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Introduction of Mutant APOBEC3G Into CD4+ T-Cells to Resist HIV-1

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Abstract

Introduction: Human immunodeficiency virus 1 (HIV-1) is a potentially lethal retrovirus with high genetic diversity and replication ability. HIV-1 mainly targets human CD4+ T-cells, causing a rapid decline in CD4+ T-cell count after infection. Apolipoprotein B mRNA Editing Enzyme Catalytic Subunit 3G (APOBEC3G or A3G) is a human anti-viral enzyme that blocks early stages of HIV-1 replication by deaminating the cytosine residues to uracil in the viral minus-strand DNA during reverse transcription. Nevertheless, HIV-1 overcomes A3G anti-viral activity by employing Vif accessory protein to degrade A3G. Due to a proline/aspartate point mutation in residue 129 (P129D), an artificial A3G mutant, also known as A3G-P129D, was found to be resistant against diverse Vif variants, including HIV-1 Vif, thereby shedding light on a potential A3G-mediated gene therapy for HIV-1.

Methods: We propose that, using CRISPR-Cas9 tools, the P129D mutation will be introduced into A3G expressed by human hematopoietic stem cells. Engineered stem cells will be transplanted into Non-Obese Diabetic Severe Combined Immunodeficient Gamma (NSG) mice, which will later be infected with HIV-1. Following infection, flow cytometry will be used to compare the CD4+ T-cell count in mice that express A3G-P129D versus control mice transplanted with unmodified human CD4+ T-cells.

Results: We expect a notably higher CD4+ T-cell count in mice carrying the P129D mutation than in the control group, which would be attributed to the efficient inhibition of HIV-1 replication by the mutant A3G.

Discussion: Transforming human A3G to A3G-P129D will prevent Vif-mediated degradation of A3G, allowing A3G to efficiently target HIV-1. Thus, this treatment would ultimately block HIV-1 replication and prevent CD4+ T-cell depletion.

Conclusion: Overall, our novel gene therapy approach could open the door to a potential one-time treatment for HIV-1 infected patients.

Keywords: human immunodeficiency virus 1; AIDS; APOBEC3G; CD4+ T-cells; Vif; gene therapy

Introduction

Human immunodeficiency virus 1 (HIV-1) is a retrovirus that hijacks CD4+ T lymphocytes and their replication machinery, enduing itself with high replication capabilities that can lead to the depletion of the reservoir CD4+ T-cells, and possibly progression to Acquired Immune Deficiency Syndrome (AIDS) [1,2]. APOBEC3s are nucleic acid editing enzymes deployed by various mammal cells to produce lethal mutations in viral genomes, providing an endogenous defence mechanism against viral pathogens [3]. Specifically, APOBEC3s are DNA-editing cytidine deaminases that deaminate cytosine residues to uracil in the minus strand of the viral DNA during reverse transcription, therefore, disrupting early stages of HIV-1 replication [4]. Human lymphocytes such as T cells and B cells express 7 APOBEC3 enzymes, out of which APOBEC3D, APOBEC3F, APOBEC3G, and APOBEC3H possess an anti-viral activity against HIV-1 [5]. Interestingly, APOBEC3F (A3F) and APOBEC3G (A3G) were found to be the most widely expressed in the human population, making them significant targets for HIV-1 therapies [6]. However, HIV-1 employs the Vif accessory protein to degrade the defensive cellular APOBEC3s, inhibiting the innate anti-viral activity of the APOBEC enzymes [4]. Viral Vif forms an assembly with E3-ubiquitin ligase complex, which enables Vif to mark the anti-viral A3G with a ubiquitin for degradation [7]. As a result, Vif prevents A3G from deterring HIV-1 replication and accompanying the viral progeny budding from the cell [7]. Although A3F is less sensitive to Vif-mediated degradation than A3G, A3G is more efficient in mutating the viral genome and is expressed at levels that are ten times higher than A3F [8,9]. Therefore, we hypothesize enhancing A3G anti-viral activity as a promising strategy for HIV-1 therapies.

