Preclinical In Vivo Evaluation of the Safety of a Multi-shRNA-based Gene Therapy Against HIV-1

Mireille Centlivre1,4, Nicolas Legrand2-7, Sofieke Klamer1, Ying Poi Liu1, Karin Jasmin van Eijj1, Martino Bohne2, Esther Siteur-van Rijnstra1, Kees Wijer2,6, Bianca Blom2, Carlijn Voermans3, Hergen Spits2,5 and Ben Berkhout1

Highly active antiretroviral therapy (HAART) has significantly improved the quality of life and the life expectancy of HIV-infected individuals. Still, drug-induced side effects and emergence of drug-resistant viral variants remain important issues that justify the exploration of alternative therapeutic options. One strategy consists of a gene therapy based on RNA interference to induce the sequence-specific degradation of the HIV-1 RNA genome. We have selected four potent short hairpin RNA (shRNA) candidates targeting the viral capsid, integrase, protease and tat/rev open-reading frames and screened the safety of them during human hematopoietic cell development, both in vitro and in vivo. Although the four shRNA candidates appeared to be safe in vitro, one shRNA candidate impaired the in vivo development of the human immune system in Balb/c Rag2−/− IL-2Rγc−/− (BRG) mice. The three remaining shRNA candidates were combined into one single lentiviral vector (LV), and safety of the shRNA combination during human hematopoietic cell development was confirmed. Overall, we demonstrate here the preclinical in vivo safety of a LV expressing three shRNAs against HIV-1, which is proposed for a future Phase I clinical trial.

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Subject Category: siRNAs, shRNAs, and miRNAs

Introduction

HIV-1 and AIDS remains as a major public health problem with more than 34 millions infected individuals worldwide in 2011. The development of highly active antiretroviral therapy (HAART) has dramatically reduced both mortality and morbidity, and improved life expectancy and quality of life of the treated individuals. Despite its success, HAART does not fully suppress viral replication, especially in some critical sites (e.g., gut), and allows the persistence of a latent viral reservoir.2,3 The efficacy of HAART relies on daily adherence to drug regimens, but this life-long treatment is associated with side effects and emergence of drug-resistant viral variants, therefore promoting the search for alternative therapeutic options.

Among these, gene therapy has emerged as new therapeutic option. Ideally, such a gene therapy approach should target the CD34+ human hematopoietic progenitor cells (hHPC), allowing the generation of a HIV-resistant multilineage immune system with constant supply of HIV-resistant cells that will colonize the blood and tissues. Such approaches were reinforced by the recent clinical success described for the “Berlin patient”. In early 2007, a HIV-positive individual with acute myeloid leukemia received a compatible CD34+ hHPC transplant from one of the rare individuals who are naturally resistant to HIV-1 (CCR5Δ32) in an effort to eradicate both diseases at once.4 The patient was declared “functionally cured” as no sign of viral replication was found even 3–4 years after cessation of HAART.5 Although successful, this approach is impractical for widespread use due to the difficulty of finding suitable CCR5-negative donors and the high risk of the required whole body irradiation.

To circumvent allogeneic transplantation, ex vivo modification of individuals own hHPC have been proposed, using new antiviral gene therapy approaches based on RNA interference (RNAi).6–8 Gene silencing through RNAi can be induced by expression of double-stranded RNA that will lead to sequence-specific degradation of the target RNA.9 Anti-HIV-1 short hairpin RNA (shRNA) expression in CD4+ T cells strongly inhibits HIV-1 replication in vitro.10,11 The shRNA-expressing gene cassettes are delivered to autologous hHPC via transduction with a lentiviral vector (LV), which stably integrates into the host genome. There is growing evidence from longitudinal studies in patients that LV can be used safely without signs of abnormal cell expansion induced by vector integration-mediated activation of proto-oncogenes.12

To study safety issues of gene therapy approaches, mouse models of the human immune system (HIS) have been used.13,14,15 Over the last two decades, several humanized mouse models have been generated and optimized for the experimental study of HIS development and function.15–17 We and others have transplanted CD34+CD38− hHPC into immunodeficient newborn mice, such as BALB/c Rag2−/− IL-2Rγc−/− (BRG) or NOD.scid IL-2Rγc−/− (NSG) mice, resulting in the development of a multilineage HIS in vivo.18–20 Since target

The first two authors contributed equally to the work.

