Molecular Therapy
Nucleic Acids
Original Article

Removal of HIV DNA by CRISPR from Patient Blood Engrafts in Humanized Mice

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We used NOD/SCID mice, also known as NRG, to assess the ability of lentivirus-mediated intravenous delivery of CRISPR in editing the HIV-1 genome from the circulating PBMC engrafts, some of which homed within several animal solid tissues. Lentivirus-mediated delivery of a multiplex of guide RNAs accompanied by Cas9 endonuclease led to the excision of the targeted region of the viral genome positioned within the HIV-1 LTR from the *in-vitro*-infected human peripheral blood mononuclear cells (PBMCs) embedded in the spleens of NRG mice. Similarly, the treatment of NRG mice harboring PBMC engrafts derived from HIV-1-positive patients with the therapeutic lentivirus eliminated the presence of the viral DNA fragment in the blood, as well as in the spleen, lung, and liver, of the engrafted animals. Sanger sequence analysis of the viral DNA after treatment with the lentiviral vectors expressing Cas9 and gRNAs verified the editing and removal of the proviral DNA fragment from the viral genome at the predicted sites. This proof-of-concept study, for the first time, demonstrates successful excision of the HIV-1 proviral DNA from patient immune cell engrafts in humanized mice upon treatment with lentivirus-expressing CRISPR and causes a decline in the level of replication-competent virus.

INTRODUCTION

After more than 3 decades, HIV-type 1 (HIV-1) remains a major public health concern that affects over 35 million people worldwide, with new infections continuing at a steady rate of greater than 2 million per year. Antiretroviral therapy (ART) effectively controls the viral load for most individuals yet fails to eliminate the virus.[1,2] The major obstacle in curing HIV-1 infection relates to the presence of integrated proviral DNA that persists in a dormant state but can be reactivated to produce replication-competent virus. Indeed, interruption of ART often results in rapid viral rebound, requiring most HIV-1-infected persons to maintain a lifelong treatment with ART, including those who respond very well to therapy.

During latency, HIV-1-infected cells produce little or no viral protein, thereby avoiding both viral cytopathicity and host antiviral immune clearance. This results in the development of a long-lived reservoir of viral persistence that is believed to be composed, largely, of resting memory $CD4^+$ T cells and tissue macrophages, including microglia in brain.[3–5] Earlier efforts to eliminate HIV-1 from these reservoirs have focused on the "shock-and-kill" approach that includes steps for reactivation of latent HIV-1 to purge virus-producing cells by cytolysis or immune attack.[6] Considering the multiple limitations of this approach, this strategy showed insufficient efficacy in a clinical setting.[7] Thus, it is evident that a new strategy is required to identify and eliminate infected cells and/or eradicate the HIV-1 genome from infected cells without perturbation of host cell homeostasis.

CRISPR and its associated endonuclease (Cas9) provide a powerful tool for editing eukaryotic genes.[8,9] The method is easy to use, very efficient, and flexible, so it can be adapted to several targets within the gene of interest.[8] Recently, we have modified and used the CRISPR/Cas9 apparatus with the ability to recognize specific DNA sequences within the HIV-1 promoter spanning the long terminal repeat (LTR), as well as several other DNA sequences within viral DNA coding sequences, and precisely excised segments and/or the entire proviral DNA from infected cells *in vitro* and *in vivo*.[10–12]

In accord with this observation, several other laboratories have assessed the efficacy of CRISPR technology in editing the HIV-1 genome in a variety of cell models.[13,14] An important consideration that arose from these recent studies was the appearance of mutant virus that may stem from insertions or deletions (indels) generated by non-homologous end joining (NHEJ) repair mechanisms after CRISPR editing.[15,16] This concern, however, can be avoided by targeting multiple sites within the viral genome to excise large segments of viral DNA, resulting in a permanent inactivation of viral replication in the infected cells.[17,18]

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While the gene editing strategy has shown an unprecedented ability to eliminate HIV-1 DNA from PBMCs in laboratory cell models and suppression of viral infection in primary human blood cell cultures *in vitro*, its efficacy in editing HIV-1 DNA in cells and tissues that support viral reservoirs *in vivo* requires further investigation. Clearly, the most convenient path for application of CRISPR/Cas9 is a gene therapy strategy using a vehicle that can be directly administered to the subject. In this regard, recent studies have shown that adeno-associated virus (AAV)-mediated intravenous delivery of HIV-1-targeting CRISPR/Cas9 to transgenic animals harboring partial segments of HIV-1 genome or humanized mice infected with HIV-1 results in the editing of integrated copies of the viral genome from a broad range of tissues.[11,19] Here, we used non-obese diabetic/severe combined immunodeficiency (NOD/SCID, or NRG) mice engrafted with an *in-vitro*-infected primary culture of PBMCs or PBMCs isolated from a small cohort of (three) HIV-1-positive patients and demonstrated that intravenous administration of lentiviral vectors (LVs) for the delivery of CRISPR/Cas9 caused excision of segments of the HIV-1 genome in circulating human PBMCs, as well as the infected blood cells homing to spleen—one of the major sites serving as a reservoir for viral latency—and several other organs. Sanger sequencing was used to confirm the expected cleavage of the viral genome in these treated humanized mice.

