JVI Accepts, published online ahead of print on 7 March 2012
J. Virol. doi:10.1128/JVI.06300-11

Generation of an HIV-1 resistant immune system with CD34+ HSCs transduced with a triple combination anti-HIV lentiviral vector

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Running title: In vivo safety and efficacy of anti-HIV HSCs

Abstract word count: 250

Text word count: 5589

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ABSTRACT:

HIV gene therapy has the potential to offer an alternative treatment strategy for HIV infected individuals compared to the use of current small molecule antiretroviral drugs. Therapies designed to administer HIV-resistant stem cells to an infected patient may also provide a functional cure, as observed in a bone marrow transplant performed with hematopoietic stem cells (HSCs) homozygous for the CCR5-Δ32 bp allele. In our current studies, preclinical evaluation of a combination anti-HIV lentiviral vector was performed, *in vivo*, in humanized NOD-RAG1-/-IL2rγ-/- knockout mice. This combination vector, which displays strong pre-integration inhibition of HIV-1 infection *in vitro,* contains a human/rhesus macaque TRIM5alpha isoform, a CCR5 shRNA, and a TAR decoy. Multi-lineage hematopoiesis from anti-HIV lentiviral vector transduced human CD34+ HSCs was observed in the peripheral blood and in various lymphoid organs including the thymus, spleen, and bone marrow of engrafted mice. Anti-HIV vector transduced CD34+ cells displayed normal development of immune cells including T cells, B cells, and macrophages. The anti-HIV vector transduced cells also displayed knockdown of cell surface CCR5 due to the expression of the CCR5 shRNA. After *in vivo* challenge with either an R5-tropic BaL-1 or X4-tropic NL4-3 strain of HIV-1, maintenance of human CD4+ cell levels and a selective survival advantage of anti-HIV gene modified cells was observed in engrafted mice. The data provided from our study confirms the safety and efficacy of this combination anti-HIV lentiviral vector in a hematopoietic stem cell gene therapy setting for HIV and validates its potential application in future clinical trials.

INTRODUCTION:

HIV gene therapy offers a potential alternative treatment strategy for HIV infected individuals compared to current antiretroviral drugs which, after prolonged use, can become toxic and allow for the generation of escape mutants (7,11,20,24,26,29). A recent hematopoietic stem cell (HSC) transplant for acute myeloid leukemia in an HIV infected patient was performed utilizing allogeneic cells from an individual homozygous for the delta-32 CCR5 deletion (12,14,19). HIV-1 suppression has been observed in the recipient, to date, even after halting antiretroviral drug therapy (16). The success of this stem cell transplant is the first report to describe a functional cure of an HIV infected individual and brings about a realization that stem cell therapies for HIV infected patients can have a dramatic impact on the outcome of their disease (15). Therefore, HIV stem cell gene therapy offers the possibility to mimic the results of this transplant by engineering a patient's autologous HSCs to express anti-HIV genes thus conferring resistance to infection (27). Advantages in utilizing HSCs for HIV gene therapy include the reconstitution of an HIV-resistant immune system, the potential for lifelong protection from further HIV replication, and the possibility of a one-time treatment upon transplantation of anti-HIV gene modified HSCs (27).

Numerous anti-HIV genes have been designed to inhibit HIV replication, however, the use of a single anti-HIV gene may not be sufficient to protect cells long-term from infection due to the high mutation rate of HIV (1,3-4,13,18,21-23). This has been proven through the use of monotherapy small molecule antiretroviral drugs which eventually select for viral escape mutants (7,20,26). Therefore, similar to combination approaches with small molecule drugs, multiple anti-HIV genes inserted into a single gene therapy vector could potentially confer

stronger protection from HIV infection in the long-term while also preventing the generation of viral resistance (2,5,9-10).

Anti-HIV genes targeted to block the early stages of HIV infection including attachment and entry, reverse transcription, and integration offer a number of advantages over molecules which act at later stages of infection including preventing the generation of provirus and the continued replenishment of viral reservoirs which are major reasons for the failure to cure HIV infected individuals (1,3-4,25,28). In this regard, by combining multiple pre-integration anti-HIV genes into a single vector, potent pre-integration protection from HIV infection could be conferred (5,17). In a previous report by our group, strong pre-integration protection from HIV-1 infection, *in vitro*, was established by a triple combination anti-HIV lentiviral vector containing a human/rhesus macaque TRIM5α isoform, a CCR5 shRNA, and a TAR decoy (5). This vector not only prevented HIV integration in challenged cells but also blocked the generation of escape mutants.

For the preclinical analysis of anti-HIV genes and vectors, it is necessary to utilize an appropriate *in vivo* model capable of demonstrating safety and efficacy of the novel therapy (6,8). The NOD-RAG1-/-IL2rγ-/- double mutant (NRG) mouse model offers the potential to evaluate multi-lineage human hematopoiesis from intrahepatic injection of human CD34+ HSCs into newborn mice. Three months after transplantation, functional human T cells, B cells, and macrophages can be detected in lymphoid organs including the spleen, thymus, and bone marrow (6). Mice successfully engrafted with a human immune system can be infected with HIV and display normal HIV disease characteristics including CD4+ cell depletion and an increase

in plasma viremia (6). This mouse model offers a unique pre-clinical *in vivo* system to evaluate anti-HIV gene therapy molecules in human cells at a level acceptable to regulatory agencies.

In our current studies, the preclinical safety and efficacy of a combination anti-HIV lentiviral vector was evaluated, *in vivo*, in a humanized NRG mouse model. Here we demonstrate multi-lineage human hematopoiesis from anti-HIV lentiviral vector transduced CD34+ HSCs in the peripheral blood and in various lymphoid organs including the thymus, spleen, and bone marrow. After *in vivo* challenge with either an R5-tropic BaL-1 or X4-tropic NL4-3 strain of HIV-1, maintenance of human CD4+ cells and a selective survival advantage was observed in mice containing the anti-HIV vector transduced cells. The data provided here confirms the utility of this combination anti-HIV lentiviral vector in inhibiting HIV infection in a stem cell gene therapy setting and validates its potential for application in a future human clinical trial.