Based on the literature, the Vif-A3G interface crucially needs A3G residues 128 to 130 to carry out Vif-mediated degradation of A3G [10,11]. Consequently, mutations in these residues of the A3G gene were shown to grant resistance against the viral Vif. As such, the mutant A3G-P129D demonstrated the most potent resistance against Vif-mediated degradation due to its ability to change the structure of A3G at the critical residues and block A3G-Vif interface [10,11]. In this research protocol article, we hypothesize that the introduction of the mutant A3G-P129D will confer resistance against HIV-1 replication and prevent the depletion of CD4+ T-cells. At first, CRISPR-Cas9 will be utilized to genetically engineer human fetal thymic and liver Hematopoietic Stem Cells (HSCs) to express the mutant A3G by mutating the Proline at the 129 residue into Aspartate. Subsequently, we propose the combined intravenous transplantation and subrenal implantation of the engineered hematopoietic stem cells to generate humanized NSG mice. Using the humanized NSG mice, we aim to examine the efficiency of introducing the mutant A3G against HIV-1 infection, providing a novel one-time treatment for HIV-infected patients.

Methods

Engineering Multiple Human HSCs Carrying Mutant APOBEC3G

Thymus and liver HSCs will be drawn from ~20 gestational weeks old human fetuses in accordance with Chapter 12 regulations of the Tri-Council Policy Statement (TCPS2) on the use of human biological material [12,13]. The obtained fetal thymic and liver human HSCs will be sorted for the primitive huCD34+ HSC subpopulation via fluorescence-activated cell sorting (FACS) and cultured in StemPro HSC Expansion medium for later introduction of lentiviral vectors and gene therapy before transplantation [14,15]. The timepoints for huCD34+ HSCs cultures will be determined after conducting pilot experiments and assessing the cells' growth and proliferation on a daily basis using inverted microscopy. The primitive huCD34+ HSC subpopulation will be selected for transduction and later transplantation due to its gradual repopulation and engraftment for longer intervals compared to other HSC subpopulations [16,17]. Cytomegalovirus promoter (CMV), guide RNA sequence (gRNA), Cas9 gene, donor template sequence, Puromycin N-acetyltransferase (pac) gene, and Green Fluorescent Protein stain (GFP) will be integrated in a lentiviral vector, respectively. The antibiotic resistance gene (pac) will strengthen the selection pressure for the genetically engineered huCD34+ HSCs by granting them antibiotic resistance and enabling their isolation from the non-transduced HSCs after Puromycin treatment [18]. Internal Ribosome Entry Sites (IRES) will interpose each two genes to coordinate the expression of the multiple genes within the viral vector [19]. The following single-stranded oligodeoxynucleotide (ssODN), GCCAAGGATG AAGCCTCACTTCAGGTACCGCTGCGACCTCTACCC ACTGGGCCCCTCTGCTGCCCCT, was designed as a donor template to knock-in the A3G-P129D mutation using homologous-directed repair (HDR) [20]. The proposed ssODN sequence aligns in close proximity to the CRISPR-Cas9 cutting site and consists of two 30-nucleotide homology arms, which enhance the efficiency of HDR and optimize the knock-in of the intended mutation [20]. Additionally, the candidate gRNA sequences are shown in Table 1 and were designed using ATUM gRNA designing tool. Pseudotyped HIV-1 vectors devoid of all accessory proteins except for gag, pol, and rev will be constructed for later delivery of the CRISPR-Cas9 complex and introduction of the mutant A3G gene into the collected human HSCs [21]. HIV-1 based vectors are a very well-understood lentivirus model utilized in the integration of new genes into target host cells without associating any viral genes. HIV-1 vectors are capable of gene transduction into dividing and non-dividing cells, besides the vector's delivery efficiency and high cloning capacity [22,23]. Similarly, a control HIV-1 based vector will be constructed however excluding the Cas9 gene, donor template sequence, and gRNA sequence. To harvest concentrated viral supernatants for infection, we will infect HEK293T cells with either one of the HIV-1 based vectors and lyse them after 48h to collect the supernatants [10,24]. Following, two human HSC cultures will be infected with either control or gRNA-containing viral supernatants *in vitro*. After 24h, we will screen for the reporter gene (GFP) via FACS, which will also isolate GFP+ huCD34+ HSCs for further culturing and later Hematopoietic Stem Cells Transplantation (HSCT) in four cohorts of NSG mice [14,25]. Randomly selected GFP+ huCD34+ HSCs will be used to extract DNA for amplification by Polymerase Chain Reaction (PCR) and further Sanger Sequencing to ensure that the mutant and wildtype A3G genes are present in the corresponding HSC cultures. Using allele-specific primers, Sanger Sequencing can generate the nucleotide sequence for each A3G allele and provide the ratio of mutant to wildtype copies in the sequenced HSCs, enabling us to evaluate the success of CRISPR-Cas9 gene editing [26]. Additionally, Western blotting will be conducted using GFP+ huCD34+ HSC lysates to confirm not only the presence of the mutant and wildtype A3G genes but also their expression into proteins. Following the confirmation of successful transduction, two cohorts of mice will receive the engineered HSCs, while two other cohorts will receive the control HSCs. Using pilot experiments, we will calculate the number of mice needed per group to sufficiently power our subsequent analysis with an alpha set to <0.05 at a beta level of 0.8. This method will ensure that we are not using extra unnecessary animal resources or insignificant sample sizes [27].

