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Synthesis and use of an amphiphilic dendrimer for siRNA delivery into primary immune cells

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Abstract

Genetically manipulating immune cells using siRNAs is important for both basic immunological studies and therapeutic applications. However, the siRNA delivery is challenging because primary immune cells are often sensitive to the delivery materials and generate immune responses. We have recently developed an amphiphilic dendrimer, which is able to deliver siRNA to a variety of cells including primary immune cells. We provide here the protocols for the synthesis of

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Author contributions

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Conflicts of Interest

The authors declare no competing interests.

Related links

Key references using this protocol

Liu, X. et al. Angew. Chem. Int. 53, 11822–11827 (2014): https://doi.org/10.1002/anie.201406764

Ellert-Miklaszewska, A. et al. Nanomedicine (Lond) 148, 2441–2458 (2019): https://doi.org/10.2217/nnm-2019–0176

Garofalo, S. et al. Nat Commun 11, 1773 (2020): https://doi.org/10.1038/s41467–020-15644–8

this dendrimer and siRNA delivery into immune cells such as primary T- and B-cells, natural killer cells, macrophages, and primary microglia. The dendrimer synthesis entails straightforward click coupling followed by amidation reaction, and the siRNA delivery protocol requires simple mixing of siRNA and dendrimer in buffer, with subsequent application to the primary immune cells to achieve effective and functional siRNA delivery. This dendrimer-mediated siRNA delivery outperforms largely the standard technique of electroporation, opening a new avenue for functional and therapeutic studies of the immune system. The whole protocol encompasses the dendrimer synthesis which requires 3 weeks, the primary immune cell preparation which takes 3–10 days depending on the tissue source and cell type, the dendrimer-mediated siRNA delivery and subsequent functional assays which take an additional 3–6 days.

INTRODUCTION

RNA interference (RNAi) is a powerful tool for manipulation of gene expression in basic research and an emerging therapeutic strategy to treat various diseases.[1, 2] The power of RNAi lies in its ability to potently and specifically silence any gene of interest with small interfering RNAs (siRNAs), allowing functional study of the target gene and/or effective inhibition of the disease-associated gene for therapeutic intervention. The breakthrough success of RNAi therapeutics came in 2018, when Patisiran (Alnylam Pharmaceuticals) became the first siRNA drug approved by the USA Federal Drug Administration (FDA).[3] Also, the recent success of cancer immunotherapy has fueled a tremendous interest in genetic manipulation of immune cells using siRNAs to address basic immunological questions and for potential therapeutic applications.[4, 5] However, the major obstacle in implementing RNAi in those cells has been poor delivery of the siRNA,[6, 7] in particular in primary immune cells, such as lymphocytes (B-cells, T-cells, natural killer (NK) cells), mononuclear phagocytes (monocytes, macrophages, microglia, dendritic cells) and granulocytes (neutrophils, eosinophils, basophils). This is because siRNA molecules are hydrophilic and highly negatively charged, and cannot readily cross cell membranes to reach the RNAi machinery within the cytoplasm for gene silencing. In addition, naked siRNA is not stable and can be rapidly degraded by enzymes such as nucleases. If administered at high concentration, naked siRNA will often generate off-target effects and activate innate immunity,[8, 9] which may induce severe adverse effects. Also, siRNA can induce the activation of the innate immune response through various possible pathways, such as Toll-like receptors (TLR3, TLR7/8), dsRNA-dependent protein kinase (PKR) and retinoic acid-inducible gene-I (RAI1)[10]. Although using modified siRNA chemistry and optimized siRNA sequences can stabilize siRNA and lessen the unwanted effects,[10] siRNA delivery to primary immune cells remains a special challenge. Immune cells such as T lymphocytes and NK cells are small, with limited cytoplasm. Macrophages, dendritic cells and microglia, as professional phagocytes, are endowed with many potent degradative enzymes that can disrupt nucleic acid integrity and make gene transfer into these cells an inefficient process.[11] In addition, primary immune cells are often very sensitive to the delivery materials, generating non-specific immune responses. On the other hand, most common methods used to deliver siRNA into primary immune cells, which do not require any additional carriers, such as electroporation and nucleofection, lead to excessive cell death and low transfection efficiency, and hence are unsuitable for general applications in both basic and

translational research. Consequently, there is a high demand for safe and effective delivery systems, which are able to protect siRNA from degradation, deliver it to the target cells, and ultimately achieve gene silencing to facilitate genetic manipulation of immune cells for functional and therapeutic studies.

Both viral and nonviral delivery vectors have been explored for siRNA delivery [6, 7], in particular into immune cells.[5, 11–14] Viral delivery is more effective; however, increasing concerns over the immunogenicity and safety of viral vectors urge the development and improvement of non-viral delivery systems. Non-viral vectors offer more flexible options, with lipid and polymer vectors being the most commonly used. For example, the first human trial of siRNA therapeutics used a polymer vector,[15] whereas the first FDA-approved siRNA drug, Patisiran®, employs a lipid nanoparticle (LNP) delivery formulation. Although some well-studied lipid and polymer vectors perform well for the majority of established immortalized cells, they have marginal efficacy in siRNA delivery to primary immune cells, and very often induce non-specific immune responses.

To circumvent these problems, we have been developing innovative amphiphilic dendrimers for effective siRNA delivery. The rationale behind these dendrimer vectors is that they combine the multivalent cooperativity of dendritic polymer vectors with the self-assembly property of lipid vectors, hence capitalizing on the advantageous delivery characteristics of both lipid and polymer vectors, while overcoming their limitations, for more effective and potent siRNA delivery.[16–21] One of these dendrimers, **AD** (Figure 1), exhibits particularly high performance for siRNA delivery to a wide range of cell types, including highly challenging primary immune cells, such as human peripheral blood mononuclear cells (PBMCs), human B- and T-lymphocytes, NK cells (human and mouse), primary monocyte-derived macrophages and primary microglial cells (rat and mouse)[17, 20, 21]. Notably, this **AD** dendrimer is able to form small and stable nanoparticles with siRNA, thus protecting the siRNA from degradation and facilitating cellular uptake of siRNA[17, 20]. The subsequent siRNA-mediated gene silencing is specific and effective at both the mRNA and protein levels, leading to consequential biological effects. Remarkably, this dendrimer does not induce apparent cellular toxicity or non-specific immune responses under experimental conditions. Consequently, it constitutes a promising tool for siRNA delivery into immune cells and provides a new outlook for functional and therapeutic studies of the immune system.

We provide here the procedures for the robust synthesis of this dendrimer **AD** and the **AD**-mediated delivery of siRNA into immune cells using primary T cells, natural killer cells, macrophages and microglial cells as the model cells. The dendrimer synthesis is easy to follow and reproduce. Also, the final purification of the dendrimer **AD** is achieved through simple dialysis in water, giving **AD** in high yield and purity. Most importantly, the formation of the siRNA/dendrimer complexes requires only simple mixing of the siRNA with **AD** in solution at room temperature (at 25°C). The complexes can then be readily applied to the immune cells, such as T-lymphocytes, B-lymphocytes, monocytes/macrophages, brain innate immune cells (microglia), and NK cells, for transfection and gene silencing assays.

Overview of the procedures

In this protocol, we firstly describe the synthesis and molecular characterization of the dendrimer **AD**. Using 21/21-mer siRNAs and 27/29-mer Dicer substrate siRNAs (DsiRNAs) [22, 23] as examples, we explain the procedure and the conditions for the **AD**-mediated transfection assays to deliver siRNA into primary immune cells (T-cells, NK cells, macrophages and microglia) in various plate formats, and the subsequent validation of gene knockdown. Typically, the chemical synthesis of **AD** takes 20 days including purification. The whole transfection procedure, from cell seeding, preparation of the siRNA/**AD** complex and cell transfection to validation tests and functional assays takes 7 days. Time frames for preparation of primary cell cultures may vary as described in the Supplementary Methods. Details about the isolation and maintenance or/and expansion of primary immune cells are also provided in the Supplementary Methods.

Experimental design

Chemical synthesis: Synthesis of **AD** is achieved by coupling the hydrophobic chain **1** and the hydrophilic PAMAM dendron **2** via "click" reaction using copper-catalyzed azide-alkyne cycloadditions (CuAAC), followed by amidation with ethylenediamine (EDA) (Figure 2). The starting materials **1** and **2** can be easily prepared using well-established protocols.[16, 17] However, **1** is not well soluble in dimethylformamide (DMF), and it is necessary to raise the reaction temperature to 60 °C in order to solubilize **1** and drive the reaction to completion, thus producing **3** in high yield. It is also critical to keep the amount of **1** and **2** at the molar ratio of 1:1.05 for the click reaction, because it is difficult to separate the remaining excess **1** and/or **2** from the product **3** if the reaction is performed without respecting the stoichiometry. For the amidation reaction, the amount and concentration of EDA must be controlled in order to maximally suppress the retro-Michael side reaction and cyclization byproducts.[24–26] Different from our previously published protocol,[17] here we use MeOH instead of MeOH/CH_2Cl_2 as solvent for the amidation. This is because CH_2Cl_2 can react with EDA to generate oligomeric impurities,[27] and at the same time, increase the formation of cyclization at dendrimer terminals. The optimized conditions therefore allow more reliable amidation. The final dendrimer **AD** is purified using dialysis against water, and obtained as a pale powder after lyophilization.

Formation of the siRNA/AD complexes—As shown in Figure 3, the siRNA/dendrimer complexes are formulated by simply adding the siRNA solution to the dendrimer solution at 25 °C in PBS buffer or serum-free Opti-MEM®, followed by gentle vortexing and incubation. The complexes are then applied to the primary immune cell culture. It is preferable to use a freshly prepared solution of siRNA/**AD** complexes for transfection.

Calculation of the ratio of siRNA and the dendrimer AD in a transfection assay.—The formulation of siRNA/dendrimer complexes depends critically on the dendrimer-to-RNA charge ratio, which is defined as the "*N/P ratio*". It is calculated as [total terminal amino groups in the cationic dendrimer] / [total phosphates in the siRNA]. We always calculate the amount of **AD** according to the amount of siRNA in the final volume of cell culture media and the N/P ratio, as follows:

$$Amount\ of\ \mathbf{AD} = (amount\ of\ siRNA) \times (number\ of\ phosphates\ in\ siRNA) \times (N/P\ ratio) / (number\ of\ amino\ groups\ in\ \mathbf{AD}).$$

In our previous work, we established an optimal N/P ratio of either 5 or 10 for **AD**-mediated siRNA delivery into immune cells, including PBMC-CD4$^+$ T cells, natural killer cells, macrophages and microglial cells. In Table 1, we present the steps for **AD**-mediated transfection of a siRNA or a Dicer-substrate siRNA (DsiRNA) in various plate formats. Unlike a conventional siRNA duplex which is composed of a sense and antisense 21-mer oligonucleotides, the DsiRNA used in this protocol, also called 27/29-mer DsiRNA, contains a 27-mer sense oligonucleotide and a 29-mer antisense oligonucleotide. Such DsiRNAs have been demonstrated to enhance RNAi potency and efficacy[22, 23]. As an example, in a 24-well plate format, with 500 μL of cell culture medium in each well, and using a 21/21-mer siRNA or a 27/29-mer DsiRNA at 50 nM concentration, the amount of **AD** with 8 terminal amino groups is calculated at an N/P ratio of 5 as follows:

For a 21/21-mer siRNA, the amount of **AD** = (50 nM × 500 μL) × (21+21) × (5) / (8) = 656.25 pmol

For a 27/29-mer DsiRNA, the amount of **AD** = (50 nM × 500 μL) × (27+29) × (5) / (8) = 875 pmol

Consequently, 2.73 or 3.65 μL of 240 μM **AD** stock solution is needed for transfection of a 21-mer siRNA or a 27-mer DsiRNA, respectively, in a 24-well plate.

siRNA delivery and testing efficacy of gene silencing—For siRNA delivery using dendrimer **AD**, the prepared stock solution containing the siRNA/**AD** complexes is added to primary immune cells with an appropriate dilution to reach the required final siRNA concentration. The effects of the transfection procedure on gene and protein expression are analyzed after 2 or 3 days by quantitative real-time PCR (qRT-PCR) and western-blotting (WB) or FACS, respectively. Several key controls should be included in the experimental design: 1) cells without any siRNA/**AD** transfection (as a mock control); 2) cells transfected with an unrelated or scrambled siRNA/**AD** complex (as a negative control); or 3) cells transfected with the experimental siRNA/commercial transfecting agent (as a positive control or as a comparison control). By including these controls and comparing their effect on target gene and protein expression, the **AD**-mediated delivery and siRNA-mediated knockdown activity is defined and validated. Generally, siRNAs serving as negative controls that do not target any human' or animal' genome can be purchased from commercial vendors with the validation test in which the negative control siRNA has been functionally proven to have no significant effect on cell proliferation, viability or morphology (https://www.thermofisher.com/us/en/home/life-science/rnai/synthetic-rnai-analysis/controls-for-rnai-experiments.html#2).

