Optimized Lentiviral Vectors for HIV Gene Therapy: Multiplexed Expression of Small RNAs and Inclusion of MGMT<sup>P140K</sup> Drug Resistance Gene

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Gene therapy with hematopoietic stem and progenitor cells is a promising approach to engineering immunity to human immunodeficiency virus (HIV) that may lead to a functional cure for acquired immunodeficiency syndrome (AIDS). In support of this approach, we created lentiviral vectors with an engineered polycistronic platform derived from the endogenous MCM7 gene to express a diverse set of small antiviral RNAs and a drug resistance MGMT<sup>P140K</sup> marker. Multiple strategies for simultaneous expression of up to five RNA transgenes were tested. The placement and orientation of each transgene and its promoter were important determinants for optimal gene expression. Antiviral RNA expression from the MCM7 platform with a U1 promoter was sufficient to provide protection from R5-tropic HIV in macrophages and resulted in reduced hematopoietic toxicity compared with constructs expressing RNA from independent RNA polymerase III promoters. The addition of an HIV entry inhibitor and nucleolar TAR RNA decoy did not enhance antiviral potency over constructs that targeted only viral RNA transcripts. We also demonstrated selective enrichment of gene-modified cells in vivo using a humanized mouse model. The use of these less toxic, potent antis-HIV vectors expressing a drug selection marker is likely to enhance the in vivo efficacy of our stem cell gene therapy approach in treating HIV/AIDS.

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INTRODUCTION

The human immunodeficiency virus type 1 (HIV-1) causes acquired immunodeficiency syndrome (AIDS) that affects millions of individuals worldwide.<sup>1</sup> The current standard of care is combinatorial antiretroviral therapy which consists of a cocktail of small molecule drugs that target multiple stages of viral replication. Although significantly improving patient survival and quality of life, combinatorial antiretroviral therapy is a lifelong, expensive treatment that is associated with an increased incidence of cardiovascular disease, lipodystrophy, and neurological complications among other clinical sequelae.<sup>2–6</sup> Furthermore, with the emergence of drug-resistant viral mutants and the persistence of latent viral reservoirs, an alternative and more comprehensive approach to conventional HIV therapy is needed. Gene therapy with HIV-resistant T lymphocytes or hematopoietic stem and progenitor cells (HSPCs) has been proposed as an alternative or supplemental treatment that could lead to a functional cure for AIDS.<sup>7–9</sup> We postulate that generating a lifelong supply of HIV-resistant cells is a critical step in achieving a functional cure for HIV/AIDS.

We are currently developing autologous stem cell transplantation protocols in which gene-modified (HIV-resistant) HSPCs are used to transplant HIV patients in an attempt to provide a persistent source of viral-resistant CD4<sup>+</sup> progeny. Our approach includes the use of small (<200 nucleotides) RNAs to confer HIV resistance to HSPCs and their progeny. These antiviral small RNAs can be grouped into three major classes based on the mechanism of action: small interfering RNAs (siRNAs) to induce posttranscriptional gene silencing; hammerhead ribozymes that catalytically cleave targets with sequence specificity; and nucleolar-localizing RNA decoys or aptamers that sequester targets in a stoichiometric fashion. Similar to traditional combinatorial antiretroviral therapy, RNA-based gene therapy can also be combinatorial with antiviral agents that target multiple stages of viral entry and replication to increase efficacy and reduce the likelihood of viral resistance. Various trigger formats have been used in combinatorial RNA interference (RNAi) approaches, including short hairpin RNAs (shRNAs),<sup>10–12</sup> polycistronic microRNA (miRNA) clusters,<sup>13,14</sup> and long hairpin RNAs,<sup>15</sup> with transgenes expressed from RNA polymerase III (Pol III) and/or RNA polymerase II (Pol II) promoters. Furthermore, multiplexing strategies with different classes of small RNAs to avoid saturation of any processing pathway have also been developed, including our own<sup>16</sup> and others.<sup>17</sup>

We previously reported the use of a clinical vector in which we coexpressed a tat/rev siRNA, a nucleolar TAR RNA decoy, and a CCR5-targeting ribozyme from independent Pol III promoters to ensure persistent antiviral transgene expression in all hematopoietic cells.<sup>16,18</sup> This combinatorial vector potently inhibited HIV...
replication in cord blood HSPC-derived progeny with greatly enhanced efficacy and reduced development of viral resistance relative to vectors with single antiviral RNA constructs. We conducted a pilot clinical trial in four AIDS patients undergoing salvage therapy and autologous stem cell transplantation for lymphoma in which a portion of the HSPCs were modified with this combinatorial vector. We observed rapid engraftment of gene-modified cells with no product-related serious adverse events, demonstrating that the treatment was safe and feasible. Importantly, detection of integrated vector in multiple cell lineages and persistent transgene expression for at least three years following transplantation provide evidence that long-term repopulating cells were modified, and this type of treatment could thus be long lasting. However, the frequency of gene marking in peripheral blood mononuclear cells ranged from 0.02 to 0.32% (200–3,200 copies per million cells) and was too low for clinical assessment of antiviral efficacy. It is unclear how much of this low gene marking was the result of the infusion of a 50- to 100-fold excess of unmanipulated HSPCs (for patient safety) versus limitations in the level of transduction of long-term engrafting cells or potential toxicity of the construct to those cells or their progeny. These concerns led us to develop and test second-generation combinatorial lentiviral vectors that expressed lower levels of small RNA transgenes to reduce potential toxicity, while incorporating a drug selection marker for chemical enrichment of gene-modified cells, should the level of transduction of long-term engrafting cells be low.

We used an endogenous polycistronic miRNA cluster located in the intron of the protein encoding MCM7 gene, previously engineered as a platform to express small RNAs including RNAi triggers and small nucleolar RNAs (snoRNAs) as a strategy to express more physiological levels of anti-HIV small RNA transgenes. The MCM7 platform allows coordinate expression of three antiviral RNAs from a Pol II U1 promoter, eliminating the need for multiple promoters. We previously demonstrated that MCM7 was a versatile platform for multiplexing different combinations and classes of small RNA antivirals, including tat/rev, rev, and tat RNAi triggers, a nucleolar-localizing TAR RNA decoy (U16TAR), and a nucleolar U5-targeting ribozyme (U16U5RZ) with several combinations strongly inhibiting replication of the NL4-3 strain of HIV in stably transduced CEM T lymphocytes.

