consistent with our previous studies [16,22].

The virus stocks produced in C33A cells were used to infect the HIV-susceptible leukemic T-cell line SupT1. Virus replication was followed by sampling of the culture supernatant and measurement of the CA-p24 concentration (Fig. 3, left panel). Surprisingly, replication of the minimized rtTAΔ6^A and rtTAΔ6^B variants is significantly faster than that of the parental HIV-rtTA virus and even faster than the wild type LAI virus. Similar results were obtained in multiple replication assays that were initiated either by virus infection or by transfection of the molecular clones (results not shown). Direct virus competition assays confirmed this ranking order, with rtTAΔ6^A being slightly more fit than rtTAΔ6^B, and both much more fit than HIV-rtTA (results not shown). This surprise finding

will be dealt with in detail later in this paper. As expected, virus replication is fully dependent on dox addition.

The T-cell cultures were also analyzed for the cell killing capacity of these viruses. A time-limited FACS analysis was used to determine the relative number of live cells in the infected cultures and a mock-infected SupT1 culture as the control. The cell killing capacity was determined by dividing the number of cells in the infected culture by the number of cells in the control culture (Fig. 3, right panel). The LAI virus and the different HIV-rtTA variants are able to kill all SupT1 cells. The cell killing kinetics correlate nicely with the replication capacity of the respective viruses. There was no decrease in the number of live cells when the HIV-rtTA virus was tested without dox, confirming that the increase in cell death is the result of active virus replication.

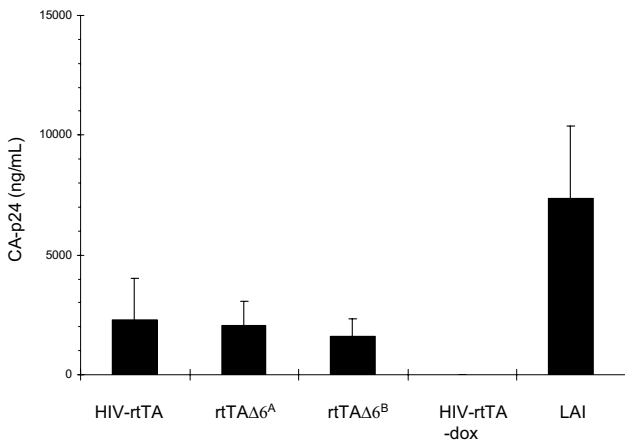


Figure 2
Virus production of HIV-rtTA constructs. The non-susceptible C33A cell line was transfected with five microgram of the indicated plasmids. The culture supernatant was harvested after three days and used in a CA-p24 Elisa to determine virus production. All rtTA samples were cultured with 1000 ng/mL dox unless indicated otherwise. The figure is representative for three independent transfections.

We tried to set up experiments with patient derived primary leukemic T-cells but the high death rate of these cells in in vitro culture experiments (without any virus) prevented any significant conclusions to be reached about virus-induced cell killing (results not shown).

Switching virus replication on and off at will

Dox-regulation should allow strict control over replication of the therapeutic viruses. To demonstrate the regulatory possibilities of this system, we followed several rtTAΔ6A cultures with different dox regimens, ranging from no to continuous dox treatment. We also tested delayed dox addition and dox-withdrawal near the peak of infection. Virus infections were started with dox (Fig. 4, upper left panel) or without dox (Fig. 4, lower left panel) and virus production was followed by measurement of the CA-p24 concentration in the supernatant. After nine days, the cultures were split and either continued with the same treatment (Fig. 4, left panels) or switched from dox to no dox (Fig. 4, upper right panel) or vice versa (Fig. 4, lower right panel). The results show that virus replication is completely controllable by dox. In the cultures with dox a productive infection is started that can be turned off by withdrawal of dox. In the cultures that were started without dox, a single round of infection takes place that leads to the establishment of an integrated but silent provirus,

