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ABSTRACT (< 250 words)

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 this dendrimer and its siRNA delivery into immune cells such as primary T- and B-cells, natural killer cells, macrophages, and primary microglia. It is noteworthy that the dendrimer synthesis is straightforward, and the siRNA delivery protocol is easy, requiring 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 assay which takes additional 3-6 days.

KEYWORDS:

dendrimer, gene silencing, immune cells, non-viral vector, siRNA delivery,

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) was 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 the case of 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), retinoic acid-inducible gene-I (RAI1), etc.[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. Standard transfection techniques such as electroporation and nucleofection show low transfectability, yet impacting negatively viability. 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 non-viral delivery vectors have been explored for siRNA delivery in general [6, 7] and into immune cells in particular.[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 working on 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 general protocols 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 straightforward and 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 formulation of the siRNA/dendrimer complexes is very convenient, and 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. We present below an overview of these procedures, their validation, and their applications in siRNA delivery into primary immune cells using T-cells, NK cells, macrophages and microglia as examples.

Overview of the procedures

In this protocol, we firstly describe itemized steps for the synthesis and molecular characterization of the dendrimer **AD**. Using 21/21-mer siRNAs and 27/29-mer Dicer substrate siRNAs (DsiRNAs) as examples, we explain the procedure and the conditions for the **AD**-mediated transfection assays to deliver siRNA into primary immune cells such as 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 may take 7 days. Time frames for preparation of primary cell cultures may vary as described in the supplementary materials. Details about the isolation and maintenance or/and expansion of primary immune cells are also provided. Some key points relating to the protocols are discussed below.

Experimental design

<u>Chemical synthesis:</u>

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 50 °C in order to solubilize **1** and drive the reaction to completion, thus offering **3** in high yield. It is also critical to keep the stoichiometric ratio of **1** and **2** at 1/1.05 for 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.[22-24] Different from our previously published protocol, we have used MeOH instead of MeOH/CH_2Cl_2[17] as solvent for the amidation. This is because CH_2Cl_2 can react with EDA to generate oligomeric impurities,[25] and at the same time, increase the formation of the defected dendrimer with cyclization 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. 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.

It should be noted that 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 **AD** = (amount of siRNA) × (number of phosphates in siRNA) × (N/P ratio) / (number of amino groups in **AD**).*

In our previous experiment, we established an optimal N/P ratio of 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. Different from the conventional siRNA which has oligonucleotide of 21-mer for both sense and antisense strains, the DsiRNA used in this work contains 27-mer oligonucleotide in the sense strain and 29-mer oligonucleotide in the antisense strand, which is also called 27/29-mer DsiRNA. Such DsiRNAs have been demonstrated to enhance RNAi potency and efficacy[26, 27]. For 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 concentrations. The effects of the transfection procedure on gene and protein expression are analyzed after 2 or 3 days by quantitative real-time PCR and western-blotting (WB) or FACS, respectively. To define an effective RNAi activity, several key controls should be included in the experimental design: 1) a cell alone control without any siRNA/**AD** transfection (as a mock control); 2) a cell control transfected with a unrelated or scrambled siRNA/**AD** complex (as a negative control); or 3) a cell control transfected with the experimental siRNA/commercial transfecting agent (as a positive control or as a comparison control). By setting these proper 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.

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] The procedure for formulation of the siRNA/dendrimer complexes is very easy, and requires only simple mixing of the siRNA with **AD**. The siRNA/**AD** complexes can then be readily applied to the primary immune cells, such as T-cells, NK cells, macrophages and microglia, for functional siRNA delivery. Therefore, this dendrimer **AD** constitutes the long-searched-for vector to delivery siRNA safely and effectively for the purpose of functional study and therapeutic applications in primary immune cells. Details for siRNA transfection and functional assays were presented below in the "Procedure" section and key results are summarized in "Anticipated Results" section.

Limitations

This protocol provides a facile and robust 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, play a key role in 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