Table 1. Sequences of Three Complimentary Guide RNAs

List of Complimentary gRNAs	Sequence of gRNA	On Target score*
Candidate gRNA 1	**TCCTGGACGTGATTCCCTTTTGG**	**100**
Candidate gRNA 2	**TATGGCCACGTTCCTGGCCGAGG**	**97.52**
Candidate gRNA 3	**CAGAGATGAGATTCTTCCACTGG**	**85.77**

* Higher scoring gRNA implies lower off-target hits

Construction of Humanized NSG Mice

The transduced liver-derived huCD34+ HSCs will be transplanted by intravenous injection in two cohorts of NSG mice following a subrenal xenograft of transduced fetal thymic and liver huCD34+ HSCs [14, 25,28]. This combined transplantation enhances and sustains higher T-cell repopulation as opposed to either Peripheral Blood HSCT (PBHSCT) or fetal tissue implantation alone [14, 25,28]. Likewise, the latter steps will be followed with the non-transduced HSCs to generate a control group consisting of two cohorts of untreated NSG mice. Post-transplantation, we will evaluate the human leukocyte engraftment, huCD4+ T-cells in particular, on a weekly basis using flow cytometry analysis. Previous studies on similar immunodeficient mice models exhibited an enhanced human cells' engraftment of ~70% around 12 weeks post-transplantation, during which the model was ready for HIV-1 intraperitoneal inoculation [14,29]. Before HIV-1 infection, DNA will be isolated and PCR will be conducted on both treated and untreated mice to amplify the mutant and wildtype A3G genes, respectively, followed by Sanger Sequencing to check the presence of either gene in the corresponding cohorts. The following forward and reverse primers, 5'-GGGTTCCTGTCCTGTGTGTC-3' and 5'-GGTTAACTCTTGCCACACCC-3' respectively, were designed similarly for both the wildtype and mutant A3G genes using the Primers Blast machine of the National Library of Medicine. Thereafter, Western Blot will be applied to all cohorts using FACS-isolated huCD4+ T-cells to ensure not only the presence of the wildtype and mutant A3G genes but also the expression of these genes and translation of the corresponding proteins.

HIV-1 Infection of Humanized NSG Mice

Following 70% engraftment of human cells as determined by flow cytometry, one cohort of hu-NSG mice of each the treated and untreated groups will be inoculated with HIV-1 by intraperitoneal injection, while the other cohort of each group is left uninfected [29]. Under normal conditions, HIV-1 infection enters the acute phase after a few weeks of the introduction of the virus, which is characterized by a peak in viral load and a rapid decline in CD4+ T-cells followed by a latent chronic state [30]. Due to the shorter lifespan of SCID mice relative to humans, the sudden drop in huCD4+ T-cells is expected to occur at an earlier time, within 7-14 days post-infection, in the infected untreated mice with quick progression into the acute phase following HIV-1 introduction [31].