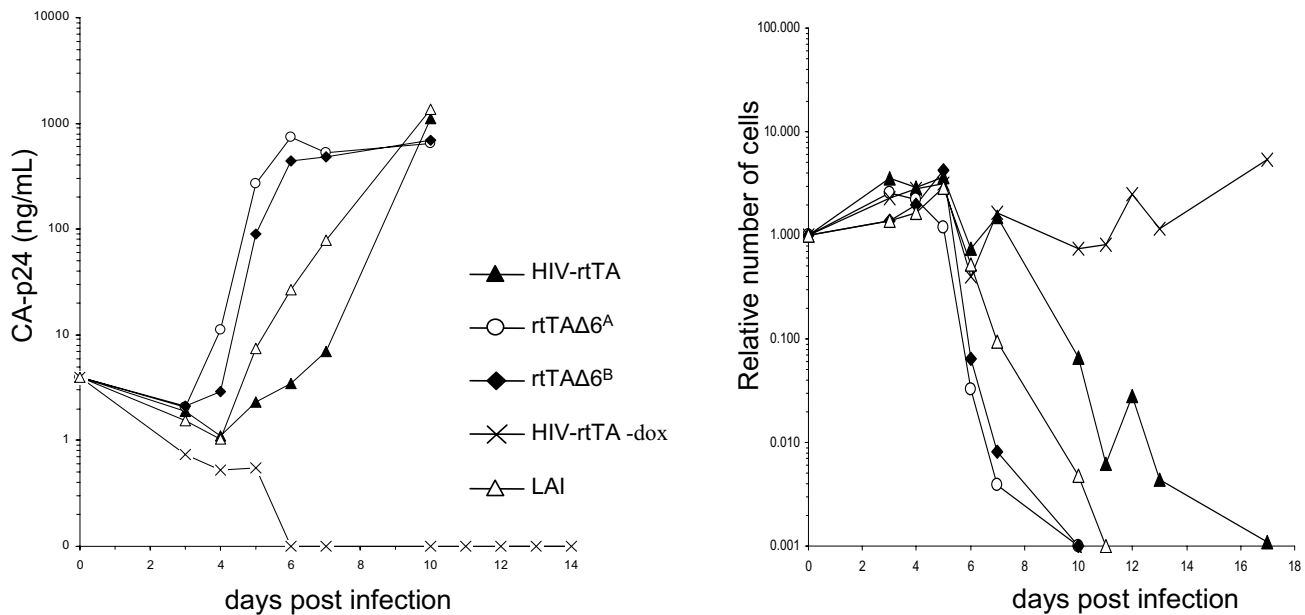


Figure 3
Replication and cell killing capacity of dox-inducible viruses in SupT1 cells. (Left) Virus replication of LAI (Δ), HIV-rtTA (▲), HIV-rtTAΔ6A (○), HIV-rtTAΔ6B (◆) and HIV-rtTA without dox (×) was determined by measuring of the supernatant CA-p24 concentration after infection with virus (20 ng CA-p24) in a 5 mL SupT1 culture. (Right) The number of cells in each culture was determined by a 30 sec time limit FACS analysis. The cell killing capacity of the viruses was determined as the ratio of SupT1 cells present in the infected culture versus the uninfected control culture.

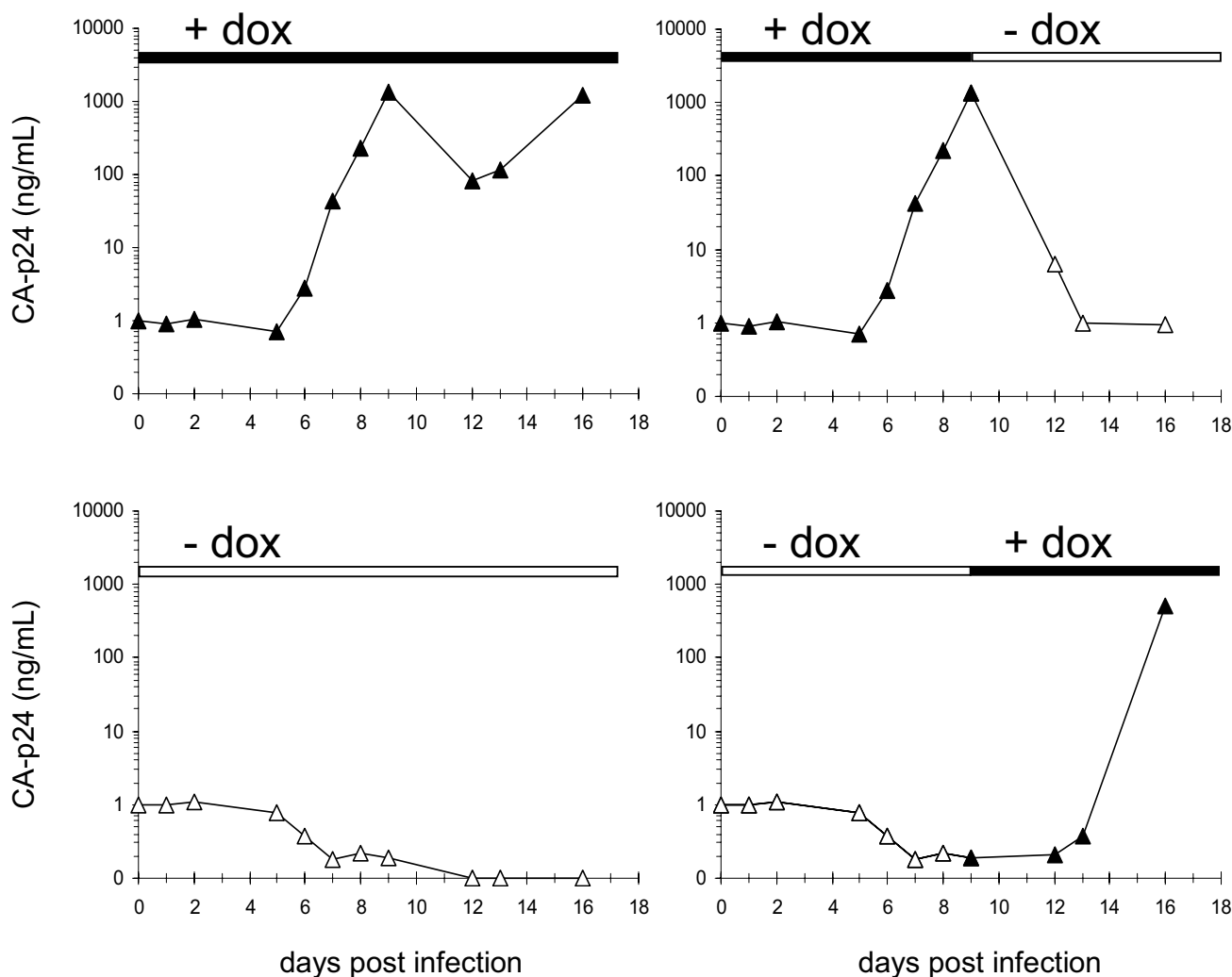


Figure 4
Dox regulated replication of the mini-rtTA virus rtTA Δ 6^A. SupT1 cells were infected with rtTA Δ 6^A virus (1 ng CA-p24). The culture was split and the cells were cultured with dox (upper panels) or without (lower panels). Virus replication was monitored by CA-p24 Elisa on the culture supernatant. At day 9 post infection, both cultures were washed and each culture split into one culture with dox (left panels) and one without (right panels). Filled triangles indicate cultures without dox and open triangles indicate cultures with 1000 ng/mL dox.

which can subsequently be activated by the addition of dox.