Validation and applications

To demonstrate the validation and application of siRNA delivery into immune cells using the dendrimer **AD**, we have selected four examples (Figure 4) in which **AD** has been used to

successfully deliver: 1) anti-HIV siRNAs into primary $CD4^+$ T-cells for efficient suppression of HIV-1 replication;[17] 2) an anti-NKG2D siRNA into NK cells to reduce their cytotoxic activity towards tumor cells and motor neurons in a model of amyotrophic lateral sclerosis (ALS); [21] 3) an siRNA targeting JAK1 gene in primary mouse macrophages to regulate their inflammatory activities; and 4) an siRNA targeting the transcription regulator ID1 into microglia for functional exploration of ID1.[20]

Limitations

This protocol provides a reliable and easy to use transfection agent, **AD**, for siRNA delivery into various primary immune cells. It will be important to determine whether the chemical composition of **AD**, or the size and surface charge of the siRNA/**AD** complexes, affect the efficacy of siRNA delivery and gene expression interference in various types of immune cells. Furthermore, detailed bio-distribution studies in *ex vivo* white blood cells (WBCs) or *in vivo* animal models may be necessary to establish how cells at the different anatomic sites of the body would be exposed to the siRNA/**AD** complexes. Also, cell-specific targeting strategies can be developed for **AD** with the goal of targeting immune cell subsets for specific siRNA delivery.[19] We are actively working in this direction.

MATERIALS BIOLOGICAL MATERIALS

- Blood **!CAUTION** The research involves blood specimens from anonymous human subjects with no identifiers of age, race, ethnicity, or gender. Use of such specimens does not need to be approved nor does it need to undergo continuing review by the Institutional Review Board (IRB) at the authors' institute (J. H. Zhou and J.J. Rossi: City of Hope. REF#: 97071 / 075546). Human tissue was obtained and used in accordance with the Declaration of Helsinki, and the human subjects Ethical Committee of Sapienza University approved the selection process and technical procedures (reference 3314/25.09.14; protocol no. 1186/14). For human samples, blood should be obtained according to a protocol approved by ethical committee and after obtaining consent from subjects. All blood samples should be treated as infectious materials.
- Rats and mice **!CAUTION** Ethics statement: All animal care and procedures were performed according to protocols reviewed and approved by the corresponding Institutional Animal Care and Use Committee (IACUC) held by the authors, if required. Experiments described in the present work were approved by the Italian Ministry of Health (authorization n. 78/2017-PR) in accordance with the guidelines on the ethical use of animals from the European Community Council Directive of September 22, 2010 (2010/63/EU), and from the Italian D.Leg 26/2014. All possible efforts were made to minimize animal suffering, and to reduce the number of animals used per condition by calculating the necessary sample size before performing the experiments.
- C57BL/6J Mice (Charles River, Ref. C57BL/6 JFEMELLESPF3)
- HIV-1 isolate **!CAUTION** HIV-1 IIIB and Bal (the NIH AIDS Research and Reference Reagent Program, Division of AIDS, IIIB cat. no. 398; Bal cat. no.

510). HIV-1 is a class 2/3 human pathogen and it should be handled in a BSL3 level facility.

- L929 Cell Line from mouse (ECACC cat. no. 85011425; RRID: CVCL_0462, https://scicrunch.org/resolver/CVCL_0462) **[CE: Please embed the scicrunch URLs beneath the RRID codes, e.g., "CVCL_0462", similarly to accession codes.] !CAUTION** The cell lines used in the research should be regularly checked to ensure they are authentic and are not infected with mycoplasma. Mycoplasma test: The cell lines were checked regularly using MycoAlert Mycoplasma detection kit from Lonza or Universal Mycoplasma detection kit from ATCC. Cell lines that were mycoplasma contaminated were discarded or treated using Plasmocin from InVivoGen or BM-Cyclin from Sigma-Aldrich.

▲CRITICAL: L929 is a fibroblast cell line. These cells produce natural M-CSF (macrophage colony-stimulating factor) which is essential for the maturation of mouse bone marrow cells to macrophages. The use of L929-conditioned medium for mouse macrophage culture is well documented in the literature.[28]

REAGENTS Chemicals

!CAUTION Because most of the chemicals and organic solvents used for dendrimer synthesis are potentially hazardous to human health, we recommend performing the click reaction and the amidation reaction as well as handling organic solvents inside an efficient chemical fume hood while wearing personal protective equipment (lab coat, gloves and goggles) to prevent exposure.**1** and **2** should be synthesized according to the reported protocols[16, 17]

- N,N-Dimethylformamide (DMF; Sigma-Aldrich, cat. no. 227056)
- Dichloromethane (CH_2Cl_2; Sigma-Aldrich, cat. no. 32222-M)
- Methanol (MeOH; Sigma-Aldrich, cat. no. 179337)
- Triethylamine (TEA; Sigma-Aldrich, cat. no. 471283)
- Copper(I) iodide (CuI; Sigma-Aldrich, cat. no. 215554)
- 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU; Sigma-Aldrich, cat. no. 139009)
- Sodium sulfate anhydrous (Na_2SO_4; Sigma-Aldrich, cat. no. 798592)
- Magnesium sulfate monohydrate (Mg_2SO_4 *H_2O; Sigma-Aldrich, cat. no. 434183)
- Ammonium chloride (NH_4Cl; Sigma-Aldrich, cat. no. 213330)
- Sodium bicarbonate ($NaHCO_3$; Sigma-Aldrich, cat. no. S6014)
- Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S9888)
- Silica gel for flash chromatography (Sigma-Aldrich, cat. no. 227196)
- Ethylenediamine (EDA; Merck, cat. no. S7392647 745) **!CAUTION** To ensure the purity of the reagent, EDA should be distilled before using.[29]

- Chloroform-d (99.8 atom % D; Sigma-Aldrich, cat. no. 416754)
- Methanol-d_4 (99.8 atom % D; Sigma-Aldrich, cat. no. 151947)

Cell culture & biological reagents

- 1 × RBC lysis buffer solution (eBioscience, cat no 00433357)
- Universal negative siRNA controls (Integrated DNA Technologies, IDT, Negative control DsiRNA, cat. no. 51–01-14–03; Scrambled negative control DsiRNA, 51–01-19–08).

▲CRITICAL It is crucial to have an appropriate negative control against which one can compare experimental results in gene silencing and functional studies using siRNA or DsiRNA duplexes. A scrambled siRNA does not target any part of the human, mouse or rat transcriptomes.

- Custom-made siRNAs (Integrated DNA Technologies, Table 2) ▲CRITICAL It is crucial to select an optimal siRNA sequence for effective gene silencing. According to our previous experience, we designed one 21-mer siRNA and one 27-mer DsiRNA per target sequence with appropriate 3′-overhangs and chemical modifications.
- Rat ID1 siRNA (Dharmacon, ON-TARGET plus SMART pool; cat. no: L-080165–02-0005).
- Custom primers for qRT-PCR analysis (IDT or Genescript; Table 3).
- Negative control siRNA which does not target any human, mouse or rat gene products (Dharmacon, ON-TARGET plus non-targeting pool; cat. no: D-001810–10-05).
- AllStars Negative Control siRNA (Qiagen, AllStars Neg. Control siRNA; cat. no./ID: 1027281)
- Milli-Q ultrapure water (Millipore)
- Opti-MEM medium (Gibco, cat. no 31985–047)
- Dulbecco’s PBS without calcium and magnesium (DPBS, Corning, cat. no. 21–031-cv)
- Dulbecco’s PBS with calcium and magnesium (Thermo Fisher, cat. no.14040091)
- Ficoll-PAQUE plus (GE healthcare Pharmacia, cat. no. 17–1440-02)
- Ficoll (Sigma-Aldrich (Milan, Italy)
- Trypsin inhibitor (Sigma-Aldrich, cat. no. T6522)
- DNase (Sigma-Aldrich, cat. no. DN25)
- RPMI-1640 (Corning, cat. no. 15–040-cv)
- RPMI-1640 with GlutaMAX (Gibco, cat. No 61870036)

- Skimmed milk powder (non-fat) (Merk, cat. no. 70166)
- DMEM with GlutaMAX, high glucose (Thermo Fisher, cat. no 31966–021, 61965026)
- MEM Non-Essential Amino Acids Solution (100×; Thermo Fisher, cat. no.11140050)
- Sodium Pyruvate (100 mM,Thermo Fisher, cat. no. 11360070)
- 2-Mercaptoethanol (50 mM, Thermo Fisher, cat. no. 31350010).
- HEPES (1 M, Thermo Fisher, cat. no. 11360070)
- Fetal bovine serum (FBS, Gibco, cat. no. 10082–147, 10270–106)
- L-Glutamine (200 mM, 100×, IrvineScientific, cat. no. 9317–100 mL)
- Penicillin/streptomycin, 10,000 units of penicillin per mL/10,000 μg of streptomycin per mL (Gibco, cat. no. 15140–122-100 mL)
- Phytohemagglutinin-L (PHA, Roche, cat. no. 11 249 738 001)
- Recombinant interleukin-2 (IL-2, Teceleukin, Hoffmann–La Roche, cat. no. Ro 23–6019)
- ActiCyte - TC Medium kit (CytoMedical Design Group, cat. no. TCM1000)
- Trypan Blue Stain 0.4% (wt/vol) (Invitrogen, cat. no. T10282)
- EasySep Human $CD4^+$ T Cell Isolation Kit (StemCell Technologies, cat. no. 17952)
- RoboSep Buffer (StemCell Technologies, cat. no. 20104) **!CAUTION** Potential irritant to eyes, respiratory system and skin. Wear suitable protective clothing, glasses and gloves.
- TRIZOL agent (Thermo Fisher) **!CAUTION** Harmful if inhaled or comes in contact with skin. Toxic if swallowed. Irritant to eyes. Evidence of a carcinogenic effect. Wear suitable protective clothing, glasses and gloves and use in a fume hood. This material and its container must be disposed of as hazardous waste.
- Chloroform/Isopropanol 24/1 solution (Sigma, cat. no. C0549) **!CAUTION** Harmful if inhaled or comes in contact with skin. Toxic if swallowed. Irritant to eyes. Evidence of a carcinogenic effect. Work under a fume hood and wear suitable protective clothing, glasses and gloves. This material and its container must be disposed of as hazardous waste.
- Glycogen (Roche, cat. no. 10 901 393 001)
- RNA isolation Kit (RNeasy Mini Kit, QIAGEN, cat. no. 74104)
- QuantiNova Reverse Transcription kit (QIAGEN, cat. no. 205411) or SuperScript™ III Reverse Transcriptase (Thermo Fisher, cat. no 18080–044) and a set of dNTPs (Promega, cat. no U1330)

- SsoAdvanced Universal SYBR Green Supermix (BIO-RAD, cat. no. 172–5271) or Fast SYBR Green Master Mix (Applied Biosystems, cat. no 4385612)
- Pacific Blue-conjugated CD4 antibody (clone RPA-T4) (BD Biosciences cat. no. 558116; RRID: AB_397037, https://scicrunch.org/resolver/AB_397037)
- ID1 antibody (clone B-8) (Santa Cruz Biotechnology cat. no. sc-133104; RRID: AB_2122863, https://scicrunch.org/resolver/AB_2122863)
- GAPDH antibody (Millipore cat. no. MAB374; RRID: AB_2107445, https://scicrunch.org/resolver/AB_2107445)
- Anti-mouse IgG peroxidase conjugated secondary antibody (Vector Laboratories cat. no. PI-2000; RRID: AB_2336177, https://scicrunch.org/resolver/AB_2336177)
- iNOS Antibody (Cell signaling Technology, cat. no. 13120S; RRID: AB_2798613, https://scicrunch.org/resolver/AB_2798613)
- Arginase-1 Antibody (Cell signaling Technology, cat. no. 93668S; RRID: AB_2800207, https://scicrunch.org/resolver/AB_2800207)
- α/β-Tubulin Antibody (Cell Signaling Technology, cat. no. 2148S; RRID: AB_2288042, https://scicrunch.org/resolver/AB_2288042)
- NKG2D Antibody (eBioscience, PE 12–5882-82; RRID: AB_465996, https://scicrunch.org/resolver/AB_465996)
- Fixation/Permeabilization solution kit (BD Biosciences, cat. no. 554714) **! CAUTION** Limited evidence of a carcinogenic effect. May cause sensitization if it comes in contact with skin. Work under a fume hood and wear suitable protective clothing and gloves.
- Alliance HIV-1 p24 Antigen ELISA Kit (PerkinElmer, cat. No. NEK050A001KT)
- Recombinant interleukin-15 (IL-15, eBioscience)
- BCA Protein Assay Kit (Thermo Scientific, cat. no. 23225)
- 8–16% Tris-Glycine precast polyacrylamide gels (e.g., Thermo Scientific) **! CAUTION** Harmful if inhaled, swallowed or comes in contact with eye or skin. Contains material which may cause damage to kidneys, the nervous system, liver, upper respiratory tract and skin.
- TRIS (BioShop, Burlington, ON, Canada; cat. no. TRS001)
- Glycine (BioShop, Burlington, ON, Canada; cat. no. GLN001)
- Bovine Serum Albumin (BSA; Sigma-Aldrich, cat. No. 9048–46-8)
- Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L3771) **!CAUTION** Harmful if inhaled. May cause damage to upper respiratory tract.
- 4–15% SDS-PAGE precasted gel (BioRad, cat. no. 64280120)

