RNAi based gene therapy for HIV-1, from bench to bedside

Von Eije, K.J.

Citation for published version (APA):
Von Eije, K. J. (2009). RNAi based gene therapy for HIV-1, from bench to bedside

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
9.1 Abstract

RNA interference (RNAi) gene therapy against HIV-1 by stable expression of antiviral short hairpin RNAs (shRNAs) can potently inhibit viral replication in T cells. Recently, a mouse model with a human immune system (HIS) was developed that can be productively infected with HIV-1. In this in vivo model, in which Rag-2−/−γc−/− mice are engrafted with human CD34+CD38− hematopoietic precursor cells, we evaluated an anti-HIV RNAi gene therapy. Human hematopoietic stem cells were transduced with a lentiviral vector expressing an shRNA against the HIV-1 nef gene (shNef) or the control vector. We observed normal development of the different cell subsets of the immune system. However, although initial transduction efficiencies were similar for both vectors, a reduced percentage of transduced human immune cells was observed for the shNef vector after establishment of the HIS in vivo. Further studies are required to fully evaluate the safety implications. When we infected the mature human CD4+ T cells from the HIS mouse ex vivo with HIV-1, potent inhibition of viral replication was scored in shNef-expressing cells, confirming efficacy. When challenged with an shNef-resistant HIV-1 variant, equal replication was scored in control...
and shNef-expressing cells, confirming sequence-specificity of the RNAi therapy. We thus demonstrated that an antiviral RNAi-based gene therapy on blood stem cells leads to HIV-1-resistant T cells in vivo, an important proof of concept in the clinical development of RNAi against HIV-1.

9.2 Introduction

Multiple anti-retroviral drugs are available that inhibit HIV-1 replication. Currently, patients are treated with a combination of drugs known as highly active anti-retroviral therapy (HAART), which can effectively control the infection [74, 242, 258]. However, adherence to the drug regimen is an important prerequisite for the success of HAART, which can be further complicated by severe side effects. Suboptimal levels of medication, resulting from irregular intake, can result in the selection of drug-resistant virus variants and treatment failure [74]. Thus, there is a need for alternative therapeutic strategies to treat HIV-1 infection. Gene therapy approaches, in which HIV-1-susceptible cells are rendered resistant to the virus in a single treatment, have the potential to significantly improve the quality of life of HIV-1-infected individuals [276]. In such a gene therapy, the antiviral products are exclusively produced in the genetically modified cells, and toxic side effects linked with the systemic application of drugs should thus be avoided.

The discovery of RNA interference (RNAi) [95] provides a potent sequence-specific gene regulatory mechanism that can be added to the gene therapy arsenal against HIV [276, 325]. Efficient and durable inhibition of HIV-1 replication can be achieved when an RNAi inducer directed against the virus is stably expressed as a short hairpin RNA (shRNA) in T cell lines [61, 70, 141, 290]. Several target cells for HIV infection are found in the immune system, in particular CD4+ T lymphocytes, monocytes, macrophages, and dendritic cells. These cellular subsets derive from a self-maintained pool of human hematopoietic stem cells, which differentiate to give rise to progenitors of both the myeloid and lymphoid lineages. It is therefore attractive to consider hematopoietic stem cell transduction with lentiviral vectors as a gene therapy approach for shRNA delivery [276, 325]. Engraftment of autologous transduced hematopoietic stem cells will eventually result in the steady production of HIV-1-resistant immune cells that may lead to (partial) reconstitution of the immune system.

We have previously characterized the effects of the potent antiviral shNef that targets a sequence present in all viral transcripts [70, 318]. Using a retroviral vector-transduced T cell line, we observed that shNef potently inhibited HIV-1 replication in vitro. However, the in vivo safety and efficacy of such an approach still remains elusive. The SIV/macaque model [166], although mainly used for vaccine studies, may be considered for a preclinical safety test. However, this model has severe limitations because HIV-based lentiviral vector transduction is restricted by TRIM5α in macaque cells [277]. In addition, as RNAi is a sequence-specific mechanism, anti-HIV shRNAs should either be changed to anti-SIV shRNAs or HIV-1 target sequences have to be
incorporated into the SIV genome. These limitations of the expensive SIV/macaque model do not apply to humanized mouse models \cite{78}. We and others have recently established BALB/c Rag-2\(^{-/-}\gamma_c^{-/-}\) newborn mice as a new xenograft model for the \textit{in vivo} study of development and function of the human immune system (HIS) \cite{101,295}. In this HIS (BALB-Rag/\(\gamma\)) mouse model, all major human myeloid and lymphoid cellular compartments develop and mature \cite{176,198,268}, giving access to \textit{in vivo} and \textit{ex vivo} experimentation on human T cells \cite{174}. It has already been demonstrated that HIS (BALB-Rag/\(\gamma\)) mice support both R5- and X4-tropic HIV-1 infection, including rectal and vaginal transmission routes, which result in typical pathogenesis of CD4\(^{+}\) depletion and viremia, similar to infected patients \cite{12,22,33,34,312,335}. Here, we tested the shNef inhibitor in the HIS (BALB-Rag/\(\gamma\)) mouse model as a relevant \textit{in vivo} model for an eventual anti-HIV gene therapy.