Replication characteristics of the HIV-rtTA viruses in PBMC

The different HIV-rtTA viruses were further analyzed by testing their replication capacity on PBMC (Fig. 5, left panel). Killing of the CD4+ target cells was plotted as the CD4+/CD8+ ratio relative to that of the control culture without dox (Fig. 5, right panel). The wild type HIV-1 LAI isolate replicates efficiently, resulting in a high peak of CA-p24 production and complete removal of the CD4+

cells from the PBMC culture within 5 days. Due to the removal of target cells, the CA-p24 concentration reaches a maximum at 3 days post infection and subsequently levels off. The parental HIV-rtTA virus replicates slowly, but eventually reaches CA-p24 values similar to that of the wt virus. In this culture, a gradual reduction in CD4+ cells is scored, but HIV-rtTA replication is completely dependent on dox addition. No production of CA-p24 was measured in the PBMC cultures infected with the minimized rtTA Δ 6^A and rtTA Δ 6^B variants, and no significant reduction in the CD4+/CD8+ ratio was observed. Thus, these viruses are unable to cause a spreading infection in PBMC.

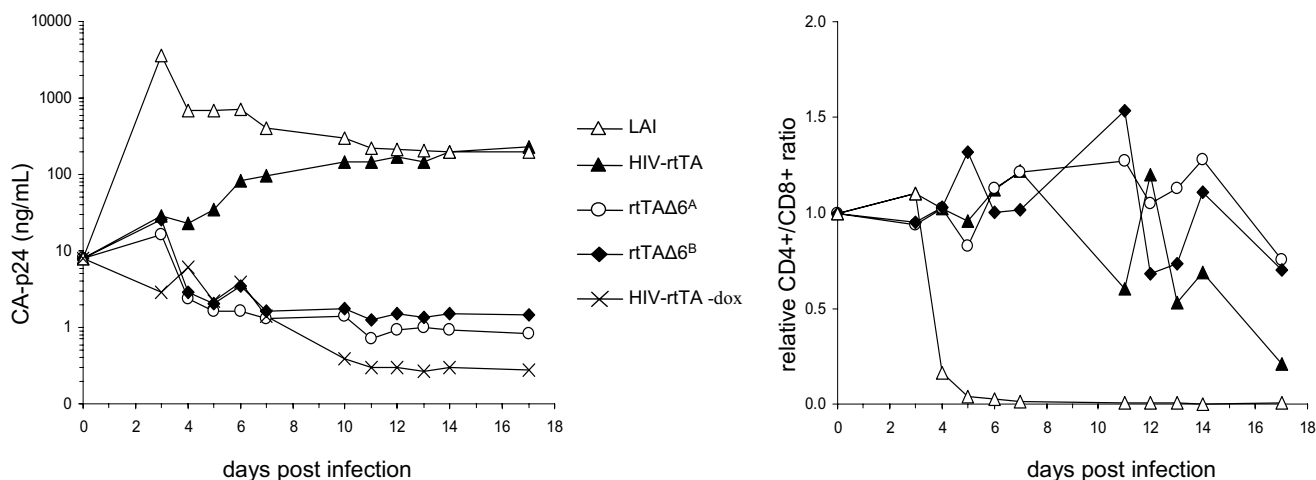


Figure 5
Replication and cell killing capacity of dox inducible viruses in PBMC. (Left) Virus replication of LAI (Δ), HIV-rtTA (\blacktriangle), rtTA Δ 6^A (\circ), rtTA Δ 6^B (\blacklozenge) and rtTA without dox (\times) was determined by monitoring the supernatant CA-p24 concentration after virus infection (40 ng CA-p24) in a 5 mL PBMC culture. (Right) The CD4⁺ and CD8⁺ cell populations in the infected cultures and an uninfected control culture were quantified by a 30 sec time limit FACS analysis and the CD4/CD8 ratio was calculated. The figure shows the CD4/CD8 ratio in the infections normalized for the control uninfected PBMC culture.

Extending the time for replication by feeding these cultures with fresh PBMC did also not result in a spreading infection.

It cannot be excluded that the rtTA Δ 6^A and rtTA Δ 6^B viruses replicate at an extremely low level, and thus stay below the CA-p24 detection limit. To test for this, we used a very sensitive SupT1-based rescue assay to screen for viable virus in the PBMC cultures. PBMC were harvested at day 13, washed and subsequently co-cultured with SupT1 cells. Virus replication is readily observed in the control co-cultures derived from the LAI and HIV-rtTA infections. No virus could be detected in the cultures derived from the rtTA Δ 6^A or rtTA Δ 6^B infections, even with 1000-fold more input sample compared to the LAI or HIV-rtTA samples.

Selective removal of leukemic T-cells from a mixed culture

In a virotherapy setting, the blood of a patient will contain a mixture of leukemic and untransformed cells. The viral therapeutic agent should selectively replicate and kill the leukemic target cells without affecting the untransformed cells. To mimic this situation in our *in vitro* culture system, we started co-cultures of the SupT1 cell line and PBMC. These cells can easily be distinguished by FACS analysis using the CD4 and CD8 surface markers. SupT1 cells are double positive T-cells (CD4⁺CD8⁺), whereas PBMC contain a mixture of single positive CD4⁺CD8⁻ and CD4⁻CD8⁺ cells (Fig. 6, left). A PBMC-SupT1 culture was split in five samples. These cultures were infected with an equal amount of HIV-rtTA, rtTA Δ 6^A or rtTA Δ 6^B virus. The two

remaining cultures were used for a mock infection and a control rtTA Δ 6^A infection without dox. The cell composition was followed over time by FACS analysis, showing the more rapid proliferation of leukemic SupT1 cells versus PBMC in the uninfected control (mock, upper panels). The same result was obtained for the rtTA Δ 6^A control without dox (rtTA Δ 6^A-dox, lower panels). In contrast, the SupT1 cells are selectively depleted in 8 days from the cultures containing rtTA Δ 6^A or rtTA Δ 6^B virus with dox. In agreement with the slower replication kinetics of HIV-rtTA in SupT1 cells (Fig. 3), SupT1-depletion is delayed for this virus. These results indicate that it is possible to selectively remove leukemic T-cells from a mixture with untransformed cells by the use of a dox-controlled mini-HIV-1 variant.