- Pre-stained protein markers for SDS-PAGE electrophoresis (eg. Thermo Scientific)
- SuperSignal® West Pico Chemiluminescent Substrate (Thermo Scientific) or similar reagent containing HRP substrate for chemiluminescent protein detection in western blot analysis
- RIPA Buffer (Sigma, R0278)
- cOmplete™, Mini Easypack (Roche, Ref:04693124001)
- SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher, cat no. 34095)
- Accutase® (Biowest, Product code: L0950)
- CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Promega, cat no. G7890)
- MycoAlert Mycoplasma detection kit (Lonza, cat. no. LT07–318)
- Plasmocin (Invivogen, cat code. ant-mpt-1)
- BM-Cyclin (Sigma, cat no. 10799050001)

EQUIPMENT

- Round bottom flasks (Synthware, 250 mL, 100 mL, 50 mL, 25 mL)
- Dialysis tubing, benzoylated (MWCO 2000; Sigma-Aldrich, cat. no. D7884–10FT)
- Dialysis tubing closures (Sigma-Aldrich, cat. no. Z371068)
- Compressor (nessicare 30, cat. No. CNST-300D)
- Nitrocellulose membrane (eg. Hybond–ECL, Amersham)
- Equipment for chemical synthesis and characterization: rotary evaporator (Heidolph), water bath, oil bath, and reflux condenser
- Magnetic stirrer (IKA)
- Stainless-steel needle (Sterican, cat. no. 466 5643)
- Silica gel plates for thin-layer chromatography (Merck, cat. no. 1.0554.0001)
- NMR spectrometer (JEOL, 400M; Bruker, 500M)
- NMR tube (5 mm; Wilmad)
- MS–ESI (Waters)
- FTIR spectrometer (Bruker)
- HIV-1 BSL2/3 lab **!CAUTION** HIV-1 is a class 3 human pathogen and all the HIV-1-related procedures (HIV-1 infection, cell transfection and isolation of samples) should be carried out in a BSL2/3 level facility.

- EasySep Magnet (StemCell Technologies, cat. no. 18000)
- Flash-chromatography column
- Countess II FL Automated Cell Counter (Invitrogen, cat. no. AMQAF1000)
- Nucleocounter (Chemometec, cat. no. NC-100)
- 25 and 75 cm^2 cell culture flask with vent cap
- Falcon 100 mm × 15 mm Not TC-treated Bacteriological Petri Dish (Corning, product no. 351029)
- 15- and 50-mL centrifuge tubes
- 1.5- and 2-mL screw cap microtubes
- 1.7-mL Eppendorf safe-lock microcentrifuge tube
- 14-mL Falcon Polystyrene Round-Bottom Tubes
- 5-mL round-bottom cytometer tube
- Flat-bottom 6-, 24-, 12-, 48- and 96-well tissue culture plates
- Round-bottom 96-well tissue culture test plates (Corning, Product number CLS3367, or similar)
- Sterile 5-, 10- and 25-mL disposable pipettes
- Disposable glass Pasteur pipette
- Nuclease-free tips (0.5–10, 2–20, 20–200, 100–1000 μL)
- Pipettes (0.5–10, 2–20, 20–200, 100–1000 μL)
- Multichannel pipette (50–200, 1000–1000 μL)
- Benchtop centrifuge with sealed buckets and plate carriers (Eppendorf, 5810R, 5424, 5425) or similar
- Mini vortex (Thermolyne, maxi Mix plus) or similar
- Microbiological safety cabinet
- Plate shaker
- CO_2 cell culture incubator (Thermal Fisher) or similar
- Hemocytometer (Fisher Scientific, cat. no. 0267110) or similar
- NanoDrop Microvolume Spectrophotometers (Thermo Fisher, 2000) or similar
- Optical microscope (Nikon Elipse, TE2000-S) or similar
- Confocal microscope (ZEISS, LSM880) or similar
- ELISA microplate reader (with kinetic reading capabilities) (BioTek, Cytation 5 Cell Imaging Multi-Mode Reader, or Molecular Devices, SpetraMax iD5) or similar

- Multicolor Flow cytometer (BD Biosciences, LSRFortessa) or similar
- Real-time PCR (Bio-Rad, CFX96 Touch Real-Time PCR Detection System) or similar
- Thermocycler (Mastercycler gradient, Eppendorf or similar)
- MACS Separators for magnetic cell isolation (Miltenyi Biotec)
- Large (LS) columns for magnetic cell isolation (Miltenyi Biotec)
- Protein electrophoresis and wet electroblotting system (Mini Trans–Blot® Electrophoretic Transfer Cell, Bio-Rad or similar) with power supply
- X-ray film processor (Fuji FPM 800A or similar) or chemiluminescence detection system (ChemiDoc, BioRad or similar)

REAGENT SETUP

siRNAs

Re-suspend siRNAs (double-stranded RNA duplexes) in sterile, double-distilled, nuclease-free water as a 500 μM stock solution. Before transfection, dilute siRNA stock solution with 1×PBS solution to 10 μM for the transfection assay. siRNA stocks can be stored in aliquots at −20 °C for up to 4 weeks or at −80 °C for up to 6 months. **!CAUTION** Since freezing and thawing repeatedly (freeze-thaw cycles) can degrade RNA, store the RNA solution in aliquots of 40 μl.

Separation buffer

Supplement PBS 1× with 2.0 mM EDTA and 0.5% (vol/vol) FBS. This buffer can be stored at 4 °C for up to 3 weeks.

Labeling buffer

Supplement PBS 1×supplemented with 1.0% (vol/vol) FBS. This buffer can be stored at 4 °C for up to 2 months.

Primary T-cell culture basic medium

Supplement RPMI-1640 with 2 mM L-glutamine, penicillin-streptomycin (100 units per mL of penicillin /100 μg per mL of streptomycin) and 10% (vol/vol) FBS. The medium can be stored at 4 °C for up to 4 weeks.

Primary microglia culture basic medium

To high glucose DMEM containing Glutamax, add penicillin-streptomycin (100 units per mL of penicillin /100 μg per mL of streptomycin) and 10% (vol/vol) FBS. This medium can be stored at 4 °C for up to 4 weeks.

Activation/expansion medium

Supplement RPMI-1640 culture medium with 1.0 μg/mL PHA and 100 μL/mL IL-2. This medium should be freshly prepared or stored for no longer than 1 week at 4 °C.

IL-2 medium

Supplement RPMI-1640 culture medium with 100 μL/mL IL-2. This medium can be stored at 4 °C for up to 2 weeks.

Solution of sodium acetate, pH 5.2, 3.0 M

Dissolve 24.6 g of sodium acetate (anhydrous) in 70 mL of Milli-Q water in a 100 mL Duran bottle. Adjust the pH to 5.2 by adding glacial acetic acid. Top up the solution to 100 mL with Milli-Q water. Filter the solution using a 0.20 μm filter membrane. This solution can be stored at room temperature for several months.

NK cell culture basic medium

Supplement RPMI-1640 containing GlutaMAX, with penicillin-streptomycin (100 units per mL of penicillin /100 μg per mL of streptomycin), 10% (vol/vol) FBS, and 50 ng/mL IL-15. This medium can be stored at 4 °C for up to 4 weeks without IL-15 and up to 2 weeks with IL-15.

BMDM cell culture basic medium

Supplement high glucose DMEM containing GlutaMAX, with penicillin-streptomycin (100 units per mL of penicillin /100 μg per mL of streptomycin), 1.0 % Non-Essential Amino Acids (vol/vol), 1.0 mM Sodium Pyruvate, 1.0 % (vol/vol) HEPES, 0.25 mM Beta-Mercaptoethanol and 10% (vol/vol) heat inactivated FBS. This medium can be stored at 4 °C for up to 4 weeks. Supplemented BMDM culture medium with 20% (vol/vol) L929 conditioned media just before adding to cell culture and use immediately.

MACS buffer

Supplement PBS 1× with 2.0 mM EDTA and 0.5% (vol/vol) BSA. This buffer can be stored at 4 °C for up to 2 weeks.

$MgSO_4$ solution

Prepare a 3.8% (w/v) solution in 1×PBS. Store at −20 °C for up to 2 months.

DNase solution

Prepare a 2.0 mg/mL solution in 1×PBS. Aliquot and store at −20 °C for several months.

Trypsin inhibitor solution

Prepare a 20 mg/mL solution in 1×PBS. Aliquot and store at −20 °C for several months

BSA solution

Prepare a 4.0% (w/v) solution in 1×PBS. Aliquot and store at −20 °C for several months.

Cell lysis buffer for protein isolation

This buffer is 20 mM Tris HCl, pH 6.8, 137 mM sodium chloride, 25 mM β-glycerophosphate, 2.0 mM sodium pyrophosphate, 2.0 mM EDTA, 1.0 mM sodium

orthovanadate, 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 5.0 μg/mL leupeptin, 5.0 μg/mL aprotinin, 2.0 mM benzamidine, 0.5 mM DTT. Stored at −20 °C for several months. Add 1:100 of 100 mM PMSF just prior to use. **!CAUTION** PMSF is unstable in aqueous solutions.

Sample loading buffer 1×

The final composition of this buffer is 60 mM Tris-Cl pH 6.8, 2.0% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.5 mM DTT, 0.01% (wt/vol) bromophenol blue. Prepare as 4× concentrate without DTT and store at room temperature for several months. Prior to use, add 4× concentrated DTT (i.e. 2 mM) to 4× concentrate stock to get 4× sample loading buffer with DTT. **!CAUTION** DTT and SDS powder are hazardous. Prepare solution in a ventilated fume hood. SDS can precipitate from the solution over time. Warm up the buffer for 3–5 min to at least 55°C to dissolve the SDS.

Running buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 1×

This buffer is 25 mM Tris, 192 mM glycine and 0.1% (wt/vol) SDS, pH approx. 8.6. **! CAUTION** SDS powder is hazardous. Prepare solution in a ventilated fume hood. This buffer can be stored at 4 °C for up to 8 weeks.

Electrophoretic transfer buffer 1×

This buffer is 25 mM Tris, 192 mM glycine, 0.05% (wt/vol) SDS and 20% (vol/vol) Methanol. **!CAUTION** SDS powder is hazardous. Prepare solution in a ventilated fume hood. Methanol is hazardous chemical that is highly flammable and toxic. It must be used by people that have been properly trained in its handling in a ventilated fume hood.

Membrane wash buffer TBS-T

Combine Tris-buffered saline (TBS 1×: 25 mM TRIS, 130 mM NaCl, pH 7.6) with 0.1% (vol/vol) Tween 20. This buffer can be stored at 4 °C for up to 8 weeks.

Blocking buffer

Dissolve 5.0 g skimmed milk powder (non-fat) in 100 mL membrane wash buffer (TBS-T). Prepare freshly before use. Alternatively, blocking buffer can be prepared with 5.0 g of BSA in 100 mL of TBS-T.

EQUIPMENT SETUP

qRT-PCR system:

Configure as follows: 10 min setting-up time per plate per parameter, 1.5–2 h run time.

Flow cytometry:

Configure as follows: 30 min pre-clean time, 15 min per parameter setting-up time, 30–60 min run time (will vary according to the number of samples), 30 min cleaning time.

ELISA microplate reader:

Configure as follows: 15 min run time, 30 s of time interval to read at 490 nm wavelength.

Chemiluminescence detection instrument (X-ray film processor or chemiluminescence detection system)—Configure as follows: 10 min pre-warming time, 5–30 min signal acquisition time.

PROCEDURE

Synthesis of 3

● TIMING 0.5 h for set-up, 1.5 h for the reaction, 3 h for work-up and purification

1. Weigh 95 mg (0.102 mmol) of **1** in a 25-mL round-bottom flask.
2. Weight and add 5.7 mg (0.030 mmol) CuI to the round-bottom flask. **! CAUTION** The CuI is easily oxidized, weigh it quickly.
3. Add a magnetic stir bar, cap with a rubber septum and wrap the flask with Parafilm.
4. Create a vacuum inside the round-bottom flask and flush with argon from a balloon.

   ▲CRITICAL STEP As the Cu^{+} is easily oxidized, the argon protection is mandatory.
5. Inject a solution of 143 mg (0.10 mmol) **2** in 5.0 mL of DMF into the round-bottom flask through a stainless-steel needle.
6. Inject 70 μL 1.8-diazabicyclo(5,4,0)undec-7-ene (DBU) through a stainless-steel needle into the round-bottom flask while stirring.
7. Place the flask in a preheated oil bath (60 °C) on a hot-plate magnetic stirrer and stir the reaction solution for 90 min under nitrogen atmosphere.
8. Monitor the progress of the reaction by taking an aliquot of 0.05 mL via a stainless-steel needle and running TLC using silica-gel-coated plates and MeOH/CH_2Cl_2 1/9 (vol/vol) (R_f is 0.6 for **1** (starting material)). Detect the eluted product and the starting material by I_2[30].
9. After completion of the reaction, which generally takes 90 min, remove the stopper and the magnetic stir bar.