MATERIALS and METHODS:

Lentiviral vector design and production:

The construction of the combination anti-HIV lentiviral vector has been described previously (5). Briefly, a third-generation self-inactivating lentiviral vector, CCLc-MNDU3-x-PGK-EGFP (control vector), which contains an EGFP reporter gene and was used as the EGFP-alone control vector was utilized to construct the combination anti-HIV vector (5). The chimeric human/rhesus macaque TRIM5α gene under the control of the MNDU3 promoter, a CCR5 shRNA under the control of a human polymerase-III U6 promoter, and a TAR decoy under the

control of a human polymerase-III U6 promoter were inserted upstream of the PGK driven EGFP reporter gene to derive CCLc-Combination-Anti-HIV (Fig. 1a) (3-5,22,25).

Lentiviral vectors were generated in the packaging cells, HEK-293T. Twenty-five micrograms of the packaging construct, pΔ8.9 (containing *gag* and *pol* genes), 25ug of CCLc-MNDU3-x-PGK-EGFP (EGFP-alone control empty vector) or CCLc-Combination-Anti-HIV (transfer vector), and 5ug of VSVG (envelope) were transfected into HEK-293T packaging cells in T225 flasks by lipofection. Vector supernatants were collected at 48 hours post-transfection and concentrated by ultrafiltration 100-fold. Vectors were subsequently tittered on HEK-293T cells and titers obtained ranged from $2x10^9$ to $6x10^9$ transducing units/ml.

Transduction of primary human CD34+ HSCs:

CD34+ hematopoietic stem cells (HSCs) were isolated from umbilical cord blood (NDRI, Philadelphia, PA) by Ficoll-Paque (GE Healthcare, Piscataway, NJ) and purified by magnetic bead column separation (Miltenyi Biotec, Auburn, CA). CD34+ cell isolation purity (>93%) was routinely obtained. Total CD34+ cells were cultured in complete IMDM media containing 10% FBS and supplemented with 50 ng/ml stem cell factor (SCF), Flt-3 ligand, and thrombopoietin (TPO). Cells were transduced with the lentiviral vectors EGFP-alone or the anti-HIV combination vector (MOI 10) for three hours at 37°C with 8 μg/ml protamine sulfate.

Transplantation and screening of NRG mice:

NOD-RAG1-/-IL2rγ-/- double mutant (NRG) mice (stock number 007799) were obtained from The Jackson Laboratory (Sacramento, CA) and were used in compliance with institutional

and IACUC guidelines and regulations. Two to five day old newborn NRG pups were sublethally irradiated with 200 cGy of gamma irradiation. Nontransduced, EGFP-alone transduced (control), or anti-HIV vector transduced HSCs ($3*10^5$ total cells/mouse) were injected intrahepatically into irradiated pups. Three months post-transplantation, mice were bled retro-orbitally and the peripheral blood was analyzed by FACS for EGFP and human leukocytes with a PE-CY7 conjugated anti-human CD45 antibody (clone HI30), an APC-conjugated CD3 (clone HIT3A), and a PE-conjugated CD4 antibody (clone RPA-T4) (BD Biosciences, San Jose, CA).

FACS analysis of engrafted human immune cells:

To evaluate multi-lineage hematopoiesis in transplanted NRG mice, cells from the peripheral blood and various lymphoid organs including the thymus, spleen, and bone marrow were stained with anti-human antibodies and analyzed by FACS. T cells were stained with an APC-conjugated CD3 (clone HIT3A), a PE-conjugated CD4 antibody (clone RPA-T4), or APC-conjugated CD8 antibody (clone RPA-T8) (BD Biosciences, San Jose, CA). B cells were stained with a PE-conjugated CD19 antibody (clone HIB19) (BD Biosciences, San Jose, CA). Macrophages were stained with a PE-conjugated CD14 antibody (clone M5E2) (BD Biosciences, San Jose, CA). To detect cell surface expression of CCR5, cells were stained with a PE-conjugated anti-human antibody (clone 2D7) (BD Biosciences, San Jose, CA). Cells were also evaluated for EGFP expression to determine the levels of engraftment of vector transduced cells. Isotype controls used were a PE-conjugated mouse IgG1 (clone MOPC-21) (BD Biosciences, San Jose, CA), a PE-conjugated mouse IgG2a (clone IC003P) (RND Systems,

Minneapolis, MN), and an APC-conjugated mouse IgG1 (clone MOPC-21) (BD Biosciences, San Jose, CA). FACS analysis was performed on a Beckman Coulter FC-500.

***In vivo* HIV-1 challenge of engrafted NRG mice:**

To determine whether the anti-HIV gene modified cells were resistant to HIV-1 infection, engrafted mice were challenged *in vivo* with either an R5-tropic BaL-1 or an X4-tropic NL4-3 strain of HIV-1. BaL-1 virus was obtained from the NIH AIDS Research Reference and Reagent Program and grown in CD34+ cell derived macrophages. To grow the stock virus, macrophages were infected with an MOI of 1.0 and cell culture supernatants were collected on various days post-infection. Viral titers were obtained using the Ghost cell assay. Briefly, Ghost-R5-X4-R3 cells were infected with serial dilutions of stock virus. Forty-eight hours post-infection the Ghost cells were analyzed by flow cytometry for EGFP expression to obtain an infectious viral titer. For NL43, an infectious clone was obtained from the NIH AIDS Research Reference and Reagent Program and transfected into 293T cells. Seventy-two hours post-transfection, cell culture supernatants were collected and tittered on Ghost-R5-X4-R3 cells by the Ghost cell assay.

Mice were infected intravenously with 200,000 total infectious units. On various weeks post-infection, peripheral blood draws were taken and analyzed for total human CD4+ cell percent by FACS and for HIV plasma viremia by quantitative-PCR (QPCR). For FACS analysis, the cells were stained with a PE-CY7-conjugated CD45 antibody (clone HI30), an APC-conjugated CD3 antibody (clone HIT3A), and a PE-conjugated CD4 antibody (clone RPA-T4) (BD Biosciences,

San Jose, CA). EGFP percent was also analyzed to determine the levels of vector transduced cells. FACS analysis was performed on a Beckman Coulter FC-500.