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cells for HIV-1 infection are present in the HIS mice, such animal models have become popular for assessing human pathogenesis and addressing new HIV therapeutic strategies. Considering a future RNAi-based gene therapy approach in the clinic, CD34+ hHPC would be exposed ex vivo to LV and reinfused into the patient, where they would home to the bone marrow and produce mature hematopoietic cells that are protected against HIV-1 infection. Thus, generating HIS mice with genetically engineered hHPC for the expression of anti-HIV shRNA is likely to represent a sensitive safety screen for human hematopoiesis consistent with the envisioned clinical strategy.

In this study, we have selected four shRNA candidates targeting conserved regions of the viral genome for the development of the combinatorial shRNA-based gene therapy against HIV-1. These four shRNA were first tested individually in HIS mice and the safe shRNAs were combined into a single LV that was evaluated for antiviral activity in vitro and for safety in HIS mice.

Results
Selection of effective anti-HIV-1 shRNAs targeting highly conserved HIV-1 regions
We have previously identified 21 shRNAs targeting eight highly conserved regions of the HIV-1 genome that exhibit potent inhibition of HIV-1 replication. Based on long-term in vitro culture experiments, we selected the four most effective shRNAs, shGag5, shPol1, shPol47, and shR/T5, respectively targeting the viral capsid, integrase, protease, and tat/rev open-reading frames (Figure 1a). The respective four shRNA cassettes were cloned individually in a self-inactivating LV (Figure 1b).

No signs of toxicity of shRNA expression in a human colony-forming cell assay
We first evaluated the safety of the four shRNAs by performing a human colony-forming cell assay (hCFCC). This in vitro assay is commonly employed for determining the colony- and burst-forming capacity of hematopoietic progenitor cells and is widely used for measurement of drug toxicity on human hematopoietic progenitor cells (hHPC). We transduced CD34+CD38− hHPC with the different LVs expressing a single shRNA candidate or the empty control vector JS1. Transduced GFP+ hHPC were isolated by fluorescence-activated cell sorting and cultured for 2 weeks in the appropriate conditions driving development of colony-forming unit-granulocytic-macrophages (CFU-GM), colony-forming unit-granulocytic-erythroid-macrophages-megakaryocytes (CFU-GEMM) and burst-forming unit-erythroid (BFU-E) (Figure 2a).

CFU-GM, CFU-GEMM, and BFU-E were then counted in four independent experiments. For all the different LV conditions analyzed, transduced hHPC gave rise to the three types of colony (BFU). We counted between 13 and 54 CFU-GM, 5 and 16 BFU-E and 0 and 4 CFU-GEMM for all the conditions analyzed, with high variability between the experiments and between the LV conditions tested, which likely reflects the interdonor hHPC variability. Still, the LV-transduced hHPC – encoding a single or no shRNA candidate – generated comparable numbers of CFU-GM, BFU-E, and CFU-GEMM (Figure 2b–d), indicating no obvious toxicity of shRNA expression on the CFU/BFU capacity of CD34+ human hematopoietic progenitor cells in this relatively short-term in vitro assay.

In vivo monitoring of the shRNA candidates reveal some toxicity for shGag5 during human hematopoiesis
We next assessed the in vivo safety of single shRNA treatment in the humanized BRG-HIS mouse model. CD34+CD38− hHPC were transduced with LVs expressing a single shRNA or the empty control LV prior to xenograft transplantation in immunodeficient BRG newborn mice. The BRG mice were injected with the bulk of cultured hHPC, i.e., they received a mixture of transduced and nontransduced hHPC, providing for each animal an internal control for human immune system development and maturation. An aliquot of the transduced hHPC was kept in culture in vitro for 3–4 days to determine the transduction efficiency based on GFP expression.

BRG-HIS mice were killed between 10 and 13 weeks post-hHPC transplantation and we first analyzed the absolute human cell number in the blood and the lymphoid organs (bone marrow, thymus, spleen, and liver) of the animals. Similar numbers of human cells were detected in all the analyzed organs of BRG-HIS mice transplanted with shRNA-transduced hHPC as compared with the control JS1 group (Table 1). This highlights that all four shRNA treatments do not have a negative impact on human hematopoiesis at a global level.