Leukocyte Cell Count and Viral Load Analysis

Using PCR-based Plasma Viral Load Test (PVL Test), the viral load in all cohorts will be evaluated on a daily basis starting from day 0 until the expected rapid decline of huCD4+ T-cells in the infected untreated NSG mice [31]. Simultaneously, huCD4+ T-cell count as well as other leukocyte counts including B cells and NK cells will be monitored in both treated and untreated NSG groups by daily flow cytometry starting from day 0 until the expected decline point. Following the peak in viral load and drop in huCD4+ T-cells in the infected untreated mice, the chronic latent phase of the infection will be monitored once every week using the previous methods on all cohorts of mice. In addition, Western Blotting will be employed using huCD4+ T-cells post-infection from day 0 on a weekly manner in an attempt to validate the endurance and degradation of the mutant and wildtype A3G, respectively, in the presence of HIV-1 Vif protein.

Post-Infection Mice Health Assessments

A health assessment will be conducted on all cohorts of mice on a daily basis post-infection. The vital signs of the mice will be monitored and their weight will be recorded. A decrease in mice weight along with unstable vitals is a measure that indicates the endpoint of our experiment. Additionally, the immune responses of the treated and untreated mice groups will be examined by challenging the mice with contaminated water with heat-killed microorganisms for one week pre- and post-infection [32]. Antibody Titer Test will be used to examine the immune responses and ensure the absence of any effects from the introduction of the mutant A3G on the mice immunocompetence.

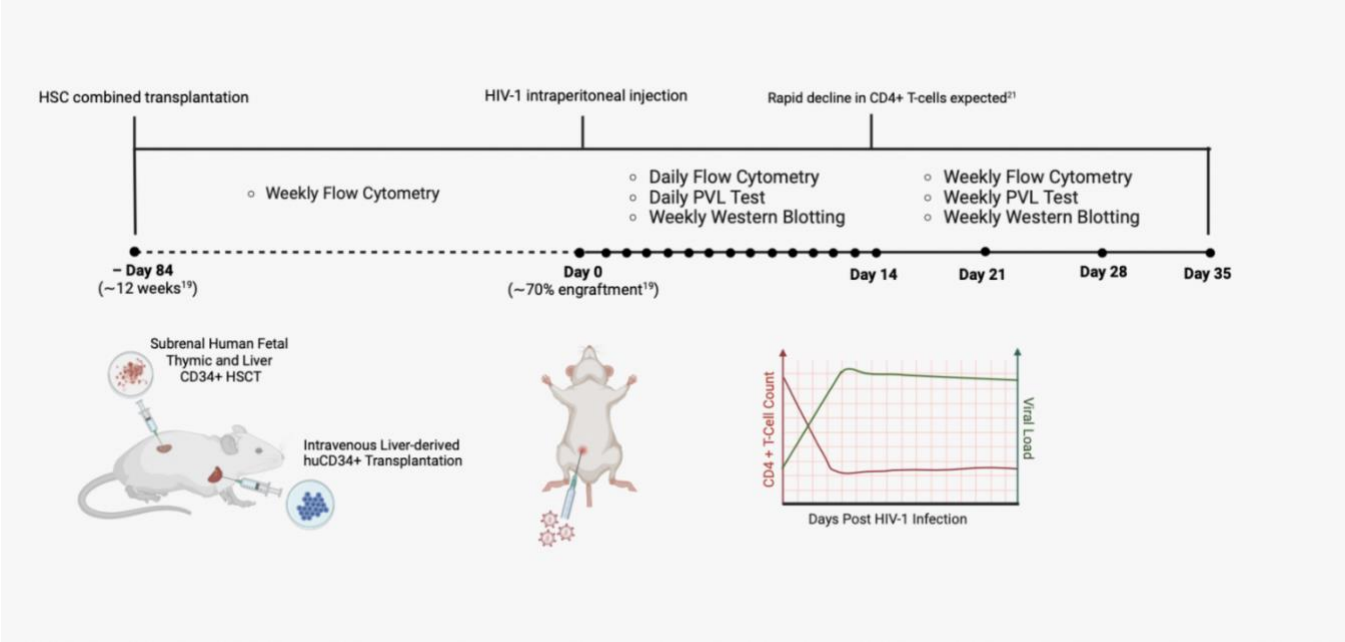

Figure 1. Schematic timeline of various experimental assessments after the construction of hu-NSG mice and HIV-1 inoculation. Mice are infected at ~70% engraftment. Depletion of huCD4+ T-cells and increase of viral load are expected around day 14 post-infection. Illustrations created using BioRender.com.