Effects of different Tat proteins on the replication of the dox-inducible mini-rtTA viruses

We constructed two dox-regulated viruses that specifically target leukemic T cells. A surprising finding was that these viruses, with many deletions (Δ 6), replicated much better in SupT1 cells than the parental construct HIV-rtTA (Fig. 3). In the construction of rtTA Δ 6^A and rtTA Δ 6^B, the wild-type tat open reading frame is restored when compared to the rtTA virus that carries the Y26A inactivating Tat mutation. Although Tat-mediated transcriptional activation is not needed for replication of the dox-controlled virus, it is possible that Tat restoration enhances virus replication by other means, which may explain the enhanced replication of rtTA Δ 6 variants.

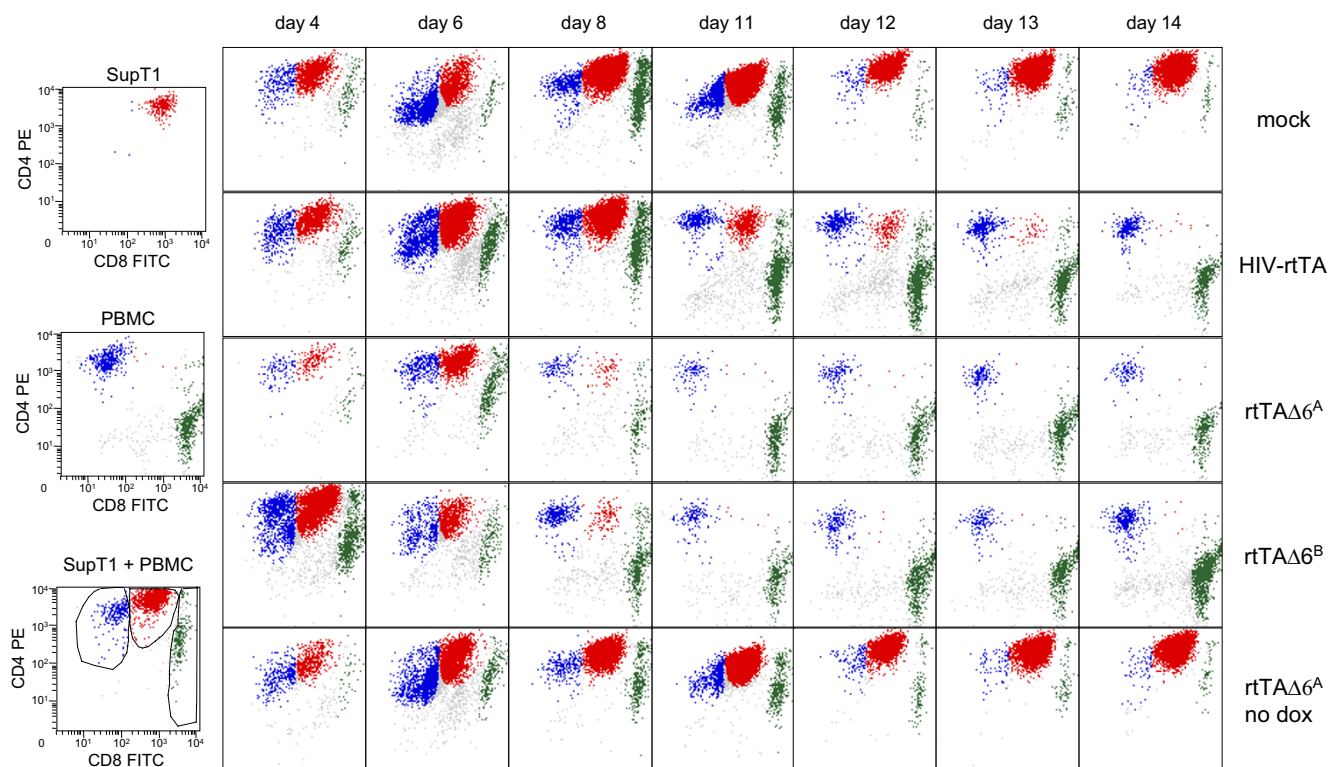


Figure 6

Selective SupT1 killing in SupT1/PBMC co-cultures by mini-rtTA viruses. Infections were started with virus corresponding to 40 ng CA-p24 or mock infected. The FACS dot plot of the initial PBMC + SupT1 cell mixture is in the lower left corner, and the separate cultures are shown above. The gates for CD4+ PBMC (blue), SupT1 (red) and CD8+ PBMC (green) are indicated. The composition of the PBMC + SupT1 culture was followed over time upon infection with the indicated viruses.

To test this hypothesis, the Y26A mutation was reintroduced in the $rtTA\Delta6^A$ and $rtTA\Delta6^B$ background, yielding the $rtTA\Delta7^A$ and $rtTA\Delta7^B$ viruses. For comparison, the mutant *tat* gene (Y26A) in the HIV-*rtTA* virus was also replaced by the wild-type *tat* gene from the LAI isolate, yielding $rtTA\Delta3$ (LAI), or the *tat* gene from the NL4-3 isolate, yielding $rtTA\Delta3$ (NL4-3). This set of viruses was used to infect SupT1 cells to test their replication capacity (Fig. 8). Comparison of the replication capacity of $rtTA\Delta6^A$ versus $rtTA\Delta7^A$, $rtTA\Delta6^B$ versus $rtTA\Delta7^B$ (Fig. 8, left) and $rtTA\Delta3$ (LAI) versus HIV-*rtTA* (Fig. 8, right) demonstrate that the Y26A *Tat* mutation causes a small decrease in replication. Thus, a wild-type *tat* gene improves replication. The introduction of the NL4-3 *tat* gene in HIV-*rtTA*, however, improved replication much more than introduction of the *tat* gene of the LAI isolate (Fig. 8, left panel, compare $rtTA\Delta3$ (NL4-3) with HIV-*rtTA* and $rtTA\Delta3$ (LAI)). In fact, the $rtTA\Delta3$ (NL4-3) variant replicated consistently better than the wild type LAI virus. Similar results were obtained in repeated infections and in replication studies that were initiated by DNA transfection (results not shown).