In this report, we further extend the capability of the MCM7 platform in combinatorial gene therapy by incorporating additional antiviral RNAs. We demonstrate expression and processing of up to five antiviral transgenes without concomitant vector toxicity while providing strong antiviral potency in protecting gene-modified HSPC-derived macrophages. In addition, we incorporate a mutant form of the human methylguanine methyltransferase gene (MGMT) in our constructs to allow prospective chemical selection of gene-modified cells. Endogenous MGMT repairs DNA damage from alkylating agents, such as bis-chloroethyl-nitrosourea (BCNU), but this activity is inhibited in the presence of O6-alkylguanine (O6-BG). A naturally occurring P140K mutation in MGMT (MGMTP140K) confers resistance to O6-BG and thus only cells expressing transgenic MGMTP140K survive treatment with the combination of O6-BG/BCNU. This positive selection strategy increases gene modification allowing evaluation of clinical efficacy. Large animal studies demonstrated that this selection strategy effectively increases the percentage of gene-modified cells in vivo after repeated drug administration. We propose that this selection strategy would also work in the human clinical setting and demonstrate the ability of our constructs to protect gene-modified human blood cells during in vivo O6-BG/BCNU treatment using an immunodeficient (NSG) mouse model. These vector designs have increased antiviral efficacy and reduced toxicity along with the potential to confer selective protection of transduced cells with clinically relevant drugs, providing a foundation for advancing RNA-based stem cell gene therapy into proof-of-concept studies.

RESULTS

Lentiviral vector design to incorporate a polycistronic MCM7 platform and a drug selection marker (MGMTP140K) for combinatorial RNA-based gene therapy

Lentiviral vectors are efficient gene delivery vehicles with the ability to transduce nondividing cells, such as HSPCs, resulting in long-term expression of the therapeutic transgenes in differentiated progeny. We modified a third-generation, self-inactivating lentiviral vector, pHIV7, that previously demonstrated high efficiency in transducing primary CD4+ T lymphocytes and HSPCs to also express MGMTP140K from a CMV promoter (Figure 1a). We observed no differences in viral titer and transduction efficiency with inclusion of the MGMTP140K transgene (data not shown).

Our earliest lentiviral vector used independent Pol III promoters (first-generation lentiviral vector (FGLV), Figure 1b) to ensure strong and persistent expression of antiviral small RNA transgenes. Subsequently, we engineered an MCM7 platform that coexpresses three small RNAs within the polycistronic cluster from a single Pol II U1 promoter (second-generation lentiviral vector, Figure 1c) to express small RNAs at more moderate levels to reduce potential vector toxicity. The MCM7 platform is designed to coexpress three small RNAs within the polycistronic cluster using a single Pol II promoter. Although any Pol II promoter can be utilized to engineer tissue-specific transgene expression in this platform, we selected the U1 promoter for ubiquitous and persistent transgene expression in all hematopoietic cells derived from HSPCs. We previously demonstrated combinations of both si- and snoRNAs can be multiplexed in this format with antiviral functionality. Because the MCM7 platform expresses small RNAs at lower levels than independent Pol III promoters, we reasoned that we could incorporate additional small RNA transgenes to further enhance antiviral potency without a significant increase in toxicity. We incorporated a CCR5-targeting siRNA driven by an independent Pol III transfer RNA Serine promoter (tRNASer, tRNAOS-CCR5sh cassette) in the 3’ intron of MCM7 in both orientations (forward (SGLV3) or reverse (SGLV4), Figure 1c) as a viral entry inhibitor. We also introduced a fifth RNA cassette, the nucleolar TAR RNA decoy, driven by the independent Pol III U6 promoter (U6-U16TAR cassette) to increase antiviral potency by inhibiting Tat-dependent viral transcription. This cassette was cloned outside of the MCM7 transgene to reduce the possibility of promoter interference that could negatively impact gene expression. These novel lentiviral vectors express up to five antiviral small RNAs to block both viral entry and replication of
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Biological activity and expression optimization of the tRNA<sup>Ser</sup>-CCR5<sub>sh</sub> cassette in the MCM7 platform

We investigated the use of transfer RNA (tRNA) promoters to express candidate antiviral RNAs due to their small size, ease in multiplexing, and independent regulation of RNA expression. This (Pol III) expression strategy utilizes the endogenous transfer RNA biogenesis pathway to express a primary tRNA-shRNA chimeric transcript which releases the shRNA upon tRNA<sub>Ser</sub> Z cleavage for further processing into mature siRNA. In the present constructs, we utilized tRNA<sup>Ser</sup> to express a CCR5 shRNA as an entry inhibitor against R5-tropic HIV. To assess the ability of this construct to downregulate surface CCR5 expression, we transiently transfected plasmids containing either the promoter sequence only (the endogenous tRNA<sub>Ser</sub> gene) or with the tRNA<sub>Ser</sub>-CCR5<sub>sh</sub> cassette into CCR5 overexpressing U373-MAGI-CCR5E cells. The reduction in CCR5 expression by flow cytometry was evaluated 72 hours after introduction of the construct. Potent and specific CCR5 knockdown was only observed with the tRNA<sub>Ser</sub>-CCR5<sub>sh</sub> cassette and not with the (empty) tRNA<sub>Ser</sub> promoter alone (Figure 2a).

We also utilized quantitative reverse transcription polymerase chain reaction (qRT-PCR) to evaluate CCR5 transcript level as the measure of functionality of our tRNA<sup>Ser</sup>-CCR5<sub>sh</sub> cassette in primary cells where CCR5 protein expression is often difficult to resolve by flow cytometry. We observed 80% downregulation of CCR5 mRNA in primary macrophages derived from in vitro differentiation of gene-modified HSPCs. These data demonstrate...
that sufficient CCR5 siRNA is produced in the context of the MCM7 platform to downregulate gene expression in primary cells (Figure 2b).