REAGENTS

- Chemical reagents **!CAUTION** Because most of the chemicals and organic solvent used for dendrimer synthesis are potentially hazardous to human health, we recommend performing all reactions in a fume hood while wearing personal protective equipment (gloves, lab coat and goggles) to prevent exposure.
- **1** and **2** were 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.
- Dialysis tubing, benzoylated (MWCO 2000; Sigma-Aldrich, cat. no. D7884-10FT)
- Chloroform-d (99.8 atom % D; Sigma-Aldrich, cat. no. 416754)
- Methanol-d_4 (99.8 atom % D; Sigma-Aldrich, cat. no. 151947)
- 1 × RBC lysis buffer solution (eBioscience, cat no 00433357)
- Compressor (nessicare 30, cat. No. CNST-300D)
- Universal negative siRNA controls (Integrated DNA Technologies, IDT, Negative control DsiRNA, cat.no. 51-01-14-03; Scrambled negative control DsiRNA, cat.no. 51-01-19-08). ▲CRITICAL STEP 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, IDT: HIV-1 tat/rev site I 27-mer DsiRNA, CD4 21-mer siRNA and 27-mer DsiRNA, TNPO3 21-mer siRNA and 27-mer DsiRNA) ▲CRITICAL STEP 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.
- HIV-1 tat/rev site I 27 mer DsiRNA. Sense: 5' -GCG GAG ACA GCG ACG AAG AGC UCA UCA-3'; Antisense: 5'-UGA UGA GCU CUU CGU CGC UGU CUC CGC dTdT-3'.
- CD4 21-mer siRNA. Sense: 5'-GAU CAA GAG ACU CCU CAG U dGdA-3'; Antisense: 5'-ACU GAG GAG UCU CUU GAU C dTdG-3'.
- CD4 27-mer DsiRNA. Sense: 5'-GAU CAA GAG ACU CCU CAG UGA GAA G-3'; Antisense: 5'-CUU CUC ACU GAG GAG UCU CUU GAU CUG-3' (2′-OMe modified U was underlined.)
- TNPO3 21-mer siRNA. Sense: 5'-CGA CAU UGC AGC UCG UGU AUU-3'; Antisense: 5'-UAC ACG AGC UGC AAU GUC GUU-3'.
- TNPO3 27-mer DsiRNA. Sense: 5'-CGA CAU UGC AGC UCG UGU ACC AG dGdC-3'; Antisense: 5'-GCC UGG UAC ACG AGC UGC AAU GUC GUU-3'.
- Custom-made siRNAs (Horizon Discovery,JAK1 siRNA)
- JAK1 mouse siRNA. Sense: 5'-GAA UAA AUG CAG UAU CUA AAU-3'; Antisense:5'-UUA GAU ACU GCA UUU AUU CGG-3'.
- Rat Id1 siRNA (Dharmafect, ON-TARGET plus SMART pool; cat. no: L-080165-02-0005).
- Custom made primers for qRT-PCR analysis (Integrated DNA Technologies, IDT or Genescript).
- Negative control siRNA which does not target any human, mouse or rat gene products (Dharmafect, ON-TARGET plus non-targeting pool; cat. no: D-001810-10-05).
- AllStars Negative Control siRNA is currently the most thoroughly tested and validated negative control siRNA. This siRNA has no homology to any known mammalian gene (Qiagen, AllStars Neg. Control siRNA; Cat No./ID: 1027281).

- HIV-1 tat/rev forward primer: 5'-GGC GTT ACT CGA CAG AGG AG-3'; tat/rev reverse primer: 5'-TGC TTT GAT AGA GAA GCT TGA TG-3';
- CD4 forward primer: 5'-GCT GGA ATC CAA CAT CAA GG-3'; CD4 reverse primer: 5'-CTT CTG AAA CCG GTG AGG AC-3';
- TNPO3 forward primer: 5'-CCT GGA AGG GAT GTG TGC-3'; TNPO3 reverse primer: 5'-AAA AAG GCA AAG AAG TCA CAT CA-3';
- GAPDH forward primer: 5'-CAT TGA CCT CAA CTA CAT G-3'; GAPDH reverse primer: 5'-TCT CCA TGG TGG TGA AGA C-3'.
- ID1 forward primer: 5'-AGTCTGAAGTCGCGACCGCC-3'; ID1 reverse primer 5'-CTGGAACACATGCCGCCTCGG-3'.
- NKG2D forward primer: 5'-TACTGTGGCCCATGTCCTAA-3'; NKG2D reverse primer 5'-CTTTCAGAAGGCTGGCATTT-3'.
- 18S RNA forward primer: 5'-CGGACATCTAAGGGCATCACA-3'; 18SRNA reverse primer 5'-AACGAACGAGACTCTGGCATG-3'.
- 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)
- 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)
- 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 Inc., cat.no. Ro 23-6019)
- ActiCyte® - TC Medium kit (CytoMedical Design Group, cat.no. TCM1000)
- Trypan Blue Stain 0.4% (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. 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# 558116, RRID:AB_397037)
- Id1 antibody (clone B-8) (Santa Cruz Biotechnology Cat# sc-133104, RRID:AB_2122863)
- GAPDH antibody (Millipore Cat# MAB374, RRID:AB_2107445)

- Anti-mouse IgG peroxidase conjugated secondary antibody (Vector Laboratories Cat# PI-2000, RRID:AB_2336177)
- iNOS Antibody (Cell signaling Technology, cat.# 13120S, RRID: AB_2798613)
- Arginase-1 Antibody (Cell signaling Technology, cat.# 93668S, RRID: AB_2800207)
- α/β-Tubulin Antibody (Cell signaling Technology, cat.# 2148S, RRID: AB_2288042)
- 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 (Perkin Elmer, cat. No. NEK050A001KT)
- Recombinant interleukin-15 (IL-15, eBioscience Inc., San Diego, CA)
- BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA, cat no 23225)
- 8-16% Tris-Glycine precast polyacrylamide gels (eg. 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)
- Nitrocellulose membrane (eg. Hybond™–ECL, Amersham)
- 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)
- cOmpletes, 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)

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) in 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 patients' samples, blood should be obtained according to a protocol approved by ethical committee and after obtaining consent from patients. All blood samples should be treated as infectious materials.
- Animal specimens from laboratory animals **!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 Mouse (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 (Mouse C3H/An connective tissue, ECACC Cat# 85011425, RRID:CVCL_0462) **!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 will be check regularly using MycoAlert Mycoplasma detection kit from

Lonza or Universal Mycoplasma detection kit from ATCC. Cell lines that were mycoplasma contaminated will be discarded or cured if necessary using Plasmocin from InVivoGen or BM-Cyclin from Sigma-Aldrich.