Discussion

We describe the development of therapeutic viruses based on HIV-1 for virotherapy against T-ALL. We combined mini-HIV-1 variants [13] that lack several accessory proteins with the dox-controllable HIV-*rtTA* virus approach [16]. One molecular clone, $rtTA\Delta6^A$, has four deletions (*vif*, *vpR*, *nef* and *U3*) and two motifs with inactivating mutations (*TAR* and *vpU*). The molecular clone $rtTA\Delta6^B$ is similar, but the *vpU* gene is deleted instead of having the inactivating mutation. These mini-*rtTA* viruses replicate efficiently in leukemic T-cell lines and virus replication results in cell death. These viruses do not replicate in PBMC, even in co-cultures with susceptible SupT1 cells that continuously produce new infectious virus particles (Fig. 6). The results are summarized in Table 1. Most importantly, virus replication is strictly dox-dependent.

The viral *Vif* protein counters the potent antiviral activity of APOBEC3G in some cells including PBMC [reviewed in [23]], and the absence of *Vif* may therefore be the main contributor to the replication defect in primary cells. Nevertheless, the other accessory proteins (*vpR*, *vpU* and *Nef*)

Table 1: Virus replication and cell killing capacity

virus	SupT1		PBMC	
	replication	cell killing	replication	CD4+ killing
LAI	++	++	++	++
HIV-rtTA	+	++	+	±
rtTAΔ6A	++	++	-	-
rtTAΔ6B	++	++	-	-

also have important roles *in vivo* [24-26] and *in vitro* [13,27-29]. The presence of multiple gene deletions will not only increase safety of the therapeutic virus, but may also provide synergistic effects. For instance, it was recently demonstrated that the combined elimination of the *vif* and *vpR* genes, unlike the individual mutants, renders the virus incapable of causing cell death and G2 cell cycle arrest [30].

A surprising finding is that removal of the genes encoding the accessory proteins *Vif*, *VpR* and *VpU* appeared to have a positive effect in the context of the dox-controlled HIV-rtTA virus, whereas the same deletions have a negative impact when introduced into the wild-type HIV-1 isolate [13]. This observation enabled us to make HIV-1 variants that replicate extremely fast in leukemic cells, yet are fully replication-impaired in primary cells. This result, combined with the strict dox-regulation, suggests to us that a safe therapeutic use of these virus variants is feasible. In a therapeutic setting, the minimized virus can be used to target the leukemic cells in the presence of dox. This will result in a self-limiting viral infection since the target cells are killed by the virus. Withdrawal of dox provides an additional safety feature to block ongoing replication after the leukemic cells are removed. It may be possible to add therapeutic short interfering RNAs (siRNAs) to this viral vector system [31]. We plan to set up a T-ALL model in severe combined immunodeficiency (SCID) mice to test the capacity of these therapeutic viruses to selectively remove leukemic cells *in vivo*.

HIV-rtTA was originally designed as a novel attenuated virus vaccine candidate. To minimize the possibility of reversion to normal TAR-Tat regulated transactivation, inactivating mutations were made in both the TAR hairpin and the Tat protein (Y26A). In our minimized Δ6 deletion variants, a wild type NL4-3 *tat* gene was introduced due to the cloning procedure. Restoration of a wild type Tat function could explain the observed fast replication kinetics of these viruses. However, reintroduction of the Y26A mutation in these viruses (rtTAΔ7^A and rtTAΔ7^B) caused only a small decrease in replication capacity, which is consistent with previous results [16]. The TAR hairpin in these constructs is inactivated by multiple point mutations, which

are sufficient as individual point mutation to block Tat-mediated transcription [16,32-34] and virus replication [35]. Restoration of the normal Tat-TAR transcription axis is therefore an unlikely scenario in the dox-dependent virus. Thus, the absence of the Y26A mutation does not provide an explanation for the improved replication, but the results demonstrate that the Y26A mutation, apart from abolishing Tat-TAR mediated transcription, has an additional (small) negative effect on the replication of HIV-rtTA.

Another possible explanation for the improved replication of the mini-HIV-rtTAs is provided by inspection of the sizes of these viral genomes. The RNA genome of the wild-type HIV-1 LAI isolate is 9,229 nt, but the HIV-rtTA genome is extended to 9,607 nt due to the insertion of the *rtTA* gene and *tetO* DNA binding sites. The latter genome size may be sub-optimal for replication, *e.g.* due to restricted RNA packaging in virion particles, and removal of the *vif-vpR-vpU* genes may thus be beneficial in this context. Deletion of these genes reduces the RNA genome to 8989 for rtTAΔ6^A and to 8872 for rtTAΔ6^B. One would nevertheless expect a reduction of viral fitness due to removal of three accessory genes, unless these viral-protein functions do not add significantly to virus replication in T cell lines. In fact, we consistently measured that the rtTAΔ6 variants replicate significantly faster than the wild-type virus in T-cell lines, perhaps indicating that some of the accessory HIV-1 genes have a negative impact on virus replication in these leukemic cells. Consistent with this idea is the frequent selection of inactivation mutations in these open reading frames upon prolonged culturing in T cell lines. Alternatively, these viral functions may have lost significance in the context HIV-rtTA, in which Tat-TAR mediated transcription is taken over by the *rtTA-tetO* elements. For instance, *VpR* has been reported to have a transcriptional component [36], and this transcriptional contribution may be less important in the HIV-rtTA context.