We next monitored the relative frequency of shRNA-transduced hematopoietic cells (GFP+ hCD45+ cells). The in vivo frequency of human GFP+ cells in the blood and lymphoid organs of the animals was measured and compared with the original in vitro transduction efficiency (Figure 3a). An in vivo/in vitro GFP+ ratio around 1.0 indicates no selection bias with a good recovery of human GFP+ cells in the animals as compared with the initial transduction efficiency. We observed a similar efficient GFP+ recovery in the analyzed organs of the BRG-HIS mice that received hHPC expressing shPol1, shPol47, or shR/T5 (Figure 3b–f). In contrast, shGag5 expression appeared to be detrimental for the recovery of human GFP+ cells in vivo in all lymphoid organs.

Figure 1 Anti-HIV-1 shRNA target regions and cloning strategy.
(a) The shGag5, shPol1, shPol47, and shR/T5 target positions within the HIV-1 genome are indicated. (b) The third generation self-inactivating lentiviral vector JS1 expresses the green fluorescent protein (GFP) reporter. Single shRNA vectors express in addition a shRNA targeting the HIV-1 genome from the human polymerase III H1 promoter.
B cells, monocytes, and pDC (Figure 4d) did not affect the recovery of human BRG-HIS mice (Figure 4c) as compared with the control JS1 LV. The results (mean ± SD) are pooled from four independent experiments with duplicates for each experiment. Good GFP recovery with a ratio around 1.0 was observed for human T cells, B cells, monocytes, and pDC (Figure 4a).

Figure 2 Impact of in vitro shRNA expression in early human hematopoietic progenitors. (a) Human fetal liver CD34-CD38- hHPC were transduced with JS1, shGag5, shPol1, shPol47, or shR/T5-expressing lentiviral vector. Transduced (GFP+) hHPC were sorted and a human colony-forming cell assay was performed. (b-d) Graphs show the relative colony counts of CFU-GM (b), BFU-E (c), and CFU-GEMM (d), as compared with control JS1 transduced human progenitor cells.

Table 1 Absolute number of human CD45+ cells in the organs of shRNA-expressing BRG-HIS mice

| (×10^5) | Bone marrow | Thymus | Spleen | Liver | Blood |
|---------|-------------|--------|--------|-------|-------|
| JS1     | 47.2 ± 31.4 | 14.7 ± 10.3 | 21.1 ± 18.6 | 5.2 ± 3.4 | 1.53 ± 1.86 |
| shGag5  | 25.4 ± 21.6 | 10.1 ± 3.2 | 8.5 ± 6.0 | 4.8 ± 2.5 | 0.91 ± 0.53 |
| shPol1  | 72.4 ± 56.8 | 16.3 ± 10.9 | 39.0 ± 25.5 | 5.7 ± 2.6 | 8.22 ± 14.70 |
| shPol47 | 34.9 ± 18.9 | 14.4 ± 10.9 | 16.5 ± 14.0 | 5.1 ± 2.9 | 0.71 ± 0.35 |
| shR/T5  | 41.9 ± 38.5 | 9.1 ± 6.2 | 18.5 ± 15.0 | 4.8 ± 2.5 | 1.17 ± 1.40 |

Suggesting a negative impact of this specific shRNA on the development of the transduced human hematopoietic cells.

We further analyzed the GFP expression in several human leukocyte subsets of the spleen of BRG-HIS mice, namely man CD45+ cells and compared with control animals (empty JS1-transduced n=17) or shR/T5 (n=9), shPol47 (n=11) were analyzed 10–13 weeks post-hHPC transplantation for absolute number of human CD45+ cells and compared with control animals (empty JS1-transduced hHPC; n=26). Bone marrow analysis was performed on two femurs per animal.

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| shR/T5  | 41.9 ± 38.5 | 9.1 ± 6.2 | 18.5 ± 15.0 | 4.8 ± 2.5 | 1.17 ± 1.40 |

Looking at the relative colony counts of CFU-GM and BFU-E, we observed a lower recovery of human GFP+ T cells in shPol47-expressing mice (Figure 4c), suggesting that seeding of the thymus by human progenitors was hindered by shPol47 expression.