To determine the levels of plasma viremia, viral RNA was extracted from the plasma of infected mice using a Qiagen Viral RNA extraction kit (Qiagen, Valencia, CA). Reverse transcription with oligo dT primers was then performed using Taqman RT Reagents (Applied Biosystems, Carlsbad, CA). Quantitative-PCR was then performed using SYBR Green (Applied Biosystems, Carlsbad, CA) and a primer/probe set specific for the HIV *pol* gene: 5'-CTGGCTACTATTTCTTTTGCTA-3' and 5'-TGGCATGGGTACCAGCACA-3' and probe 5'-TTTATCTACTTGTTCATTTCCTCCAATTCCTT-3' (IDT DNA Technologies, Coralville, IA). Q-PCR was performed on an Applied Biosystems 7200 analyzer.

***In vitro* challenge of anti-HIV gene modified cells:**

To determine the level of HIV-1 resistance in a purified population of anti-HIV gene modified cells, splenocytes were collected from engrafted mice and sorted for EGFP expression. The sorted cells were further purified for human CD3+ T cells by magnetic bead separation (Miltenyi Biotec, Auburn, CA). CD3+ T cells, either control nontransduced or EGFP+ anti-HIV gene modified cells ($5x10^5$ cells/well) were stimulated with 1μg/ml IL-2 and 1μg/ml phytohemagglutinin. On day 3 post-stimulation, the cells were challenged at an MOI of 0.05 with either an R5-tropic BaL-1 or an X4-tropic NL43 strain of HIV-1. On various days post-infection culture supernatants were collected and analyzed by p24 antigen ELISA.

Cytokine secretion from *in vivo* derived T cells:

Splenocytes from engrafted mice were isolated and sorted based on EGFP expression. These sorted cells were further purified for human CD3+ T cells by magnetic bead separation (Miltenyi Biotec, Auburn, CA). CD3+ T cells, either control nontransduced or EGFP+ anti-HIV gene modified cells ($1x10^6$ cells/well) were stimulated with 1μg/ml IL-2 and 1μg/ml phytohemagglutinin. On day 3 post-stimulation, culture supernatants were collected and analyzed by FACS for expression of IL-4, IL-6, IL-10, TNFα, and IFNγ using a BD Cytokine Bead Array kit (BD Biosciences, San Jose, CA).

Karyotyping:

To determine if the anti-HIV vector transduced mobilized peripheral blood CD34+ HSCs maintained their chromosomal and genetic stability, karyotyping was performed. Upon transduction, EGFP positive cells were sorted based on EGFP expression and further cultured for 5 days in Methocult semi-solid methylcellulose media enriched with cytokines (Stem Cell Technologies, Vancouver, Canada) to promote robust proliferation. The cells were washed twice with complete DMEM with 10% FBS to dissolve the methylcellulose and then treated with Colcemid, a mitotic inhibitor, for 30 minutes at 37°C to arrest the cells in metaphase. Cells were subsequently treated with cell stripper for 10 minutes at 37°C followed by treatment with a KCL hypotonic solution and 3:1 methanol:acetic acid fixative solutions. Karyotyping slides were made and Giemsa banded. Karyotyping was performed on an Olympus Bx41 microscope with a DP20 camera. Analysis was performed with an Applied imaging System.

Statistical Analysis:

T-tests were used to compare the differences between different treatments of the study interest. The statistical analyses were conducted in R (version 2.10.1) for Windows. A significance level of 0.05 was used in hypothesis testing.

RESULTS:

Successful engraftment of anti-HIV vector transduced CD34+ HSCs:

A third generation self-inactivating combination anti-HIV lentiviral vector expressing a human/rhesus macaque chimeric TRIM5α, a CCR5 shRNA, a TAR decoy and an EGFP reporter gene was utilized in our experiments (Fig. 1a). Expression of all three anti-HIV genes has been previously demonstrated in vector transduced cells (5). The control empty vector, EGFP-alone, does not contain any anti-HIV genes but does contain the PGK-EGFP reporter gene (5). To evaluate the potential of combination anti-HIV vector transduced CD34+ HSCs to engraft NRG mice, FACS analysis was performed on transplanted mice. As displayed in Figure 1b, successful engraftment of transduced cells was observed in the peripheral blood. The average engraftment of EGFP-alone vector transduced cells was 21.9% with a standard deviation of 9.4 and the average engraftment of anti-HIV vector transduced cells was 17.5% with a standard deviation of 8.0. The percentages obtained were from total human leukocytes stained with an anti-human CD45 antibody. No significant difference ($p=0.213$) between engraftment of EGFP-alone and anti-HIV vector transduced cells was observed.

To determine the levels of CD4+ T cell development in anti-HIV vector transduced cell engrafted mice, peripheral blood was stained with anti-human CD3 and CD4 antibodies (Fig.

1c). No significant difference (p=0.063 and p=0.420, respectively) in CD4+/CD3+ T cell development in the peripheral blood was observed in anti-HIV vector transduced engrafted cells as compared to control EGFP-alone vector transduced and nontransduced engrafted cells. The average level of anti-HIV vector transduced CD4+ T cells was 55.0% of CD3+ T cells (standard deviation of 10.3) compared to nontransduced cells (average of 59.2% CD4+ T cells with a standard deviation of 16.4) and EGFP-alone vector transduced cells (average of 47.6% CD4+ T cells with a standard deviation of 9.0). Representative flow cytometry plots are displayed below the bar graphs. Table 1 displays peripheral blood engraftment results from nontransduced (NT), EGFP-alone, and anti-HIV vector transduced cell transplanted mice. These results demonstrate that no cytotoxic effects were observed with the anti-HIV vector transduced cells in the peripheral blood of engrafted mice.