### 9.3 Results

We constructed a third-generation self-inactivating lentiviral vector \cite{87,266} JS1-shNef, which contains an expression cassette for an shRNA against the HIV-1 \textit{nef} gene (Figure 9.1). The SupT1 T cell line, which is fully susceptible to HIV-1 replication, was transduced with the lentiviral vectors JS1 and JS1-shNef at a low multiplicity of infection, and transduced cells were selected with live sorting for green fluorescent protein (GFP) expression. Subsequently, the SupT1 cell lines were infected either with wild-type HIV-1 (HIV-1\(_{\text{wt}}\)) or an HIV-1 variant that is resistant to shNef due to two point mutations within the target sequence (HIV-1\(_{R3}\); Figure 9.1) \cite{70,318}.

The JS1-shNef SupT1 cells infected with the wild-type virus were viable and no syncytia were observed, whereas massive virus-induced syncytia and cell death was observed 10 days post infection in control JS1 and untransduced SupT1 cells. This observation was confirmed by measuring CA-p24 levels in the supernatant as a read-out for viral replication. Wild-type viral production in JS1-shNef cells was 3 orders of magnitude reduced compared with control and JS1 cells (Figure 9.2A, left panel). In contrast, all cells infected with the shNef-resistant virus HIV-1\(_{R3}\) showed massive syncytia and cell death, and similar kinetics of CA-p24 production (Figure 9.2A, right panel).

Next, we transduced stimulated human primary CD4\(^{+}\) T cells with the JS1-shNef vector. Two days after transduction, cells were sorted for GFP expression and positive and negative cells were subsequently challenged with HIV-1\(_{\text{wt}}\) or HIV-1\(_{R3}\). HIV-1\(_{\text{wt}}\) was potently inhibited in the JS1-shNef-transduced cells, but replicated efficiently in the control cells (Figure 9.2B, left panel). In contrast, HIV-1\(_{R3}\) replicated equally well in JS1-shNef and control cells (Figure 9.2B, right panel). Taken together, the results obtained with the SupT1 cell line and primary CD4\(^{+}\) T cells demonstrate that shNef selectively inhibits wild-type HIV-1 replication, confirming the sequence-specificity that is typical for RNAi. Moreover, it also demonstrates that the expression of shNef did not intrinsically affect the capacity of transduced cells to support HIV-1 replication, as the shNef-resistant virus replicated equally well in JS1-shNef, JS1 and
CHAPTER 9. EVALUATION OF SAFETY AND EFFICACY OF RNAI AGAINST HIV-1 IN THE HUMAN IMMUNE SYSTEM (RAG-2\(^{-/-}\)\(\gamma_C\)^{-/-}) MOUSE MODEL

**Figure 9.1: Lentiviral vectors and HIV-1 target viruses.** (A) The third-generation self-inactivating lentiviral vectors. JS1 expresses the green fluorescent protein (GFP) reporter and the JS1-shNef vector expresses in addition an shRNA against the HIV-1 nef gene from the human polymerase III H1 promoter. Lentiviral vectors were produced by co-transfection of vector constructs and packaging constructs pMDLg/pRRE, RSV-rev and pVSVg in 293T cells with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), as previously described. (B) The shNef target position within the HIV-1 viral genome is indicated for HIV-1\(^{wt}\) and HIV-1\(^{R3}\). (C) The shNef target sequence is shown with the mutations in the HIV-1\(^{R3}\) highlighted.
control cells.