▲CRITICAL STEP: 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]

EQUIPMENT

- All equipment for chemical synthesis / characterization: rotary evaporator / water bath / oil bath / reflux condenser
- Rotary evaporator (Heidolph)
- Magnetic stirrer (IKA)
- 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
- 25 and 75 cm^2 cell culture flask with vent cap
- Falcon® 100 mm x 15 mm Not TC-treated Bacteriological Petri Dish (Corning, Product Number 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
- 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 microscopy (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 cytometry (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

- **Solution of experimental AD** Re-suspend the experimental **AD** in sterile, double-distilled, nuclease-free water as a 2.0 mM stock solution. Can be stored in aliquots at -80 °C for up to 12 months. Before assay, dilute with 1×PBS solution to 240 μM for transfection assay. 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.
- **Solution of experimental RNAs** Re-suspend the experimental RNA in sterile, double-distilled, nuclease-free water as a 500 μM stock solution. Dilute with 1×PBS solution to 10 μM for the transfection assay. 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.
- **Separation buffer** PBS 1× supplemented with 2.0 mM EDTA and FBS 0.5% (vol/vol). Can be stored at 4 °C for up to 3 weeks.
- **Labeling buffer** PBS 1×supplemented with FBS 1.0% (vol/vol). Can be stored at 4 °C for up to 2 months.
- **Primary T-cell culture basic medium** RPMI-1640 supplemented with 2 mM L-glutamine, penicillin-streptomycin (100 units per mL of penicillin /100 μg per mL of streptomycin) and FBS 10% (vol/vol). Can be stored at 4 °C for up to 4 weeks.
- **Primary microglia culture basic medium** DMEM with 4.5 g/L glucose supplemented with Glutamax, penicillin-streptomycin (100 units per mL of penicillin /100 μg per mL of streptomycin) and FBS 10% (vol/vol). Can be stored at 4 °C for up to 4 weeks.
- **Activation/expansion medium** Culture medium supplemented with PHA (1.0 μg/mL) and IL-2 (100 μL/mL). Should be freshly prepared or stored for no longer than 1 week at 4 °C.
- **IL-2 medium** Culture medium supplemented with IL-2 (100 μL/mL). 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 value 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. Can be stored at room temperature (at 25°C) for several months.

- **NK cell culture basic medium** RPMI supplemented with Glutamax, penicillin-streptomycin (100 units per mL of penicillin /100 µg per mL of streptomycin) and FBS 10% (vol/vol). Can be stored at 4 °C for up to 4 weeks. Culture medium is supplemented with IL-15 (50 ng/mL). Can be stored at 4 °C for up to 2 weeks.
- **BMDM cell culture basic medium** DMEM supplemented with high glucose, GlutaMAX, (100 units per mL of penicillin /100 µg per mL of streptomycin), 1 % Non-Essential Amino Acid (vol/vol), 1 mM Sodium Pyruvate, HEPES 1% (vol/vol), 0.25 mM Beta-Mercaptoethanol and heat inactivated FBS 10% (vol/vol). Can be stored at 4 °C for up to 4 weeks. BMDM culture medium is supplemented with L929 conditioned media 20% (vol/vol). BMDM culture medium is prepared just before the cell culture and used immediately.
- **MACS buffer** PBS 1× supplemented with 2.0 mM EDTA and BSA 0.5% (vol/vol). Can be stored at 4 °C for up to 2 weeks.
- **$MgSO_4$ solution** 3.8% w/v in PBS. Can be stored at -20 °C for up to 2 months.
- **DNase solution** 2.0 mg/mL in PBS. Aliquot and store at -20 °C for several months.
- **Trypsin inhibitor solution** 20 mg/mL in PBS. Aliquot and store at -20 °C for several months
- **BSA solution** 4.0% w/v in PBS. Aliquot and store at -20 °C for several months.
- **Cell lysis buffer for protein isolation** 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% Triton X-100, 10% glycerol, 5.0 µg/mL leupeptin, 5.0 µg/mL aprotinin, 2.0 mM benzamidine, 0.5 mM DTT. Can be 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**× 60 mM Tris-Cl pH 6.8, 2.0% SDS, 10% glycerol, 5.0% DTT, 0.01% bromophenol blue. Prepare as 4× concentrate without DTT and store at room temperature (at 25°C) for several months. Add DTT just prior to use. **!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^{o}C to dissolve the SDS.

- **Running buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 1**× 25 mM Tris, 192 mM glycine and 0.1% SDS, pH approx. 8.6. **!CAUTION** SDS powder is hazardous. Prepare solution in a ventilated fume hood.
- **Electrophoretic transfer buffer 1**× 25 mM Tris, 192 mM glycine, 0.05% SDS and 20% Methanol. **!CAUTION** SDS powder is hazardous. Prepare solution in a ventilated fume hood.
- **Membrane wash buffer TBS-T** Tris-buffered saline (TBS 1×: 25 mM TRIS, 130 mM NaCl, pH 7.6) with 0.1% Tween 20.
- **Blocking buffer** Dissolve 5.0 g skimmed milk powder (non-fat) in 100 mL membrane wash buffer (TBS-T).