To further assess the processing of the tRNA\textsuperscript{Ser}-CCR5sh cassette in combination with the other anti-HIV elements, we incorporated this expression unit into the 3′ intron of the MCM7 platform (“inside MCM7”) in both orientations (forward (SGLV3) and reverse (SGLV4), Figure 1c) with respect to the parental U1 promoter. When transiently transfected into human embryonic kidney (HEK) 293 cells, we observed the reverse orientation (SGLV4) gives 2.4-fold greater transgene expression based on northern blotting with a radioactive probe specific for detecting the guide strand of the CCR5 shRNA (Figure 3a). Moreover, this analysis further distinguishes products in various stages of processing including the primary tRNA\textsuperscript{Ser}-shRNA chimeric transcript, the released shRNA, and the mature Dicer-processed siRNA sequence capable of gene silencing. The siRNA is the dominate product suggesting efficient processing, but some saturation of the processing pathway due to overexpression from the transient transfection is also evident (Figure 3a). Interestingly, northern blotting analysis shows that HIV7 containing only the tRNA\textsuperscript{Ser}-CCR5sh RNA cassette (no MCM7) drives dramatically higher expression levels in stably transduced CEM T lymphocytes than the above vector where the transgenes are inside the MCM7 3′ intron (Figure 3b), even though the vector is present at only one to two integrated copies per cell. These high levels of expression result in the accumulation of incompletely processed tRNA\textsuperscript{Ser}-shRNA chimeric transcript and large quantities of shRNAs. Based on these results, we concluded

![Figure 2 Biological activity of tRNA\textsuperscript{Ser}-CCR5sh cassette. (a) Potent CCR5 knockdown in U373-MAG1-CCR5E cells. Plasmid with only the tRNA\textsuperscript{Ser} promoter sequence (solid black line) or with the tRNA\textsuperscript{Ser}-CCR5sh cassette (dashed gray line) was transiently transfected into CCR5 overexpressing U373-MAG1-CCR5E cells\textsuperscript{27} with knockdown estimated by flow cytometry 72 hours later. Potent and specific downregulation of surface CCR5 expression was only observed with the construct containing CCR5sh. (b) Potent CCR5 RNA transcript degradation in gene-modified macrophages. Adult hematopoietic stem and progenitor cells were transduced with the indicated lentiviral vectors, sorted based on enhanced green fluorescent protein expression, and then differentiated into macrophages as described in Material and Methods. CCR5 transcript knockdown was measured by quantitative reverse transcription polymerase chain reaction with normalization to GAPDH housekeeping gene and expressed relative to the untransduced control. tRNA\textsuperscript{Ser}-CCR5sh cassette in the context of MCM7 platform induced potent silencing in gene-modified macrophages. tRNA\textsuperscript{Ser}, transfer RNA Serine promoter.](image)
that the tRNA\textsuperscript{Ser}-CCR5sh cassette in reverse orientation located within the 3′ intron of MCM7 platform would provide a more optimal level of siRNA expression.

**Flexible MCM7 platform expresses up to five antiviral small RNAs with efficient processing**

Combinatorial therapy has inherent challenges including various types of interference or competition between the individual elements that can negatively impact the potential therapeutic outcome. For example, when a vector encodes multiple RNAi triggers, competition can arise during processing and for incorporation into the RNA-induced silencing complex. To ensure that all the transgenes are expressed and processed into functional forms, we performed northern blotting with CEM T lymphocytes that stably express the transgenes. To ensure that the expression level resembles the physiological condition of one to two copies of integrated vectors in HSPCs, we only sorted transduced populations that were lower than 30% enhanced green fluorescent protein positive (EGFP\textsuperscript{+}).

Northern expression analyses indicate that incorporation of additional RNA cassettes such as tRNA\textsuperscript{Ser}-CCR5sh did not interfere with expression and processing of other transgenes in the MCM7 platform (Figure 4). We also confirmed correct and efficient processing of all RNAi triggers precursors (S1, S2M, S3B, and CCR5sh) into functional mature siRNAs that are 20-23 nucleotides and the small nucleolar RNAs (U16US5RZ and U16TAR) into 132-nucleotide processed products (Figure 4). We did not observe accumulation of pri- and pre-miRNAs suggesting that the endogenous RNAi pathway is not saturated and that it is possible to coexpress four siRNAs safely in a combinatorial approach. Therefore, MCM7 serves as a versatile tool in a multiplexing approach with the capacity for incorporating small RNA cassettes driven by both Pol II and Pol III promoters.

Comparing transgene expression levels between combinatorial strategies with two different classes of RNA promoters, SGLVs with the Pol II–driven MCM7 platform express transgenes at lower levels than FGLV with independent Pol III promoters (lanes 3–5 versus lane 6 in Figure 4). While the lower levels resulting from the U1 promoter may reduce toxicity, we questioned whether these levels were sufficient for efficacy. Therefore, we next turned to assessing the anti-HIV activity of the current combinations.

**The MCM7 platform produces sufficient small RNA expression to protect gene-modified cells from R5-tropic HIV**

We first assessed antiviral functionality by viral challenge of gene-modified CEM T lymphocytes with R5-tropic JR-FL HIV virus for 42 days, monitoring replication by p24 capsid levels in the culture supernatant (Supplementary Figure S1 and Supplementary Materials and Methods). All the therapeutic constructs (FGLV, SGLV4, SGLV5, and SGLV6) provided long-term protection, with up to a 5-log reduction in p24 capsid production in comparison with pLV and untransduced (unprotected) cells. Notably, SGLV4 and SGLV5 had almost no detectable levels of p24 capsid during this long-term challenge, providing evidence that the MCM7 platform expresses sufficient amount of small RNAs for functionality.