Results

The potential results obtained from this study will shed light on the anti-viral activity of the mutant A3G-P129D against HIV-1 replication *in vivo*. This section is a descriptive analysis devoid of numerical data as it is confined to analysing hypothetical outcomes based on literature as demonstrated in Figure 2. The infected treated hu-NSG mice cohort with the mutant A3G is expected to sustain huCD4+ T-cell and other leukocyte counts, jointly with a low viral load. The mutant A3G-P129D is not expected to degrade with the presence of HIV-1 Vif protein [10]. Opposingly, the infected untreated hu-NSG mice cohort is expected to show the normal pathogenic stages of HIV-1 infection of CD4+ T-cell depletion, peak in viral load due to rapid replication and the degradation of the wildtype A3G in the presence of the viral Vif protein [11]. Future studies would need to be conducted to provide numerical data analyzing the efficiency of APOBEC3G gene therapy in hu-NSG mice to inhibit HIV-1 replication and huCD4+ T-cell depletion. Using the one-way analysis of variance (ANOVA) with the treated and untreated hu-NSG mice groups as two independent samples and a 95% confidence interval, statistical significance in differences between the two groups is achieved when $P<0.05$. Statistical significance is aimed to confirm that the difference in huCD4+ T-cell count among both the infected treated and untreated NSG cohorts is due to the introduction of the mutant APOBEC3G.

Discussion

This experimental protocol proposes gene therapy on human stem cells as a one-time treatment for HIV-1 infection. We intend to introduce APOBEC3G-P129D utilizing CRISPR-Cas9 gene therapy on various human HSCs to be transplanted in a novel humanized mice model that can be widely exploited as an efficient model for studying HIV-1 infection [25].

NSG mice were utilized instead of the classical NOD-SCID mice due to their insusceptibility to develop early lymphomas that shortens the lifespan of the NOD-SCID model [33]. Similarly, PBL-SCID mice, unlike their NSG counterpart, were not preferred experimentally due to the progression of the fatal Graft-Versus-Host-Disease (GVHD) shortly after transplantation, which speeds the experimental timeline and leads to early sacrifice of the mice in most cases [34]. Therefore, NSG mice strain was found to be the most successful for human HSCs transplantation due to their long lifespan and lower susceptibility to various post-transplantation diseases [33].