   ? TROUBLESHOOTING
10. Remove the DMF with a rotary evaporator at ~30 °C in a water bath.
11. Pour 15 mL CH_2Cl_2 into the round-bottom flask.
12. Transfer the mixed solution to a 125-mL separating funnel, and subsequently add 15 mL saturated NH_4Cl solution.

13. Gently shake the mixed solution and let it sit for 5 min until a clear layer appears.

14. Open the valve of the separating funnel and collect the lower organic phase (CH_2Cl_2 layer) in a 250-mL conical flask.

15. Extract the aqueous phase three times with 15 mL CH_2Cl_2 each time and combine the organic phase.

16. Transfer the CH_2Cl_2 layer to a 125-mL separating funnel, then wash the organic phase twice with 15 mL saturated NH_4Cl solution, twice with 15 mL saturated $NaHCO_3$ solution, and once with 20 mL of brine solution.

17. Collect the CH_2Cl_2 layer in a 250-mL conical flask and dry the layer by adding anhydrous Na_2SO_4 until the powder stops aggregating.

18. Gently shake the solution by hand, and filter it using a fritted filter funnel under vacuum into a 250-mL round-bottom flask.

19. Remove the solvent with a rotary evaporator at ~30 °C in a water bath.

20. Dissolve the residue in 1.0 mL of MeOH/CH_2Cl_2 1:9 (vol/vol).

21. Purify the compound by flash column chromatography (diameter of the column is 2.4 cm and height of the silica gel in the column is 20 cm), using MeOH/CH_2Cl_2 1:9 (vol/vol) to remove the impurities and unreacted starting materials, then MeOH/CH_2Cl_2 1:9 (vol/vol) with 1% (vol/vol) TEA to elute the product.

22. Collect eluents in fractions of 15 mL using 20 mL test tubes. Analyze the collected fractions by TLC using MeOH/CH_2Cl_2 1:9 (vol/vol) as the eluent.

23. Pool the pure fractions into a new 100 mL round-bottom flask. Evaporate the solvents with a rotary evaporator at ~30 °C in a water bath.

24. Remove the residual solvent under high vacuum overnight. Weigh the pure **3** and calculate its yield.

? TROUBLESHOOTING

▮ PAUSE POINT **3** can be stored at −20 °C for a week.

Synthesis of AD

● **TIMING** 0.5 h for the set-up, 72 h for the reaction, 6 d for purification

25. Weigh 110 mg (0.046 mmol) of **3** in a 25-mL round-bottom flask.

26. Add a magnetic stir bar, cap with a rubber septum and wrap the flasks with Parafilm.

27. Create a vacuum inside the round-bottom flask and flush with argon from a balloon.

▲CRITICAL STEP As there are amine functionalities in the reaction, the argon protection is mandatory.

28. Inject 4.0 mL of MeOH into the round-bottom flask through a stainless-steel needle.

? TROUBLESHOOTING

29. Wrap the round-bottom flask in black paper to protect the reaction from light.

▲CRITICAL STEP As there are amine functionalities in the reaction, it is suggested to protect the reaction from light to avoid unnecessary byproducts.

30. Inject 4.0 mL (60 mmol) of ethylenediamine (EDA) drop-by-drop while stirring.

31. Place the flask in a preheated oil bath (30 °C) on a hot-plate magnetic stirrer and stir the reaction solution for 72 h under nitrogen atmosphere.

32. Monitor the progress of the reaction by taking an aliquot of 0.05 mL via a stainless-steel needle and running infrared spectroscopy (IR) (look for disappearance of the peak at 1750 cm^{-1}, which corresponds to the carbonyl function in the ester group).

33. After completion of the reaction, which generally takes 72 h, remove the stopper and the magnetic stir bar. Evaporate the MeOH and excess EDA with a rotary evaporator at ~30 °C in a water bath.

34. Dissolve the residue in 3.0 mL ultrapure water.

35. Transfer the solution to a dialysis tube (MW=2000). Before transferring the liquid, clamp one side of the tubing with a dialysis tubing closure. After filling the tubing, close it with a dialysis tubing closure which has a plastic foam tied to it. Attach a dialysis buoy to the top side of the tubing (the side with the light clamp), and suspend the tube in a separate 2-liter glass beaker filled with ultrapure water and containing a magnetic stirrer.

▲CRITICAL STEP The pore size of the dialysis membrane must be selected according to the molecular weight of the product. Typically, the product is subjected to three rounds of dialysis and lyophilization to ensure its purity. The NMR spectrum is used to monitor the purity. The volume of product solution may increase to at most 2 times during the dialysis. It is suggested to use a larger than needed amount of dialysis tube.

36. Dialyze the contents for 8 h at room temperature with stirring. Replace the ultrapure water every hour to remove the EDA.

37. Transfer the contents of the dialysis tube into new 15-mL Falcon tubes (~5 mL per tube). Wrap the tube with aluminum film to protect the sample from light exposure, and fasten with a rubber band.

38. Freeze the samples by keeping them immersed for 5 min in a tank of liquid nitrogen fitted with a tube rack. Transfer the frozen samples to a lyophilizer and dry them for 1 d.

39. Dissolve the lyophilates in 3.0 mL ultrapure water and repeat steps 35–38 twice to obtain pure **AD**, which appears as a white to a faint-colored foam-like solid after lyophilization.

▲CRITICAL STEP In order to remove water completely, a lyophilization procedure is required. If there is still water/ice in the product after lyophilization, dissolve the product in ultrapure water again and repeat step 38.

PAUSE POINT Dried **AD** can be stored at −20 °C for months. Alternatively re-suspend the **AD** in sterile, double-distilled, nuclease-free water as a 2.0 mM stock solution which can be stored in aliquots at −80 °C for up to 12 months. Before using in transfection, dilute with 1×PBS to 240 μM. Diluted **AD** can be stored in aliquots at −20 °C for up to 4 weeks. **!CAUTION** Since freezing and thawing repeatedly (freeze-thaw cycles) can degrade **AD**, store the **AD** solution in aliquots of 40 μl.

AD-mediated siRNA transfection into HIV-1-infected CD4$^+$ T cells

● TIMING 1 h for seeding the HIV-1-infected CD4$^+$ T cells, 1.5 h for forming the siRNA/**AD** complex, and 2–3 days for the complex-mediated gene silencing and HIV-1 suppression.

40. *Counting the HIV-1 infected CD4$^+$ T cells.* A schematic of the dendrimer-mediated siRNA delivery into HIV-1-infected primary human CD4$^+$ T cells is shown in Figure 5. Grow CD4+ T cells and challenge with HIV-1 as outlined in the Supplementary Methods. Wash off the free virus and after the last wash, pipet off the supernatant and loosen the cell pellet by adding 1.0 mL PBS and gently resuspend cells with the 1.0 mL pipette. Mix 10 μL cells with 10 μL Trypan Blue Stain 0.4% (wt/vol) and count cells (e.g. in a hemocytometer). Count unstained cells as live cells and determine the cell density. Take the desired amount of cells, and add IL-2 medium to adjust the density as desired.

▲CRITICAL STEP Cell viability is calculated as the number of viable cells divided by the total number of cells within the grids on the hemocytometer. If cells take up trypan blue (turn blue), they are considered non-viable. Long processing time and poor technique may adversely affect and the cell viability.

41. *Seeding the HIV-1-infected CD4$^+$ T cells*. Using a 24-well plate format as an example, seed 400 μL of cells at 5×10^5 cells per mL into each well (2×10^5 cells per well). Incubate the cells at 37 °C in a humidified incubator under 5% CO_2.

42. *Formation of the siRNA/**AD** complex* (Fig 3). As shown in Table 1, calculate the total amount of **AD** and HIV-1 tat/rev siRNA using the formula provided in the Experimental Design and taking into account the number of samples to be transfected. Dilute the **AD stock** solution (240 μM in PBS buffer from step 39) with Opti-MEM® medium and mix well by pipetting up and down for 15 seconds (Step ii in Fig 3). Dilute siRNA solution (10 μM stock solution in PBS) with Opti-MEM® medium and mix well by pipetting up and down for 15 seconds (Step iii in Fig 3). Add the diluted siRNA solution to the diluted **AD** solution, mix well by pipetting up and down for 20 seconds (Step iv in Fig 3). Incubate the mixture for 25 min at room temperature to allow the nanoparticles to form (Step v in Fig 3). For a 24-well plate format, mix 50 μL of diluted siRNA and 50 μL of **AD** to get 100 μL complex in total.

43. ***AD****-mediated siRNA transfection* Gently mix the complexes by pipetting up and down. Carefully distribute the solution containing the siRNA/**AD** complex drop by drop into each well and mix by gently rocking the plate back and forth (Step vi in Fig 3). Incubate the cells at 37 °C in a humidified incubator under 5% CO_2 for 24–72 h (Step vii in Fig 3) before further functional assays.

▲CRITICAL STEP Proceed to complex addition immediately once the complex is formed. Evenly distribute the complex onto the cells and do not mix vigorously the complex with the cells.

Functional evaluation of the gene silencing and anti-HIV activity

● TIMING 4 h for collecting the cell-free supernatant, collecting cells and isolating total RNA, 4 h for cDNA synthesis and qRT-PCR assay, 4 h for surface $CD4^+$ staining and flow cytometry analysis, and 6 h for HIV-1 p24 ELISA assay.

44. Using a 1-mL pipette, carefully transfer the HIV-1-infected $CD4^+$ T cells from the wells of the 24-well plate to 1.7 mL Eppendorf Safe-Lock microcentrifuge tubes. Centrifuge at 400 × g for 5 min at room temperature and gently transfer the cell-free supernatant into a new 1.7 mL tube. Store the supernatant at −80 °C until the p24 ELISA test (Steps 54–62). Cells can be used for either total RNA extraction (Steps 45–47) or antibody staining (Steps 51–53) as described below.

▲CRITICAL STEP After centrifugation, the HIV-1-infected cells are concentrated at the bottom of the tube. For aspiration, tilt the tube and place the tip of the aspirator on the wall of the tube, well above the cell pellet, to avoid loss of cells.

45. *Determination of siRNA-mediated silencing of target genes by qRT-PCR assay (Steps 45–50)* Total RNA extraction by TRIZOL reagent: Add an appropriate amount of TRIZOL® reagent and lyse the cells by gently pipetting up and down for 15 seconds. Incubate the homogenized samples for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. Add 0.2 mL of chloroform/isopropanol 24/1 solution per 1 mL of TRIZOL reagent. Vortex tubes vigorously for 15 seconds and incubate them for 3 min at room temperature. Centrifuge the samples at more than 12000 × g for 15 min at 2–8 °C. Carefully transfer the clear upper aqueous phase to a new 1.7 mL tube. The volume of this phase is about 60% of the volume of TRIZOL reagent used for homogenization.

▲CRITICAL STEP 1 mL of TRIZOL reagent is enough for up to 10^7 cells. To limit biohazard use and improve total RNA extraction, 0.5 mL of TRIZOL reagent is used for one well of a 24-well plate.

46. Precipitate the RNA by mixing with 0.5 mL of isopropanol per 1.0 mL of TRIZOL reagent used for the initial homogenization. Incubate samples at room temperature for 10 min and centrifuge at no more than 12000 g for 20 min at 2–8 °C.

▲CRITICAL STEP Prior to precipitating the RNA with isopropanol, add 5–10 μg RNase-free glycogen as carrier to the aqueous phase to improve RNA recovery.

47. Wash the RNA pellet once with at least 1.0 mL of 75% (vol/vol) ethanol per 1.0 mL of TRIZOL® reagent used for the initial homogenization. Centrifuge at no more than 7500 × g for 5 min at 2–8 °C. Remove and discard Ethanol and dry pellet for 5 min.

48. Dissolve RNA in 20 μl of RNase-free water and quantify RNA concentration using a NanoDrop Microvolume Spectrophotometer.

49. cDNA synthesis: Equilibrate kit reagents to room temperature before use. As recommended by the manufacturer (QuantiNova Reverse Transcription kit, QIAGEN, or similar), combine 0.5–1.0 μg of total RNA with other reaction components in a 20 μL reaction on ice. Mix thoroughly by pipetting up and down several times. Incubate the complete reaction mix in a thermal cycler following the manufacturer's protocol.

50. Real-time qPCR: Thaw SsoAdvanced Universal SYBR Green Supermix (2×) (or similar) and gene-specific primers to room temperature. Determine the number of PCR reactions to use based on the total number of samples plus standards and controls. For a 20 μL PCR reaction, combine 10 μL of SYBR Green Supermix (2×), 2.0 μL of cDNA, gene-specific forward and reverse primers (400 μM working concentration; Table 3; HIV-1 tat/rev primer set for target gene; GAPDH primer set for housekeeping gene) and nuclease-free water on the ice. Mix thoroughly to ensure homogeneity and dispense into the wells of a PCR plate. Program the thermal cycling protocol into the real-time PCR instrument according to the manufacturer's instructions (see EQUIPMENT SETUP).

