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EDITING OF SIV IN NONHUMAN PRIMATES BY CRISPR-CAS9 IN VIRAL RESERVOIRS

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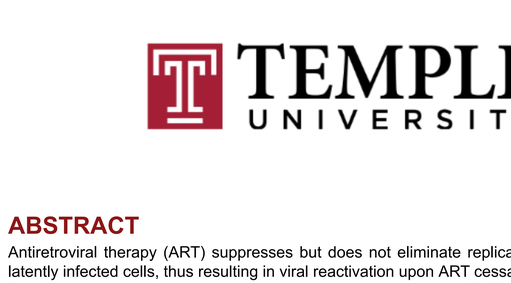
ABSTRACT

Antiretroviral therapy (ART) suppresses but does not eliminate replication competent HIV proviral DNA from latently infected cells, thus resulting in viral reactivation upon ART cessation. Therefore, removal of HIV proviral DNA from infected individuals is needed. We have assessed a CRISPR-Cas9 based gene editing strategy for the elimination of SIV proviral DNA in the rhesus macaque model.

An all-in-one AAV9 gene therapy vector was constructed to deliver CRISPR-Cas9 plus two gRNAs targeting sequences within the 5' and 3' viral LTRs and the Gag gene to excise the intervening proviral DNA fragment. Ten adult Indian rhesus macaques were i.v. infected with SIVmac239 then treated daily with a drug regimen of tenofovir, emtricitabine and dolutegravir (5.1/50/2.5mg/kg daily s.q.). Animals were randomized into groups to receive low versus high dose of AAV9-CRISPR-Cas9 in a single i.v. infusion (low dose: 1.4x10^12 GC/kg n=4; high dose: 1.4x10^13 GC/kg n=3) as well as control SIV infected animals (n=3). Longitudinal blood samples and lymph node biopsies were collected, and animals were necropsied at 3 (n=8) or 6 months (n=2) after CRISPR treatment.

SIV-infected animals treated with AAV9-CRISPR-Cas9 at both high and low doses showed in vivo excision of viral DNA from serial blood samples. Results from Sanger sequencing confirmed the precise breakpoint of the viral DNA in samples in which excision was detected. Biodistribution of the AAV9-CRISPR-Cas9 vector was assessed by PCR to detect the presence of the Cas9 gene sequence. DNA scope was performed on lymph nodes and spleen to detect the AAV9-CRISPR-Cas9 viral vector and expression of the Cas9 gene. Broad excision of SIV proviral DNA was observed in blood, lymph nodes, and other tissues known to be viral reservoirs including spleen, gut, and brain. A dose response between low and high doses, as well as temporal distribution between 3 and 6 months, was observed for AAV9-CRISPR-Cas9 viral DNA in the blood.

Here we demonstrate broad SIV DNA excision in viral reservoirs leading to permanent inactivation of SIV proviral DNA in a one shot CRISPR molecule. We observed biodistribution of AAV9-CRISPR-Cas9 in the blood in a dose and time dependent manner for the elimination of SIV DNA. These findings support the utilization of AAV9-CRISPR-Cas9 as a potential therapeutic strategy for in vivo gene editing of HIV proviral DNA from latent tissue reservoirs.



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Figure 1. Excision strategy for the elimination of SIV proviral DNA. A. Schematic of the CRISPR-Cas9 approach to viral inactivation and generation of escape mutants. Our strategy uses the CRISPR-Cas9 endonuclease to cut the SIV proviral genome using multiple gRNAs. When multiple gRNAs are used, this allows the creation of a large deletion with no protein. This avoids the possibility of escape mutants, which could arise with a single gRNA due to a base substitution, or InDel mutations which may occur during repair of the double-strand DNA break by error-prone non-homologous end-joining (NHEJ). **B.** We utilized one gRNA targeting both the 5' and 3' viral LTRs (i.e., one 5'LTR cut site and one 3'LTR cut site) and a second gRNA targeting the Gag gene. gRNA sequences, cut sites within the SIV genome, and the three potential resulting excision products are shown.