Figure 4 Delayed seeding of HIS mice thymus by shPol47-expressing human progenitor cells

Reduced accumulation of shRNA-expressing T cells was noticed in 10–13-week old BRG-HIS mice as compared with control LV-transduced T cells, except for shPol47. Still, we questioned whether shPol47-expressing hHPC also showed hindered capacity for T-cell generation at earlier time points, i.e., during early events of BRG-HIS mouse thymus colonization and human T-cell differentiation. We transduced CD34-CD38- hHPC with JS1 or shPol47-encoding LV prior to xenograft transplantation in BRG newborn mice. At 1-week post-hHPC transplantation, the site of hHPC injection and major hematopoietic cell niche at this age, i.e. the liver of the BRG-HIS mice, was analyzed for the presence of human hematopoietic GFP+ cells. We did not observe any difference in the absolute number of human cells nor in the in vivo/in vitro ratio of human GFP+ cells between JS1 control and shPol47-expressing BRG-HIS mice. At 4 weeks post-hHPC transplantation, the bone marrow and the thymus of BRG-HIS mice are the major sites of human hematopoietic cell accumulation. No difference in the absolute number of human cells nor in the in vivo/in vitro ratio of human GFP+ cells was noted between the two groups of mice in the bone marrow at this age (Figure 5a,b). By contrast, the seeding of the thymus by shPol47-transduced (GFP+) human progenitors was almost abolished whereas a GFP+ ratio around 1.0 was observed in the thymus of control animals (Figure 5b). A similar number of human CD45+ thymocytes was retrieved from the two BRG-HIS mouse groups, suggesting that seeding of the thymus by human progenitors was hindered by shPol47 expression.
Simultaneous expression of three nontoxic shRNAs is safe in vitro and in vivo

As for HAART, combination of multiple shRNAs into a single LV resulted in additive inhibition of HIV-1 in vitro. Even more importantly, the combinatorial RNAi approach prevented the selection of RNAi-escape variants. We combined three shRNAs (shPol1, shPol47, and shRT5; R3) or four shRNAs candidates (shGag5, shPol1, shPol47, and shRT5; R4) into the JS1 LV (Figure 6a) and evaluated the in vitro safety of these combinatorial LV in the hCFC assay (Figure 2a). The numbers of CFU-GM, BFU-E, and CFU-GEMM were comparable between R3 LV and JS1 control LV (Figure 6b). The hHPC transduced with the shGag5-encoding R4 LV gave rise to a reduced number of CFU-GM as compared with control hHPC. We next assessed the in vivo safety of a combinatorial shRNA treatment in BRG-HIS mice. As for the single shRNA treatment experiments, similar numbers of human cells were detected in all the analyzed organs of BRG-HIS mice transplanted with shRNA-transduced hHPC as compared with the control JS1 group (Supplementary Table S1, online).

When the in vivo frequency of human GFP+ cells in the blood and lymphoid organs of the animals was measured and compared with the original in vitro transduction efficiency, we observed a severely reduced recovery of human GFP+ cells in vivo in the R4 animals (Figure 6c–g). This recovery was even lower than observed for the single shGag5 experimental group (Figure 3). The recovery of GFP+ cells from R3-treated BRG-HIS mice was lower than in the JS1 control BRG-HIS mice, although it was significantly lower only for the spleen and blood. This reduced global recovery in the spleen was explained in the analysis of different human immune cell subsets, where we measured a reduced frequency of...
GFP+ cells in the human B- and T-cell subsets (Figure 6h). Still, R3-transduced hHPC gave rise to multilineage in vivo reconstitution of the human immune system, demonstrating the in vivo safety of the R3 construct. When compared with the shGag5 group, the R3-treated BRG-HIS mice exhibited higher recovery of GFP+ cells in the primary lymphoid organs (bone marrow, thymus) and the liver, suggesting that the reconstitution kinetics by the R3-transduced cells might be delayed, as observed with shPol47.