▲CRITICAL STEP Set up a no-RT control with the same amount of total RNA but no reverse transcriptase and a no-template control (use the same volume of water) for accurate detection of genomic DNA amplicons. The volume of cDNA synthesis reaction used must not exceed 10% of the qPCR volume. For optimal results, assemble the reaction components on ice.

? TROUBLESHOOTING

51. *Determination of anti-HIV-1 (CD4 or HIV-1 Tat/Rev) siRNA-mediated knockdown of target protein by flow cytometry (Steps 51–54).* Cell-surface staining with anti-CD4 antibody: In the case when CD4 siRNA is used for the transfection experiment, the surface CD4 receptor can be assayed by flow cytometry. After 2–3 days of transfection, wash the pelleted cells from Step 44 twice with 500 μL of PBS buffer as described in Step 40. Resuspend the cell pellet with 50 μL of labeling buffer for each tube. Add the fluorescent dye-labeled anti-CD4 antibody (5 μl per 1 million cells) and gently mix by pipetting up and down for 15 seconds. Wrap the tubes in aluminum foil and incubate at room temperature for 30 min. Add 1.0 mL of labeling buffer to each tube and centrifuge the tubes at 400 g for 5 min at room temperature Discard supernatants.

52. Fixation of HIV-1-infected $CD4^+$ T cells: Loosen the cell pellets and add 250 μL of BD Cytofix/cytoperm solution. Incubate in the dark at 4 °C for 20 min. Add

1.0 mL of BD Perm/Wash buffer to the tube and centrifuge the tubes at 400 g for 5 min at room temperature. Discard supernatants and resuspend cell pellets in 350 μL of labeling buffer. Store samples at 4 °C in the dark while setting up the flow cytometry with non-stained control (cells alone without anti-CD4 antibody). Proper fluorescence compensation controls will be used to correct for possible emission spectra overlap in applied.

53. Flow cytometry analysis: Proceed to acquisition of experimental data (surface CD4 expression level) by flow cytometry (see EQUIPMENT SETUP for flow cytometer settings).

54. *Determination of siRNA-mediated HIV-1 suppression by HIV-1 p24 ELISA (Steps 54–62)* Equilibrate kit reagents (HIV-1 p24 antibody coated microplate, 5% Triton X-10, Detector antibody, Streptavidin-HRP diluent, Streptavidin-HRP concentrate, substrate diluent, OPD tablets) to room temperature before use. Dilute 20 × plate wash concentrate to 1× with distilled, deionized water and determine the number of antibody-coated strips to use based on the total number of samples plus standards.

55. Prepare p24 standards using POSITIVE CONTROL provide by the Kit as a virology quality assurance, which covers 6 concentrations from 4,000 pg/mL to 12.5 pg/mL.

56. Prepare samples by diluting the cell-free supernatant from Step 44 with RPMI medium to the appropriate concentration.

▲CRITICAL STEP It is recommended to set up a pre-test with non-transfected samples. This will help to determine the dilution ratio of the supernatant. If the supernatant is too diluted, the readout will be negative; if the supernatant is too concentrated, the readout will be saturated.

57. Label the ELISA plate and add 20 μL of Triton X-100 to all wells except the substrate blank.

58. Add 200 μL of standards, negative control (RPMI medium) or diluted samples to the appropriate wells. Seal plate and incubate for 2 h at 37 °C.

59. Wash plate in cell washer. Add 100 μL of detector antibody to all wells, except the blank. Seal plate and incubate for 1 h at 37 °C.

60. Wash plate in cell washer. Add 100 μL of SA-HRP 1:100 working dilution to all wells, except blank. Seal plate and incubate for 0.5 h at room temperature

61. Wash plate in cell washer. Add 100 μL of OPD substrate solution to all wells, except blank. Seal plate and incubate for 0.5 h at room temperature.

▲CRITICAL STEP Add one OPD tablet to 11 mL of substrate diluent and protect from light. OPD should be freshly made and used.

62. Stop the reaction by adding 100 μL of stop solution to all wells. Immediately read the plate at 490 nm on a preconfigured plate reader (see EQUIPMENT SETUP).

? TROUBLESHOOTING

AD-mediated siRNA transfection into NK cells

● TIMING 1.5 h for sorting NK cells from mouse spleen, cell counting and seeding, 1 day for resting of NK cells in medium supplemented with IL-15, 1.5 h for formulating the siRNA/**AD** complex, and 2 days for the complex-mediated gene silencing and functional studies.

▲CRITICAL The same procedure also applies to human NK cells.

63. Isolate and sort NK cells from mouse spleen as described in the Supplementary Methods. On the experimental day, when cells are sorted, count the NK cells with a hemocytometer. Add IL-15 medium to adjust the concentration as desired.

64. Seeding NK cells: As an example, for a 24-well plate format, seed 1.0 mL of cells at 5×10^5 cells into each well. Incubate the cells at 37 °C in a humidified incubator under 5% CO_2 for 24h.

65. Formation of the NKG2D siRNA/**AD** complex: follow the procedure as described for $CD4^+$ T cells (Step 42).

66. **AD**-mediated siRNA transfection: follow the procedure as described for $CD4^+$ T cells (Step 43).

Functional evaluation of NKG2D gene silencing in NK cells

● TIMING 4 h for cell collection and total RNA extraction, 4 h for cDNA synthesis and qRT-PCR assay, and 4 h for flow cytometry analysis.

67. Carefully transfer the transfected NK cells (Step 66) from the wells to 1.7 mL Eppendorf Safe-Lock microcentrifuge tubes with a 1.0-mL pipette. Centrifuge at 400 g for 5 min at room temperature and proceed to total RNA extraction or antibody staining as described below.

▲CRITICAL STEP After centrifugation, the NK cells are concentrated at the bottom of the tube. For aspiration, tilt the tube and place the tip of the aspirator on the wall of the tube, well above the cell pellet, to avoid loss of cells.

68. *Determination of siRNA-mediated silencing of the target gene by qRT-PCR assay (Steps 68–70).* RNA isolation: Follow the procedure as described for CD4+ T cells (Steps 45–47) or alternatively the manufacturer's instructions for the RNA isolation kit (RNeasy Mini Kit, QIAGEN).

69. Elute RNA with RNase-free water and quantify the RNA concentration using a NanoDrop Microvolume Spectrophotometer.

70. cDNA synthesis and qRT-PCR: follow the procedure as described for $CD4^+$ T cells (Steps 49–50) using NKG2D primers (Table 3).

71. *Determination of siRNA-mediated knockdown of NKG2D protein by flow cytometry (Steps 71–73).* In the case when NKG2D siRNA is used for the transfection experiment, surface expression of the NKG2D receptor can be

assayed by flow cytometry. To stain cell surface with anti-NKG2D antibody, after 2–3 days of transfection, wash the cells from Step 67 twice with 500 μL of PBS buffer. Re-suspend the cell pellet with 50 μL of labeling buffer for each tube. Add the fluorescent dye-labeled anti-NKG2D antibody (0.25 μg/test) and gently mix by pipetting up and down for 15 seconds. Wrap the tubes in aluminum foil paper and incubate at room temperature for 30 min. Add 1.0 mL of labeling buffer to each tube and centrifuge the tubes at 400 g for 5 min at room temperature. Discard supernatants.

72. Fixation of NK cells: Loosen the cell pellets and add 250 μL of BD Cytofix/cytoperm™ solution. Incubate in the dark at 4 °C for 20 min. Add 1 mL of BD Perm/Wash™ buffer to the tube and centrifuge the tubes at 400 g for 5 min at room temperature. Discard supernatants and resuspend cell pellets in 350 μL of labeling buffer. Store samples at 4 °C in the dark while setting up the flow cytometry with non-stained control cells.

73. Flow cytometry analysis: Proceed to acquisition of experimental data (surface NKG2D expression level) by flow cytometry (see EQUIPMENT SETUP for flow cytometer setup).

AD-mediated siRNA transfection into primary mouse macrophages (BMDM, bone marrow derived macrophages)

● TIMING 1.5 h for detaching the BMDM from petri dish, cell counting and seeding them in a 12-well plate, 1 h for formation of the siRNA/**AD** complex, and 2 days for the complex-mediated gene silencing.

74. Detaching the BMDMs from Petri dish and cell counting: Isolate BMDMs as described in the Supplementary Methods. On day 7, wash the cells with 5 mL of PBS, then add 2 mL of accutase for 5 min at 37°C in the incubator. Recover the cells by flushing the culture Petri dish and collect in a 50 mL conical tube containing 5 mL BMDM cell culture basic medium as described in the Reagent Setup section. Centrifuge at 150 g for 5 min at room temperature. Aspirate the supernatant and re-suspend the cells in BMDM cell culture basic medium and count the number of cells using a Countess™ II FL Automated Cell Counter using Trypan blue as a viability dye.

75. Seeding of macrophages: Seed 1×10^6 cells per well in 1 mL BMDM cell culture basic medium on a 12-well plate format. Incubate the cells at 37 °C in a humidified incubator under 5% CO_2 for 1 h to allow the cells to attach on the plate. After 1 h, centrifuge the plate at 150 g for 5 min at room temperature, remove the medium and refill with 800 μL of OPTI-MEM medium.

76. Formation of the siRNA/**AD** complex: Follow the procedure with JAK1 siRNA as described for $CD4^+$ T cells (Step 42).

77. **AD**-mediated siRNA transfection: Gently mix the complexes by pipetting up and down. Carefully add dropwise the solution containing the siRNA/**AD** complex onto cells and rock gently the Petri dish 10 seconds to homogenize siRNA/**AD**

complex dispersion on the cells. Incubate the cells at 37 °C in a humidified incubator under 5% CO_2 for 8 h. Then centrifuge the plate at 150 g for 5 min at room temperature, remove the OPTI-MEM and replace with DMEM base media. Incubate for another 40 h before further functional assays.

Functional evaluation of JAK1 gene silencing in primary macrophages

● TIMING 1 h for collecting cell lysates and preparing protein samples, 6 h for SDS-PAGE and electrophoretic transfer, 16 h for protein detection by western blotting.

78. Wash the cell monolayer (from Step 77) once with ice-cold DPBS with calcium and magnesium. For protein isolation, add 1 tablet of cOmplete™ in 10 mL of RIPA buffer to prepare the cell lysis buffer and add 150μL of cell lysis buffer per well to evenly cover the plate well surface.

▲CRITICAL STEP When collecting cell lysates for protein analyses, keep the culture plate and tubes on ice.

■ PAUSE POINT At this stage the culture plate can be stored at −20 °C until required for further processing for western blot analysis.

79. *Determine siRNA-mediated knockdown of target protein in macrophages by western blotting (Steps 79–84).* Remove the cell lysates from −20°C, defrost them on ice and centrifuge at 15,000 to 20,000 g for 15 min at 4°C. Transfer the supernatants to fresh tubes and discard the pellets.

80. Determine the protein concentration with the BCA assay, adjust the concentration in each sample to an equal value (*e.g.* 0.5 μg/mL) and add an appropriate volume of 4× LDS buffer and 10× sample reducing buffer. Boil at 95°C for 10 min to denature the proteins.

? TROUBLESHOOTING

81. Mount the precast polyacrylamide (4–15%) gels in the electrophoresis system and add the running buffer for SDS-PAGE. Load equal amounts of protein (*e.g.* 10 μg) into the wells of the gel along with the pre-stained protein marker. Run the gel for the initial 10 min at 80 V and then increase the voltage to 120–170 V and continue for 1–1.5 h until the protein samples are resolved in the gel.

82. Transfer the proteins from the gel to a nitrocellulose membrane in electrophoretic transfer buffer at 100 V for 60 min.

83. Block the membranes in blocking buffer for 1 h at RT and incubate overnight at 2–8 °C with primary anti-JAK1 antibodies diluted at 1/1000, or anti-GAPDH antibodies diluted at 1/2000, in 5% BSA in TBS-T. The primary antibody reaction is followed by several TBS-T washes, then by incubation with the peroxidase-conjugated secondary antibody diluted at 1/10000 for 1 h.

84. Detect the immunocomplexes using a chemiluminescence detection kit and instrument of choice. Estimate the molecular weight of the detected proteins according to pre-stained protein markers.

? TROUBLESHOOTING

AD-mediated siRNA transfection into primary microglia

● TIMING 1.5 h for separation of microglia from monolayer mixed glial culture, cell counting and seeding, 1–2 days for resting of the microglia culture, 1.5 h for formulation of the siRNA/**AD** complex, and 2–3 days for the complex-siRNA mediated gene silencing and functional studies.

85. Isolate and culture primary microglia from newborn pups as described in the Supplementary Methods

86. Seeding of microglia cells: Using a 24-well plate format as an example, seed 1×10^5 cells per well in 0.5 mL culture medium. Incubate the cells at 37 °C in a humidified incubator under 5% CO_2 for 48 hours to reach quiescence.

87. Formation of the siRNA/**AD** complex: follow the procedure as described for $CD4^+$ T cells (Step 42) using rat ID1 siRNAs.

88. **AD**-mediated siRNA transfection: follow the procedure as described for $CD4^+$ T cells (Step 43).