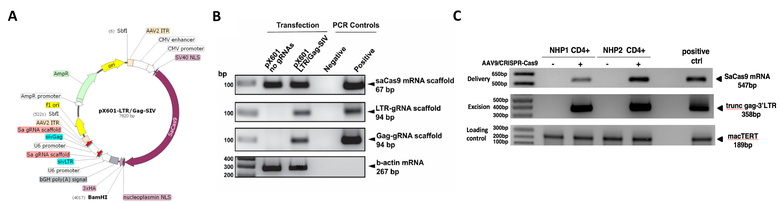


Figure 2. Validation of EBT-001 expression by plasmid transfection in HEK293T cells and AAV9 transduction in primary human CD4+ T cells. A. Map of EBT-001 plasmid (AAV pX601-SaCas9-2xgRNA). **B.** Confirmation of SaCas9 and gRNA expression by RT-PCR analysis of HEK293T cells transfected with the EBT-001 plasmid. **C.** Confirmation of SaCas9 and gRNA expression by RT-PCR analysis of primary human CD4+ T cells transduced with AAV-CRISPR-Cas9 (EBT-001).

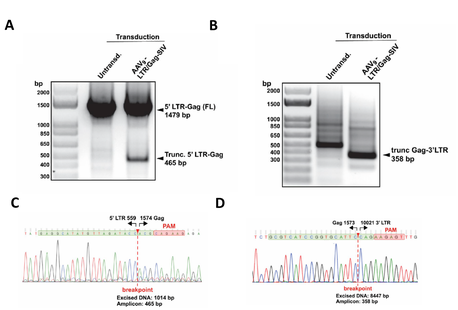


Figure 3. Ex vivo confirmation of EBT-001 cleavage of target SIV proviral DNA. A. Gel electrophoresis analysis of PCR reactions for detection of SIV DNA after the treatment of cells with EBT-001. Results from PCR analysis showed a clear band of 465 bp in size in cells that were transduced with EBT-001 representing the 5'LTR to Gag excision product. **B.** Results from PCR analysis showed a clear band of 358 bp in size in cells that were transduced with EBT-001 representing the Gag to 3'LTR excision product. **C.** Results from sequencing of the 465 bp amplicon showed the breakpoint of the viral DNA, where the truncated 5'LTR is joined to the residual Gag gene after the removal of the 1014 bp DNA fragment. **D.** Results from sequencing of the 358 bp amplicon showed the breakpoint of the viral DNA, where the truncated Gag is joined to the residual 3'LTR after the removal of the 8447 bp DNA fragment.

Figure 4. Study design and timeline – Effect of EBT-001 on SIVmac239-infected ART-treated Indian rhesus macaques

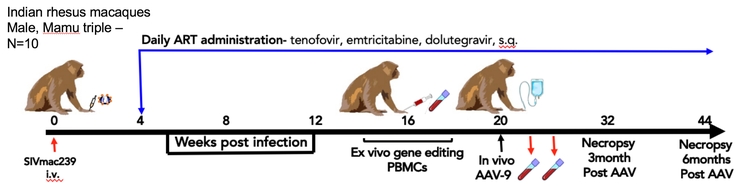


Table 1. Animal groups, dosing, and numbers per group:

Group	EBT-001 Treatment Dose (vg/kg)	SIV infection SIVmac239 i.v.	ART treatment s.q.	Total Number of Males	Post-Treatment Termination Interval 3 months	6 months
1	0	200 $TCID_{50}$	Daily starting 1 month post SIV	3	3	--
2	1.4 x 10^12 vg/kg	200 $TCID_{50}$	Daily starting 1 month post SIV	3	3	--
3	1.4 x 10^13 vg/kg	200 $TCID_{50}$	Daily starting 1 month post SIV	4	2	2

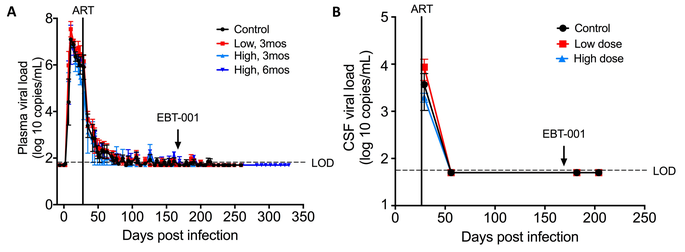
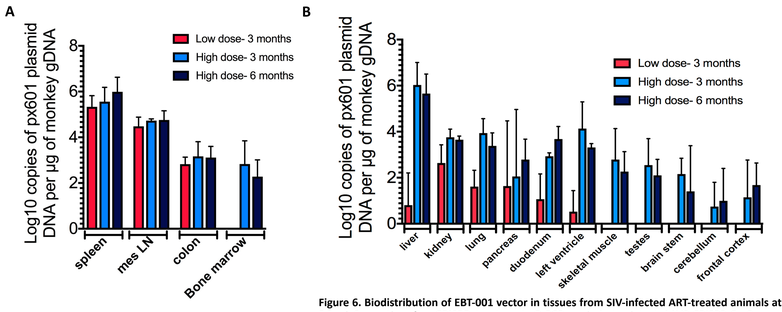