Figure 9.2: Sequence-specific inhibition of HIV-1 in JS1-shNef-transduced T cells. (A) The SupT1 T cell line and (B) human primary CD4\(^+\) T cells transduced either with JS1-shNef (■) or JS1 (▲), and control untransduced cells (□) were infected with wild-type HIV-1 (HIV-1\(^{wt}\)) and shNef-resistant (HIV-1\(^{R3}\)) viruses. The experiment was performed as described previously [290]. Briefly, SupT1 cells and human primary CD4\(^+\) T cells were transduced with JS1 or JS1-shNef lentiviral vector at a low multiplicity of infection, and green fluorescent protein (GFP)-positive cells were sorted with live fluorescence-activated cell sorting. Transduction percentages for the SupT1 cells with the JS1 and JS1-shNef vector were 41 and 40%, respectively, and for the CD4\(^+\) cells it was 34%. Virus was produced by transfection of the HIV-1 molecular clones [318] in 293T cells; virus production was measured by CA-p24 ELISA of the supernatant. SupT1 cells cultured in advanced RPMI (Invitrogen, Carlsbad, CA, USA) supplemented with 1% fetal calf serum (1.5 × 10\(^6\) cells in 2 ml) were infected with virus supernatant containing 0.2 ng CA-p24. Human primary CD4\(^+\) cells (CD8\(^+\)-depleted PHA-stimulated PBMCs) cultured in RPMI supplemented with IL-2 (100 U/ml) and 10% fetal calf serum (1 × 10\(^5\) in 0.2 ml) were infected with 0.05 ng CA-p24. Virus spread was monitored by measuring CA-p24 production with ELISA.

As transduction with the JS1-shNef vector did not exhibit apparent negative effects on SupT1 and T cells \textit{ex vivo}, we next studied whether shNef expression in human hematopoietic stem cells would have an impact on hematopoiesis \textit{in vivo}. For this purpose, human fetal liver CD34\(^+\)CD38\(^-\) hematopoietic progenitors were isolated by
fluorescence-activated cell sorting and transduced with the JS1-shNef or JS1 vector. Both vectors exhibited equivalent transduction efficiencies, as assessed 3–4 days later by GFP expression (Figure 9.3A). The hematopoietic progenitors, comprising a mixture of transduced and nontransduced cells, were inoculated into newborn BALB/c Rag-2\(^{-/-}\)\(\gamma\)\(^{-/-}\) c recipient mice (13 to 14 animals per group in two independent experiments). After 7 weeks, the two groups of animals were analyzed for the presence of human hematopoiesis-derived CD45\(^{+}\) cells in the blood (Figure 9.3B). The frequency of human cells was similar between the two groups (JS1: 31.2 \(\pm\) 6.9\% vs JS1-shNef: 37.0 \(\pm\) 7.5\%; \(n=13\) to 14 mice per group). However, when we analyzed the percentage of transduced (GFP\(^{+}\)) human cells, a significant difference was observed between the JS1 and JS1-shNef groups. For the JS1 group we observed a similar percentage of transduced cells (28.7 \(\pm\) 4.1\%) as in the initial transduction of the progenitors, whereas with JS1-shNef this percentage was reduced (17.9 \(\pm\) 2.5\%).

Next, we analyzed the respective frequency of different GFP\(^{+}\) cell subsets of the human immune system, including the CD4\(^{+}\) and CD8\(^{+}\) T lymphocytes (CD3\(^{+}\)), the B lymphocytes (CD19\(^{+}\)) and the non-T and non-B cells (CD3\(^{-}\)CD19\(^{-}\)), which include mostly CD56\(^{+}\) natural-killer cells, CD14\(^{+}\)CD11c\(^{+}\) monocytes, CD14\(^{-}\)CD11c\(^{+}\) conventional dendritic cells and BDCA2\(^{+}\)IL-3R\(^{+}\) plasmacytoid dendritic cells. This analysis revealed that JS1-shNef transduction did not have any effect on the relative proportion of these three cell populations, as compared with the control JS1 (Table 9.1). Similar frequencies were also observed between the nontransduced (GFP\(^{-}\)) populations of the two groups. Overall, our results indicate that shNef expression in hematopoietic progenitors in vivo does not impact on the relative proportion of the major hematopoiesis-derived cell populations, although the percentage of transduced cells in the HIS (BALB-Rag/\(\gamma\)) mice was lower than what was expected based on the initial transduction efficiency.