EQUIPMENT SETUP

- Configure confocal microscopy as follows: fluorescent dye-labeled siRNA/**AD** complex is imaged in live cells by confocal microscopy through a high-magnification (40×) special water-immersion objective in a spectral window.
- Configure qRT-PCR system as follows: 10 min setting-up time per plate per parameter, 1.5-2 h run time.
- Configure flow cytometry 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.
- Configure ELISA microplate reader as follows: 15 min run time, 30 s of time interval to read at 490 nm wavelength.
- Configure chemiluminescence detection instrument (X-ray film processor or chemiluminescence detection system) 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 it 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 it 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.
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 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 with 15 mL CH_2Cl_2, and repeat the extraction twice.
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) and MeOH/CH_2Cl_2 1:9 (vol/vol) with 1% (vol/vol) TEA.

    ▲CRITICAL STEP For the column chromatography, the possible unreacted starting materials and impurities can be easily removed by MeOH/CH_2Cl_2 1:9 (vol/vol) and the product can be obtained by MeOH/CH_2Cl_2 1:9 (vol/vol) with 1% (vol/vol) TEA.
22. Collect eluents in 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 ^{o}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 it 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 avoid 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 it via a stainless-steel needle and running IR (look for disappearance of the peak at 1750 cm^{-1}, which corresponds to the carbonyl function in the ester group). Generally, the expected product will show disappearance of the ester group peak at ~1750 cm^{-1} in IR spectrum.
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 'heavy' plastic clamp. After filling the tubing, close it with a 'light' plastic clamp. 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. Generally, the product is subjected to dialysis three times to ensure its purity. The NMR spectrum is used to monitor the purity.
36. Dialyze the contents for 8 h at room temperature (at 25°C) with stirring. Replace the ultrapure water every hour to remove the EDA.
37. Transfer the contents of the dialysis tube into new 50-mL Falcon tubes (~10 mL per tube). Close the tube with aluminum film, 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 afford 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, redissolve the product in ultrapure water again and repeat step 38.

■ PAUSE POINT **AD** can be stored at -20 ^{o}C for months.

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 formulating the siRNA/**AD** complex, and 2-3 days for the complex-mediated gene silencing and HIV-1 suppression. (**Figure 5**)

40. Counting the HIV-1 infected $CD4^+$ T cells: 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% and count cells (e.g. in a hemocytometer). Unstained cells are counted as live cells and the cell density is determined. 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. Formulation of the siRNA/**AD** complex: As shown in **Table 1**, calculate the total amount of **AD** and siRNA on the basis of experimental samples and design. Dilute **AD** solution (240 μM stock solution in PBS buffer) with Opti-MEM® medium and mix well by pipetting up and down for 15 seconds. Dilute siRNA solution (10 μM stock solution in PBS) and mix well by pipetting up and down for 15 seconds. Add the diluted siRNA solution to the diluted **AD** solution, mix well by pipetting up and down for 20 seconds. Incubate the mixture for 25 min at room temperature (at 25°C) to allow the nanoparticles to form. In a 24-well plate format, 50 μL of diluted siRNA and 50 μL of **AD** is mixed to get 100 μL of complex in total.
43. **AD**-mediated siRNA transfection: Gently mix the complexes by pipetting up and down. Carefully drop the solution containing the siRNA/**AD** complex into cells and mix them by gently rocking the plate back and forth. Incubate the cells at 37 °C in a humidified incubator under 5% CO_2 for 24-72 h before further functional assays.

▲CRITICAL STEP Proceed to complex addition immediately once the complex is formed. Evenly drop the complex into the cells and do not vigorously mix 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 wells to 1.7 mL Eppendorf Safe-Lock microcentrifuge tubes. Centrifuge at 400 g for 5 min at room temperature (at 25°C) 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. Cells can be used for either total RNA extraction or antibody staining 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

    i. 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 (at 25°C) 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 (at 25°C). Centrifuge the samples at more than 12000 g for 15 min at 2-8 °C. Carefully transfer the upper aqueous phase (clear) 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.

    ii. 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 (at 25°C) 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.

iii. Wash the RNA pellet once with at least 1.0 mL of 75% 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.

iv. Dissolve RNA in RNase-free water and quantify RNA concentration using a NanoDrop Microvolume Spectrophotometer.

v. cDNA synthesis: Equilibrate kit reagents to room temperature (at 25°C) before use. As recommended by the manufacturer (QuantiNova Reverse Transcription kit, QIAGEN, or similar), assemble 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.

vi. Real-time qPCR: Thaw SsoAdvanced Universal SYBR Green Supermix (2*) (or similar) and gene-specific primers to room temperature (at 25°C). 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, assemble 10 µL of SYBR Green Supermix (2*), 2.0 µL of cDNA reaction, forward and reverse primers (400 µM working concentration) 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 and a no-template control 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