Figure 5. Plasma and CSF SIV viral loads in SIV-infected ART-treated infected animals +/- EBT-001 during the course of study. A. Plasma viral loads of the SIV-infected ART-treated rhesus macaques. **B.** CSF viral loads of the SIV-infected ART-treated rhesus macaques. All animals were infected with SIVmac239 and received daily ART administration. Animals in red received low dose EBT-001 and were euthanized at 3 months post-EBT-001 treatment. Animals in light blue received high dose EBT-001 and were euthanized at 3 months post-EBT-001 treatment. Animals in dark blue received high dose EBT-001 and were euthanized at 6 months post-EBT-001 treatment. Animals in black were not treated with EBT-001. The threshold of the assay was 83 copies/mL.

Table 2. Clinical observations and gross necropsy findings of SIVmac239-infected ART-treated animals +/- EBT-001.
Note: No significant findings noted which were attributable to EBT-001 treatment.

	Animal number	Date of necropsy	Final weight (kg)	Body weight change (%)	External observations	Gross necropsy results
Group 1 Untreated 3 months	1T6	02/04/19	3.2	-10.3	very thin; 7 to 10% dehydrated, body condition score 2/5	dehydrated, thin body condition (2/5 BCS), findings consistent with SIV infection
	7S0	02/04/19	4.7	+2.1	subcutaneous abscess at ART injection sites	no significant findings
	CF53	02/11/19	3.9	+8.1	no significant findings	no abnormal or significant findings
Group 2 Lose dose 3 months	CF63	02/11/19	4.1	+7.5	no significant findings	no abnormal or significant findings
	CI77	02/12/19	5.2	+23.7	no significant findings	no abnormal or significant findings
	MA285	02/12/19	5.1	+13.6	no significant findings	no abnormal or significant findings
Group 3 High dose 3 months	CK10	02/05/19	4.2	-12.8	no significant findings; weight loss of 10%; body condition score was in normal range 3/5	no abnormal or significant findings (weight loss noted)
	CK49	02/05/19	5.2	+14.3	no significant findings	no abnormal or significant findings
Group 3 High dose 6 months	CF27	04/15/19	6.0	+11.3	no significant findings	no abnormal or significant findings
	CH97	04/15/19	4.7	+7.1	no significant findings	no abnormal or significant findings



Both panels:
The linear range = 5 validation runs
Lower limit of detection (LOD) = 10 copies (1 log10 copy)
Lower limit of quantification (LLOQ) = 25 copies (1.4 log10 copies)
Upper limit of quantification (ULOQ) = 10^7 copies (7 log10 copies)

Figure 6. Biodistribution of EBT-001 vector in tissues from SIV-infected ART-treated animals at 3 and 6 months after CRISPR treatment. Q-PCR analysis to detect AAV9-CRISPR-Cas9 gene therapy vector EBT-001 in tissues from animals treated with high and low doses of EBT-001. Q-PCR amplification detected a portion of the Cas9 transgene in genomic DNA extracted from various tissues of the EBT-001 treated animals collected at necropsy. **A.** Levels of EBT-001 detected in lymphoid tissues known to be major HIV reservoirs are shown. **B.** Levels of EBT-001 detected in other tissues are shown. Assay detection limits are shown at the left.