Inhibition of HIV-1 replication on in vivo generated human CD4+ T cells expressing three shRNA against HIV-1

To evaluate the capacity of human shRNA+ cells to inhibit HIV-1 replication, we isolated mature human CD4+ T cells from NSG-HIS mice produced either with control JS1 or with R3-transduced hHPC. At sacrifice, splenocytes were harvested and GFP+ human CD4+ T cells were isolated by fluorescence-activated cell sorting. The sorted CD4+ T cells were challenged ex vivo by either a wild-type HIV-1 strain (LAI) or a shPol1-resistant HIV-1 virus (HIV-1 LAI-D30N) that was selected during mono-RNAi therapy. HIV-1 LAI replication was inhibited in transduced GFP+ CD4+ T cells from R3-expressing NSG-HIS mice but not in the control GFP+ CD4+ T cells from JS1-expressing NSG-HIS mice (Figure 7a). When the sorted cells were challenged with the shPol1-resistant HIV-1 virus, viral inhibition was still observed for the transduced GFP+ CD4+ T cells from R3-expressing NSG-HIS mice but not in the control GFP+ CD4+ T cells from JS1-expressing NSG-HIS mice.
LV and injected into newborn BRG mice. At 1 and 4 weeks post-
hHPC were transduced with JS1-control or shPol47-expressing

**Discussion**

T cells that express R3 are resistant to HIV-1 replication. Fur-
these results demonstrate that

in vivo

generated human CD4+

based gene therapy. Here, we selected four anti-HIV-1 shRNA
candidates that target essential HIV-1 genes and highly con-
served regions of the HIV-1 genome among all HIV-1 subtypes
(>73% target conservation of the shRNA target sequence in all
virus isolates present in the Los Alamos database), in par-
ticular subtype B isolates (>80%). We evaluated the preclini-
cal safety profile in vivo in view of further development of this
RNAi-based gene therapy towards clinical trial phase I.

Evaluation of the safety of shRNA expression by hHPC was ini-
itially performed using the classical human colony-
forming cell assay. No apparent toxicity was scored in this
assay for single shRNA expression. In contrast, when safety
of shRNA expression by human hematopoietic cells was as-
essed in vivo in mice humanized for the immune sys-
tem, a single shRNA (shGag5) exhibited a negative impact
on the development of human hematopoietic cells. Only
the shGag5-expressing cells were affected, as develop-
ment of nontransduced cells occurred normally in these
animals to reach absolute number of human cells similar
to control humanized mice. These results demonstrate that
mice humanized for the human immune system constitute
a valuable and sensitive in vivo preclinical model to evalu-
ate the hematopoietic safety of gene therapy strategies, for
several reasons. First, the hHPCs that are engrafted in the
immunodeficient newborn mice are similar to the ones that
would be genetically engineered for ex vivo gene therapy of
HIV-infected patients. Second, as the hHPCs transplanted
in the mice consist of a mixture of transduced (GFP+) and
nontransduced hHPC (GFP−), the human immune system
in the animals is thus constituted of shRNA-expressing cells
(GFP+) and nontransduced cells (GFP−). Besides evaluating
the control mice vs. the shRNA-treated mice, this approach
provides a perfect internal control to test for adverse effects
of shRNA expression on hematopoiesis by simply following
the frequency of GFP− cells over time, as compared with the
original hHPC inoculum. This approach turned out to be more
sensitive than a simple screen of the total human cell popu-
ation. We previously used such a strategy to develop a sensi-
tive cell culture system to detect transgene toxicity. Finally,
HIS mice allow for the long-term evaluation of associated tox-
icity, which could for instance not be observed in the relatively
short-term colony-forming cell assay with shGag5.

We demonstrate in HIS mice that shPol1, shPol47, and shR/
T5 were safe, as they did not affect the in vivo development of
the human immune system, both in terms of absolute human
cell numbers and shRNA-expressing cell recovery. When com-
bined into a single LV, simultaneous expression of these three
shRNAs did not affect the in vivo generation of a multilineage
human immune system, even though GFP recovery is less
effective for R3 than the vectors that express a single shRNA
from this combination. Still, GFP recovery with the R3 vector
was better than with shGag5 alone, and similar or close to the
JS1 control in the primary organs. Moreover, we provide proof-
of-concept for the capacity of human shRNA+ CD4+ T cells
harvested from R3-treated HIS mice to resist HIV-1 infection.
As for HAART, combinatorial shRNA treatment exhibits addi-
tive HIV-1 inhibition and prevents the selection of escape virus
variants. In fact, we demonstrated that R3-expressing cells
remain nonsusceptible for an HIV-1 variant with an escape
mutation in a single shRNA target sequence. These data
should be extended in the future by long-term HIV infection