To determine the levels of CCR5 down regulation of anti-HIV vector transduced cells (cells which express the CCR5 shRNA), flow cytometry was performed on total human (CD4+/EGFP+ splenocytes from mice engrafted with EGFP-alone or anti-HIV vector transduced cells. As displayed in Figure 1d, anti-HIV vector transduced cells expressed significantly (p=0.002) decreased levels of cell surface CCR5 (7.4% positive) compared to control EGFP-alone vector transduced cells (46.4% positive).

Multi-lineage human hematopoiesis from anti-HIV vector transduced cells in lymphoid organs:

To determine if normal engraftment and multi-lineage hematopoiesis of anti-HIV vector transduced cells had occurred in the lymphoid organs of transplanted NRG mice, flow

cytometry was performed (Fig. 2). Single cells were isolated from the thymus, spleen, and bone marrow of engrafted mice and stained with respective antibodies as described in the Methods section. Table 2 displays lymphoid organ engraftment results from nontransduced (NT), EGFP-alone, and anti-HIV vector transduced cell transplanted mice. No significant difference ($p>0.05$) was observed with engraftment or development of anti-HIV gene modified T cells (CD3+) in the peripheral blood, thymus, or spleen compared to nontransduced or EGFP-alone cell engrafted mice. No observed differences in the size of the thymus grafts were noted between the engrafted mice. No significant difference ($p>0.05$) was observed with engraftment or development of anti-HIV gene modified B cells in the spleen or bone marrow compared to nontransduced or EGFP-alone cell engrafted mice. The only significant difference observed in lymphoid organ engraftment of anti-HIV gene modified cells (average=11.88, standard deviation=1.78) compared to nontransduced cells (average=29.17, standard deviation=3.95) was with CD14+/CD45+ cells in the bone marrow ($p=0.005$). However, no significant difference ($p=0.112$) in engraftment of bone marrow CD14+/CD45+ cells was observed when comparing the levels of EGFP-alone (average=6.3, standard deviation=2.43) and anti-HIV vector transduced cells. These results demonstrated that no cytotoxic effects were observed with the anti-HIV vector transduced HSCs as they were capable of engrafting and undergoing multi-lineage hematopoiesis in various lymphoid organs of transplanted NRG mice at levels equivalent to control HSCs. Representative flow cytometry plots are displayed in Figure 2.

Maintenance of CD4+ T cell levels in anti-HIV cell engrafted mice upon *in vivo* HIV-1 infection:

To determine whether resistance to HIV-1 infection was conferred in anti-HIV vector transduced cellengrafted mice, the levels of total human CD4+ cells in the peripheral blood were analyzed by flow cytometry on various weeks post-infection. Maintenance of normal human CD4+ levels was observed in anti-HIV cell engrafted mice (solid lines) upon challenge with either an R5-tropic BaL-1 (Fig. 3a) or an X4-tropic NL43 (Fig. 3b) strain of HIV-1. In the anti-HIV gene modified cell engrafted mice (solid lines), total human CD4+ T cell percentages gated on human CD45+ leukocytes ranged between 46.2-65.4% and 37.5-79.2% at the end of the challenge experiment with BaL-1 or NL43 HIV-1, respectively. This was in comparison to control EGFP-alone vector transduced cell engrafted mice (dashed lines) where total human CD4+ T cell percentages gated on human CD45+ cells declined over the course of infection and ranged from 19.1-29.1% and 5.9-15.1% at the end of the challenge experiment with BaL-1 or NL43 HIV-1, respectively. The average levels of CD4+ T cells pre-infection and at the end of the challenge experiments are displayed in Figure 3c and 3d for BaL-1 and NL4-3 infections, respectively. In EGFP-alone cell engrafted mice infected with BaL-1, CD4+ T cell levels decreased to an average of 25.8% which was significantly different ($p=0.002$) compared to anti-HIV cell engrafted mice where average CD4+ T cell levels were 55.1% (Fig. 3c). A significant difference ($p=0.001$) was also observed in the average of CD4+ T cells in EGFP-alone cell engrafted mice (9.1%) infected with NL4-3 compared to the average of anti-HIV CD4+ T cell levels (58.4%) (Fig. 3d). Representative flow cytometry plots demonstrating CD4+ cell levels in the infected mice are displayed below the bar graphs. These results demonstrated that the

combination anti-HIV vector significantly enhanced survival of human T cells in the face of an HIV infection.

To evaluate the levels of human CD4+ cells in the lymphoid organs of infected mice, mice were sacrificed at the end of the challenge experiments and human CD4+ T cells were analyzed from the spleen. As displayed in Figure 4a, upon infection with BaL-1, human CD4+ T cell levels were maintained in the spleen (average of 53.8% of human CD3+ T cells with a standard deviation of 8.6) in anti-HIV vector transduced cell engrafted mice. This was in comparison to mice engrafted with control EGFP-alone vector transduced cells which displayed a significantly ($p=0.002$) decreased level of CD4+ human T cells in the spleen (average of 23.3% with a standard deviation of 7.5). As displayed in Figure 4b, upon infection with NL4-3, human CD4+ T cell levels were maintained in the spleen (average of 67.4% of human CD3+ T cells with a standard deviation of 9.3) in anti-HIV vector transduced cell engrafted mice. This was in comparison to mice engrafted with control EGFP-alone vector transduced cells which displayed a significantly ($p=0.001$) decreased level of CD4+ human CD3+ T cells in the spleen (average of 3.2% with a standard deviation of 1.4). Representative flow cytometry plots demonstrating CD4+ cell levels in the infected mice are displayed below the bar graphs. These results demonstrate that normal CD4+ cell levels could be maintained during the course of an HIV infection in a cell population which contained CD4+ cells derived from anti-HIV gene modified HSCs.