RESULTS AND DISCUSSION

Bioinformatic screening of DNA sequences corresponding to the U3 region of the HIV-1 LTR identified several specific stretches of fragments (20 nt in size) with the essential trinucleotide motif known as the protospacer-adjacent motif (PAM), which can serve as a template for the development of guide RNAs (gRNAs) and cleavage by spCas9.[10] Additional bioinformatic analyses predicted relatively no off-target effects on the cellular genes of two motifs that we have designated "LTR A" and "LTR B" and selected for the creation of gRNAs.[10,12] Here, we utilized a cocktail of LVs, each containing a DNA fragment corresponding to protospacers of the gRNA LTR-A, gRNA LTR-B, or spCas9 gene, hereinafter called LV-CRISPR/Cas9, to assess their combined ability to edit the HIV-1 genome in a system *in vivo*. As described previously,[10,12] the use of two gRNA configurations to excise large fragments of HIV-1 DNA mitigates the emergence of mutant virus with a small nucleotide indel mutation that becomes immune from CRISPR/Cas9.[16,18] It is also important to note that the use of two or more gRNAs, in addition to the excision of the intervening DNA fragments, more frequently introduces an indel mutation at each of the target sites and leads to the inactivation of the target gene.[16] During the past several years, we have been able to optimize our editing strategy, and developing pairs of gRNAs significantly enhanced the frequency of viral DNA excision by a multiplex of gRNAs.[10,12,19,20] For our experiments *in vivo*, we selected an NRG mouse model, which has multiple immunodeficiencies that are due, largely, to the absence of T, B, and NK cells and the presence of dysfunctional dendritic cells and macrophages, as well as minimal complement activity.[21] This model offers an excellent system *in vivo* for hosting human immune cells and can be used for either long-term studies using $CD34^+$ human cells or short-term studies using primary PBMCs. In most cases, graft versus host does not appear in these animals until months 6–9, making them an ideal model for both short- and long-term studies.

In the initial experiments, PBMCs prepared from buffy coat blood were cultured in media containing interleukin (IL)-2. After infection with HIV-1_{JR-FL}, cells were treated with IL-7 in the media containing ART to outgrow PBMCs. At 7 days post-infection, cells were treated with LV-CRISPR/Cas9, and the level of viral DNA corresponding to LTR was determined by digital droplet PCR (ddPCR) 6 days later. Our results, which were normalized to human β-actin gene copy number, showed that viral LTR DNA is significantly decreased in cells transduced with LVs expressing CRISPR/Cas9 and gRNAs (Figure 1A). This removes a 190-bp LTR DNA sequence positioned between gRNAs A and B, resulting in a 167-bp truncated LTR that can be identified by conventional PCR and gel electrophoresis (Figure 1B). The position of the breakpoints is also confirmed by direct sequencing of the amplicon (Figure 1C). Additionally, this strategy allows cleavage on both 5′ and 3′ LTRs, which results in the removal of the entire HIV genome positioned between the two LTRs. Rejoining of the residual DNA sequences from both LTRs by NHEJ, in turn, creates a single LTR at the integration site, which is defined by the absence of central DNA sequences corresponding to gag-pol-env. This allows the use of qPCR and ddPCR to determine the efficiency of the excision of the viral DNA by CRISPR/Cas9, as shown in Figure 3.

To explore the efficacy of LV-CRISPR/Cas9 *in vivo*, approximately 5×10^6 PBMCs infected *in vitro* were intraperitoneally (i.p.) injected into each 8-week-old NRG mouse. After 1 week, animals were subjected to intravenous administration of LV-CRISPR/Cas9 and sacrificed for HIV-1 DNA analysis 1 week later. Comparison of the viral DNA levels, corresponding to the target sequence, by ddPCR in the three control animals that did not receive lentivirus treatments with those treated with gRNAs and spCas9 showed a drastic decline in the viral DNA in the animals that received the cocktail of LVs (Figure 1D).

Amplification of viral DNA in spleens of the control group by standard PCR verified the presence of the expected 357-bp amplicon corresponding to the full-length LTR DNA segment between the PCR primers (Figure 1E, lanes 2–4). In contrast, animals that received LV-CRISPR/Cas9 (n = 3) showed a significant decrease in the intensity of the 357-bp fragment, as well as the presence of a smaller DNA fragment 167 bp in size (Figure 1E, lanes 5–7), which is not seen in the non-treated animals. Sequencing of the 167-bp amplicon verified the removal of a 190-bp DNA fragment positioned between LTR A and LTR B and identified the site of breakpoint (Figure 1F). LV-CRISPR/Cas9-treated and non-treated TZM-bl cells, which have integrated copies of HIV-1 DNA, served as a control for the excision of the 190-bp DNA from the LTR by CRISPR/Cas9 (Figure 1E, lanes 8 and 9).