![Figure 5 Impaired seeding of BRG-HIS mouse thymus by shPol47-expressing human progenitor cells. CD34+CD38− hHPC were transduced with JS1-control or shPol47-expressing LV and injected into newborn BRG mice. At 1 and 4 weeks post-
hHPC transplantation, JS1 (white boxes; n = 4) and shPol47 (grey boxes; n = 5) BRG-HIS mice were analyzed in the major sites of
hematopoiesis for the absolute number of human CD45+ cells (a) and for GFP+ ratio in human hematopoietic cells (b). The GFP+ ratio
is the ratio between the frequency of human GFP+ cells measured
in the animals (in vitro) and the frequency of GFP− hHPC injected in the
newborn mice (in vitro transduction efficiency). The results are
pooled from four independent experiments, and each dot represents
an individual animal. *P < 0.05 (Mann–Whitney test). BM, bone
marrow; LIV, liver; THY, thymus.](image)
experiments in the HIS mouse to analyze the potential emergence of HIV escape mutants over time.

Analysis of the early events of tissue colonization and cell differentiation taking place in the BRG-HIS mice engrafted with shRNA-hHPC revealed that seeding of the thymus by human bone marrow GFP+ progenitors was delayed, with kinetics varying depending on the respective shRNA. This observation could reflect several nonmutually exclusive mechanisms affecting e.g., generation of thymus seeding progenitor cells,23–25 migration of thymus seeding progenitor cells in the circulation, their entry into the thymus or the earliest steps of T-cell lineage commitment. Consequently, such a delayed capacity of shRNA-transduced cells to initiate thymopoiesis and to accumulate in secondary lymphoid organs may cause a reduced frequency of human GFP+ T cells in the periphery of adult HIS mice.

One explanation for this phenomenon could be off-target effects. The shGag5 may target unrelated mRNAs that are involved in hematopoiesis. Transcriptional interference of the shRNA-cassette with GFP transcription from the PGK polymerase II promoter seems unlikely as all single shRNA constructs were driven from the same H1 polymerase III promoter, yet exhibited a different outcome with only shGag5 showing toxic effects. Similarly, as all tested shRNA constructs were designed in the same JS1 backbone, reduced LV integration can be ruled out. Alternatively, a more general mechanism such as saturation of the miRNA pathway can be considered.11,36–39 The miRNA pathway is involved in hematopoiesis...
A reduction of the number of modified human T cells seems to be a common feature of diverse treatments in the clinical setting, using hHPC transduced with a LV encoding the three anti-HIV small RNA and infused back into the patients.\textsuperscript{45} This gene therapy was well tolerated with no signs of toxicity and shRNA expression was detected in PBMCs. Interestingly, the relative number of transduced cells increased in two patients after a spike of HIV-1 viremia, suggesting selective expansion of the protected cells.\textsuperscript{46}

What would be the significance of the temporary reduction of human shRNA-expressing T cells for a clinical application? Based on the latter clinical study and our data showing potent virus inhibition with the shRNA combination, it is likely that the developmental disadvantage of shRNA-expressing cells will be counterbalanced by the survival advantage upon HIV-1 infection. Even with maintenance of a high plasma viral load, such a selective survival advantage of treated human CD4\(^+\) T cells has already been observed in HIS mice treated with either a triple-combination anti-HIV LV or a dual shRNA LV and subsequently infected by HIV-1 (both R5- and X4-tropic strains).\textsuperscript{43,44} In this scenario, HIV-1 resistant shRNA-expressing cells are likely to be positively selected, as the unprotected cells will be removed by the immune system upon HIV-1 infection, leading to a clinical benefit for the patient. Still, this hypothesis remains to be formally proven, and alternative therapeutic strategies can also be envisioned, e.g., using clinical-grade cell sorting of modified cells – as already performed in HIS mice\textsuperscript{47} – or in vivo delivery of T cell-targeted shRNA.\textsuperscript{13} It should be emphasized that human T-cell homeostasis is particularly sub-optimal in BRG-HIS mice.\textsuperscript{15,18,19} Thus it remains unclear whether a similar delayed seeding of the thymus and peripheral lymphoid organs will be seen in a human clinical setting. Overall, the R3 LV that encodes the shPol1, shPol47, and shR/T5 shRNAs – respectively targeting the HIV-1 open-reading frames for integrase, protease and Tat/Rev – might represent an attractive prototype for further clinical development, which requires further evaluation, especially concerning the potential delay in reconstitution by R3-expressing T cells.