Selective survival advantage of anti-HIV vector transduced cells upon *in vivo* HIV-1 infection:

During HIV-1 infection of a mixed population of protected anti-HIV gene expressing cells and unprotected nontransduced cells, a selective survival advantage should be observed with the anti-HIV gene modified cells. This selective advantage would be demonstrated with an increas in the number of anti-HIV vector transduced cells during the course of infection due to killing of the infected nontransduced. To evaluate whether this had occurred *in vivo* in HIV-1 infected mice, the total percentages of EGFP+ human CD4+ cells were analyzed by flow cytometry on various weeks post-infection in the peripheral blood. As demonstrated in Figure 5, anti-HIV vector transduced cell engrafted mice displayed an increase in total EGFP+/CD4+ cells when challenged *in vivo* with either a BaL-1 (Fig. 5a) or an NL43 (Fig. 5b) strain of HIV-1 from pre-infection to the time of euthanizing the mice. During infection with BaL-1 (Fig. 5a), the levels of anti-HIV gene modified CD4+ cells increased, on average, 0.95-fold with a standard deviation of 0.35. This was a significant increase ($p=0.006$) compared to control EGFP-alone vector transduced cells which decreased 0.03-fold with a standard deviation of 0.17. In mice infected with NL4-3 (Fig. 5b), the levels of anti-HIV gene modified CD4+ cells increased 1.60-fold with a standard deviation of 0.54. This was a significant increase ($p=0.005$) compared to control EGFP-alone vector transduced cells which increased 0.05-fold with a standard deviation of 0.30. These results demonstrate that in the presence of an HIV-1 infection, *in vivo*, cells expressing anti-HIV genes have a selective survival advantage and increase in their percentage of total cells due their ability to resist infection and the killing of unprotected cells.

Detection of plasma viremia and resistance of anti-HIV gene modified cells upon *ex vivo* HIV-1 challenge:

A hallmark of successfully engrafted NRG mice is their ability to display plasma viremia upon infection with HIV-1 (6). Therefore, to determine if the levels of plasma viremia had decreased in HIV-1 infected NRG mice engrafted with anti-HIV vector transduced HSCs, Q-PCR was performed on the plasma from mice on various weeks post-infection. As demonstrated in Figure 6, the mice engrafted with anti-HIV vector transduced cell did not display a decrease in HIV-1 plasma viremia over the course of infection. Levels of HIV-1 RNA copies/ml remained between 7 and 8 logs in the anti-HIV cell engrafted mice which were similar to control cell engrafted mice (Fig. 6). These results were observed for both the R5-tropic BaL-1 (Fig. 6a) and X4-tropic NL43 (Fig. 6b) challenge experiments.

To evaluate whether HIV-1 resistance could be conferred when a pure population of anti-HIV vector transduced cells was challenged with HIV-1, EGFP+ and EGFP- human CD3+ T cells were sorted from the spleens of successfully engrafted mice. Upon stimulation with IL-2 and PHA for 3 days post-sorting, the cells were challenged with either R5-tropic BaL-1 (Fig. 6c) and X4-tropic NL43 (Fig. 6d) at an MOI of 0.05. On various days post-infection cell culture supernatants were collected and analyzed for HIV-1 p24 by antigen ELISA. As displayed in Figure 6, strong reduction in HIV output (>4 logs) was observed in the sorted anti-HIV vector transduced cell culture as compared to the nontransduced cell culture. These results demonstrate that pure populations of anti-HIV gene transduced cells isolated from engrafted animals, when faced with an HIV-1 viral load, display potent resistance to infection, coupled with strongly diminished HIV-1 production.

Anti-HIV gene modified cells are functional and retain a normal karyotype:

To determine whether the anti-HIV gene modified cells were functionally normal, the levels of secretion of specific cytokines were measured. T cells from the spleens of engrafted mice were purified and sorted based on EGFP and human CD3 expression. The T cells were stimulated with IL2 and PHA and cultured for three days. On day 3 post-stimulation, culture supernatants were collected and analyzed by FACS for expression of IL-4, IL-6, IL-10, TNFα, and IFNγ using a BD Cytokine Bead Array. As displayed in Fig. 7a, no significant difference ($p>0.05$) in the secretion of any of the cytokines measured was detected in the anti-HIV gene modified cell cultures as compared to control nontransduced cells. These data show that transduction had not affected normal cytokine secretion from the T cells.

With the transduction of CD34+ HSCs with a combination anti-HIV lentiviral vector and their subsequent differentiation toward hematopoietic lineages, it is possible that chromosomal and genetic abnormalities could arise. Therefore, karyotyping analyses were performed on anti-HIV vector transduced CD34+ HSCs. As displayed in Figure 7b, the anti-HIV cells retained a normal chromosome profile. Normal banding was observed and no translocations or other detectable chromosomal abnormalities were detected.

The results obtained from these experiments demonstrated that the anti-HIV T cells appear functionally normal and that the transduction and expression of the anti-HIV genes did not incur any gene rearrangements or abnormalities in chromosome organization.

392 **DISCUSSION:**

393 Stem cell gene therapy for HIV has the potential to offer an alternative therapeutic
394 approach for HIV infected individuals (27). With the possibility of a one-time treatment by
395 transducing self-renewing and self-repopulating HSCs with potent anti-HIV genes, the removal
396 of small molecule anti-retroviral drug therapy and the control of HIV replication could occur
397 with the development of a complete HIV resistant immune system. This has been
398 demonstrated with a bone marrow transplant in Berlin, Germany where an HIV infected
399 individual received allogeneic HSCs from a donor who was homozygous for the CCR5-Δ32 bp
400 allele (14). The recipient is currently free from HIV replication while also discontinuing anti-
401 retroviral drug therapy (16). The results from this study highlight the potential use of HIV
402 resistant stem cells to provide a functional cure for HIV infected patients (15).

403 In our current study, we evaluated the *in vivo* safety and efficacy of triple
404 combination anti-HIV lentiviral vector transduced CD34+ HSCs in a NOD-RAG1-/-IL2rγ-/- (NRG)
405 double mutant mouse model. This anti-HIV vector contains three highly potent anti-HIV genes
406 which individually confer strong resistance to HIV-1 infection. However, due to the high
407 mutation rate of HIV, its ability to generate escape variants, and the various tropisms of the
408 virus, it is critical to develop combination therapies which act at multiple stages of the HIV life
409 cycle. This has been demonstrated with the use of monotherapy small molecule antiretroviral
410 drugs which eventually give rise to viral escape mutants (7,20,26). Therefore, similar to
411 combination approaches with antiretroviral drugs, multiple anti-HIV genes inserted into a single
412 gene therapy vector may offer stronger protection from HIV infection and will have a greater
413 chance in preventing viral resistance.