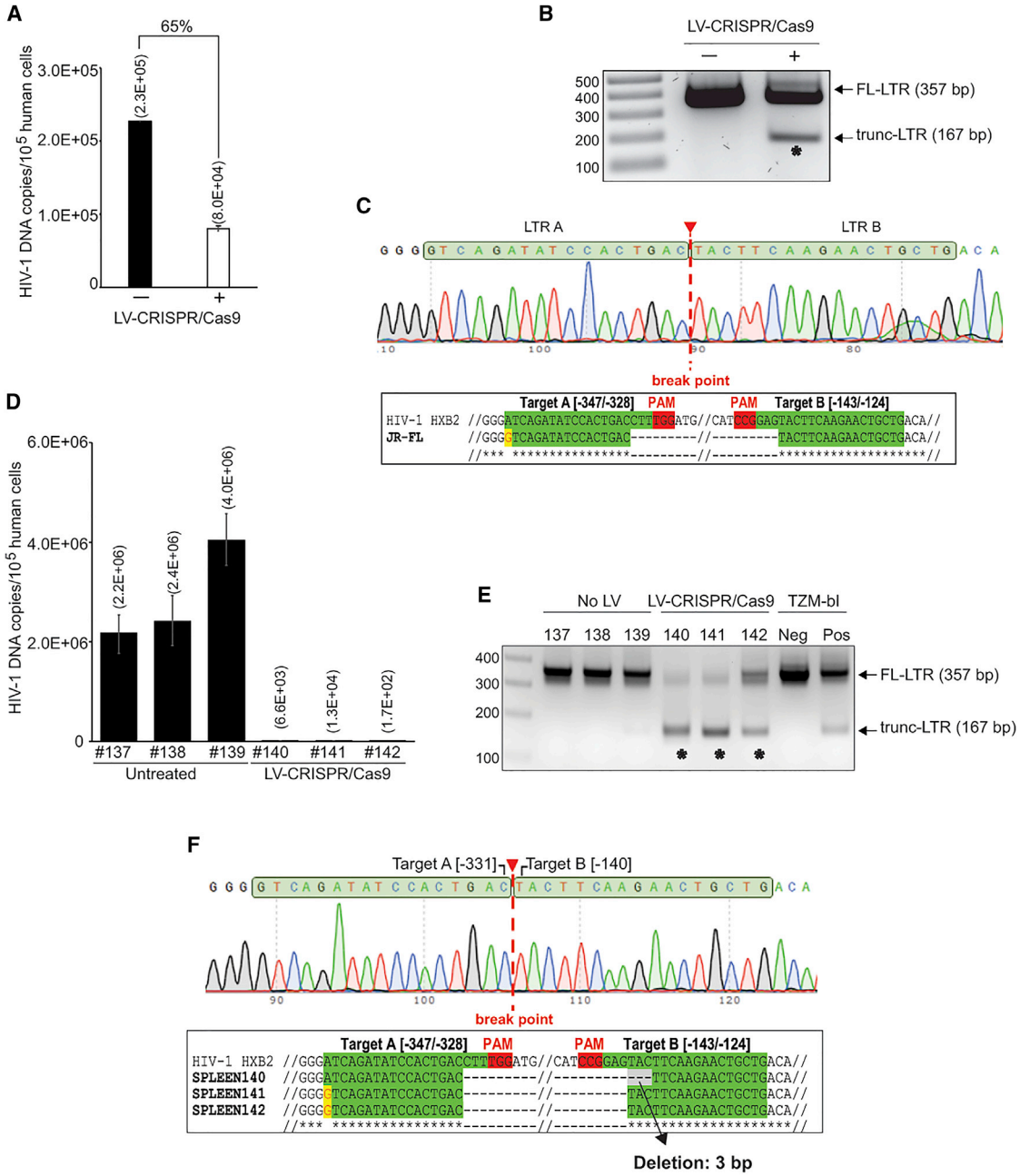

Figure 1. Editing of HIV-1 DNA in *In Vitro*-Infected PBMCs by LV-CRISPR/Cas9

PBMCs isolated from the blood of healthy donors were infected *in vitro* with HIV-1_{JR-FL}. After 1 week, IL-7 and ART were added. Two weeks later, half of the cells were treated with an LV-CRISPR/Cas9 cocktail *in vitro*, and the other half were engrafted into NRG mice followed by injection with LV-CRISPR/Cas9. Genomic DNA was extracted from cells treated *in vitro* at day 6 after lentiviral transduction and from the spleens of engrafted animals after 1 week from lentiviral injection. (A) HIV-1 DNA levels in cells treated *in vitro* were quantified by digital droplet PCR (ddPCR) using TaqMan primer/probe sets specific to the U3 region of viral LTRs and normalized to human β-actin gene copy number. (B) Agarose gel electrophoresis of conventional PCR for the detection of truncated LTR DNA in the cells treated with LV-CRISPR/Cas9. (C) Top: alignment analysis of sequences obtained from Sanger sequencing of truncated amplicons. HIV-1 HXB2 5′-LTR sequence was used as a reference. Target sites are highlighted in green, PAM sequences are indicated in red, mismatches are indicated in yellow, and deletions are indicated in gray. Bottom: representative Sanger sequence tracing result. The position of the CRISPR/Cas9-specific double-cleaved/end-joined site is shown as a breaking point. (D) ddPCR analysis for detecting the HIV-1 sequence in circulating human PBMCs isolated from the spleens of treated and untreated NRG mice engrafted with the aforementioned PBMCs. (E) Gel analysis of conventional PCR to reveal excision of viral DNA isolated from the tissues of engrafted NRG mice. (F) Sanger sequencing of the 167-bp amplicon amplified from an LTR sequence showed breakpoint (top) and deletion of the DNA fragment between the target sites of LTR-A and B (bottom), while some minor nucleotide variations were also detected. The asterisks below the bands indicate the specificity of the cleavage by CRISPR, as verified by sequencing.