**Materials and methods**

**Constructs, LV preparation, virus titer determination and cell culture.** LV – JS1, R/T5, Gag5, Pol1, Pol47, R3, and R4 – were previously described.\textsuperscript{10,11} The JS1 LV carries the GFP reporter gene that allows for distinction between transduced (GFP\(^+\)) cells and nontransduced (GFP\(^-\)) cells. LV\textsuperscript{48} were produced by cotransfection of vector constructs and packaging constructs pSYNGP, pRSV-rev, and pVSVg in 293T cells with human embryonic kidney 293T cells with lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA), as previously described.\textsuperscript{49} The virus stocks were concentrated on a 5% sucrose layer by ultracentrifugation (32 K, 90 minutes) and aliquots of 350 µl were stored at −80 °C. Virus production was determined by cotransfection of vector constructs and packaging constructs pSYNGP, pRSV-rev, and pVSVg in 293T cells with lipofectamine-2000 (Invitrogen, Carlsbad, CA, USA), as previously described.\textsuperscript{49} The virus stocks were concentrated on a 5% sucrose layer by ultracentrifugation (32 K, 90 minutes) and aliquots of 350 µl were stored at −80 °C. Virus production was determined by co-infection of CA-p24 ELISA and virus titers were determined by serial dilution of virus and transduction on SupT1 cells. Human embryonic kidney 293T cells were grown at 37 °C and 5% CO\(_2\) in DMEM (Gibco BRL) and SupT1 cells were grown in RPMI 1640 (Gibco BRL). Both media were supplemented with 10% fetal calf serum, minimal essential medium nonessential amino acids, penicillin (100 U/ml) and streptomycin (100 µg/ml).
Isolation of human hematopoietic progenitor cells and transduction procedure. Human hematopoietic progenitor cells (hHPC) were isolated from human fetal liver tissue samples obtained from elective abortions, with gestational age of 14–18 weeks. The use of fetal liver tissues was approved by the Medical Ethical Committee of the Academic Medical Center of the University of Amsterdam and was contingent on informed consent. Single-cell fetal liver suspensions were prepared and hHPC were isolated as previously described.26 In brief, CD34+ cells were enriched by immunomagnetic sorting (indirect CD34 human progenitor cell isolation kit, Miltenyi Biotech) and the CD34+CD38- hHPC were sorted using a FACSAria (BD Biosciences, Franklin Lakes, NJ) to purity ≥99%.

The sorted CD34+CD38- hHPC were transduced with the different LVs after overnight culture in IMDM (Invitrogen, Carlsbad CA, USA) supplemented with Yssel’s medium, 5% normal human serum (NHS), 20 ng/ml human stem cell factor (huSCF; PeproTech, Rocky Hill, NJ, USA), 20 ng/ml human thrombopoietin (huTPO; PeproTech), 20 ng/ml human interleukin-3 (huIL-3, PeproTech) and 20 ng/ml human interleukin-7 (huIL-7; Cytheris). The following day, the cells were incubated for 6–8 hours with virus supernatant in fibronectin-coated plates (30 µg/ml; Takara Biomedicals, Otsu, Shiga, Japan).

Colon-forming cell assay. CD34+CD38- hHPC were transduced by the different LVs. GFP+ transduced CD34+CD38- hHPC were sorted with live fluorescence-activated cell sorting and plated in duplicate in 12-well tissue culture plates at concentrations of 100 cells per well in methylcellulose medium (MethoCult H4435, StemCell Technologies, Vancouver, BC, Canada). Cultures were incubated for 12–14 days at 37 °C in a 5% CO₂-humidified atmosphere. BFU-E and colony-forming units-granulocyte/macrophage and CFU-granulocyte/erythroid/macrophage/megakaryocyte (CFU-GM and CFU-GEMM, respectively) were scored by microscopy according to manufacturer’s instructions. CFC assay was repeated four-times with duplicates for each condition tested per experiment.

Generation of BRG-HIS and NSG-HIS mice. BALB/c (H-2d) rabbits for each conditions tested per experiment. respectively) were scored by microscopy according to manu-

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and completion of this study. The other authors declare no competing financial interests.

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