To further demonstrate the feasibility of this stem cell–based approach in protecting gene-modified progenies, we evaluated antiviral potencies of our constructs using macrophages derived from HSPCs. Macrophages are myeloid cells particularly suited for demonstrating antiviral potency in HIV challenge assays with vectors expressing CCR5 inhibitors because of the requirement of CCR5 coreceptor usage for infection with R5-tropic virus. We wished to design an assay where adult HSPCs are the substrate for gene modification as they are the eventual clinical target cell population for a stem cell–based gene therapy approach. After transduction, HSPCs can be differentiated into myeloid cells in vitro, and the efficacy of the candidate constructs evaluated by challenge with R5-tropic Bal HIV virus. To this end, we developed a novel single cell flow cytometric assay of intracellular staining with an antibody specific to HIV-1 core antigens (55, 39, 33, and 24-kD proteins) to monitor viral replication. The 55-kD protein is the primary precursor, while the 39- and 33-kD proteins are the intermediates of the mature 24-kD core protein.\textsuperscript{28} We used uninfected macrophages to establish nonspecific staining and background signal in the flow cytometric assay. Using this novel method, we were able to follow the kinetics of HIV infection on a cellular basis during the 42-day challenge in primary macrophages.
Representative intracellular HIV staining results at day 18 (D18) of the viral challenge are shown in Figure 5. We observed a 2-log difference in fluorescence intensity comparing nonspecific background staining of uninfected cells (Figure 5a) to an actual HIV-infected culture (Figure 5b), validating that our novel flow cytometric assay has excellent sensitivity for HIV detection. Furthermore, we found that 76.5% of untransduced (unprotected) macrophages are infected with HIV at D18 (Figure 5b) and as high as 95% at later time points (e.g., D28) (Figure 6, unTDX trace), providing good dynamic range as a vector screen to distinguish constructs with varying antiviral activities. For example, this flow cytometric assay can distinguish constructs with intermediate protection (31.7% HIV infection in SGLV1 (Figure 5d) and 56.4% in SGLV6 (Figure 5h)) from constructs with the best protection (16.7% HIV infection in FGLV (Figure 5c); 1.6% in SGLV2 (Figure 5e); 3.9% in SGLV4 (Figure 5f); 3.9% in SGLV5 (Figure 5g); and 8.9% in SGLV7 (Figure 5i)). When comparing constructs with the highest antiviral activities, we observed that SGLV2 had better antiviral activity (1.6% infection, Figure 5e) compared with small RNAs expressed from independent strong Pol III promoters such as in FGLV (16.7% infection, Figure 5c). There results emphasize that the higher levels of small RNA transgene expression are not necessary for anti-HIV potency, as lower RNA expression from the MCM7 platform with a single Pol II U1 promoter is sufficient for functionality. When comparing different RNA combinations in the MCM7 platform (SGLVs), we observed that antiviral protection also increases in constructs containing at least two anti-HIV siRNAs (i.e., SGLV4 and SGLV5). This could

| Vectors | Gated on live population | Uninfected | Untransduced | FGLV |
|---------|--------------------------|------------|--------------|------|
| EGFP    |                          |            |              |      |
| SGLV1   | 67.06%                   | 31.67%     |              |      |
| SGLV2   | 94.50%                   | 1.56%      |              |      |
| SGLV4   | 93.12%                   | 3.94%      |              |      |
| SGLV5   | 89.66%                   | 3.92%      |              |      |
| SGLV6   | 38.53%                   | 56.40%     |              |      |
| SGLV7   | 81.22%                   | 8.91%      |              |      |

Figure 5 Intracellular human immunodeficiency virus type 1 (HIV-1) staining demonstrates potent antiviral protection of macrophages derived from gene-modified hematopoietic stem and progenitor cells (HSPCs). Macrophages differentiated from adult HSPCs transduced with indicated lentiviral vectors were challenged with HIV-1 Bal at a multiplicity of infection of 0.01. Viral infection was monitored by intracellular staining by flow cytometry with an antibody specific to HIV-1 core proteins. Data from 18 days postinfection are shown. Background signal for intracellular staining was established with an uninfected control with identical culture and staining protocol. Intracellular staining showed a high degree of infection in untransduced macrophages, with some constructs with intermediate protection while differentiating some with excellent protection. EGFP, enhanced green fluorescent protein; SGLV, second-generation lentiviral vector. (a) Untransduced and uninfected, (b) untransduced and infected with HIV, (c-i) cells transduced with the indicated lentiviral vector and infected with HIV.
be due to the additive nature and efficiency of multiple siRNAs in cleaving HIV targets to inhibit replication.

To our surprise, we observed no added benefit with the inclusion of the tRNA<sup>ser</sup>-CCR5sh cassette as an entry inhibitor (3.9% in SGLV4, Figure 5f) and the nucleolar TAR RNA decoy (8.9% in SGLV7, Figure 5i) to the parental SGLV2 construct (1.6% in Figure 5e), perhaps because the parental construct already has potent antiviral activity with very low HIV infection. Another measure of potency is the duration of protection from HIV-1 replication. We therefore continue to follow infection for 42 days with the aforementioned intracellular HIV staining methodology to identify construct(s) with persistent protection (Figure 6). In this group, we observed an increase in the frequency of infected cells in some constructs over time: SGLV5 (D28) > FGLV (D35) > SGLV4 = SGLV7 (D35), but SGLV2 had no significant increase in viral infection through 42 days of culture. Our long-term results further confirm that overexpression of small RNA is not a guarantee of long-term antiviral protection and that the MCM7 platform can provide lower but sufficient level of RNA expression for functionality.

Lower small RNA expression reduces potential vector-related toxicity

Having established that sufficient levels of small RNAs are produced from the MCM7 platform for potent antiviral protection, we continued to explore the issue of vector-related toxicity on hematopoietic potential using an in vitro colony forming unit (CFU) assay. The CFU assay measures the hematopoietic potential of uni- and multipotent myelo-erythroid progenitors and therefore can be used to assess toxicity of multiplexed RNA expression in gene-modified HSPCs. We normalized the total number of CFUs to the untransduced control with respect to each donor to account for differences in hematopoietic potential between donors.

We found that the transduction process for gene modification with the empty vector had minimal impact on hematopoietic potential (83 ± 11% for pLV, Figure 7), which is relevant for clinical translation of stem cell–based gene therapy. In contrast, the levels of therapeutic small RNA expressed from independent strong Pol III promoters in FGLV had a negative impact on hematopoietic potential (54 ± 4%, Figure 7). However, when the small RNA expression is lowered with the MCM7 platform (e.g., SGLV4), we observed a recovery of hematopoietic potential to a level similar to that of the empty vector (77 ± 5 versus 83 ± 5%, respectively, Figure 7). Surprisingly, incorporating the U6-U16TAR cassette expressing the nucleolar TAR RNA decoy (SGLV7) created a sharp decline in CFU formation (from 77 ± 5 to 59 ± 4%, Figure 7). It is therefore possible that the U6-U16TAR cassette alone may be responsible for the reduction in hematopoietic potential with FGLV observed in this assay. Further experiments are required to establish the relative roles of higher overall small RNA levels versus the specific effect of the U6-U16TAR moiety on the loss CFU potential observed with FGLV.