46. Determination of anti-HIV-1 (CD4 or HIV-1 Tat/Rev) siRNA-mediated knockdown of target protein by flow cytometry.

i. In the case that CD4 siRNA is used for the transfection experiment, the surface CD4 receptor can be assayed by flow cytometry.

ii. Cell-surface staining with anti-CD4 antibody: After 2-3 days of transfection, wash the cells twice with 500 µL of PBS buffer as described. Resuspend the cell pellet with 50

μL of labeling buffer for each tube. Add the fluorescent dye-labeled anti-CD4 antibody and gently mix by pipetting up and down for 15 seconds. Wrap the tubes in aluminum foil paper and incubate at room temperature (at 25°C) 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 (at 25°C). Discard supernatants.

iii. 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 (at 25°C). 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 and compensation controls in applied.

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

47. Determination of siRNA-mediated HIV-1 suppression by HIV-1 p24 ELSIA

i. Equilibrate kit reagents to room temperature (at 25°C) before use. Dilute plate wash concentrate 20 × to 1×, and determine the number of antibody-coated strips to use based on the total number of samples plus standards.

ii. Prepare p24 standards using POSITIVE CONTROL, which covers 6 concentrations from 4000 pg/mL to 12.5 pg/mL.

iii. Prepare samples by diluting the cell-free supernatant 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. If the supernatant is too diluted, the readout will be negative; if the supernatant is too concentrated, the readout will be saturated.

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

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

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

vii. 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 (at 25°C).

viii. 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 (at 25°C).

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

ix. Stop the reaction by addition 100 μL of stop solution to all wells. Immediately read the plate at 490 nm wavelength 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.

48. Counting the NK cells: On the experimental day, count the NK cells with a hemocytometer. Add IL-15 medium to adjust the concentration as desired.
49. 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.
50. Formulation of the siRNA/**AD** complex: follow the procedure as described for $CD4^+$ T cells.
51. **AD**-mediated siRNA transfection: follow the procedure as described for $CD4^+$ T cells.

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.

52. Carefully transfer the NK cells from 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 (at 25°C), 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.

53. Determination of siRNA-mediated silencing of the target gene by qRT-PCR assay

i. RNA isolation: Follow the procedure as described for $CD4^+$ T cells or alternatively the manufacturer's instructions for the RNA isolation kit.

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

iii. cDNA synthesis and qRT-PCR: follow the procedure as described for $CD4^+$ T cells.

54. Determination of siRNA-mediated knockdown of NKG2D protein by flow cytometry

i. In the case that NKG2D siRNA is used for the transfection experiment, surface expression of the NKG2D receptor can be assayed by flow cytometry.

ii. Cell surface staining with anti-NKG2D antibody: After 2-3 days of transfection, wash the cells twice with 500 μL of PBS buffer as described. Re-suspend cell pellet with 50 μL of labeling buffer for each tube. Add the fluorescent dye-labeled anti-NKG2D antibody and gently mix by pipetting up and down for 15 seconds. Wrap the tubes in aluminum foil paper and incubate at room temperature (at 25°C) 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 (at 25°C). Discard supernatants.

iii. 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 (at 25°C). 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 and compensation controls in applied.

iv. Flow cytometry analysis: Proceed to acquisition of experimental data (surface NKG2D expression level) by flow cytometry (see EQUIPMENT 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 formulation of the siRNA/**AD** complex, and 2 days for the complex-mediated gene silencing.

55. Detaching the BMDM from Petri dish and cell counting: On day 7, detach cells from the culture petri dish using accutase as a detaching solution. Collect the cells in a 50 mL conical

tube and centrifuge at 150 g for 5 min at room temperature (at 25°C). Aspirate the supernatant and re-suspend the cells in DMEM base media and count the number of cells using a Countess™ II FL Automated Cell Counter using Trypan blue as a viability dye.

56. Seeding of macrophages: Seed 1×10^6 cells per well in 1 mL culture medium on a 12-well plate format. Incubate the cells at 37 °C in a humidified incubator under 5% CO_2 for 1 h for the attachment of the cells on the plate. After 1 h, centrifuge cells at 150 g for 5 min at room temperature (at 25°C) and remove the medium and refill with 800 µL of OPTI-MEM medium.
57. Formulation of the siRNA/**AD** complex: Follow the procedure as described for $CD4^+$ T cells.
58. AD-mediated siRNA transfection: Gently mix the complexes by pipetting up and down. Carefully drop the solution containing the siRNA/AD complex into cells and mix them by pipetting. 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 (at 25°C) and remove the OPTI-MEM and replace with DMEM base media and 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.

59. Wash the cell monolayer once with ice-cold DPBS with calcium and magnesium. For protein isolation, add 150 uL of complete supplemented RIPA buffer as cell lysis buffer 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.

60. Determine siRNA-mediated knockdown of target protein in macrophages by western blotting
61. 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 supernatant to a fresh tube and discard the pellet for cytoplasmic protein of interest.