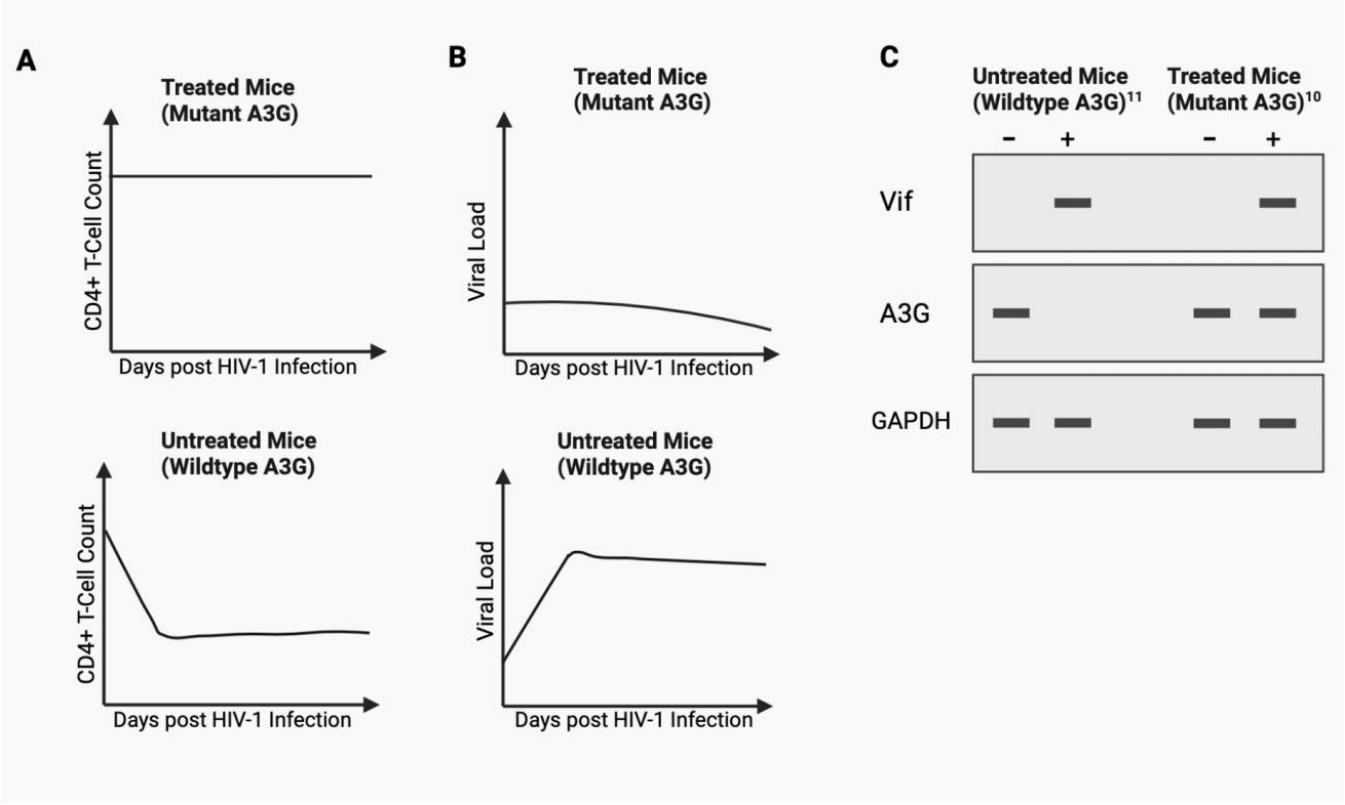

Figure 2. Responses of treated (Mutant A3G) and untreated (Wildtype A3G) hu-NSG mice to HIV-1 infection A) Evaluating CD4+ T-cell count in HIV-1 infected treated and untreated hu-NSG mice B) Viral load in HIV-1 infected treated and untreated hu-NSG mice C) Western Blot analysis of the viral Vif and cellular A3G proteins using huCD4+ T-cells in HIV-1 infected (+) and uninfected (-) treated and untreated hu-NSG mice. Illustrations created using BioRender.com

Peripheral Blood HSCs (PBHSCs) were not chosen as a source for hematopoietic stem cells to be transplanted in any SCID mice strain due to the PBHSCT-related anergic effect that appears as early as 3 weeks, which ultimately ends with the progression of fatal GVHD at 4-8 weeks post-transplantation in most SCID mice strains [35, 36]. Although NSG mice were successful in delaying the onset of GVHD, PBHSCs transplantation was not favoured in our methodology to eliminate potential limitations [37]. Alternatively, fetal thymic and liver HSCs have been implanted in NOD-SCID mice, however, the humanized mice lacked any effective immune responses despite the successful human thymopoiesis [25]. Likewise, the transplantation of liver-derived huCD34+ HSCs exclusively in NSG mice did not result in the highest levels of engraftment that can be possibly obtained [28]. As a result, we propose the intravenous transplantation of liver huCD34+ HSCs following the subrenal xenograft of fetal thymic and liver huCD34+ HSCs in the NSG mice strain [14,25]. This mice model was designed to minimize the potential post-transplantation implications and maximize the efficiency of the engrafted human immune system.