In vivo O<sup>6</sup>-BG/BCNU drug selection allows for enrichment of gene-modified cells in humanized NSG mice

One of the current hurdles in stem cell–based gene therapy for HIV is the low frequency of gene-modified cells typically generated by
and developed a protocol to test for enrichment of gene-modified cells. Therefore, an MGMTP140K-expressing vector (FGLV) and used to transduce gene-modified monocytes in the spleen of treated mice (gated on CD45 + population). * Frequency of CD45 +/EGFP + gene-modified T lymphocytes in the spleen of treated mice (gated on CD45 + population). (a) Frequency of CD45 + cells in the spleens of treated mice. (b) Frequency of CD45+/CD3+/CD4+ T lymphocytes in the spleen and bone marrow was enriched 10- and 15-fold in the 2× and 3× treated cohorts, respectively (Figure 8b,d). The average frequency of EGFP+/CD3+/CD4+ T lymphocytes in the spleen increased 3-fold only when O6-BG/BCNU treatment was performed three times (Figure 8e). Similarly, EGFP+/CD4+/CD14+ monocytes were increased 3-fold in the spleens of mice following three O6-BG/BCNU treatments (Figure 8f). No deficiencies in lineage development were noted in the drug treated mice (data not shown). These data demonstrate that selective enrichment of HIV-target cells occurred in vivo and thus may allow for posttransplant increase in the frequency of HIV-resistant and 8th weeks (2× treatment cohort) or at the 7th, 8th, and 9th weeks (3× treatment cohort) following transplantation. Two or three weeks after completion of the O6-BG/BCNU treatment (i.e., 11 weeks following transplantation), animals were necropsied, and the level of engraftment and frequency of gene-modified cells in the spleen and bone marrow were evaluated.

Our results demonstrate that engraftment of the bone marrow and spleen with human (CD45+) cells was significantly reduced in treated animal cohorts relative to the control cohort (P < 0.001) (Figure 8a,c, respectively), but the frequency of EGFP+/CD45+ cells in the bone marrow and spleen was enriched 10- and 15-fold in the 2× and 3× treated cohorts, respectively (Figure 8b,d). The endogenous MGMT enzyme is responsible for repairing DNA damage caused by alkylating agents such as BCNU. O6-BG deactivates the endogenous MGMT enzyme so that cells cannot repair BCNU-induced DNA damage resulting in cell death. However, gene-modified cells that express a modified MGMT (MGMTP140K) are not sensitive to O6-BG treatment and therefore can repair DNA damage from BCNU treatment and survive. The net result of the O6-BG/BCNU selection is increased frequency of gene-modified cells. Therefore, we included this drug resistance gene in our anti-HIV constructs and developed a protocol to test for enrichment of gene-modified HSPC and CD4+ progeny in vivo.

In order to establish that a sufficient level of MGMTP140K was expressed in the relevant cell types, HSPCs were transduced with an MGMTP140K-expressing vector (FGLV) and used to transplant immunodeficient NSG mice as described in Materials and Methods. Animals were treated with O6-BG and BCNU at the 7th and 8th weeks following transplantation, animals were necropsied, and the level of engraftment and frequency of gene-modified cells in the spleen and bone marrow were evaluated.

Figure 8 In vivo drug selection enhances the frequency of gene-modified cells in the bone marrow and spleen of humanized NSG mice expressing MGMTP140K. Analysis of NSG mice transplanted with gene-modified hematopoietic stem and progenitor cells (HSPCs) expressing MGMTP140K and treated with two or three doses of O6-benzylguanine (O6-BG)/bis-chloroethyl nitrosourea (BCNU) as described in Material and Methods. Each animal received 1×10⁶ HSPCs following transduction with FGLV at the date of transplantation and 20 µg of Fc/IL-7 protein weekly for 11 weeks. (a) Frequency of CD45+ cells in the bone marrow of treated mice. (b) Frequency of CD45+/EGFP+ cells in the bone marrow of treated mice. (c) Frequency of CD45+ cells in the spleens of treated mice. (d) Frequency of CD45+/EGFP+ cells in the spleen of treated mice. (e) Frequency of CD3+/CD4+/EGFP+ gene-modified T lymphocytes in the spleen of treated mice (gated on CD45+ population). (f) Frequency of CD14+/CD4+/EGFP+ gene-modified monocytes in the spleen of treated mice (gated on CD45+ population). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. EGFP, enhanced green fluorescent protein; IL, interleukin.
cells in patients where engraftment of gene modified cells may be suboptimal.

DISCUSSION

Gene therapy with HIV-resistant HSPCs holds great promise in achieving a functional cure for HIV/AIDS. Among the various avenues of engineering HIV immunity, small RNA agents offer advantages over antiviral proteins due to their lower potential immunogenicity. Similar to combinatorial antiretroviral therapy, multiplexed RNA-based approaches inhibit viral entry and replication via multiple mechanisms and targets to enhance efficacy and to reduce the potential for viral escape. Our previous studies demonstrated that RNA-based gene therapy can be durable with detection of integrated vector in multiple differentiated cell lineages and persistent expression of small RNA transgenes three years after transplantation. However, the low frequency of gene-modified cells in vivo prevented clinical assessment of efficacy. Therefore, improvements in vector design and prospective enrichment of gene-modified cells are likely needed to advance RNA-based stem cell gene therapy and demonstrate proof of concept in controlled clinical trials.

We recently engineered an endogenous miRNA cluster located in an intron of the protein encoding MCM7 gene as an improved platform for providing more physiological amounts of therapeutic small RNAs to reduce potential vector-related toxicity. We demonstrate here that MCM7 is a flexible and versatile platform for coexpressing combinations of up to three si- and snoRNAs and can support additional independent RNA cassettes (both inside and outside of MCM7) for a total of five small RNAs. To our knowledge, this is the first example of multiplexing both classes of RNA promoters in a combinatorial approach. We observed expression and complete processing of all small RNA transgenes into functional forms without saturation of processing pathways or promoter interference that could negatively impact transcription. Interestingly, although the tRNA^A^-CCR5sh cassette is expressed at high levels in the pLV without MCM7, its expression is much weaker in the context of MCM7. This result may be related to the requirement for splicing of the intronic MCM7 cluster prior to transgene expression. Moreover, expression is also related to orientation of the cassette in MCM7, highlighting the importance of optimizing placement in multiplexing strategies.

The level of small RNA expression from the MCM7 platform driven by a single Pol II U1 promoter is much weaker compared to amounts driven by independent Pol III promoters. Nonetheless, we established that sufficient amounts of antiviral small RNA were produced from the MCM7 platform to protect gene-modified CEM T lymphocytes and primary macrophages derived from gene-modified HSPCs from R5-tropic HIV, with the duration and level of protection highly dependent on the RNA combination. This suggests that the optimal level of RNA expression is more important than achieving maximal levels of expression for a given modality. It is likely that the overall amount of small RNA processing that can occur in a cell at any given time is limited. In theory, because of the catalytic nature of siRNAs and ribozymes in turning over multiple substrates, it should take less RNA to achieve the same therapeutic effect compared to agents that sequester their target in stoichiometric ratio such as TAR RNA decoys.