62. Determine the protein concentration with the BCA assay, adjust the concentration in each sample to 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

63. 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.
64. Transfer the proteins from the gel to the nitrocellulose membrane in electrophoretic transfer buffer at 100 V for 60 min.
65. Block the membranes in blocking buffer for 1 h and incubate overnight at 2-8 °C with primary antibodies diluted 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 for 1 h.

    ? TROUBLESHOOTING

66. 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.

67. Separation of microglia from a monolayer mixed glial culture and cell counting: Grow the mixed glial cell cultures from newborn pups for 8-9 days. When bright microglial cells appear at the top of cell layer, shake the culture flasks on a vertical shaker at 100 rpm at 37 °C for 50 min, collect the medium and centrifuge at 150 g for 10 min at room temperature (at 25°C). Aspirate the supernatant leaving a small volume of culture medium to

re-suspend the cells, then count the cells using a Nucleocounter according to the manufacturer's procedure.

68. 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.
69. Formulation of the siRNA/**AD** complex: follow the procedure as described for $CD4^+$ T cells.
70. **AD**-mediated siRNA transfection: follow the procedure as described for $CD4^+$ T cells.

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.

71. Wash the cell monolayer twice with ice-cold PBS. To collect the cell lysates for total RNA extraction, follow the procedure as described for $CD4^+$ T cells 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 total RNA extraction or further processing for western blot analysis.

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

72. Determination of siRNA-mediated silencing of the target gene by qRT-PCR assay
    i. RNA isolation: Follow the procedure as described for $CD4^+$ T cells or alternatively the manufacturer's instructions for the RNA isolation kit.
    ii. Elute RNA with RNase-free water and quantify RNA concentration using a NanoDrop Microvolume Spectrophotometer
    iii. cDNA synthesis and qRT-PCR: follow the procedure as described for $CD4^+$ T cells.

    ?TROUBLESHOOTING
73. Determination of siRNA-mediated knockdown of target protein in microglia by western blotting

i. Remove the cell lysates from -80^{o}C, defrost them on ice and centrifuge at 16000 g for 15 min at 4 ^{o}C. Transfer the supernatant to a fresh tube and discard the pellet.

ii. Determine the protein concentration with the BCA assay, adjust the concentration in each sample to equal value (e.g. 0.5 μg/mL) and add an appropriate volume of 4× sample loading buffer. Boil at 98 ^{o}C for 5 min to denature the proteins.

iii. 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.

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

v. Block the membranes in blocking buffer for 1 h and incubate for 2 h at room temperature (at 25°C) or overnight at 2-8 ^{o}C with primary antibodies diluted in 5% BSA in TBS-T. The primary antibody reaction is followed by incubation with the peroxidase-conjugated secondary antibody for 1 h.

vi. 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 44-47: 3 days for functional assays of siRNA-mediated gene silencing

Steps 48-51: 1 day for AD-mediated siRNA transfection into NK cells

Steps 52-54: 3 days for functional assays of siRNA-mediated gene silencing

Steps 55-58: 2.5 hours for AD-mediated siRNA transfection into primary macrophages cells

Steps 59-66: 4 days for functional assays of siRNA-mediated gene silencing

Steps 67-70: 1 day for AD-mediated siRNA transfection into primary microglia

Steps 71-73: 3 days for functional assays of siRNA-mediated gene silencing

ANTICIPATED RESULTS

For the synthesis of **AD**, we only describe the yield. The detailed characterization is in the supplementary information. The synthesis 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 documented in the supplementary materials (**Figures SA1-4**).

AD-mediated siRNA delivery in primary T-cells was realized 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,[29] while HIV-1 Tat and Rev are essential positive regulators of virus gene expression.[30] 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. 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**). Both **AD** alone and a control DsiRNA/**AD** complex didn't generate any silencing effect (**Figure 4A**), neither affect the cell viability (**Figure SB1A**) [17]. When we compared the commercially available vectors, we found that Lipofectamine RNAiMAX (lipo) was unable to deliver the siRNA effectively (**Figure SB1B**), 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 a 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 the cytotoxic function when it recognizes ligands expressed by the target cell such as cancer cell.[31] 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 (**Figure SB2A**).[21] Also, the commercial vector Lipofectamine (lipo) was unable to deliver the siRNA in NK cells (**Figure SB2B**). 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,[32] inflammatory bowel disease,[33] and Areata alopecia.[34] Reduction of JAK1 expression in macrophages, in order to influence their activity in the inflammation, remains a challenging issue. Using **AD** as the vector for the delivery of JAK1-specific siRNA to mouse primary macrophages yielded to a significant reduction of JAK1 protein expression estimated of 67% of the normal by Western blot analyzed 48 h post-transfection with N/P ratio 5/1 (**Figures 4E** and **4F**). In addition, cell survived well during the treatment with **AD** (**Figure SB3A**), and the primary macrophages remained unpolarized upon treatment with **AD** and **AD**-complexed control siRNA (**Figure SB3B**). In contrast, commercial vector such as Lipofectamine RNAiMAX (lipo) did not decrease JAK1 protein expression level (**Figure SB3C**). Delivery of JAK1-specific siRNA to primary macrophages using **AD** as a potential vector 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 (**Figure SB4A**), remained unpolarized (**Figures SB4B** and **SB4C**) 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 (**Figure SB4B**) or Lipofectamine, induced strong inflammatory effects in primary microglia cultures.[20, 35] 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.[36, 37] Treatment of the primary microglia with the siRNA/**AD** complexes triggered effective downregulation of the target *id1* mRNA and its protein product, resulted 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-noxious siRNA delivery to primary immune cells. **AD** therefore constitutes the long-searched-for vector with the potential to delivery siRNA safely and effectively for the purpose of functional study and therapeutic applications in immune cells.