A

PID	Race	Gender	Age	HIV Diagnosis	CD4 (cells/ul)	VL (copies/ml)	ART regimen	HIV-assoc. Disorders	Non-HIV chronic health conditions	UDS Results
TUR0036	AA	M	52	2011	551	39	darunavir norvir abacavir	None	DM, neuro manif type II, uncontrolled; HTN; hyperlipidemia; hypogonadism; HAV; HBV; latent syphilis; vitamin D deficiency	THC5
TUR0094	AA	M	26	2011	490	<20	prezcobix complera	None	Asthma; HSV; urinary tract infection	THC5
TUR0095	AA	F	63	2011	1897	<20	triumeq	None	Bacterial vaginitis; breast lesion; DM: HTN; HCV; LGSIL; migraines; nicotine dependence; pneumonia; pure hypercholesterolemia; rash; weight gain	THC5

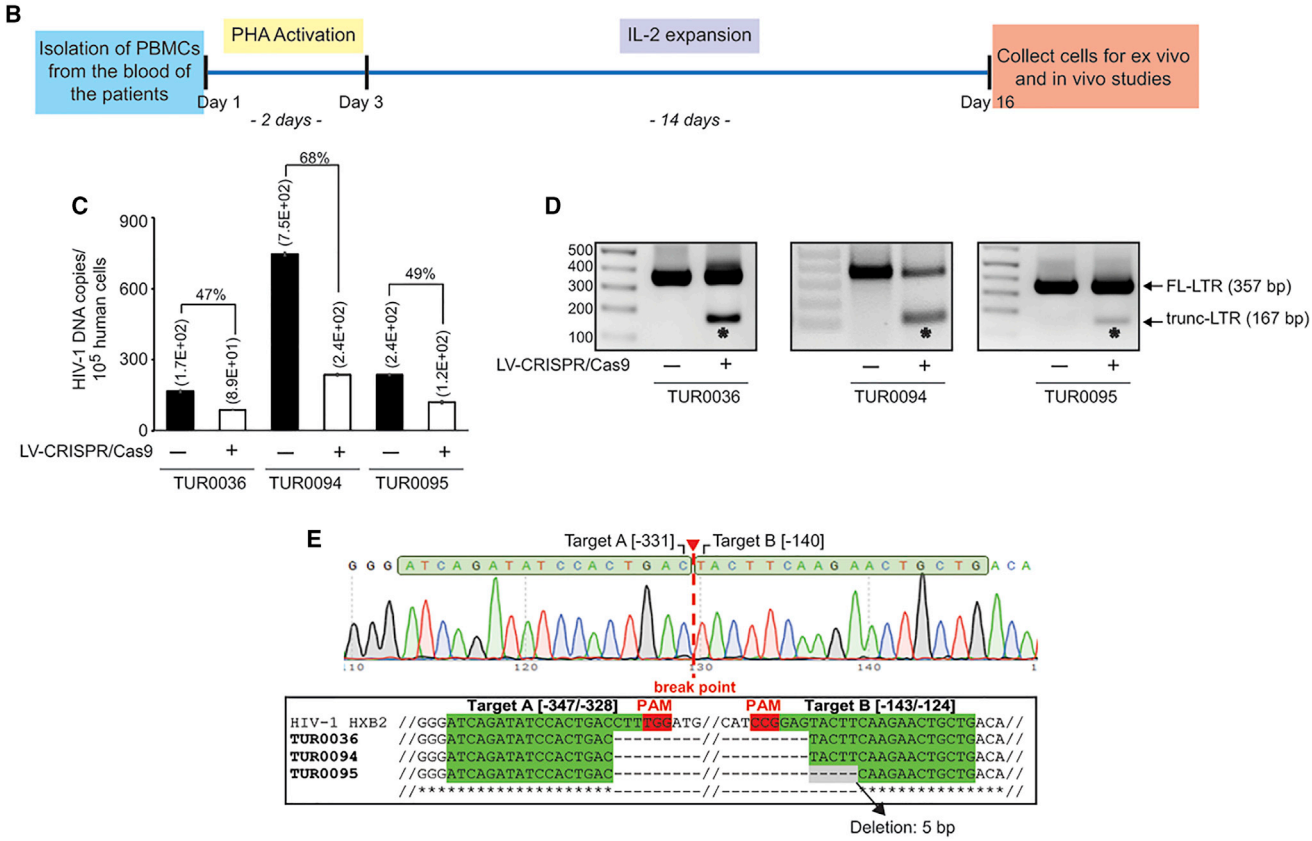

Figure 2. *Ex Vivo* Editing of HIV-1 DNA from Patient Blood

(A) PBMCs from three HIV-1-infected patients treated with ART were acquired through the Clinical Core Repository of the NIMH-funded Comprehensive NeuroAIDS Center. DM, diabetes mellitus; HTN, hypertension; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus. (B) Schematic of preparation and treatments of PBMCs. On day 16, a portion of cells was used in an *ex vivo* experiment, as described here and in (C) and (D), and some were used for engrafting NRG animals, as described in the Materials and Methods section. (C) ddPCR assessing the level of viral load from patient PBMC cultures after treatment with LV-CRISPR/Cas9 (normalized to human β-actin sequence). (D) Excision assay by PCR and gel electrophoresis revealed a 167-bp amplicon in all patient samples after treatment with LV-CRISPR/Cas9. (E) Results from Sanger sequencing showing the removal of the viral DNA sequence spanning between the target sites of LTR-A and LTR B. The position of a 5-nt deletion is seen in one of the TA clones. Target sites are highlighted in green, PAM sequences are highlighted in red, mismatches are highlighted in yellow, and deletions are highlighted in gray. Below, the representative Sanger sequence tracing result is shown. The position of CRISPR/Cas9-specific double-cleaved/end-joined site is shown as a breaking point.