Our results show that using multiple siRNAs in the MCM7 platform provides better protection against HIV, e.g., three siRNAs targeting tat and rev (SGLV2) are better than a single tat/rev siRNA (SGLV1). This could be due to the additive nature and efficiency of multiple siRNAs in cleaving HIV targets, while increasing the selective pressure against viral escape. Interestingly, the addition of two other potent anti-HIV RNAs, tRNA^A^-CCR5sh entry inhibitor and a U6-driven nucleolar TAR RNA decoy (SGLV4 and SGLV7, respectively), to the parental triple siRNA construct (SGLV2) did not provide additional benefit in inhibiting viral replication or preventing viral breakthrough. In fact, the additional small RNA cassettes actually negatively impacted potency with the observation of viral breakthrough at 35 days with the parental construct observed limited breakthrough through the 42-day culture. It is unclear why addition of the entry inhibitor and the nucleolar TAR RNA decoy was less optimal in inhibiting HIV, although it is possible that CCR5 siRNA may compete with other anti-HIV siRNAs (i.e., S1, S2M, and S3B siRNAs) for RNA-induced silencing complex factors in gene silencing; if so, then using the forward orientation of the tRNA^A^-CCR5sh inside MCM7 could alleviate competition. Alternatively, the CCR5 target or shRNA format may be responsible. Furthermore, TAR RNA has been reported as a pri-miRNA that is processed into functional miRNAs and a potent binder to TRBP (HIV-1 TAR RNA binding protein) within the endogenous RNA-induced silencing complex that negatively impacts RNAi pathway. The newly discovered toxicity associated with overexpression of the TAR RNA decoy (see below) could implicate selection of cells with overall less RNA expression translating into less optimal antiviral protection.

We performed an in vitro CFU assay to identify any vector-related myelo-erythroid toxicity. Our data demonstrate that the MCM7 platform has the capacity to safely express four siRNAs with complete processing without reducing in vitro hematopoietic potential of gene-modified HSPCs. Surprisingly, we observed a reduction of CFU potential when incorporating the U6-U16TAR cassette, which is the first report of toxicity related to overexpression of RNA decoys. It is possible that the U6-U16TAR cassette is responsible for the observed toxicity in FGLV, but further experiments including assessment of each individual Pol III RNA cassette on CFU potential are likely required. Additional studies may be needed to assess potential toxicity in other (lymphoid) cell populations not addressed in these in vitro assays.

Increasing the frequency of gene-modified cells in vivo may be required to achieve a clinically meaningful antiviral effect in cases where engraftment of gene-modified cells is low. Therefore, we incorporated a drug resistance gene (MGMT^TP140K) in our improved lentiviral vector to allow for in vivo enrichment of gene-modified cells using alkylating agents. Our results demonstrate the feasibility of this approach in increasing the frequency of gene-modified CD4^+ cells using in vivo treatment with levels of BCNU similar to those used in a non–human primate model of HIV gene therapy.

In summary, combinatorial gene therapy approaches are often most effective when targeting multiple stages of viral replication. We present here the improved MCM7 platform with enhanced flexibility, increased safety with sufficient level of transgene expression for long-term inhibition of HIV in HSPC-derived
macrophages. We demonstrate that more is not always better but rather a balance between transgene expression, mechanism of action, and target choice is required for optimizing the combinatorial approach. With the improvement in vector efficacy and strategies to increase the frequency of HIV-resistant cells, we hope to achieve sufficient gene marking and antiviral potency to move closer to a functional cure of HIV.

MATERIALS AND METHODS

Generation of improved second-generation lentiviral vectors with polycistronic MCM7 platform and selectable MGMT\(^{\text{PPTK}}\) marker. The MGMT\(^{\text{PPTK}}\) transgene was coexpressed with the EGFP marker from a polycistronic message utilizing a self-cleaving P2A peptide sequence from the CMV promoter. The MGMT\(^{\text{PPTK}}\) gene was first PCR amplified from pRSC-SMPGW2 plasmid,\(^{24} \) using the following primers (forward: 5′-GGG TCT AGA ATG GAC AAG CAT TGT GAA ATG AAA CGC-3′ and reverse: 5′-GGG GAA TTC CGT AGC TCA GTT TCG GCC AGC AGG CG-3′) flanked by XbaI and EcoRI sites. The fragment was digested with appropriate enzymes and then subcloned into NheI and EcoRI sites of the pFUG-P2A-WPRE vector (Burnett J, Unpublished data) just downstream of the P2A peptide sequence to generate pFUG-P2A-MGMT\(^{\text{PPTK}}\)-WPRE. The P2A-MGMT\(^{\text{PPTK}}\) fragment was then excised by BsrGI and BsiWI digestion and subcloned into the BsrGI site of pHIV7-GFP vector\(^{23} \) to generate a modified lentiviral vector pHIV7-GFP-P2A-MGMT\(^{\text{PPTK}}\). The same strategy was utilized to introduce the P2A-MGMT\(^{\text{PPTK}}\) fragment into FGLV\(^{18} \) to generate the modified lentiviral vector suitable for drug selection.

The cTag5-targeting siRNA is expressed from the Pol III RNA\(^{\text{a,b}}\) promoter. The tRNA\(^{\text{a,c}}\)-CCR5sh cassette was amplified from p1133-2 (Haushalter K, unpublished data) with the following primers (forward: 5′-ATG CGC CGG CAT CGA TGA AAA TGA TGC CAC GCT TAG CAT GTG AGC AGG TGG CGG AGT-3′ and reverse: ATG CGG CGG CAT TTA AAT AAA AAA GTG TCA AGT CCA ATC TAT GAT CTT GAG AAT AGA-3′) flanked by Nael and Swal sites on the 5′ end and NarI and ClaI sites on the 3′ end. The Nael-NarI and Swal-ClaI fragments were subcloned into pcDNA3-U1-MCM7-U1t plasmids\(^{25} \) containing three triple combinations of small anti-HIV RNAs with the Clal and Swal sites −70 bases upstream of the 3′ splice signal to generate clones in both forward and reverse orientations, respectively [pcDNA3-U1-MCM7-CCR5sh-U1t]. U1-MCM7-CCR5sh-U1t fragments were excised from pcDNA3-U1-MCM7-CCR5sh-U1t plasmids with NruI and NotI digestion and subcloned into pHIV7-GFP-P2A-MGMT\(^{\text{PPTK}}\) vector to generate SGLVs.