Another explanation comes from the comparison of the control viruses rtTAΔ3 (NL4-3) and rtTAΔ3 (LAI) that have the same gene deletions, yet a different *tat* gene. The introduction of the NL4-3 *tat* gene improved virus replication significantly more than insertion of the LAI *tat* gene. In fact, the replication of rtTAΔ3 (NL4-3) is similar to that of rtTAΔ6^A and rtTAΔ6^B. Thus, the presence of a fragment encoding the NL4-3 *tat* gene is the decisive determinant for the improved replication of rtTAΔ6^A, rtTAΔ6^B and rtTAΔ3 (NL4-3). As discussed above, this is not due to the Y26A mutation, which has a similar small negative effect in both sequence contexts (LAI and NL4-3). Furthermore, this effect appears to be specific for the HIV-rtTA virus since replication of the mini-HIV-1 virus, which has a wild type NL4-3 *tat* gene, is impaired [13].

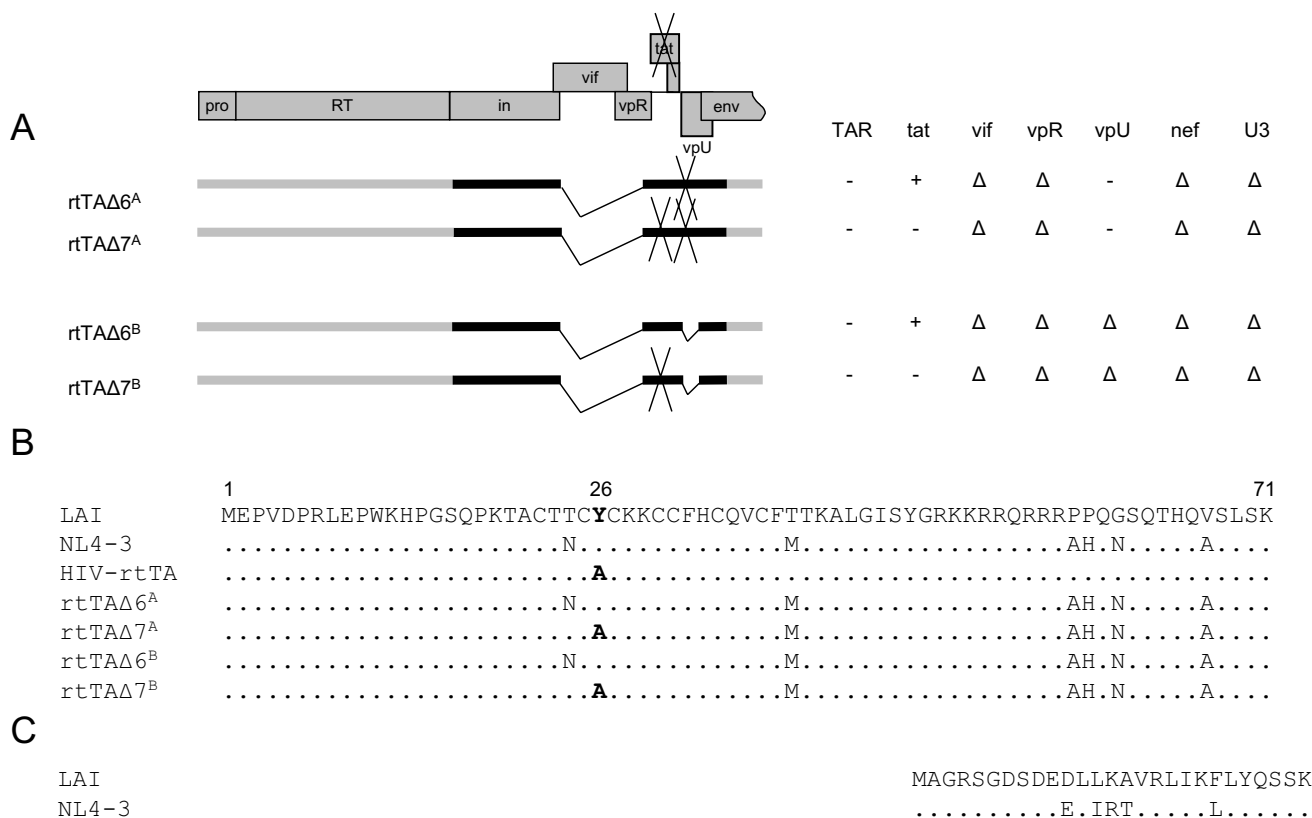


Figure 7
Overview of the different tat constructs. (A) The position of the various deletions and mutations, and a summary of the inactivated (-) or deleted (Δ) viral genes is shown. See the Materials and Methods section for construction details. (B) Sequence alignment of the different tat genes. The position of the Y26A mutation is indicated in bold. (C) Sequence alignment of the corresponding part of the rev gene. The rev startcodon overlaps the tat codon for Y47.

The differences between the fast replicating virus rtTAΔ3 (NL4-3) and the slow replicating rtTAΔ3 (LAI) are located exclusively in the 350 nt tat fragment. This fragment encodes the first exon of the tat gene, the overlapping first exon of the rev gene and part of the open reading frames for vpU and Env. The sequence differences result in six amino acid substitutions in Tat (Fig. 7B, N24T in the Cysteine-rich domain, M39T in the core domain and A58P, H59P, N61G and A67V in the C-terminal domain). In addition, these sequence differences also change the rev gene (Fig. 7C, E11D, I13L, R14K T15A and L21F). Furthermore, there are two substitutions in the vpU gene (I5Q and V60I). We can exclude some of these differences to play a role in this phenotype by comparison with the efficient replicating rtTAΔ7^A and rtTAΔ7^B viruses. These viruses lack the vpU gene and have the LAI-specific Threonine at position 24 in Tat, indicating that these motifs are not responsible for the improved phenotype. Thus, the differences are caused by one or more of the remaining substitutions in the core and/or C-terminal domain of Tat or the overlapping Rev protein. Recently, it was reported

that tat genes from different HIV-1 subtypes differentially regulate gene expression [37]. Our results demonstrate that sequence variation in this genome segment can have a profound effect on replication even when derived from the same subtype B.