Functional evaluation of *Id1* gene silencing in primary microglia culture

● TIMING 3 h for collecting cell lysates and extracting total RNA or preparing protein samples, 4 h for cDNA synthesis and qRT-PCR assay, 6 h for SDS-PAGE and electrophoretic transfer, 4 – 16 h for protein detection by western blotting.

89. Wash the cell monolayer (Step 88) twice with ice-cold PBS. For total RNA extraction, follow the procedure as described for $CD4^+$ T cells (Steps 45–47) or alternatively the manufacturer's instructions for the RNA isolation kit. For protein isolation, scrape the cells into the cell lysis buffer containing phosphatase and protease inhibitors. Add a sufficient volume of the cell lysis buffer to evenly cover the plate well surface (for example, add 50 μL to each well of a 24-well plate). Transfer the lysate to a microcentrifuge tube and store the lysates at −80 °C until required for further processing for western blot analysis.

▲CRITICAL STEP When collecting cell lysates for protein analyses, keep the culture plate and tubes on ice.

90. *Determination of siRNA-mediated silencing of the target gene by qRT-PCR assay (Steps 90–91).* Dissolve RNA in RNase-free water and quantify RNA concentration using a NanoDrop Microvolume Spectrophotometer.

91. cDNA synthesis and qRT-PCR: follow the procedure as described for $CD4^+$ T cells (Steps 49–50). Use rat ID1 and 18S RNA primers (Table 3).

?TROUBLESHOOTING

92. *Determination of siRNA-mediated knockdown of target protein in microglia by western blotting (Steps 92–97).* Remove the cell lysates (Step 89) from −80°C,

defrost them on ice and centrifuge at 16000 g for 15 min at 4 °C. Transfer the supernatant to a fresh tube and discard the pellet.

93. Determine the protein concentration with the BCA assay, adjust the concentration in each sample using cell lysis buffer to equal value (e.g. 0.5 μg/mL) and add an appropriate volume of 4× sample loading buffer with DTT to get 1× concentration. Boil at 98 °C for 5 min to denature the proteins.

94. Mount the precast polyacrylamide gels in the electrophoresis system and add the running buffer for SDS-PAGE. Load equal amounts of protein (e.g. 20 μg) into the wells of the gel along with the pre-stained protein marker. Run the gel for the initial 10 min at 50 V and then increase the voltage to 100–150 V and continue for 1–1.5 h until the protein samples are resolved in the gel.

95. Transfer the proteins from the gel to the nitrocellulose membrane in electrophoretic transfer buffer at 400 mA for 75 min.

96. Block the membranes in blocking buffer for 1 h and incubate for 2 h at room temperature or overnight at 2–8 °C with primary antibodies (anti rat ID1 diluted to 1:1000 or anti GAPDH diluted to 1:25000) in 5% BSA in TBS-T. The primary antibody reaction is followed by incubation with the peroxidase-conjugated secondary antibody diluted to 1: 10000 for 1 h.

97. Detect the immunocomplexes using a chemiluminescence detection kit and instrument of choice. Estimate the molecular weight of the detected proteins according to pre-stained protein markers.

?TROUBLESHOOTING

● TIMING

Steps 1–39: 3 weeks for chemical synthesis, purification and characterization.

Steps 40–43: 2.5 hours for AD-mediated siRNA transfection into HIV-1-infected $CD4^+$ T cells

Steps 44–62: 3 days for functional assays of siRNA-mediated gene silencing in HIV-1-infected $CD4^+$ T cells

Steps 63–66: 1 day for AD-mediated siRNA transfection into NK cells

Steps 67–73: 3 days for functional assays of siRNA-mediated gene silencing in NK cells

Steps 74–77: 2.5 hours for AD-mediated siRNA transfection into primary macrophage cells

Steps 78–84: 4 days for functional assays of siRNA-mediated gene silencing in primary macrophages

Steps 85–88: 1 day for AD-mediated siRNA transfection into primary microglia

Steps 89–97: 3 days for functional assays of siRNA-mediated gene silencing in primary microglia

TROUBLESHOOTING

Troubleshooting advice can be found in Table 4.

ANTICIPATED RESULTS

The synthesis of **AD** started with 95 mg (0.102 mmol) of **1**, which delivered 108 mg of the pure compound **3** as a colorless solid with a yield of 73%. Further transformation of **3** produced 118 mg of the pure product **AD** as a faint-colored foam-like solid at 96% yield. Detailed analytical data for **AD** are provided in Supplementary Figures 1–4 and Supplementary Results (Analytical Data of Dendrimers).

AD-mediated siRNA delivery in primary T-cells was performed in HIV-1-infected human primary PBMC-CD4$^+$-T cells using Dicer-substrate siRNAs (DsiRNAs) to target CD4 and HIV-1 tat/rev.[17] CD4 is the primary receptor for HIV-1, and transient knockdown of this receptor blocks HIV-1 entry,[31] while HIV-1 Tat and Rev are essential positive regulators of viral gene expression.[32] Using **AD** as the siRNA delivery vehicle, a substantial decrease of CD4 expression was achieved at both the mRNA and protein level in T cells.[17] Also, a considerable reduction in HIV-1 tat/rev mRNA (50–70%) was observed in the PBMC-CD4$^+$ T-cells (Figure 4a), leading to a significant reduction of viral infection (Figure 4b). **AD** alone or a control DsiRNA/**AD** complex didn't generate any silencing effect (Figure 4a), nor affect the cell viability (Supplementary Figure 5a) [17]. When we compared **AD** to commercially available vectors, we found that Lipofectamine RNAiMAX (lipo) was unable to deliver the siRNA effectively (Supplementary Figure 5b), while Trans IT-TKO (TKO) exhibited notable toxicity.[17] These results demonstrate the excellent siRNA delivery ability of **AD**, which opens up a new avenue for siRNA delivery into T cells and also for adoptive T cell therapy.

AD-mediated siRNA delivery in NK cells was carried out in both murine NK cells collected from mouse spleen and human NK cells purified from the blood of healthy donors, using an siRNA specifically targeting the Natural-killer receptor group 2, member D (NKG2D). NKG2D is an activating receptor expressed on the membrane of NK cells that triggers their cytotoxic function when it recognizes ligands expressed by a target cell such as a cancer cell.[33] The NKG2D receptor is involved in the cytotoxic function of NK cells against motor neurons.[21] The delivery of **AD** loaded with NKG2D-specific siRNA occurs 6 h after transfection, as shown using siRNA labeled with a fluorescent dye.[21] **AD**-mediated siRNA transfection reduced the *NKG2D* mRNA by ~ 60–70% in both human and murine NK cells, and the protein level by ~ 50–60% in murine NK cells, as indicated by qPCR and FACS analysis (Figures 4c and 4d). The reduction of NKG2D receptor expression reduced the cytotoxic activity of NK cells against motor neurons and tumor cells.[21] Empty **AD** or **AD** loaded with scrambled siRNA did not affect the NKG2D expression in NK cells (Figures 4c and 4d), nor the viability of NK cells (Supplementary Figure 6a).[21] Also, the commercial vector Lipofectamine (lipo) was unable to deliver the siRNA in NK cells (Supplementary Figure 6b). Taken together, these results demonstrate the excellent performance of **AD** to deliver siRNA into NK cells. The successful **AD**-mediated siRNA delivery into primary NK cells corroborated the role of NKG2D in the cytotoxic activity of NK cells against tumor cells and motor neurons in neurodegenerative diseases. This opens up the possibility of

modifying the functional activation state of NK cells and may provide a new therapeutic approach in different diseases.

AD-mediated siRNA delivery in primary macrophages has been studied using a specific siRNA targeting Janus Kinase 1 (JAK1) in mouse primary macrophages originating from the bone marrow (BMDM). JAK1 is a kinase, which is associated with several cytokine or interferon receptors, thus playing an important role in the inflammatory responses. Inhibition of JAK1 activity has been found valuable in the treatments of several diseases including rheumatoid arthritis,[34] inflammatory bowel disease,[35] and Areata alopecia.[36] Reduction of JAK1 expression in macrophages, in order to restrict their pro-inflammatory activity, remains a challenging issue. Using **AD** as the vector for the delivery of JAK1-specific siRNA to mouse primary macrophages resulted in a significant reduction of JAK1 protein expression estimated to 67% of the normal by western blot analyzed 48 h post-transfection with an N/P ratio of 5/1 (Figures 4e and 4f). In addition, a high proportion of cells survived the treatment with **AD** (Supplementary Figure 7a), and the primary macrophages remained unpolarized upon treatment with **AD** and **AD**-complexed control siRNA (Supplementary Figure 7b). In contrast, commercial vector such as Lipofectamine RNAiMAX (lipo) did not decrease JAK1 protein expression level (Supplementary Figure 7c). Delivery of JAK1-specific siRNA to primary macrophages using **AD** as a transfection reagent promises to treat various JAK1-dependent disease conditions. The capacity in the modulation of JAK1 activity in primary inflammatory cells such as macrophages opens a new window to intervene in treatment for inflammatory diseases.

Importantly, **AD** allowed effective siRNA delivery to primary microglial cells at a low siRNA concentration (12.5 nM), and had no effect either on the cell morphology or on the expression of inflammatory and pro-invasive response genes in these cells.[20] **AD** did not affect the basal function or immune gene expression in primary microglial cells.[20] Microglial cells treated with **AD** alone or **AD** complexed with control siRNA retained their ability to proliferate (Supplementary Figure 8a), remained unpolarized (Supplementary Figures 8b and 8c) and their responses to stimuli were unaffected.[20] In contrast, viral vectors, such as a lentiviral vector carrying a shRNA, and nonviral vectors, such as Viromer (Supplementary Figure 8b) or Lipofectamine, induced strong inflammatory effects in primary microglia cultures.[20, 37] We used **AD** to deliver an siRNA targeting the transcription regulator ID1 in rat microglial cells. ID1 controls the proliferation and differentiation of many cells, and has been hypothesized to act as a master switch in microglia activation by glioma-derived factors and a suppressor of anti-tumor immune responses via inhibition of myeloid cell maturation.[38, 39] Treatment of the primary microglia with the siRNA/**AD** complexes triggered effective downregulation of the target *id1* mRNA and its protein product, resulting in ~40–60% of gene silencing as analyzed by qRT-PCR and western blotting at 72 h post-transfection (Figures 4g and 4h).[20] Knockdown of the transcription regulator ID1 in microglia led to significant changes in gene expression, e.g. downregulation of cell cycle-related genes and upregulation of genes associated with the immune response as demonstrated by RNA sequencing.[20] This allowed us to explore for the first time the functions of ID1 in glioma-stimulated microglia, and specifically identify the role of ID1 in tumor-induced immunosuppression.[20]

Collectively, all these results demonstrate the excellent performance of **AD** for efficient and non-toxic siRNA delivery to primary immune cells. **AD** therefore constitutes the long-searched-for transfection reagent with the potential to deliver siRNA safely and effectively for the purpose of functional study and therapeutic applications in immune cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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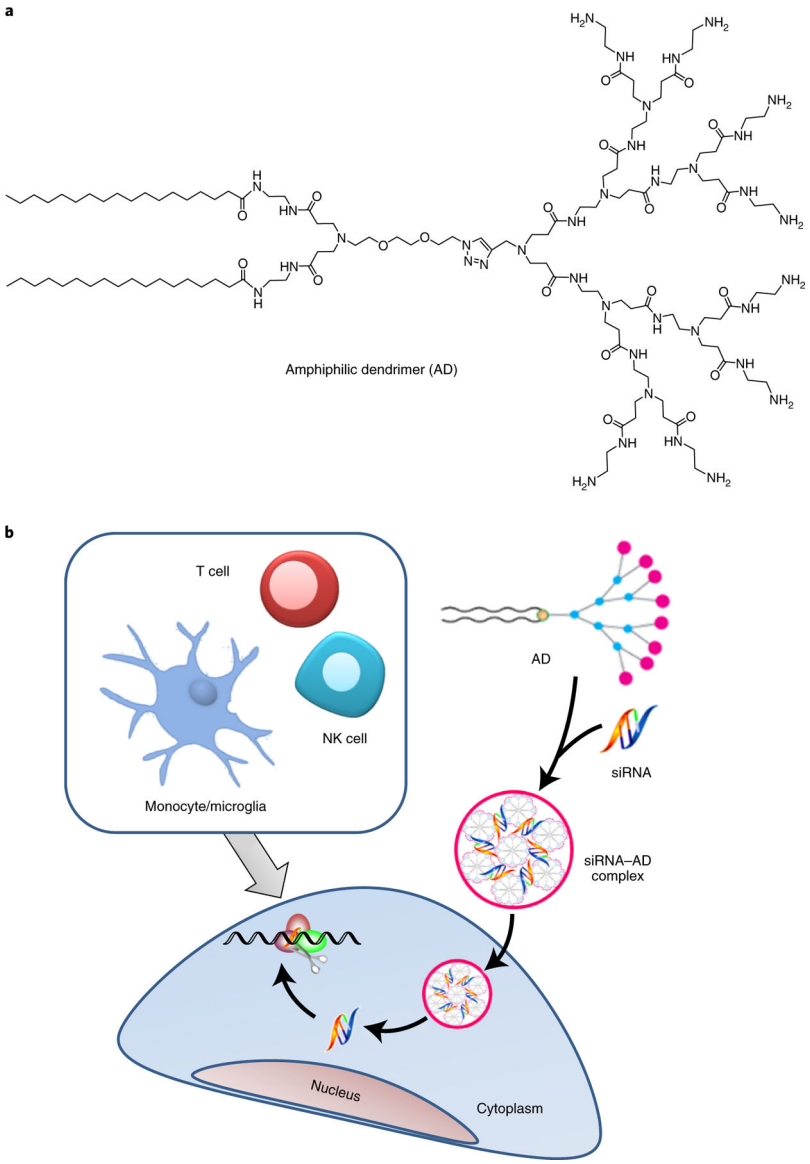

Figure 1:

Schematic presentation of the siRNA delivery mediated by the amphiphilic dendrimer **AD**. **a)** The molecular structure of **AD**. **b)** Cartoon illustration for the **AD**-mediated siRNA delivery into various immune cells, including T cells, microglia, and NK cells. (Figures adapted from Refs. 17 and 20). The pink dots represent the amine terminals, while the blue dots represent the amidoamine backbone branching units and the yellow dot represents the triazole ring in **AD**.