Conflicts of Interest

The authors declare no competing interests.

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Author contributions

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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.]

Step 1)	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 2)	Opti-MEM® Medium		10 µL	50 µL	100 µL	250 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 3)	Opti-MEM® Medium		10 µL	50 µL	100 µL	250 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 4)	Diluted **AD** + Diluted siRNA: Step 2)+ step 3) solutions		20 µL	100 µL	200 µL	500 µL
Step 5)	Incubate at room temperature (RT (at 25°C)) for 25 min to allow nanoparticles to form.					
Step 6)	Final volume: step 1) cell culture + step 4) complex		100 µL	500 µL	1000 µL	2500 µL
Step 7)	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 | 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 high boiling point and may be encapsulated within the dendrimer **3**.	Dissolve the product in CH_2Cl_2 and washed by brine solution. Collect the organic phase then remove CH_2Cl_2 to obtain the pure product
28	**3** is not well dissolved	Poor solubility in pure MeOH	After addition of EDA and increase of temperature to 30^{o}C, the dendrimer **3** can be completely dissolved and the final reaction solution will be a clear colorless solution. Sonication can be applied to help dissolve the starting material. Shake the flask gently to make sure **3** are well dissolved in the solution.
45	Suboptimal siRNA knockdown efficiency	Poor delivery or suboptimal N/P ratio	The formulation 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.
45	No significant siRNA knockdown efficiency	Poor total RNA quality	Total RNA extraction from HIV-1 infected primary CD4+ T cells may be challenging. After re-suspend 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.
45	False positive signal shown in no-RT control sample	Genomic DNA contamination	Ensure the DNase treatment is effective and completed.
47	HIV p24 ELISA readout is too higher or too lower to see.	Improper sample dilution	Ensure proper sample dilution by setting up 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.
62	Low protein yield	Insufficient number of cells for transfection or insufficient quantities of proteins to be detected by Western blot	Increase the number of seeded cells and control cell viability. If cell mortality is observed, revise the method of cell collection by more gentle and rapid handling.
65	High background in Western blots	Insufficient blocking of the membrane.	Check for blocking buffer and procedure; change membrane, some batches can be not appropriate.
66	Inefficient Western blot detections	Too low quantity of loaded proteins, 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.
72	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 (eg. cell viability). Use quick but more gentle 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.
72	Suboptimal knockdown efficiency	Too low siRNA concentration 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 reverse-transcription stage and use of a proper, endogenous control gene (*18SRNA, GAPDH*) in qPCR is

			required for valid and accurate evaluation of gene expression changes.
73	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 (eg. cell viability). Use quick but more gentle method for all washing steps during collection of cell lysates, avoiding scratching or disturbing of cell monolayer.
73	Suboptimal knockdown efficiency	Too low siRNA concentration, not optimal time point for the evaluation of the expression levels of selected target gene 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 (eg. Actin, GAPDH) to ensure equal protein loading in each sample.
73	No or poor quality chemiluminescence signal in Western blotting	Inefficient transfer of proteins from the gels to nitrocellulose or antibody failure.	Stain gels after electrotransfer to ensure a complete protein transfer. Check if a primary antibody is validated for use in Western blotting and check for proper selection of a secondary antibody.
73	Non-specific signal on the blots	Non-specific binding of primary antibody to the nitrocellulose membrane	Extent incubation of the membranes in a blocking buffer for 3-4 h or increase dilution of primary antibody.

Figure Legend:

Figure 1: Cartoon illustration of the siRNA delivery mediated by the amphiphilic dendrimer **AD**. **A)** The molecular structure of **AD**. [17] **B)** The **AD**-mediated siRNA delivery into various immune cells, including T cells, microglia, and NK cells.[17, 20, 21]

Figure 2: Chemical synthesis of the amphiphilic dendrimer **AD**.[17]

Figure 3: Experimental outline. A schematic illustrating dendrimer-mediated siRNA transfection into primary cells.

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 (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). (adapted from Ref 17).

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, ** $P < 0.01$ 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, ** $P < 0.01$ versus empty **AD**, one-way ANOVA). (adapted from Ref 21).

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 or Control siRNA. Transfection was done with 50nM 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 in 3 independent experiments shows a mean reduction of 67% of JAK1 specific expression after transfection (n = 3, * $p \leq 0.05$, by paired Student's *t* test).

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). Statistically significant differences between cultures treated with siId1/**AD** and siCtrl/**AD** were evaluated using the paired Student's *t*-test. * $p<0.05$; ** $p<0.01$. In western blotting evaluation, detection of Gapdh confirmed equal protein loading. (adapted from Ref 20).

Figure 5: Experimental outline. A schematic illustrating dendrimer-mediated siRNA delivery into HIV-1-infected primary human $CD4^+$ T cells.