414 The use of multiple HIV resistance genes, however, is not the only aspect to consider
415 when designing HIV gene therapies. The specific stage of the life cycle targeted and the
416 mechanism of action of the anti-HIV gene also needs to be taken into consideration. Anti-HIV
417 genes which block early stages of HIV infection including attachment and entry (CCR5 shRNA)
418 and reverse transcription and integration (TRIM5α), will prevent the generation of provirus and
419 viral reservoirs which are main reasons for the failure to cure HIV infected individuals (1,3-
420 5,25,28). In our previous report evaluating the *in vitro* efficacy of this combination vector (CCR5
421 shRNA, human/rhesus TRIM5α, and a TAR decoy), strong pre-integration protection from HIV-1
422 infection was observed (5). This anti-HIV vector not only prevented viral integration but also
423 blocked the generation of viral escape variants which led to the next step in evaluating the *In*
424 *vivo* safety and efficacy of vector transduced CD34+ HSCs in the current studies.
425 After transduction of CD34+ HSCs with the anti-HIV vector and injection into newborn
426 NRG pups, successful engraftment of combination anti-HIV vector transduced cells was
427 achieved (Fig. 1). *In vivo* development of transduced CD45+ human leukocytes (Fig. 1b) and
428 human T cells (Fig. 1c) was observed in the peripheral blood similar to control vector
429 transduced cells. To further evaluate the safety of anti-HIV vector transduced CD34+ HSCs,
430 engraftment and multi-lineage hematopoiesis was analyzed in the spleen, thymus, and bone
431 marrow. Successful development of mature immune cells including T cells, B cells, and
432 macrophages was observed in the lymphoid organs of anti-HIV vector transduced cell engrafted
433 mice (Fig. 2). Therefore, transduction with the anti-HIV vector did not have any detrimental
434 effects on the engraftment or multi-lineage hematopoiesis of the gene modified CD34+ HSCs.

435 The major goal of HIV stem cell gene therapy is to transplant anti-HIV gene modified
436 HSCs into infected patients which would further develop into HIV resistant immune cells
437 capable of blocking HIV infection and thriving in the face of a viral load. Therefore, to evaluate
438 the levels of protection from HIV-1 infection of the anti-HIV gene modified immune cells which
439 developed in the NRG mice, both total human CD4+ cell percentages and plasma viremia were
440 measured in HIV-1 infected mice as a decline in CD4+ cells and a rise in plasma viremia are
441 hallmark characteristics of HIV disease progression. Our results demonstrated that anti-HIV
442 gene modified human CD4+ cells, indeed, were protected from HIV-1 infection in the face of a
443 viral load. Maintenance of normal human CD4+ T cell levels were observed in mice engrafted
444 with the anti-HIV vector transduced CD34+ HSCs. Even though a portion of the nontransduced
445 cell population was being killed during HIV-1 infection, the anti-HIV gene modified cells were
446 able to thrive and maintain normal levels of CD4+ cells. This was in comparison to control
447 EGFP-alone vector transduced CD34+ HSC engrafted mice which, upon infection, demonstrated
448 a steady decline of human CD4+ T cells due to their inability to resist infection (Fig. 3). The
449 ability of the anti-HIV gene modified cells to survive during HIV-1 infection was also observed in
450 the spleens of infected mice. The maintenance of human CD4+ T cells in mice engrafted with
451 the anti-HIV CD34+ HSCs was due to a selective survival advantage of the protected cells. Upon
452 challenge with either an R5 or X4-tropic strain of HIV-1, an increase in EGFP+ cell percentages
453 was observed in mice engrafted with the anti-HIV CD34+ HSCs. This was due to expansion of
454 the anti-HIV resistant cell population during infection and also from the killing of the
455 nontransduced population in the same mice which acted as an internal control. When plasma
456 viremia was measured, however, the levels of HIV-1 RNA in the blood were similar between all

457 mice studied no matter if they were engrafted with anti-HIV or control EGFP-alone vector
458 transduced CD34+ HSCs. This was due to unprotected nontransduced cells which were co-
459 transplanted with the anti-HIV vector transduced cells. The mice were transplanted with a
460 mixed population of both vector transduced and nontransduced cells and hence, upon
461 challenge, HIV-1 will be able to infect the unprotected cells and establish infection. The
462 selective pressure of HIV-1 on the anti-HIV gene modified cells enabled them to be selected for
463 expansion and proliferation, however, there were still unprotected cells for the virus to
464 continually infect and produce plasma viremia (Fig. 6). Therefore, higher transduction
465 efficiencies and *in vivo* engraftment percentages may need to be achieved to reach an optimal
466 level of HIV-resistant immune cells to suppress HIV-1 replication and decrease plasma viremia.
467 This was observed when we challenged a sorted and pure population of anti-HIV gene modified
468 human T cells from the spleen of engrafted mice. Upon *ex vivo* challenge, a pure population of
469 anti-HIV T cells was capable of resisting HIV-1 infection and displayed a significant decrease in
470 HIV-1 p24 output compared to nontransduced human T cells (Fig. 6). Also, in addition to higher
471 transduction efficiencies needed, the human immune system of an infected individual may
472 come to the aid to decrease viral load. If HIV-specific immune cells survive and are not killed off
473 by HIV, over the long term (a time period that cannot be easily tested in a short term animal
474 model) the viral load may be substantially reduced since HIV-producing cells from the viral
475 reservoirs can be safely eliminated by immune cells. Even though we did not observe a
476 decrease in viral load, we were able to demonstrate that the cells expressing the anti-HIV genes
477 were capable of resisting infection *in vivo*. These cells were able to maintain normal human
478 CD4+ cell levels due to a selective survival advantage upon HIV challenge *in vivo*. Therefore,

efficacy was observed with the population of anti-HIV gene expressing cells. If a higher transduction efficiency and in vivo engraftment of HIV resistant cells can be achieved, a marked reduction in plasma viremia could occur. However, if a pure population of anti-HIV gene expressing HSCs could be transplanted into patients, complete suppression of HIV replication could be accomplished as demonstrated with the Berlin patient. One promising method to achieve this scenario is to use clonal induced pluripotent stem cells (iPSCs) which express anti-HIV genes. This was demonstrated in a recent publication where complete protection from HIV-1 infection was observed due to the expression of a combination of anti-HIV genes in every macrophage derived from the iPS line (17).