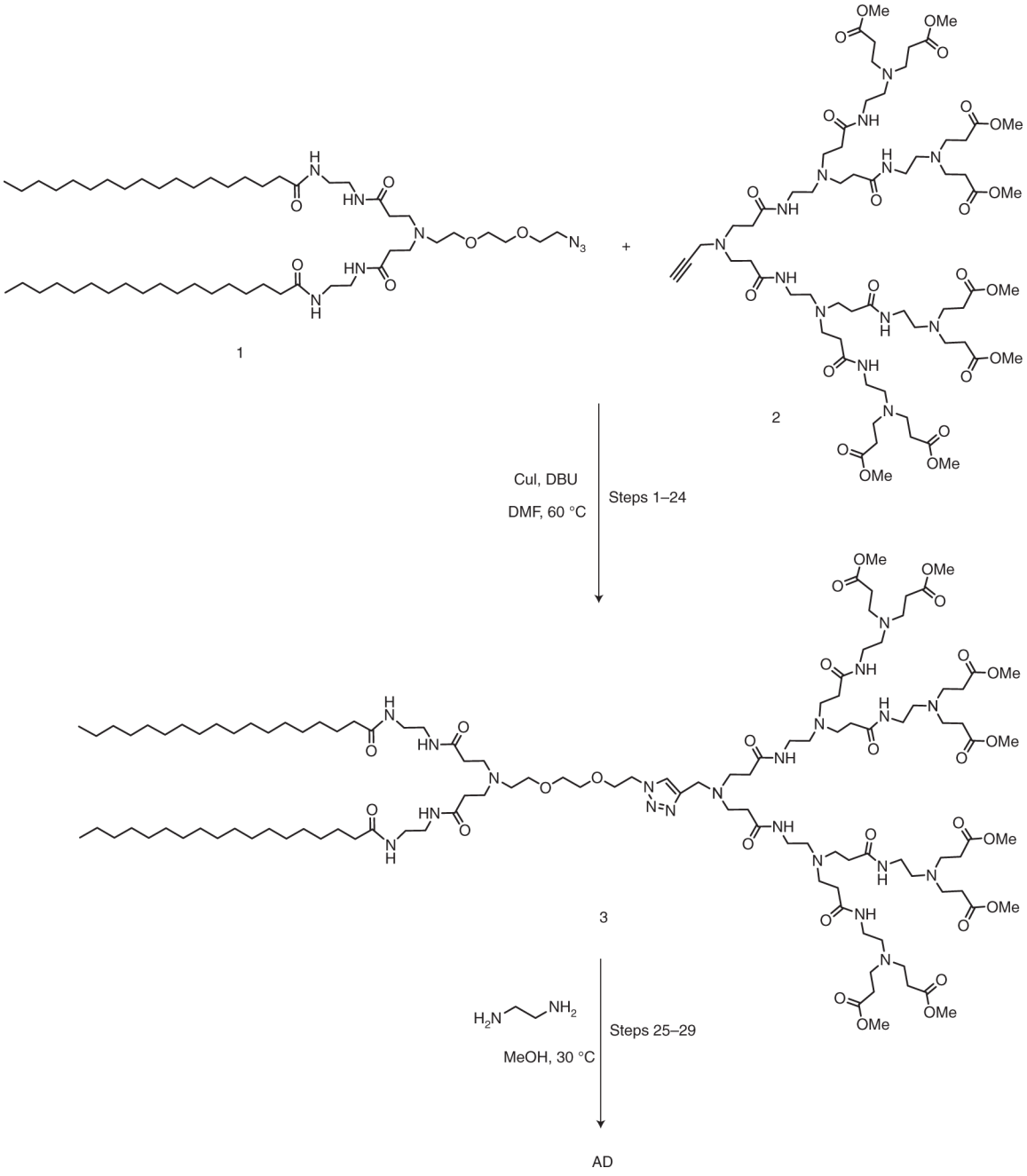

Figure 2:
Chemical synthesis of the amphiphilic dendrimer **AD**.[17] (Figures adapted from Ref. 17)

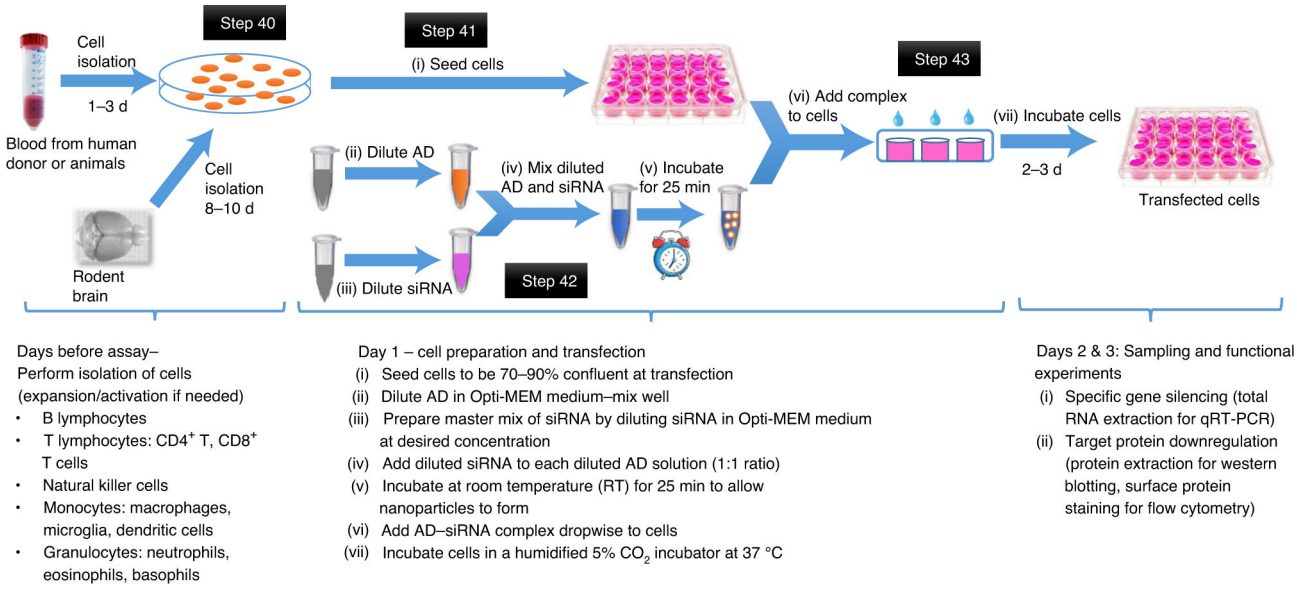

Figure 3:

Experimental outline. A schematic illustrating dendrimer-mediated siRNA transfection into primary cells. [PRODUCTION: Please change numbers 1)-7) to small roman numerals (i-vii). They should match numbering in first column of Table 1 and highlighted in yellow in Steps 42–3.]

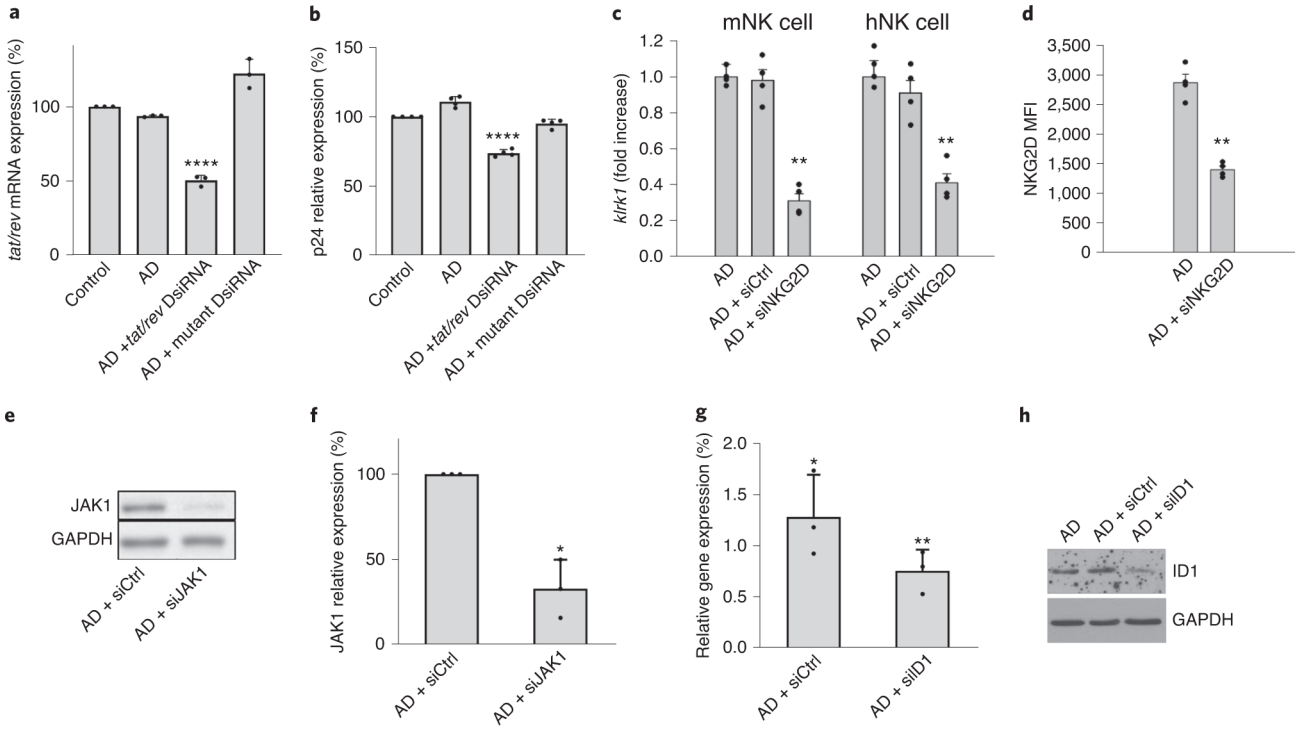

Figure 4:

Functional siRNA delivery mediated by **AD** in various primary immune cells. [17, 20, 21]

a, b) AD-mediated anti-HIV siRNA delivery in human primary $CD4^+$ T-lymphocytes. **a)** Down-regulation of *tat/Rev* mRNA expression measured by RT-qPCR (n = 3, data are expressed as mean ± SD, **** $p \leq 0.0001$ versus control, one-way ANOVA) and **b)** effective inhibition of HIV-1 replication with 50 nM dsiRNA and **AD** at N/P ratio of 5. Viral loading was assessed using HIV-1 p24 antigen ELISA at 3 days of post-treatment. PBMC-$CD4^+$ T-cells were infected by NL4–3 virus (MOI 0.001) for 5 days before transfection. (n = 4, data are expressed as mean ± SD, **** $p \leq 0.0001$ versus control, one-way ANOVA). (Figures adapted from Ref. 17). The control is cell alone without any treatment. All measurements were taken from distinct samples. Statistical significance was assessed by one-way ANOVA for parametrical data as indicated by using GraphPad Prism8.0 version.