We next evaluated the ability of our therapeutic lentivirus in editing the HIV-1 DNA present in PBMCs prepared from HIV-1-positive patients, which were provided by the Clinical Core of the Comprehensive NeuroAIDS Center (CNAC) and the Temple University Hospital AIDS Clinic. The relevant clinical history of the patients is shown in Figure 2A. Preparation and processing of the PBMCs from heparinized whole blood is schematized in Figure 2B. Although DNA sequence analyses of the viral DNA from patient samples showed minor variabilities across the LTR (Figures S1A–S1C), including the regions corresponding to the gRNAs A and B (Table S2), a significant decrease in the level of viral LTR DNA was seen in PBMCs from HIV-positive patients after transduction with the LV-CRISPR/Cas9 (Figure 2C). Gel electrophoresis of the conventional PCR reaction of the patient's PBMCs after treatment showed detection of a 167-bp amplicon, suggesting editing of the HIV LTR DNA by gRNAs and spCas9 (Figure 2D). Further, from the intensity of the 167-bp amplicon, it was evident that the efficiency of the viral DNA excision in patient #TUR0095 is far less

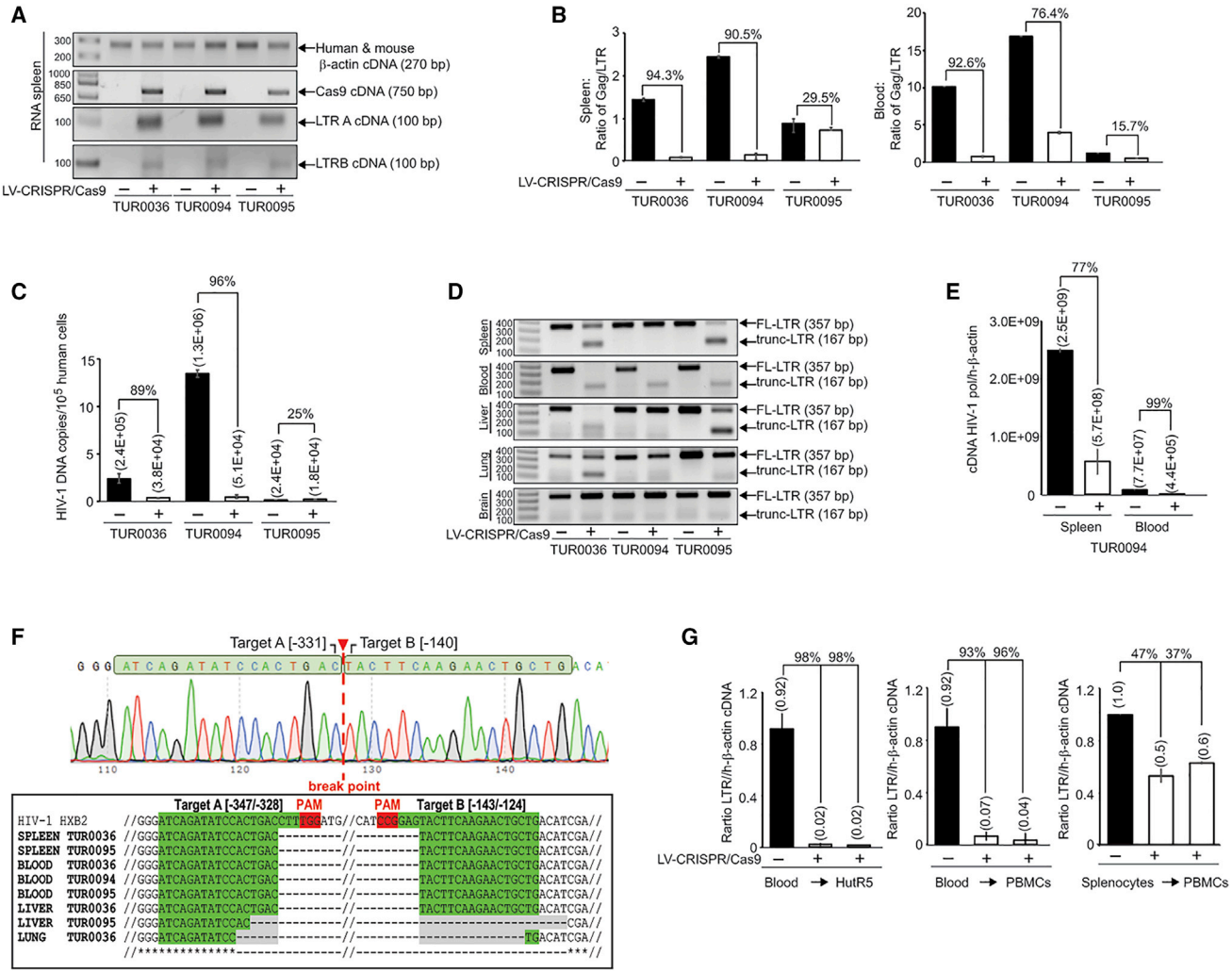

Figure 3. Removal of HIV Proviral DNA from Patient PBMCs by LV-CRISPR/Cas9 in NRG Mice