Sanger Sequencing of the PCR-amplified mutant and wildtype APOBEC3G genes obtained from the cultured GFP+ huCD34+ HSCs pre-transplantation and mice blood pre-infection confirms the successful transduction of the mutant A3G into the humanized mice and guarantees the presence of either the mutant or wildtype A3G genes in the corresponding HSC cultures pre-transplantation and NSG groups pre-inoculation. The latter would further verify the success of the proposed gene therapy conducted on various human HSCs, as well as, the prospects of combined stem cells' transplantation to humanize NSG mice and develop a complete mimicking immune system. The data from the Western Blot on GFP+ huCD34+ HSCs pre-transplantation and all cohorts of mice before HIV-1 infection ensures that the wildtype and mutant APOBEC3G genes are expressed and translated into proteins in the corresponding humanized cells.

The inhibition of huCD4+ T-cell depletion in the infected treated NSG mice in comparison to huCD4+ T-cell depletion in the infected untreated NSG mice evaluated by flow cytometry analysis proves the efficacy of A3G-P129D in blocking the collapse of the immune system susceptible to HIV-1 infection. In addition, it provides evidence for the potential of A3G-P129D to terminate the onset of HIV-1 pathogenesis without progressing into AIDS. Maintaining a stable huCD4+ T-cell count in the uninfected treated and

untreated NSG cohorts sets a control to confirm that the T-cell depletion was caused by the HIV-1 infection. Similarly, a stable cell count for various engrafted human leukocytes obtained from all cohorts of NSG mice proves that other white blood cell counts are not affected by the introduction of the mutant A3G and did not become susceptible to HIV-1 infection.

Maintaining a low viral load in the treated hu-NSG mice compared to a peaking viral load in the untreated hu-NSG mice evaluated by PVL Test after HIV-1 infection validates A3G-P129D efficient anti-viral activity in distorting HIV-1 reverse transcription, and further blocking HIV-1 replication. The mutant A3G is expected to stop the cycle of HIV-1 replication, thus, a low or undetectable HIV-1 viral load in the infected treated hu-NSG mice sheds light on the prospective use of mutant A3G in treating HIV-1 infection [38]. Our proposed methodology uses the absence of viral load in the uninfected treated and untreated NSG mice as a negative control.

Western Blotting detects the insusceptibility of the mutant A3G to HIV-1 Vif degradation. Western Blot membrane indicating a band for each the mutant A3G and HIV-1 Vif, simultaneously, proves the resistance of the mutant A3G against Vif degradation in the presence of HIV-1 Vif. Meanwhile, the absence of a band corresponding to the wildtype A3G in the presence of HIV-1 Vif band confirms the sensitivity of the wildtype A3G to Vif degradation [39].

Monitoring mice weight in all four cohorts is an essential health assessment that reflects the tolerance of the NSG model to human HSCs engraftment. Recorded weight loss greater than 15% of the initial weight in any NSG mice articulates the progression of fatal GVHD and demands sacrificing the humanized mice [35].

A3G proteins are found within the sanctity of the cytoplasm, unlike A3A which is present in the nucleus as well, thus the cell genome is unreachable to A3G and so, it lacks any potential mutagenic effects unlike A3A [40]. Furthermore, A3A transgenic mice were found to induce colorectal cancer and hepatocellular tumours more frequently than average compared to A3G transgenic mice that failed to foster mutation-mediated cancers or tumours above the control level [40]. Therefore, APOBEC3G-mediated mutations are less likely to develop carcinogenic or tumorigenic implications that deter the proposed HIV-1 therapy.

Although HIV-1 infection can be managed using life-lasting antiretroviral drugs such as integrase inhibitors and NRTIs, our proposal provides an alternative one-time gene therapy to treat HIV-1 infection. Many HIV-1 infected patients develop resistance to HIV-1 drugs, hence, the introduction of the mutant A3G can be a promising alternative [41]. Nevertheless, the introduction of the mutant A3G-P129D has the potential to confer resistance against HIV-1 replication, reversing the onset of HIV-1 infection and the progression to AIDS. Therefore, the transplantation of engineered HSCs carrying the mutant A3G into infected patients would repopulate resistant T-cells against the viral infection, replacing the T-cells prone to depletion and ultimately treating HIV-1 infection.