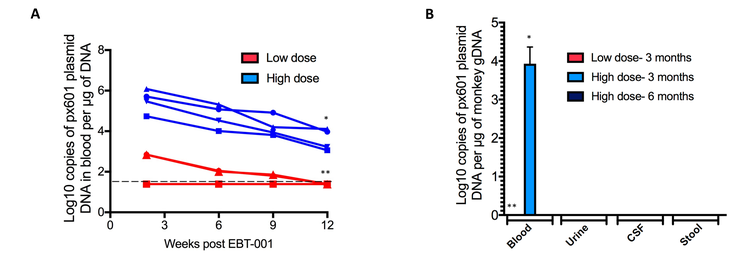


Figure 7. Biodistribution of EBT-001 vector in blood and body fluids from SIV-infected ART-treated animals after CRISPR treatment to assess vector shedding. Q-PCR analysis to detect AAV9-CRISPR-Cas9 gene therapy vector EBT-001 in blood and body fluids from animals treated with high and low doses of EBT-001. Q-PCR amplification detected a portion of the Cas9 transgene in genomic DNA extracted from whole blood, body fluids, and stool of the EBT-001 treated animals. **A.** Levels of EBT-001 detected in blood every three weeks after treatment. Dotted line represents the lower limit of detection. **B.** Levels of EBT-001 detected in body fluids and stool collected upon necropsy at 3 and 6 months after treatment. Assay detection limits are shown at the left. * and ** indicate the same time point for comparison between Panel A and Panel B.

Right panel:
The linear range = 5 validation runs
Lower limit of detection (LOD) = 10 copies (1 log10 copy)
Lower limit of quantification (LLOQ) = 25 copies (1.4 log10 copies)
Upper limit of quantification (ULOQ) = 10^7 copies (7 log10 copies)

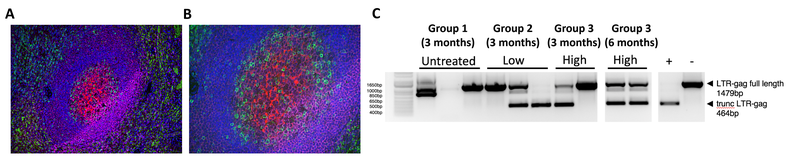


Figure 8. Detection of CRISPR-Cas9 gene therapy vector EBT-001 in spleen tissue by IHC/DNA scope and evidence of SIV excision in blood samples from SIV-infected ART-treated animals. A. and B. Triple label DNAScope and IHC was used to detect Cas9 DNA in the spleens of SIV-infected ART-treated rhesus macaques treated with EBT-001. Representative spleen of monkey CH97 (Group 3) treated with high dose of EBT-001 and necropsied at 6 months after treatment is shown. Anti-CD3 to detect T cells is shown in green; Anti-CD20 to detect B cells is shown in pink; DNAScope to detect Cas9 DNA is shown in red; Dapi counterstain is shown in blue; original magnification: 20x (Panel A) and 40x (Panel B). **D.** Gel electrophoresis analysis of PCR reaction for detection of SIV DNA in blood of all animals in Groups 1 through 3 is shown. Results from the PCR analysis showed a clear band of 464 bp in size in some animals treated with EBT-001.

CONCLUSIONS

In this proof of concept study, we observed long-term biodistribution of EBT-001 and delivery of CRISPR-Cas9 to the blood as well as sites of viral latency with minimal evidence of viral shedding at 3 and 6 months post inoculation. We observed evidence of genome editing which excised integrated SIV from the blood of EBT-001 treated SIV-infected animals on ART demonstrating the feasibility of this treatment *in vivo* using i.v. administration of an all-in-one AAV9-CRISPR-Cas9 construct. EBT-001 was broadly distributed in the cells and tissues known to be viral reservoirs and persisted for at least 6 months at the high dose. No significant safety concerns were noted in any animals which received EBT-001. These observations serve as a first step toward in vivo preclinical studies and demonstrate feasibility in employing a delivery system that has the capacity for carrying the CRISPR-Cas9 and its associated SIV-specific targeting guide RNAs (gRNAs) to known viral reservoirs. We propose the use of CRISPR-Cas9 genome editing as part of a possible HIV cure strategy. Further studies will shed light on the extent to which latent proviral DNA can be eliminated from viral reservoirs throughout the body and whether treatment with EBT-001 will delay or eliminate viral rebound upon discontinuation of ART.

CONFLICTS OF INTEREST

KK is Co-Founder and Chief Scientific Consultant, and holds equity in Excision Biotherapeutics, a biotech start-up company that has licensed the viral gene editing technology from Temple University. KK and RK are named inventors on patents that cover the viral gene editing technology. TB and JG hold equity in Excision Biotherapeutics.

ACKNOWLEDGEMENTS

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