(A) Expression of Cas9 and gRNAs A and B, and the human β-actin gene by RT-PCR in spleen tissue from the engrafted NRG mice. (B) Examination of viral load by qPCR in spleen (left), as presented by the ratio of gag/LTR DNA copies, and blood (right) of control and untreated animals. (C) ddPCR of genomic DNA isolated from the spleens of humanized NRG mice shows a decrease in the viral DNA copy numbers in the treated animals. (D) Standard PCR analysis of LTR excision using genomic DNA extracted from different tissues of patient PBMC-engrafted mice. (E) Real-time PCR showed a drastic decrease in the signal corresponding to the pol RNA sequence in the spleen of mice engrafted with PBMCs from patient #TUR0094. The values are normalized to the level of detected human β-actin sequence. (F) Representative Sanger sequencing results of the DNA fragments isolated from the gel electrophoresis shown in (D). (G) Recovery of HIV upon the co-culturing of human T cell-line-, Hut-R5-, or IL-2-activated human PBMCs with blood samples from the untreated or CRISPR/Cas9-treated NRG mice transplanted with PBMCs from patient #TUR0036 (left and middle panels) or co-culturing with splenocytes prepared from the same group of animals with the activated human PBMCs.

than that seen in the other samples, suggesting that variability in the viral DNA sequence at the target sites may contribute to the poor editing of the viral DNA sequence. Further, it is also likely that, as described previously,[16] simultaneous editing of the viral genome at the two target sites, for unknown reasons, was not as robust as in the other two patient samples. Additionally, direct sequencing of the amplicons showed the sites of the breakpoints and the cleavage of the patient viral DNAs by Cas9 at target A, i.e., 3 nt from the PAM, and the deletion of a few nucleotides at target B (Figure 2E).

Once the ability of our gRNAs and spCas9 to edit HIV-1 DNA present in the patients' blood cells was verified *ex vivo*, NRG mice were engrafted with PBMCs from the same subjects for *in vivo* editing of the viral DNA. A total of 5×10^6 human PBMCs with no detectable HIV-1 p24, as tested by ELISA, were injected i.p. into 8-week-old NRG mice (n = 3 for each blood sample obtained from each patient). At 1 week post-transplantation, animals were injected intravenously with LV-CRISPR/Cas9-carrying spCas9, LV-LTR-A-expressing gRNA LTR-A, and LV-LTR-B-expressing gRNA LTR-B. Two weeks after the injection of LVs, mice were sacrificed for measuring HIV-1

RNA and DNA by RT-PCR and ddPCR. Expression of Cas9 and gRNAs were verified by RT-PCR (Figure 3A), as well as the presence of human cells, using human β-actin as a reference. Excision efficiency was examined by qPCR, by comparing the ratios of gag/LTR DNA levels between non-treated and LV-CRISPR/Cas9-treated animals. As shown in the left panel of Figure 3B, a greater-than-90% decrease in gag/LTR was observed in spleens of treated animals engrafted with PBMCs from patient #TUR0036 or patient #TUR0094, as well as a nearly 29% decrease in the animals engrafted with PBMCs from patient #TUR0095. Similar results were observed in blood from the experimental animals (Figure 3B, right). Further evaluation of HIV-1 DNA by ddPCR confirmed the qPCR results, which showed an 89% and 96% reduction in gag sequences in the human cells isolated from spleens of treated mice engrafted with PBMCs from patient #TUR0036 or patient #TUR0094, respectively. Only a modest decrease (25%) in the viral DNA level was observed in the animals harboring PBMCs from patient #TUR0095 (Figure 3C).

After viral DNA was amplified by PCR, we used gel electrophoresis to visualize the cleavage products in several tissues recovered from the animals humanized with PBMCs from patient #TUR0036. We detected a 167-bp amplicon in spleen, blood, liver and lung, but not in brain (Figure 3D). Sanger sequencing confirmed excision of the 190-bp DNA fragment between the target sites of gRNAs LTR-A and LTR-B (Figure 3F). Examination of the excision event in the tissues from the animals engrafted with PBMCs from patient #TUR0094 showed amplification of the 167-bp fragment in blood but not any other organs, including the spleen. In light of the results from the qPCR (Figure 3B) and ddPCR (Figure 3C) showing a substantial decrease in the viral LTR DNA in the spleen of this animal, we posited that the excision, in this case, may be much broader and include the entire viral genome spanning the 5′ to 3′ LTR, as reported earlier.[10,12] In support of this notion, results from qPCR of the viral sequence corresponding to the middle of the viral genome revealed a major decrease in the level of DNA encoding the pol gene in both spleen and blood samples (Figure 3E). In the animal transplanted with PBMCs from patient TUR0095, the 167-bp amplicon was detected in spleen, blood, and liver but not in lung or brain (Figure 3D). The amplicons were sequenced to validate the specificity of the editing by CRISPR/Cas9 (Figure 3F). Throughout these studies, diverse levels of LV-CRISPR-mediated excision of viral DNA were noticed in different organs of animals separately engrafted with PBMCs from three patients. We expect that CRISPR-mediated excision efficiency of viral DNA should inversely correlate with viral burden. For example, the engraft from patient TUR0094 that exhibits very little or no apparent excision of the 190-bp DNA fragment (Figure 3D) within the 5′ or 3′ LTR in liver or lung (unlike the engrafts from the other two patients) shows the highest number of HIV-1 copies per cell, as measured by qPCR and ddPCR for blood and spleen (Figures 3B and 3C). Of note, in the humanized animals, human PBMCs remain predominantly in the circulation of engrafted animals; thus, blood and spleen are more enriched with human cells and yield more consistent outcome.