Conclusions

The introduction of the mutant A3G-P129D is intended to enhance the endogenous anti-viral activity against HIV-1 infection and provide a one-time therapy for infected patients. The mutant A3G prohibits the depletion of CD4+ T-cells and avoids the progression into AIDS by blocking HIV-1 replication. Observing CD4+ T-cell counts in treated and untreated humanized transgenic mice post-infection examines the efficacy of A3G-P129D introduction. This proposal aims to demonstrate *in vivo* the ability of A3G-P129D to protect huCD4+ T-cells from HIV-1 viral hijack. Furthermore, future studies shall be conducted to investigate and verify the absence of mutagenic effects for A3G-P129D introduction *in vivo*.

List of Abbreviations Used

A3F: APOBEC3F–apolipoprotein B mRNA editing enzyme catalytic subunit 3F
A3G: APOBEC3G–apolipoprotein B mRNA editing enzyme catalytic subunit 3G
AIDS: acquired immune deficiency syndrome
ANOVA: analysis of variance
CMV: cytomegalovirus promoter
FACS: fluorescence activated cell sorting
GFP: green fluorescent protein
gRNA: guide RNA
GVHD: graft-versus-host-disease
HDR: homologous-directed repair
HIV-1: human immunodeficiency virus 1
HSC: hematopoietic stem cells
HSCT: hematopoietic stem cells transplantation
IRES: internal ribosome entry site
NSG: non-obese diabetic severe combined immunodeficient gamma
pac: puromycin N-acetyltransferase
PBHSC: peripheral blood-derived hematopoietic stem cells
PBHSCT: peripheral blood-derived hematopoietic stem cells transplantation
PCR: polymerase chain reaction
PVL Test: plasma viral load test
ssODN: single-stranded oligodeoxynucleotide
TCPS2: tri-council policy statement

Conflicts of Interest

The authors declare they have no conflicts of interest.

Ethics Approval and/or Participant Consent

The study did not require ethics approval or participant consent as it is a research protocol. This means that the research has not been conducted and no participants were needed. This research protocol provides a potential

experimental design and discusses the hypothetical results expected to be obtained based on theoretical concepts and publicly available scientific literature.

Authors' Contributions

AS: contributed fundamentally to the experimental design and study idea, collected relevant information and analyzed data from literature, criticized the manuscript while providing solutions, connected with experts in the field, and approved the final version to be published.
AA: made substantial contributions to the design of the study, the collection of literature as well as interpretation and analysis of the data in literature, revised the manuscript critically, and gave final approval of the version to be published.
BM: made significant contributions to the design of the study, the collection, analysis and interpretation of data from scientific literature, revised the manuscript critically while pitching in with valuable thoughts and strategies to be implemented, and gave the final approval of the version to be published.
SD: made substantial contributions to the design of the study, the collection, interpretation and
analysis of the data from literature, created figures, revised the manuscript critically, and gave
final approval of the version to be published.

Acknowledgements

We would like to thank the IgNITE committee for giving us the chance to get involved and hone precious experience in the medical world by participating in the 2021-2022 IgNITE Medical Case Competition. We would also like to give our sincere thanks to our mentor Tarek Shaker, who guided us in every step of the way and provided positive feedback. Additionally, Dr. Sherri Christian provided us with valuable input and thorough constructive criticism for which we are credibly grateful. Last but not least, we would like to express our gratitude for Dr. Linda Chelico for her unhesitant support and generous information as an expert in the field.

Funding

This study was not funded.

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Article Information
Managing Editor: Jeremy Y. Ng
Peer Reviewers: Alita Gideon, Siobhan O'Brien
Article Dates: Received Sep 01 22; Accepted Dec 09 22; Published Jan 10 23

Citation
Please cite this article as follows:
Salman A, AlTawabini A, Mohamed B, Duraid S. Introduction of mutant APOBEC3G into CD4+ T-Cells to resist HIV-1. URNCST Journal. 2023 Jan 10: 7(1). https://urncst.com/index.php/urncst/article/view/433
DOI Link: https://doi.org/10.26685/urncst.433