To evaluate the efficacy of the shNef gene therapy, we isolated mature human CD4\(^{+}\) T cells from HIS (BALB-Rag/\(\gamma\)) mice produced with control JS1 or JS1-shNef-transduced hematopoietic progenitors. At 8–9 weeks post-transplantation, animals were sacrificed and lymphoid organs (thymus, spleen) were harvested. Both GFP\(^{+}\) and GFP\(^{-}\) human CD4\(^{+}\) T cells were isolated by fluorescence activated cell sorting from the two groups of reconstituted mice. The sorted CD4\(^{+}\) T cells were challenged ex vivo by HIV-1\(^{\text{wt}}\) and HIV-1\(^{\text{R3}}\) viruses. HIV-1\(^{\text{wt}}\) replication was inhibited only in transduced (GFP\(^{+}\)) CD4\(^{+}\) T cells from JS1-shNef HIS (BALB-Rag/\(\gamma\)) mice (Figure 9.4A). When the sorted cells were challenged with the shNef-resistant HIV-1\(^{\text{R3}}\) virus, replication was observed in all samples, including the JS1-shNef transduced cells (GFP\(^{+}\)) (Figure 9.4B). This indicates that these T cells have a normal phenotype, at least with respect to supporting HIV-1 replication. Taken together, these results confirm sequence-specific inhibition of HIV-1 replication in the in vivo generated human CD4\(^{+}\) T cells that express shNef.
Figure 9.3: Immune system development of JS1-shNef human immune system (HIS) (BALB-Rag/\(\gamma\)) mice is normal. (A) Human fetal liver CD34\(^+\)CD38\(^-\) SCID-repopulating cells were transduced either with JS1 control (left) or JS1-shNef-expressing vector (right) and injected into newborn BALB-Rag-2\(^{-}\)/\(\gamma_c\)^{\(-\)} mice. A fraction of the transduced cells was kept in culture for 4 days and was analyzed by flow cytometry for expression of green fluorescent protein (GFP). Transduction efficiency (%GFP\(^+\)) is indicated for each vector. (B) HIS (BALB-Rag/\(\gamma\)) mice were analyzed 7–8 weeks post-transplantation for human cells (%CD45\(^+\)) in the blood (left graph), which were also analyzed for GFP expression (right graph; %GFP\(^+\)). Data were subjected to two-tail unpaired Student’s t-test analysis; *\(P<0.05\).

9.4 Discussion

This pilot study demonstrates that the HIS mouse is a suitable model for testing the safety and efficacy of RNAi-based gene therapies against HIV-1. We observed a reduction in the percentage of transduced human immune cells in newborn Rag-2\(^{-}\)/\(\gamma_c\)^{\(-\)} mice engrafted with JS1-shNef transduced human CD34\(^+\)CD38\(^-\) hematopoietic pre-
CHAPTER 9. EVALUATION OF SAFETY AND EFFICACY OF RNAI AGAINST HIV-1 IN THE HUMAN IMMUNE SYSTEM (RAG-2−/−γc−/−) MOUSE MODEL

Table 9.1: Analysis of major subsets of the human immune system in the blood at 7–8 weeks of age. Results from two independent experiments were pooled and human cells (CD45+) were divided in nontransduced (GFP−) and transduced (GFP+) cells. For each population, the frequency of T cells (CD3+), B cells (CD19+) and non-T/B cells (CD3−CD19−) is indicated (mean % ± s.d.; n=14 for the control JS1 group, n=13 for the shNef group). The CD4/CD8 T cell ratio is also given (mean ± s.d.). There were no statistical differences between the two groups within GFP− or GFP+ cells. The HIS mice were generated as previously described [15, 16]. Human fetal liver was obtained from elective abortions, and use of this tissue was approved by the Medical Ethical Committee of the AMC-UvA and was contingent on informed consent. Purified CD34+CD38− progenitors were cultured overnight in IMDM (Invitrogen, Carlsbad, CA, USA) supplemented with Yssel’s medium, 5% normal human serum, 20 ng/ml human stem cell factor (PeproTech, Rocky Hill, NJ, USA), 20 ng/ml human thrombopoietin (PeproTech) and 20 ng/ml human interleukin-7 (PeproTech). The next day, cells were incubated for 6–8 h with control JS1 or shNef vector supernatant in fibronectin-coated plates (30 µg/ml; Takara Biomedicals, Otsu, Shiga, Japan) after which the cells (5–10×10⁵) were inoculated via intrahepatic injection into newborn (<1 week old) H-2d Rag-2−/−γc−/− mice [31] that had received sublethal (3.5 Gy) total-body irradiation with an X-ray Röntgen source. Mononuclear cells from blood were isolated by density gradient centrifugation 7–8 weeks post-transplantation. Cell suspensions were labeled with anti-human mAb targeting the following cell surface markers: CD1a (T6-RD1; Coulter-Immunotech), CD3 (SK7), CD4 (SK3), CD8 (SK1), CD19 (HIB19) and CD45(2D1; BD Bioscience, Franklin Lakes, NJ, USA) and analyzed using a LSR-II (BD Bioscience) cytometer interfaced to FACS-Diva software system.