Materials and methods

DNA-constructs

Full-length molecular HIV-1 clones are based on an improved variant of the dox-inducible HIV-1 variant described previously [38]. We first deleted the accessory proteins vif and vpR in this HIV-rtTA virus. Plasmid pDR2483 [39], which contains the 5' genome of the HIV-1 isolate NL4-3 with deletions in the genes encoding the vif and vpR proteins, was used as template in a PCR reaction with primers RJ001 (5' GGG CCT TAT CGA TTC CAT CTA 3') and 6 N (5'CTT CCT GCC ATA GGA GAT GCC TAA G 3'). The resulting PCR fragment was cut with *Clai* and *EcoR1* and ligated with a 9644 bp *BclI-EcoR1* HIV-rtTA vector fragment and a 1816 bp *BclI-Clai* fragment from pLAI-001 [13] to generate the subclone rtTAΔvifΔvpR. We

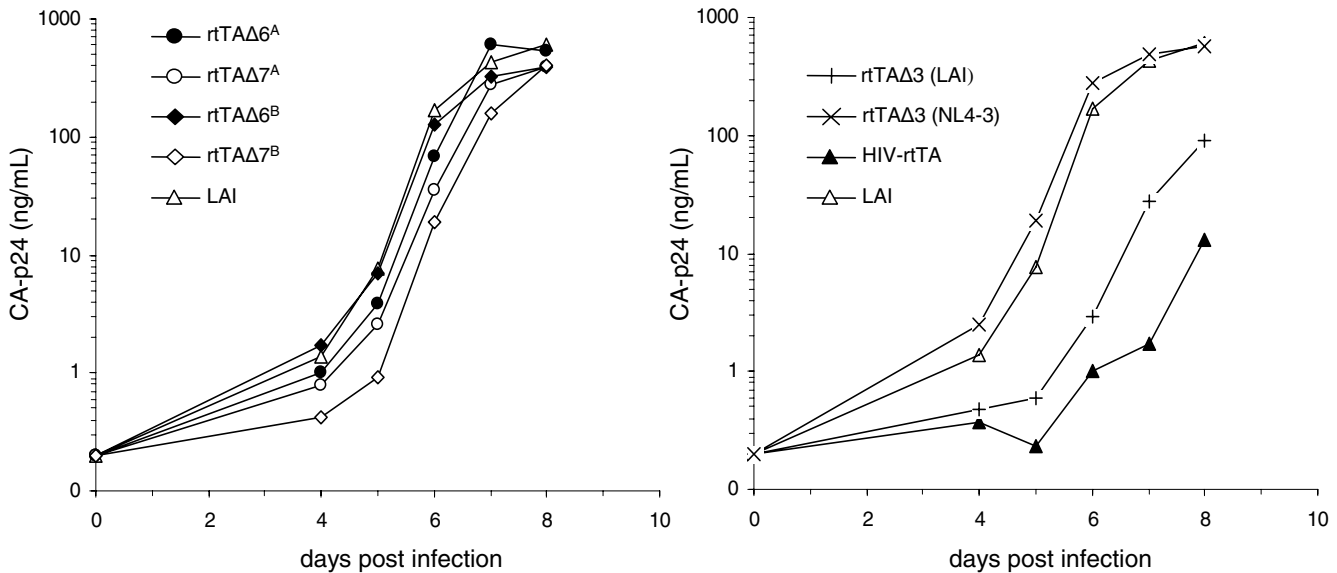


Figure 8

Effects of different Tat proteins on the replication of the dox inducible rtTA viruses. Virus replication was followed by measuring of the supernatant CA-p24 concentration after virus infection (20 ng CA-p24) in a 5 mL culture. **(Left)** Replication of the rtTA Δ 6^A (●), rtTA Δ 7^A (○), rtTA Δ 6^B (◆), rtTA Δ 7^B (◇) and LAI (△) viruses in Sup T1 cells. **(Right)** Replication of the rtTA Δ 3 (LAI, +), rtTA Δ 3 (NL4-3, X), HIV-rtTA (▲) and LAI (△) viruses in Sup T1 cells.

noticed a vpU startcodon inactivation (AUG to AUA) in one of the evolution cultures [13]. Proviral DNA was PCR amplified from total cellular DNA of this culture with the primers Pol5'FM (5'TGG AAA GGA CCA GCA AAG CTC CTC TGG AAA GGT 3') and WS3 (5'TAG AAT TCA AAC TAG GGT ATT TGA CTA AT). The same PCR was performed on DNA from a vpU-deletion construct [pDR2484, [39]]. The PCR fragments were cut with *Eco*RI and *Nde*I and ligated with a 2086 bp wild type (wt) rtTA *Bam*HI-*Nde*I fragment and the vector rtTA Δ vif Δ vpR cut with *Eco*RI and *Bam*HI. The resulting molecular clones (Fig. 1) were named rtTA Δ 6^A (vpU startcodon inactivation) and rtTA Δ 6^B (vpU deletion).