SGLV7 was generated by inserting the U6-U16TAR fragment into SGLV4. The U6-U16TAR fragment was generated from linearization of the pTZ/U6-U16TAR plasmid\(^{17} \) by BamHI digestion, followed by fill-in of the overhang by Klenow fragment to create a blunt end, and then digested with SphI. This fragment was then ligated into SGLV4 that had been similarly treated except with the initial linearization with SglI. For transduced HSPCs used for mice transplantation, HSPCs were prestimulated overnight in StemSpan-SFT6 media supplemented with 0.75 µmol/l Flt3 ligand (CellGenix, Freiburg, Germany), 10 ng/ml of thymopoietin (CellGenix), 50 ng/ml interleukin-6 (Life Technologies, Carlsbad, CA) for 48 hours prior to transduction. Lentiviral vectors adjusted to a multiplicity of infection of 20 were added to 6.4×10\(^4\) prestimulated HSPCs in 250 µl StemSpan-SFT6 media in the presence of 20 µg/ml of rapamycin (Sigma-Aldrich, St. Louis, MO) on RetroNectin (Takara, Mountain View, CA)-coated 96-well plate. After 24 hours of incubation, the lentiviral vector and rapamycin were removed, and HSPCs were cultured in 1.3-ml StemSpan-SFT6 media supplemented with 0.75 µmol/l SRI (Cellgen Technologies, San Diego, CA) for 5 days prior to sorting. HSPCs were sorted on CD34 marker, in addition to EGFP expression for the transduced population. Only CD34\(^+\) (untransduced) or CD34\(^+\)/EGFP\(^+\) (transduced) cells were used for subsequent colony forming assay setup and macrophage differentiation for HIV challenge experiments.

Generation of adult HSPC-derived macrophages. Sorted HSPCs were cultured in Iscove’s modified Dulbecco’s media with 20% FBS supplemented with 2 mmol/l of glutamine, 10% fetal bovine serum (FBS) and prestimulated in StemSpan-SFT6 media and then transduced with FGLV at a multiplicity of infection of 10 at 1×10\(^5\) cell/ml on Retromectin (Takara, Otsu-Shiga, Japan)–coated non–tissue culture T-75 flask (5 µg protein/cm\(^2\), 1.3–2.6×10\(^3\) viable cells/cm\(^2\)) for 24 hours in the presence of 20 µg/ml of rapamycin. Lentivirus and rapamycin were removed, and cells were washed once before being resuspended for transplantation in injection saline solution (APP Pharmaceuticals, Lake Zurich, IL).
Flow cytometric assay for CCR5 knockout studies in U373-MAGI-CCR5E cells. About 1 × 10^6 U373-MAGI-CCR5E cells were seeded per well in a 24-well dish 1 day prior to transfection. An equal ratio of pHIV7-EGFP to tRNA^acc construct in pBluescript with 400 ng of total plasmid DNA was transiently transfected with Lipofectamine 2000 (Life Technologies) following manufacturer’s instructions. The EGFP marker from the cotransfected pHIV7-EGFP plasmid serves as an internal control for transfection efficiency. Cells were detached 72 hours later and stained with CCR5-APC antibody (clone 2D7, BD Pharrmingen, San Jose, CA) to estimate CCR5 knockdown by flow cytometry. Data were collected on Gallios flow cytometer (Beckman Coulter, Brea, CA) and analyzed by FCS express version 4 software (De Novo software, Los Angeles, CA).

Real-time quantitative reverse transcription PCR for CCR5 knockdown studies in macrophages differentiated from adult HSPCs. Total RNA from HSPC-derived macrophages was extracted with STAT-60 reagent (Tel-Test, Friendswood, TX) according to manufacturer’s protocol and then resuspended in nuclease-free water. Residual DNA was digested with Ambion TURBO DNAse (Life Technologies) with 2 µg of total RNA in a 15-µl reaction, in accordance with manufacturer’s instructions. Complementary DNA was then synthesized with 1 µg of DNase-treated RNA with 100 ng of random primers (Invitrogen, Carlsbad, CA) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) in a 27-µl reaction according to manufacturer’s instructions. Real-time PCR was carried out with CFX96 real-time detection system with 10 ng of complementary DNA, 0.4 µmol/l of each gene-specific (CCR5 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) primers with iQ SYBR green supermix (Bio-Rad, Hercules, CA) in a final volume of 25 µl. The PCR conditions were 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 20 seconds and at 62 °C for 1 minute. A standard curve with known serial dilutions of total RNA input was utilized to calculate the ratio between CCR5 and GAPDH to estimate the percentage of CCR5 downregulation.

Primer sequences for PCR were as follows:

| Primer       | Sequence                          | Description       |
|--------------|-----------------------------------|-------------------|
| CCR5-F       | 5’-TTT ATT ACA CCT GCA GCT CTC-3’| CCR5 forward       |
| CCR5-R       | 5’-CTT GTT AGA GCT ACT GCA ATT-3’| CCR5 reverse       |
| GAPDH-F      | 5’-CGC TCT CTG CTC GTC TT-3’      | GAPDH forward      |
| GAPDH-R      | 5’-CCA TGG TGT CTG AGC GAT GT-3’  | GAPDH reverse      |

Northern blotting for small RNA expression analysis. Approximately 2 µg of pcDNA3-U1-S1/S2M/S3B-U1 with tRNA^acc-CCR5sh cassette in either orientation was transfected into HEK 293 cells with 90–95% confluency per well in six-well dish with Lipofectamine 2000 (Life Technologies) according to manufacturer’s protocol. Total RNA was extracted 48 hours later with STAT-60 (Tel-Test) according to manufacturer’s protocol. For each construct, 10 µg of total RNA was loaded onto 8% polyacrylamide gel with 8 mol/l urea and then electrophoresed onto a Hybond-N nylon membrane (Amersham, Arlington Heights, IL) and hybridized with P^-labeled DNA probe specific for small RNA. Small nuclear U2A RNA serves as an internal control.

Total RNA from sorted T lymphocytes was isolated similarly, and northern blotting was performed as described above, with the exception of 20 µg of total RNA used as input.