<table>
<thead>
<tr>
<th></th>
<th>JS1</th>
<th>JS1-shNef</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T cells 4/8 ratio</td>
<td>B cells</td>
</tr>
<tr>
<td>GFP+</td>
<td>21.9±9.1 0.90±0.26</td>
<td>56.2±7.5</td>
</tr>
<tr>
<td>GFP−</td>
<td>26.5±9.3 0.74±0.23</td>
<td>60.2±9.0</td>
</tr>
</tbody>
</table>

cursor cells, when compared with the control JS1. We observed the same effect with another shRNA directed against the HIV-1 integrase sequences (unpublished results). Similar trends can also be observed in two recent publications, although not specifically addressed by the authors. A safety and efficacy study was conducted in the SCID-hu mouse with a lentiviral vector containing three anti-HIV genes, expressing a CCR5 ribozyme, TAR-decoy and an anti-HIV shRNA [17], and in macaques with an SIV-based lentiviral vector expressing an shRNA against CCR5 [11]. This reduction may be a reason for concern from a safety point of view, especially when considering combinatorial RNAi as a gene therapy approach for HIV-1 [109, 287, 290]. Further experimentation is needed to determine the actual safety implications and the underlying mechanism, which may include an impact on the endogenous microRNA pathway, an off-target effect specific for shNef, a general shRNA effect, a reduced vector integration, silencing of GFP expression, a reduced hematopoietic stem cell viability or engraftment efficiency. Nevertheless, the in vivo development and maturation of the immune system were not affected, consistent with the results in the other animal models [32, 33]. Thus, despite an apparent reduction in the percentage of JS1-shNef-reduced cells, development of the immune system is normal, which indi-
9.5 Acknowledgements

We thank Stephan Heynen (AMC, Experimental Virology) for CA-p24 ELISA and Berend Hooibrink (AMC, Cell Biology) for live cell sorting. NL, KW and Bianca Blom are supported by the Landsteiner Blood Transfusion Research Foundation, ZonMW VIDI grant, and the Bill and Melinda Gates Foundation, through the Grand Challenges in Global Health program (Human Vaccine Consortium). MC is supported by a Marie Curie Intra-European fellowship (MEIF-CT-2007-039689). OB and KE are supported by ZonMW through a Translational Gene Therapy and VICI grant, respectively.

![Figure 9.4: Transduced JS1-shNef CD4^+ T cells from human immune system (HIS) (BALB-Rag^/-) mice inhibit HIV-1 replication ex vivo.](image)

Mature stimulated human CD4^+ T cells from JS1 and JS1-shNef-treated HIS (BALB-Rag^/-) mice were sorted into transduced (GFP^+) and untransduced (GFP^-) cells. The sorted cells were infected with HIV-1^wt (A) and HIV-1^R3^ (B). Mature human CD4^+ T cells were sorted (99% pure) from spleens and thymuses of HIS (BALB-Rag^/-) mice using an ARIA sorter (BD Bioscience) according to a mature human CD4^+ T cell phenotype (CD45^+CD3^+CD1a^-CD4^+CD8^-) and the presence or absence of GFP expression. Cells from three to six HIS (BALB-Rag^/-) mice were pooled and subsequently stimulated for 2 days with PHA (4 µg/ml) in RPMI culture medium supplemented with IL-2 (100 U/ml) and 10% fetal calf serum. The human CD4^+ T cells sorted from the HIS (BALB-Rag^/-) mice (6 × 10^5 in 0.2 ml) were infected with virus supernatant (0.2 ng CA-p24) and virus spread was monitored by measuring CA-p24 production with ELISA. The HIV-1^wt inhibition experiment was repeated four times; a representative experiment is shown. GFP, green fluorescent protein.

cates that the approach can be considered to be safe. Importantly, we show for the first time that an antiviral RNAi-based gene therapy on blood stem cells results in in vivo development of mature human T cells in which HIV-1 replication is inhibited in a sequence-specific manner. Thus, our results provide an important proof of principle for the clinical development of RNAi-based gene therapies against HIV-1.

9.5 Acknowledgements