As part of the vpU inactivation strategy, the Y26A inactivating mutation in the tat gene of HIV-rtTA is replaced by the wt tat gene of the NL4-3 isolate (first exon). The Y26A mutation was cloned back into the rtTA Δ 6^A and rtTA Δ 6^B molecular clones as follows. A PCR was done with HIV-rtTA as template and primers Pol5'FM and RJ036 (5'CTT TTG TCA TGA AAC AAA CTT GGC A 3'). The latter primer introduces a *Bsp*HI site that is also present in the wt NL4-3 sequence. The PCR product was digested with *Eco*RI and *Bsp*HI and used in a triple ligation with the 9028 bp *Eco*RI-*Bam*HI vector and either the 2545 bp *Bsp*HI-*Bam*HI fragment of rtTA Δ 6^A or the 2428 bp *Bsp*HI-*Bam*HI fragment of rtTA Δ 6^B. For comparison, we also introduced the LAI and NL4-3 tat gene into the HIV-rtTA background. For NL4-3,

this was done in a triple ligation with the rtTA vector cut with *Sph*I and *Asp*718 I, the 4378 bp *Sall*-*Sph*I rtTA fragment and the 558 bp *Sall*-*Asp*718 I fragment of pDR2480 [39]. For LAI this was done by ligation of the 9646 bp *Nco*I-*Bam*HI digested HIV-rtTA vector with the 2811 bp *Nco*I-*Bam*HI fragment of LAI.

All constructs were verified by restriction enzyme digestion and BigDye terminator sequencing (Applied Biosystems, Foster City, CA) with appropriate primers on an automatic sequencer (Applied Biosystems DNA sequencer 377). Plasmid DNA isolation was done with the Qiagen Plasmid isolation kit according to the manufacturers' protocol (Qiagen, Chatsworth, CA).

CA-p24 levels

Culture supernatant was heat inactivated at 56°C for 30 min in the presence of 0.05% Empigen-BB (Calbiochem, La Jolla, USA). CA-p24 concentration was determined by a twin-site ELISA with D7320 (Biochrom, Berlin, Germany) as the capture antibody and alkaline phosphatase-conjugated anti-p24 monoclonal antibody (EH12-AP) as the detection antibody. Detection was done with the lumiphos plus system (Lumigen, Michigan, USA) in a LUMistar Galaxy (BMG labtechnologies, Offenburg, Germany) luminescence reader. Recombinant CA-p24 expressed in a baculovirus system was used as the reference standard.

Cells and viruses

C33A cervix carcinoma cells [ATCC HTB31, [40]] were grown as a monolayer in Dulbecco's minimal essential medium supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, 20 mM glucose and minimal essential medium nonessential amino acids at 37°C and 5% CO₂. The cells were transfected by the calcium phosphate method as described previously [41].

The human T lymphocyte cell line SupT1 [ATCC CRL-1942, [42]] was cultured in RPMI 1640 (Gibco BRL, Gaithersburg, MD) supplemented with 10% (v/v) FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C and in 5% CO₂. Transfections were carried out by electroporation as described [43] using a BioRad Gene Pulser II (BioRad, Hercules, CA). Infection with C33A produced virus stocks was performed with the indicated amount of virus.

PBMC were isolated from different healthy donors, each batch consists of a mixture of four different donors. PBMC were grown as the SupT1 cells, but with the addition of 100 U/mL human IL2 after an initial PHA (5 µg/mL) stimulation for 2 days. Infections were performed with C33A produced virus stocks with the indicated amount of virus.

Virus competition assay

Virus competition experiments were done as described previously [44]. Competitions were initiated with C33A produced virus stocks. Each competition was done with virus corresponding to 6 ng virus with starting ratios of 5 to 1, 1 to 1 and 1 to 5. The competition was repeated with independent virus stocks.

Virus rescue assay

Low-level replication in PBMC was analyzed with a virus rescue assay. At day 13 post infection of PBMC, the cells from 1 mL culture were collected (4 min 4000 RPM, eppendorf centrifuge), washed once with 1 mL PBS to remove any input virus and resuspended in medium. A dilution series (1, 10×, 100×, 1000×, 10.000×) was made and each sample mixed with one million SupT1 cells. The cultures were maintained for four weeks, regularly split and inspected for virus replication by CA-p24 Elisa on the culture supernatant and visual inspection for syncytia formation.

Fluorescence-activated cell sorting analysis

Flow cytometry was performed with RPE-conjugated mouse monoclonal anti-human CD4 (clone MT310, Dako, Glostrup, Denmark) and FITC-conjugated mouse monoclonal anti-human CD8 (clone DK25, Dako). Cells from a 1 mL culture sample were collected (4 min 4000 RPM, eppendorf centrifuge), incubated with a mixture of

both monoclonal antibodies in fluorescence-activated cell sorting (FACS) buffer (PBS with 2% FCS) for 30 min at room temperature and washed with 800 µL FACS buffer. The cells were subsequently collected (4 min 4000 RPM, eppendorf centrifuge) and resuspended in 20 µL of 4% paraformaldehyde. After 5-minute incubation at room temperature, 750 µL FACS buffer was added and the suspension analyzed on a FACScalibur flow cytometer with CellQuest Pro software (BD biosciences, San Jose, CA). The machine was set for a 30-sec collection time. Cell populations were defined based on forward/sideward scattering and isotype controls were used to set markers. For mixed SupT1 plus PBMC cultures, the gates for PBMC (CD4⁺CD8⁻ and CD4⁺CD8⁺) and SupT1 (CD4⁺, CD8⁺) were set using a separate control culture.

Mixed culture SupT1/PBMC infection

Freshly isolated PBMC were stimulated for 2 days with PHA (5 µg/mL), washed twice with medium and mixed with SupT1 cells. The cell mixture was analyzed by FACS staining for CD4 and CD8 as described above. The culture was divided into equal 10 mL samples, containing approximately 1 million PBMC and 2 million SupT1 cells, which were infected with different virus variants (input 40 ng CA-p24). Daily samples were taken for CA-p24 Elisa and anti-CD4/CD8 FACS analysis.

Competing interests

The author(s) declare that they have no competing interests.

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