Here we have demonstrated the safety and efficacy of this combination anti-HIV lentiviral vector in a humanized mouse model which is capable of demonstrating multi-lineage hematopoiesis from engrafted human CD34+ HSCs. The subsequent protection and expansion of the HIV resistant immune cells in the face of an HIV viral load establishes the utility of this vector for use in future clinical trials.

ACKNOWLEDGEMENTS

This work was supported by the University of California-Davis Health System start-up funds from the Dean's office for the Stem Cell Program and by the James B. Pendleton Charitable Trust. This work was also supported in part by the Gin and Imy Mar stem cell research fund. The NIH AIDS Research and Reference Reagent Program provided many reagents and cell lines used in this work. Biostatistics support for this publication was made possible by Grant Number UL1 RR024146 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH), and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official view of NCRR or NIH. Information on NCRR is available at http://www.ncrr.nih.gov/. Information on Re-engineering the Clinical Research Enterprise can be obtained from http://nihroadmap.nih.gov/clinicalresearch/overview-translational.asp.

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FIGURE LEGENDS:

Figure 1. Combination anti-HIV lentiviral vector, peripheral blood engraftment of transduced cells, and CCR5 down regulation: A third generation lentiviral vector, CCLc-x-PGK-EGFP, was utilized to generate the combination anti-HIV construct. (a) a human/rhesus macaque TRIM5α isoform was driven under the control of the MNDU3 promoter, and a CCR5 shRNA and a TAR decoy were driven under separate human polymerase-III U6 small RNA promoters. These three anti-HIV genes were inserted upstream from the EGFP reporter gene. (b) CD34+ HSCs were transduced with the control EGFP-alone or the anti-HIV vector and transplanted into NRG pups. Transplanted mice were screened for human CD45 and EGFP expression in the peripheral blood for engraftment of transduced cells, either EGFP-alone (N=12) or anti-HIV (N=14). (c) The peripheral blood of nontransduced (NT) (N=14), EGFP-alone (N=12), and anti-HIV (N=14) cell engrafted mice was analyzed or human T cells with antibodies specific for CD3 and CD4 and also for expression of EGFP. (d) CD4+ human splenocytes were analyzed for the expression of CCR5. N=5 for EGFP-alone and N=11 for anti-HIV mice. Bar graphs display averages and standard deviations for each cohort. Statistical significance ($p<0.05$) is represented by an asterisk.

Figure 2. Engraftment of lymphoid organs in transplanted NRG mice: NRG mice were transplanted with CD34+ HSCs either nontransduced (NT) or transduced with a control EGFP-alone or the anti-HIV lentiviral vector. Upon engraftment, various lymphoid organs including the (a) spleen, (b) thymus, and (c) bone marrow were analyzed for human cell engraftment.

673 Flow cytometry was performed to detect EGFP expression along with total human leukocytes
674 (CD45), T cells (CD3, CD4, and CD8), B cells (CD19), and macrophages (CD14). Data is
675 representative of mice for each cohort. Complete data sets for each cohort of mice are
676 included in Table 2.

677

678 Figure 3. Detection of human CD4+ T cells in the peripheral blood of HIV-1 infected NRG
679 humanized mice: NRG mice successfully engrafted with either control EGFP-alone or
680 combination anti-HIV vector transduced cells were infected IV with either an (a) R5-tropic BaL-
681 1 or an (b) X4-tropic NL4-3 strain of HIV-1. On various weeks post-infection, mice were bled
682 and analyzed by FACS for total human CD4+ cell percent. Solid lines represent anti-HIV cell
683 engrafted mice. Dashed lines represent control EGFP-alone cell engrafted mice. Comparisons
684 between CD4+ T cell level averages pre-infection and post-infection were performed for both
685 the (c) BaL-1 and (d) NL4-3 infected mice engrafted with either EGFP-alone or anti-HIV vector
686 transduced cells. Bar graphs display averages and standard deviations from four mice for each
687 cohort for each set of infections. Statistical significance ($p<0.05$) is represented by an asterisk.
688 Representative flow cytometry plots are displayed.

689

690 Figure 4. Detection of human CD4+ T cells in the spleen of HIV-1 infected NRG humanized mice:
691 NRG mice successfully engrafted with either control EGFP-alone or combination anti-HIV vector
692 transduced cells were infected IV with either an (a) R5-tropic BaL-1 or an (b) X4-tropic NL4-3

693 strain of HIV-1. After completion of the *in vivo* challenge experiments, infected mice were
694 sacrificed and the spleen was analyzed by flow cytometry for CD4+ T cell (CD3+) levels. Bar
695 graphs display averages and standard deviations from four mice for each cohort for each
696 infection. Statistical significance ($p<0.05$) is represented by an asterisk. Representative flow
697 cytometry plots are displayed.

698

699 Figure 5. Selective survival advantage of anti-HIV gene modified cells in HIV-1 infected NRG
700 mice: Mice successfully engrafted with either control EGFP-alone or combination anti-HIV
701 vector transduced cells were infected IV with either an (a) R5-tropic BaL-1 or an (b) X4-tropic
702 NL4-3 strain of HIV-1. On various weeks post-infection, mice were bled and analyzed by FACS
703 for EGFP+/CD4+ human cell percent. Fold difference in EGFP+/CD4+ T cell level averages pre-
704 infection and post-infection are displayed for both the EGFP-alone and anti-HIV vector
705 transduced cells. Bar graphs display averages and standard deviations from four mice for each
706 cohort for each set of infections. Statistical significance ($p<0.05$) is represented by an asterisk.