Finally, to further assess the efficacy of our therapeutic strategy, we performed viral rescue assays using mononuclear cells recovered from the blood and spleen of the two NRG mice engrafted with PBMCs from patient TUR0036 to co-cultivate with the HuT-R5 cell line or IL2-activated PBMCs from healthy donors. In this system, significantly reduced viral outgrowth was seen from the peripheral blood PBMCs of LV-CRISPR/Cas9-treated animals, as compared to non-treated animals (Figure 3G). Conversely, isolated splenocytes from an LV-CRISPR/Cas9-treated animal did not reach the same level of significance observed with the blood PBMCs, although a significant decline (37%–47%) in virus recovery was achieved (Figure 3G). These observations demonstrate that, while CRISPR technology can permanently inactivate the patient's viral DNA in humanized animals, under the present experimental conditions, *in vivo* editing of the HIV-1 genome by CRISPR/Cas9 may not completely eliminate replication-competent virus from the solid organ, including spleen. Still, these observations, for the first time, offer a proof of principle on the ability of using LVs to effectively deliver CRISPR to various cells and organs and excise viral DNA from the PBMCs in blood of human patients who have been on ART for many years, as evidenced by, at least, a 93% decrease in the viral recovery assay. Thus, we predict that complete elimination of HIV-1 by CRISPR may require a combination therapy with additional inhibitors, such as ART, that effectively control virus replication. In some instances, one may need to personalize CRISPR/Cas9 by creating sets of gRNAs that perfectly match with the viral genome, should the universal gRNAs show less efficiency. The timing and duration of ART can play a significant role in the formation of an HIV reservoir in HIV-infected patients, which will be considered in our future plans, which will be focused on a larger cohort to determine when the CRISPR/Cas9 should be administered and what the dosage should be.

MATERIALS AND METHODS

Please see the Supplemental Information for a detailed explanation of all experimental procedures and the materials used in this study.

Statistical Analysis

The quantitative data, representing mean ± SD, were evaluated by Student's t test, with a p value < 0.05 considered as a statistically significant difference.

Animal Subject Approval

All experiments were carried out in Biosafety Level 3 (BLS-3) facilities (George Mason University, Manassas, VA, USA) in accordance with the Guide for the Care and Use of Laboratory Animals (Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, NIH Publication 86-23, revised 1996). NOD.Cg-Rag^{1tm1Mom}, Il2rgtm^{1Wjl}/SzJ mice (NRG, NOD Rag gamma) were obtained from the Jackson Laboratory (#007799, Bar Harbor, ME, USA) and were humanized as described previously.[22,23] These animals can be used either for long-term studies using CD34^{+} human cells or for short-term studies using primary PBMCs. In most of the cases, graft versus host (GVH) disease

does not appear in these animals until months 6–9, making them an ideal model for both short- and long-term studies.

Human Subject Approval

Isolated PBMCs from 3 HIV-1 infected volunteers were acquired through the Clinical Core Repository of the Comprehensive NeuroAIDS Center (CNAC; Kamel Khalili, principal investigator). Patient recruitment and blood collections were performed under protocol #21983, approved by the Temple University Institutional Review Board. Urine was collected from each subject at the time of phlebotomy and screened for amphetamines, barbiturates, benzodiazepine, cannabinoid, cocaine, methadone, methaqualone, opiate, phencyclidine, and alcohol under the same protocol (#21983). Written informed consent was obtained from all subjects at the time of enrollment into the Clinical Core Repository of the CNAC. All HIV-1-infected subjects in our study were on antiretroviral therapy with low or non-detectable virus and $CD4^+$ T cell counts near or above 500 cells/μL (Figure 2A).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods, one figure, and two tables and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.05.021.

AUTHOR CONTRIBUTIONS

Conceived and designed experiments: K.K., F.K., and R.K. Analyzed data: K.K., F.K., R.B., R.K., W.-B.Y., T.F., S.A., J.F.J., and P.F. Performed experiments: R.B., R.K., P.M., C.C., W.-B.Y., and R.S. All authors prepared the manuscript.

CONFLICTS OF INTEREST

K.K. and R.K. are named as inventors on patents that cover the viral gene editing technology that is the subject of this article. In addition to the foregoing interests, K.K. is a co-founder, board member, and scientific advisor and holds equity in Excision Biotherapeutics, a biotech start-up that has licensed the viral gene editing technology from Temple University for commercial development and clinical trials.

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The authors declare that this work was produced solely by the authors and that no other individuals or entities influenced any aspects of the work including, but not limited to, the study conception and design; data acquisition, analysis, and interpretation; and writing of the manuscript. No other entities provided funds for the work. The authors further declare that they have received no financial compensation from any other third parties for any aspects of the published work.

REFERENCES

1. Chun, T.W., Stuyver, L., Mizell, S.B., Ehler, L.A., Mican, J.A., Baseler, M., Lloyd, A.L., Nowak, M.A., and Fauci, A.S. (1997). Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc. Natl. Acad. Sci. USA *94*, 13193–13197.

2. Lorenzo-Redondo, R., Fryer, H.R., Bedford, T., Kim, E.Y., Archer, J., Pond, S.L.K., Chung, Y.S., Penugonda, S., Chipman, J., Fletcher, C.V., et al. (2016). Persistent HIV-1 replication maintains the tissue reservoir during therapy. Nature *530*, 51–56.

3. Gray, L.R., Roche, M., Flynn, J.K., Wesselingh, S.L., Gorry, P.R., and Churchill, M.J. (2014). Is the central nervous system a reservoir of HIV-1? Curr. Opin. HIV AIDS *9*, 552–558.

4. Perreau, M., Savoye, A.L., De Crignis, E., Corpataux, J.M., Cubas, R., Haddad, E.K., De Leval, L., Graziosi, C., and Pantaleo, G. (2013). Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. J. Exp. Med. *210*, 143–156.