The DNA probes for each of small RNAs are as follows:

| Primer | Sequence                          | Description       |
|--------|-----------------------------------|-------------------|
| S1     | 5’-GGG GAG ACA CCG ACC AAG ACC-3’ | Small RNA S1      |
| S2M    | 5’-GCC TGT GCC TCT TCA GCT ACC-3’ | Small RNA S2M     |
| S3B    | 5’-CAT CTC TCA TGG CAG GAA GAA-3’ | Small RNA S3B     |
| CCR5sh | 5’-AAA GTG TCA AGT CCA ATC TAT GA-3’ | CCR5 short hairpin |
| USRZ   | 5’-GAG TGC TTT TCG AAA ACT CAT CAG AA-3’ | USRZ             |
| TAR    | 5’-CCA GAG AGC TCC GAG CAG CAG-3’  | TAR               |
| CCR5R2 | 5’-GTG TCA AGT TGT GTC CAC ACG GAC TCA TCA GCA ATC GA-3’ | CCR5R2            |
| U2A    | 5’-AGA ACA GAT ACT ACA CTT GA-3’   | U2A               |

HIV challenge and intracellular HIV staining to monitor viral replication. About 1 × 10^6 HSPC-derived macrophages seeded in 48-well plate were infected in triplicate with HIV-1 Bal virus at a multiplicity of infection of 0.01 in 500 ul volume. After overnight incubation, the HIV virus was removed, and the cells were cultured in 500 ul DMEM with 10% FBS supplemented with 2 mmol/l of glutamine, 10 ng/ml of granulocyte macrophage colony stimulating factor, and 10 ng/ml of macrophage colony stimulating factor.

Viral replication was analyzed by intracellular staining of HIV core proteins at indicated time points. Cells were detached with Accutase solution (Sigma-Aldrich), viability was estimated by LIVE/DEAD fixable aqua dead cell stain kit (Sigma-Aldrich), and then fixed and permeabilized with intracellular fixation and permeabilization buffer set (eBioscience, San Diego, CA). Before staining with KC57-RD antibody (KC57-RD1 antibody, clone FH190-1-1; Beckman Coulter) that recognizes HIV-1 core proteins, and finally analyzed by flow cytometry. Data were collected on Gallios flow cytometer and analyzed by FCS express version 4 software.

In vitro CFU assay for adult HSPCs. A total of 500 sorted HSPCs were plated in triplicate in MethoCult H4435-enriched methylcellulose media (Stem Cell Technologies) according to manufacturer’s protocol. Cells were cultured for 12 to 13 days before colony scoring under inverted microscope.

Fc/IL-7 protein production. Fc/IL-7 was cloned into an OptiVext- TOPO (Invitrogen) vector, and protein was produced from a cloned transfected DG44 CHO cell line as per the methods of Lo et al. 34

Humanized NSG mouse model. NOD.Cg-Pkdcsid Il2rgtm1Wjl/SzJ (NSG) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and bred at the City of Hope Animal Resources Center according to the protocols approved by the Institutional Animal Care and Use Committee of the City of Hope. Adult (8–10 weeks old) NSG mice were irradiated at 270 cGy for 24 hours prior to transplantation. Each animal was transplanted with 1 × 10^6 HSPCs following transduction via intravenous injection. To enhance T lymphopoiesis, 20 µg of Fc/IL-7 protein was administered per animal intravenously weekly for 11 weeks.

In vivo O^6^-BG and BCNU selection for enrichment of gene-modified cells. O^6^-BG (Sigma-Aldrich) was prepared in 4% dimethylsulfoxide, 30% polyethylene glycol 400 (Sigma-Aldrich) and 66% injection saline solution. BCNU (injectable carbustine) was purchased from Bristol-Myers Squibb (New York, NY) and stock solution was reconstituted in supplied absolute ethanol at 100 mg/ml as per manufacturer’s instructions, and then diluted in injection saline solution before administration.

Drug selection was performed either at the 7th and 8th weeks (2× treatment cohort, N = 12 mice) or at the 7th, 8th, and 9th weeks (3× treatment cohort, N = 12 mice) posttransplantation. Control cohort (N = 12 mice) received saline injection. Animals in treatment cohorts received 20 mg/kg of O^6^-BG followed by 5 mg/kg of BCNU 1.5 hour later via intraperitoneal injection for each round of drug selection.

Flow cytometric analysis of engraftment and gene modification frequency. Mice were necropsied 11 weeks after transplantation for analysis of engraftment and enrichment of gene-modified cells. Single cell suspensions of bone marrow (femurs) and spleen were prepared by mechanical dissociation, and red cells were lysed using ACK lysis buffer (Sigma-Aldrich). All cell suspensions were pretreated with human immunoglobulin (GammaGard, Baxter Healthcare, Deerfield, IL) for 30 minutes to block nonspecific antibody staining. Splenocytes were stained with a human pan-leukocyte antibody to CD45-PC5 (BioLegend, San Diego, CA), and lineage-specific anti-human antibodies, CD3-ECD, CD4-APC, and CD14-APC-Alexa-750 (Invitrogen) for 20 minutes and washed two times with 1 ml of phosphate-buffered saline containing 0.1% bovine serum albumin (Sigma-Aldrich). Bone marrow cells were stained with antibodies to human CD45-PC5 (Beckman Coulter) for 20 minutes and
washed two times with 1 ml of phosphate-buffered saline containing 0.1% bovine serum albumin. To establish analytical gates and background stain-
ing, bone marrow and spleen samples from two to three untransplanted mice were stained with the same antibody panel. Data were collected on
Galios flow cytometer and analyzed by FCS express version 4 software.

**Statistical analysis**. In vitro CFU data were analyzed with statistical soft-
ware Prism version 6.01 (GraphPad Software, La Jolla, CA), using one-way analysis of variance followed by Bonferroni’s comparison test. Values of P ≤ 0.05 were considered statistically significant compared with untransduced control. The average and standard deviation were derived from two to three independent donors as indicated.

In vivo drug selection data were also analyzed with Prism software using two-way analysis of variance followed by two-tailed t-test. Values of P ≤ 0.05 were considered statistically significant compared with control animals. The average and standard deviation were derived from cohorts of 12 animals per group.

**SUPPLEMENTARY MATERIAL**

**Figure S1**. Potent antiviral activity of combinatorial RNA vectors in stably expressing CEM T lymphocytes.

**Materials and Methods**

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