c, d) AD-mediated siRNA delivery in murine and human primary NK cells. **c**) RT-PCR of *klrk1* gene in murine (left) and human (right) and NK cells treated with siRNA and **AD** at N/P ratio of 5 (n = 4, data are expressed as mean ± SEM, ** $P < 0.001$ vs empty **AD**, one-way ANOVA). **d**) Detection of NKG2d protein in murine NK cells transfected with the complexes of anti-NKG2d or scrambled siRNA with **AD** at N/P ratio 10. Protein expression was assessed by FACS 48h after transfection. (n = 4, data are expressed as mean ± SEM, ** $P < 0.001$ versus empty **AD**, one-way ANOVA). (Figures adapted from Ref. 21). All measurements were taken from distinct samples. Statistical significance was assessed by one-way ANOVA for parametrical data, as indicated; Holm–Sidak test was used as a *post hoc* test; Mann–Whitney Rank and Shapiro-wilk test for non-parametrical data, followed by Tukey's *post hoc* tests. For multiple comparisons, multiplicity-adjusted *p*-values are indicated in the corresponding figures. Statistical analyses comprising calculation of degrees of freedom were done using Sigma Plot 12.5. **e, f) AD**-mediated siRNA delivery in mouse

bone marrow derived primary macrophages (BMDM). **e)** Detection of JAK1 protein in macrophages transfected with anti-JAK1 (siJAK1) or Control siRNA (siCtrl). Transfection was done with 50 nM siRNA and **AD** at N/P ratio 5. Protein expression was assessed by western blot 48h after transfection with antibodies specific to JAK1 or specific to GAPDH as a standard for protein expression. **f)** Quantification of JAK1 expression from western blot. All measurements were taken from distinct samples. A significant reduction of JAK1 expression, with a mean at 67% of specific decrease after transfection with siJAK1/**AD** in comparison with siCtrl/**AD,** was evaluated by paired Student's *t* test (n = 3, * $p \leq 0.05$) using Graph Pad Prism version 8.4.2. **g, h) AD**-mediated siRNA delivery in rat primary microglia. **g)** The levels of mRNA and **h)** protein were analyzed 72 h post-transfection using qPCR and western blotting, respectively. Specific siRNA/**AD** complexes effectively silence the glioma-conditioned medium (GCM)-induced expression of *id1* gene in primary rat microglia. Rat microglia were transfected with **AD** alone, complexes of **AD** with the control siRNA (siCtrl/**AD**) or complexes of **AD** with the *id1*-targeting siRNA (siID1/**AD**) and next stimulated with GCM. Both siRNAs were complexed with **AD** at N/P=10 and 12.5 nM siRNA. Gene expression is presented as fold change relative to the treatment with **AD** alone (n = 3, mean ± SD). All measurements were taken from distinct samples. Statistically significant differences between cultures treated with siID1/**AD** and siCtrl/**AD** were evaluated by the paired Student's *t*-test using GraphPad Prism version 6.04. * $p<0.05$; ** $p<0.01$. In western blotting evaluation, detection of GAPDH confirmed equal protein loading. (Figures adapted from Ref. 20).

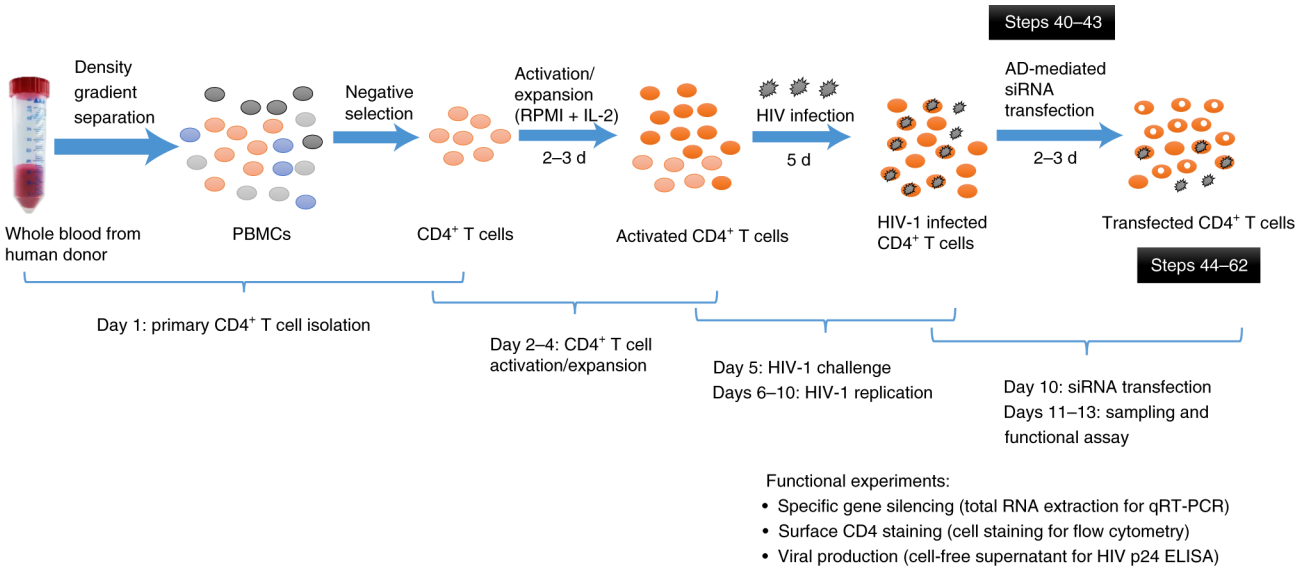

Figure 5:

Experimental outline. A schematic illustrating dendrimer-mediated siRNA delivery into HIV-1-infected primary human CD4+ T cells.

Table 1:

Summary of the volumes of reagents required for dendrimer-mediated transfection of 21/21 mer siRNA or 27/29 mer DsiRNA in various plate formats. [Example for 50 nM siRNA and N/P ratio = 5]

Steps	Component		96-well	24-well	12-well	6-well
Step i)	Experimental cells (number)		$2 – 4 \times 10^4$	$1 – 5 \times 10^5$	$0.2 – 1 \times 10^6$	$0.5 – 2 \times 10^6$
	Cell culture medium (volume)		80 µL	400 µL	800 µL	2000 µL
Step ii)	Opti-MEM Medium (in the case of 21/21-mer siRNA used)		9.45 µL	47.27 µL	94.53 µL	236.35 µL
	Amphiphilic dendrimer (**AD**) (240 µM stock solution) Here: N/P ratio = 5 as an example	21/21-mer siRNA	0.55 µL	2.73 µL	5.47 µL	13.65 µL
		27/29-mer DsiRNA	0.73 µL	3.65 µL	7.3 µL	18.25 µL
Step iii)	Opti-MEM Medium		9.5 µL	47.5 µL	95 µL	237.5 µL
	siRNA (10 µM stock solution of siRNA) 50 nM as working concentration		0.5 µL	2.5 µL	5 µL	12.5 µL
Step iv)	Diluted **AD** (Step ii) + Diluted siRNA (Step iii) solution		20 µL	100 µL	200 µL	500 µL
Step v)	Incubate at room temperature for 25 min to allow nanoparticles to form.					
Step vi)	Final volume: step i cell culture + step iv complex		100 µL	500 µL	1000 µL	2500 µL
Step vii)	Incubate cells in a humidified 5% CO_2 incubator at 37 °C					

Note: N/P ratio is defined as [total end amines in cationic dendrimer] / [phosphates in siRNA]

Table 2.

Custom siRNA sequences

siRNA	Sense (5′–3′)	Antisense (5′–3′)
HIV-1 tat/rev site I 27 mer DsiRNA (viral RNA)	GCG GAG ACA GCG ACG AAG AGC UCA UCA	UGA UGA GCU CUU CGU CGC UGU CUC CGC dTdT
Human CD4 21-mer siRNA	GAU CAA GAG ACU CCU CAG U dGdA	ACU GAG GAG UCU CUU GAU C dTdG
Human CD4 27-mer DsiRNA.	GAU CAA GAG ACU CCU CAG UGA GAA G	CUU CUC ACU GAG GAG UCU CUU GAU CUG-3’ (2′-OMe modified U is underlined.)
Mouse JAK1 21-mer siRNA	GAA UAA AUG CAG UAU CUA AAU	P-UUA GAU ACU GCA UUU AUU CGG (a phosphate is added in 5′ and underlined.)

Table 3.

Primers for qRT-PCR analysis

Name	Sequence (5′–3′)
HIV-1 tat/rev forward primer	GGC GTT ACT CGA CAG AGG AG
HIV-1 tat/rev reverse primer	TGC TTT GAT AGA GAA GCT TGA TG
Human CD4 forward primer	GCT GGA ATC CAA CAT CAA GG
Human CD4 reverse primer	CTT CTG AAA CCG GTG AGG AC
Human GAPDH forward primer	CAT TGA CCT CAA CTA CAT G
Human GAPDH reverse primer	TCT CCA TGG TGG TGA AGA C
Rat ID1 forward primer	AGTCTGAAGTCGCGACCGCC
Rat ID1 reverse primer	CTGGAACACATGCCGCCTCGG
NKG2D forward primer	TACTGTGGCCCATGTCCTAA
NKG2D reverse primer	CTTTCAGAAGGCTGGCATTT
18S RNA forward primer	CGGACATCTAAGGGCATCACA
18SRNA reverse primer	AACGAACGAGACTCTGGCATG

Table 4 |

Troubleshooting table

Step	Problem	Possible reason	Possible solution
9	Incomplete reaction	The CuI is oxidized or inactivated	Ensure proper argon protection before and during the whole reaction
		Starting material **1** or **2** is stuck on the wall of the vial	Ensure proper mixing of all the reagents. During the argon flushing procedure, flush with argon gently
24	Low isolated yield for **3**	Product is stuck on the silica gel	Increase the polarity of the eluent or increase the percentage of TEA in eluent
	Residual TEA in **3**	TEA has a high boiling point and may be encapsulated within the dendrimer **3**.	Dissolve the product in CH_2Cl_2 and wash it using saturated NaCl solution. Collect the organic phase then remove CH_2Cl_2 to obtain the pure product
28	**3** is not well dissolved in methanol	Poor solubility in pure MeOH	**3** is soluble in the mixed solvent of MeOH and EDA
50	Suboptimal siRNA knockdown efficiency	Poor delivery or suboptimal N/P ratio	The formation of siRNA/dendrimer complexes depends critically on the dendrimer-to-RNA charge ratio, which is defined as the “N/P ratio”. Ensure an optimal N/P ratio is used for effective delivery and release of siRNA
50	No significant siRNA knockdown efficiency	Poor total RNA quality	Total RNA extraction from HIV-1 infected primary CD4+ T cells may be challenging. After resuspending cell pellets in TRIZOL solution, add 1 μl glycogen to improve RNA precipitation. Ensure the ratio of 260 nm / 280 nm of total RNA is above 2.0 for RNA measurement using a NanoDrop Microvolume Spectrophotometer.
50	False positive signal shown in no-RT control sample	Genomic DNA contamination	Ensure that the DNase treatment is effective and completed
62	HIV p24 ELISA readout is too high or too lower to see.	Improper sample dilution	Ensure proper sample dilution by setting up a pre-test with non-transfected samples. This will help to determine the dilution ratio. If the supernatant is too diluted, the readout will be negative; if the supernatant is too concentrated, the readout will be saturated
80	Low protein yield	Insufficient number of cells for transfection or insufficient quantity of proteins to be detected by western blot	Increase the number of seeded cells and control cell viability. If cell mortality is observed, adjust the method of cell collection by more gentle and rapid handling.
84	Inefficient western blot detection	Quantity of loaded proteins is too low, inefficient transfer to the membrane or failure in antibody detection	Increase the quantity of proteins loaded on the gels. Control transfer efficacy with a well-established protein which can be used as internal control. Change the antibody source.
91	Low RNA yield	Insufficient number of cells for transfection, improper collection of cell lysates or improper handling of RNA samples.	Check the number of seeded cells and the condition of cells in culture (e.g. cell viability). Use a quick but gentler method for all washing steps during collection of cell lysates, avoiding scratching or disturbing of cell monolayer. Ensure that RNA isolation is performed quickly, with the use of RNase-free water and isolated RNA is handled on ice, with caution to avoid its degradation that may be caused by a contamination with RNases.
91	Suboptimal knockdown efficiency	siRNA concentration is too low for selected target gene or technical problems with qPCR detection	Increase siRNA concentration up to 25 nM, shorten or extend incubation time with **AD** complexes. A precise measurement of RNA concentration before reversetranscription stage and use of an appropriate endogenous control gene (*18SRNA, GAPDH*) in qPCR is required for accurate evaluation of gene expression changes
97	Low protein yield	Insufficient number of cells for transfection or improper collection of cell lysates	Check the number of seeded cells and the condition of cells in culture (e.g. cell viability). Use a quick but gentler method for all washing steps during collection of cell lysates, avoiding scratching or disturbing of cell monolayer

Step	Problem	Possible reason	Possible solution
97	Suboptimal knockdown efficiency	siRNA concentration is too low, time point for the evaluation of the expression levels of selected target gene is not optimal or unequal amounts of protein samples loaded on the gel	Increase siRNA concentration up to 25 nM, shorten or extend incubation time with **AD** complexes. Perform detection of a control, endogenous protein (e.g. Actin, GAPDH) to ensure equal protein loading in each sample
97	No or poor quality chemiluminescence signal in western blotting	Inefficient transfer of proteins from the gels to nitrocellulose or antibody failure.	Stain gels after transfer to ensure a complete protein transfer. Check if a primary antibody is validated for use in western blotting and check that the appropriate secondary antibody was used for detection.
97	Nonspecific signal on the blots	Non-specific binding of primary antibody to the nitrocellulose membrane	Extend incubation of the membranes in a blocking buffer for 3–4 h or increase dilution of primary antibody.