5. Wong, J.K., and Yukl, S.A. (2016). Tissue reservoirs of HIV. Curr. Opin. HIV AIDS *11*, 362–370.

6. Kim, Y., Anderson, J.L., and Lewin, S.R. (2018). Getting the "kill" into "shock and kill": strategies to eliminate latent HIV. Cell Host Microbe *23*, 14–26.

7. Delagrèverie, H.M., Delaugerre, C., Lewin, S.R., Deeks, S.G., and Li, J.Z. (2016). Ongoing clinical trials of human immunodeficiency virus latency-reversing and immunomodulatory agents. Open Forum Infect. Dis. *3*, ofw189.

8. Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819–823.

9. Mali, P., Yang, L., Esvelt, K.M., Aach, J., Guell, M., DiCarlo, J.E., Norville, J.E., and Church, G.M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823–826.

10. Hu, W., Kaminski, R., Yang, F., Zhang, Y., Cosentino, L., Li, F., Luo, B., Alvarez-Carbonell, D., Garcia-Mesa, Y., Karn, J., et al. (2014). RNA-directed gene editing specifically eradicates latent and prevents new HIV-1 infection. Proc. Natl. Acad. Sci. USA *111*, 11461–11466.

11. Kaminski, R., Bella, R., Yin, C., Otte, J., Ferrante, P., Gendelman, H.E., Li, H., Booze, R., Gordon, J., Hu, W., and Khalili, K. (2016a). Excision of HIV-1 DNA by gene editing: a proof-of-concept in vivo study. Gene Ther. *23*, 690–695.

12. Kaminski, R., Chen, Y., Fischer, T., Tedaldi, E., Napoli, A., Zhang, Y., Karn, J., Hu, W., and Khalili, K. (2016b). Elimination of HIV-1 genomes from human T-lymphoid cells by CRISPR/Cas9 gene editing. Sci. Rep. *6*, 22555.

13. Ebina, H., Misawa, N., Kanemura, Y., and Koyanagi, Y. (2013). Harnessing the CRISPR/Cas9 system to disrupt latent HIV-1 provirus. Sci. Rep. *3*, 2510.

14. Liao, H.K., Gu, Y., Diaz, A., Marlett, J., Takahashi, Y., Li, M., Suzuki, K., Xu, R., Hishida, T., Chang, C.J., et al. (2015). Use of the CRISPR/Cas9 system as an intracellular defense against HIV-1 infection in human cells. Nat. Commun. *6*, 6413.

15. Wang, Z., Pan, Q., Gendron, P., Zhu, W., Guo, F., Cen, S., Wainberg, M.A., and Liang, C. (2016). CRISPR/Cas9-derived mutations both inhibit HIV-1 replication and accelerate viral escape. Cell Rep. *15*, 481–489.

16. Wang, G., Zhao, N., Berkhout, B., and Das, A.T. (2016). A combinatorial CRISPR-Cas9 attack on HIV-1 DNA extinguishes all infectious provirus in infected T cell cultures. Cell Rep. *17*, 2819–2826.

17. Lebbink, R.J., de Jong, D.C., Wolters, F., Kruse, E.M., van Ham, P.M., Wiertz, E.J., and Nijhuis, M. (2017). A combinational CRISPR/Cas9 gene-editing approach can halt HIV replication and prevent viral escape. Sci. Rep. *7*, 41968.

18. White, M.K., Hu, W., and Khalili, K. (2016). Gene editing approaches against viral infections and strategy to prevent occurrence of viral escape. PLoS Pathog. *12*, e1005953.

19. Yin, C., Zhang, T., Qu, X., Zhang, Y., Putatunda, R., Xiao, X., Li, F., Xiao, W., Zhao, H., Dai, S., et al. (2017). In vivo excision of HIV-1 provirus by saCas9 and multiplex single-guide RNAs in animal models. Mol. Ther. *25*, 1168–1186.

20. Yin, C., Zhang, T., Li, F., Yang, F., Putatunda, R., Young, W.B., Khalili, K., Hu, W., and Zhang, Y. (2016). Functional screening of guide RNAs targeting the regulatory and structural HIV-1 viral genome for a cure of AIDS. AIDS *30*, 1163–1174.

21. Pearson, T., Shultz, L.D., Miller, D., King, M., Laning, J., Fodor, W., Cuthbert, A., Burzenski, L., Gott, B., Lyons, B., et al. (2008). Non-obese diabetic-recombination activating gene-1 (NOD-Rag1 null) interleukin (IL)-2 receptor common gamma chain (IL2r gamma null) null mice: a radioresistant model for human lymphohaematopoietic engraftment. Clin. Exp. Immunol. *154*, 270–284.

22. Sampey, G.C., Saifuddin, M., Schwab, A., Barclay, R., Punya, S., Chung, M.C., Hakami, R.M., Zadeh, M.A., Lepene, B., Klase, Z.A., et al. (2016). Exosomes from HIV-1-infected cells stimulate production of pro-inflammatory cytokines through trans-activating response (TAR) RNA. J. Biol. Chem. *291*, 1251–1266.

23. Van Duyne, R., Cardenas, J., Easley, R., Wu, W., Kehn-Hall, K., Klase, Z., Mendez, S., Zeng, C., Chen, H., Saifuddin, M., and Kashanchi, F. (2008). Effect of transcription peptide inhibitors on HIV-1 replication. Virology *376*, 308–322.