707

708 Figure 6. Detection of *in vivo* plasma viremia and *in vitro* HIV-1 challenge of sorted spleen T
709 cells: NRG mice successfully engrafted with either control EGFP-alone or combination anti-HIV
710 vector transduced cells were infected IV with either an (a) R5-tropic BaL-1 or an (b) X4-tropic
711 NL4-3 strain of HIV-1. On various weeks post-infection, mice were bled and the plasma was
712 analyzed by Q-PCR using a primer/probe pair specific for the HIV *pol* gene. *In vitro* HIV-1

challenge experiments were performed on human CD3+ T cells, both nontransduced (EGFP-) and anti-HIV vector transduced (EGFP+), with an (c) R5-tropic BaL-1 or an (d) X4-tropic NL4-3 strain of HIV-1. On various days post-infection, culture supernatants were collected and analyzed for p24 by antigen ELISA. p24 ELISA experiments were performed in triplicate.

Figure 7. Cytokine expression and karyotypic analysis of anti-HIV vector transduced cells: (a) Anti-HIV gene modified T cells from spleen were sorted based on EGFP/CD3 expression and stimulated with IL2 and PHA. On day three post-stimulation, culture supernatants were analyzed by FACS for expression of IL4, IL6, IL10, TNFα, and IFNγ. Cytokine expression experiments were performed in triplicate. (b) A representative karyotyping analysis of anti-HIV vector transduced human CD34+ HSC. Karyotypic analyses were performed in duplicate.

731 TABLE 1 Engraftment of the peripheral blood in transplanted NRG mice

Mouse	Peripheral Blood Engraftment	
	EGFP%	CD4+/CD3+
NT-1	N/A	59.9
NT-2	N/A	51.9
NT-3	N/A	56.7
NT-4	N/A	46.9
NT-5	N/A	55.2
NT-6	N/A	31.6
NT-7	N/A	62.0
NT-8	N/A	55.0
EGFP-alone-1	14.8	58.9
EGFP-alone-2	29.1	44.9
EGFP-alone-3	36.6	42.7
EGFP-alone-4	30.7	63.2
EGFP-alone-5	16.1	41.2
EGFP-alone-6	13.2	47.7
EGFP-alone-7	16.1	44.7
EGFP-alone-8	12.5	45.0
anti-HIV-1	13.9	52.0
anti-HIV-2	22.0	50.3
anti-HIV-3	15.9	69.8
anti-HIV-4	12.6	70.5
anti-HIV-5	32.0	50.3
anti-HIV-6	34.3	49.0
anti-HIV-7	16.8	34.5
anti-HIV-8	20.7	49.2

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752 TABLE 2 Engraftment of lymphoid organs in transplanted NRG mice

Mouse	Blood		Thymus		Spleen						Bone Marrow			
	CD4/ CD3	EGFP/ CD45	CD4/ CD8	EGFP/ CD3	EGFP/ CD45	CD3/ CD45	EGFP/ CD3	CD4/ CD3	CD19/ CD45	EGFP/ CD19	CD19/ CD45	EGFP/ CD19	CD14/ CD45[a]	EGFP/ CD14
NT-9	76.9	N/A	89.5	N/A	N/A	89.9	N/A	94.7	6.7	N/A	28.8	N/A	34.3	N/A
NT-10	79.0	N/A	95.4	N/A	N/A	38.3	N/A	85.0	58.8	N/A	56.9	N/A	16.3	N/A
NT-11	60.0	N/A	86.8	N/A	N/A	58.0	N/A	83.8	37.2	N/A	29.9	N/A	33.5	N/A
NT-12	31.8	N/A	89.2	N/A	N/A	28.7	N/A	72.3	57.6	N/A	37.3	N/A	35.2	N/A
NT-13	86.7	N/A	65.5	N/A	N/A	27.0	N/A	45.6	56.2	N/A	67.0	N/A	17.4	N/A
NT-14	75.5	N/A	72.5	N/A	N/A	47.2	N/A	78.7	41.6	N/A	23.8	N/A	38.3	N/A
EGFP-alone-9	63.2	39.1	72.2	45.4	40.7	31.0	35.2	60.0	41.0	33.0	81.3	36.3	13.5	32.5
EGFP-alone-10	40.7	17.2	87.0	26.3	16.3	21.8	24.5	46.2	74.0	15.5	56.7	12.3	2.9	19.2
EGFP-alone-11	37.3	21.5	79.6	12.0	17.0	35.4	17.3	52.9	59.3	20.2	24.2	24.2	4.3	25.2
EGFP-alone-12	41.2	16.1	85.6	23.4	19.9	30.6	18.8	54.1	66.2	23.3	47.0	22.6	4.5	21.7
anti-HIV-9	66.1	10.9	61.7	8.9	16.6	14.4	7.6	64.5	84.1	8.9	83.6	11.3	8.5	10.5
anti-HIV-10	52.0	13.9	83.2	17.4	8.8	19.9	9.0	54.2	62.9	10.3	53.7	9.8	10.7	10.1
anti-HIV-11	50.3	7.7	85.7	9.0	5.6	59.7	3.6	64.8	25.2	7.9	54.6	7.7	16.4	5.9
anti-HIV-12	60.8	12.3	65.1	5.8	6.6	68.1	6.0	33.1	29.6	8.3	9.6	7.8	9.7	8.5
anti-HIV-13	47.9	9.5	81.5	5.7	5.7	20.6	4.6	57.7	66.0	8.0	14.6	10.2	7.8	7.8
anti-HIV-14	66.8	22.2	53.6	21.4	11.4	89.1	9.8	75.8	7.9	10.5	77.9	11.4	18.2	14.0

753 [a] There was a statistically significant difference ($p<0.05$) between the average levels of CD14/CD45

754 positive cells in the bone marrow of anti-HIV vector transduced cell engrafted mice compared to

755 nontransduced (NT) cell engrafted mice.

756