Supplementary Information includes:

1) **Supplementary Methods**: Isolation of primary immune cells
2) **Supplementary Results A**: Analytical data of dendrimers
3) **Supplementary Results B**: Cell viability and siRNA delivery using commercial transfection vectors.

Figure SA1: ^{1}H- and ^{13}C-NMR spectra of **3**.

Figure SA2: High-resolution mass spectra of **3**.

Figure SA3: ^{1}H- and ^{13}C-NMR spectra of **AD**.

Figure SA4: High-resolution mass spectra of **AD**.

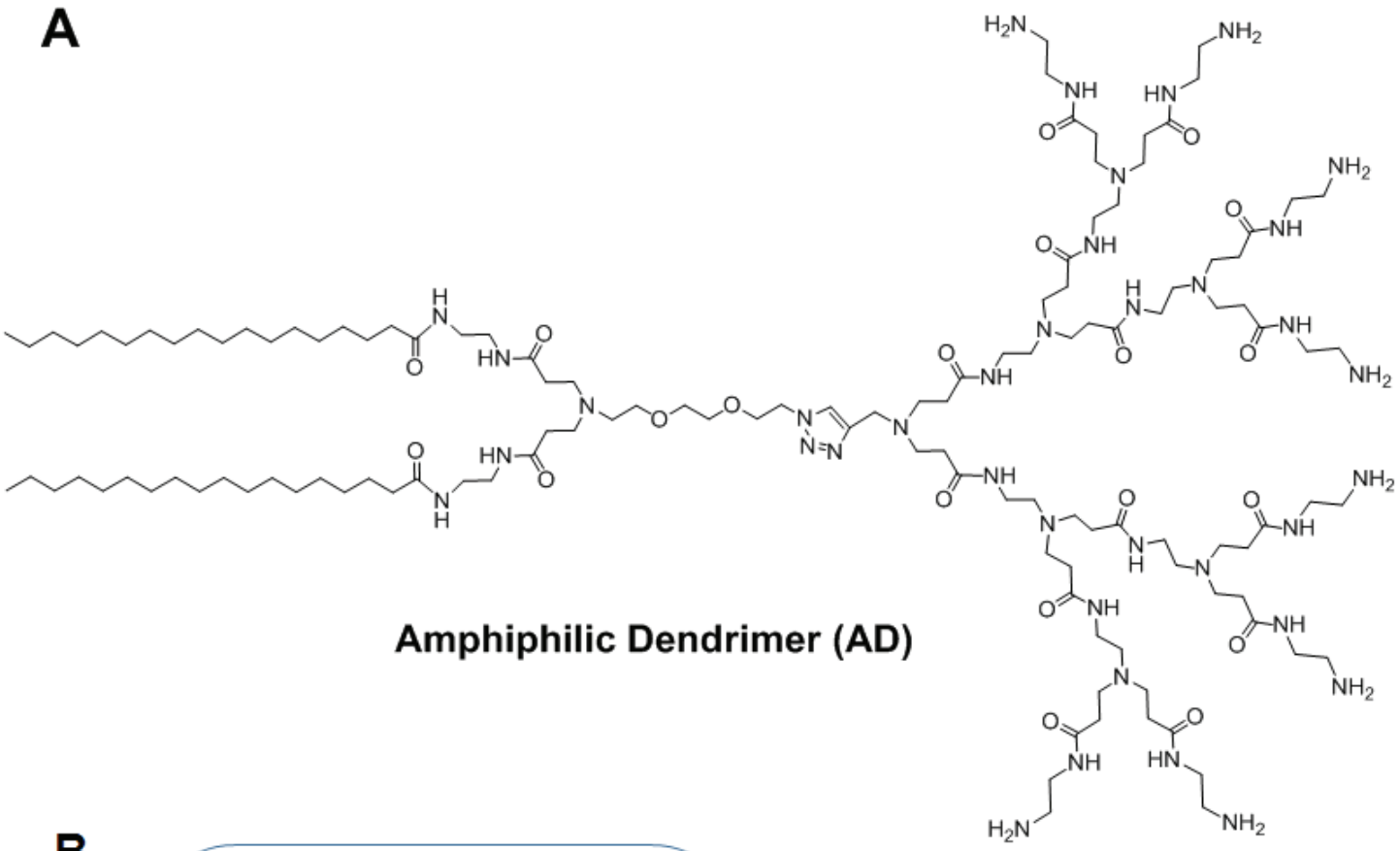
Figure SB1. Cell viability of primary T-cells after treatment with dendrimer **AD** and siRNA delivery using commercial transfection vector Lipofectamine RNAiMAX (lipo). (A) No significant toxicity was observed in PBMC CD4+ cells following treatment with **AD** and 50 nM dsiRNA at a N/P ratio of 5 (n = 4, data are expressed as mean ± SD, one-way ANOVA). (B) No effective gene silencing was achieved using commercial transfection reagent Lipofectamine RNAiMAX (lipo) and dsiRNA (50 nM) in primary PBMC CD4+ cells (n = 3, data are expressed as mean ± SD, one-way ANOVA).

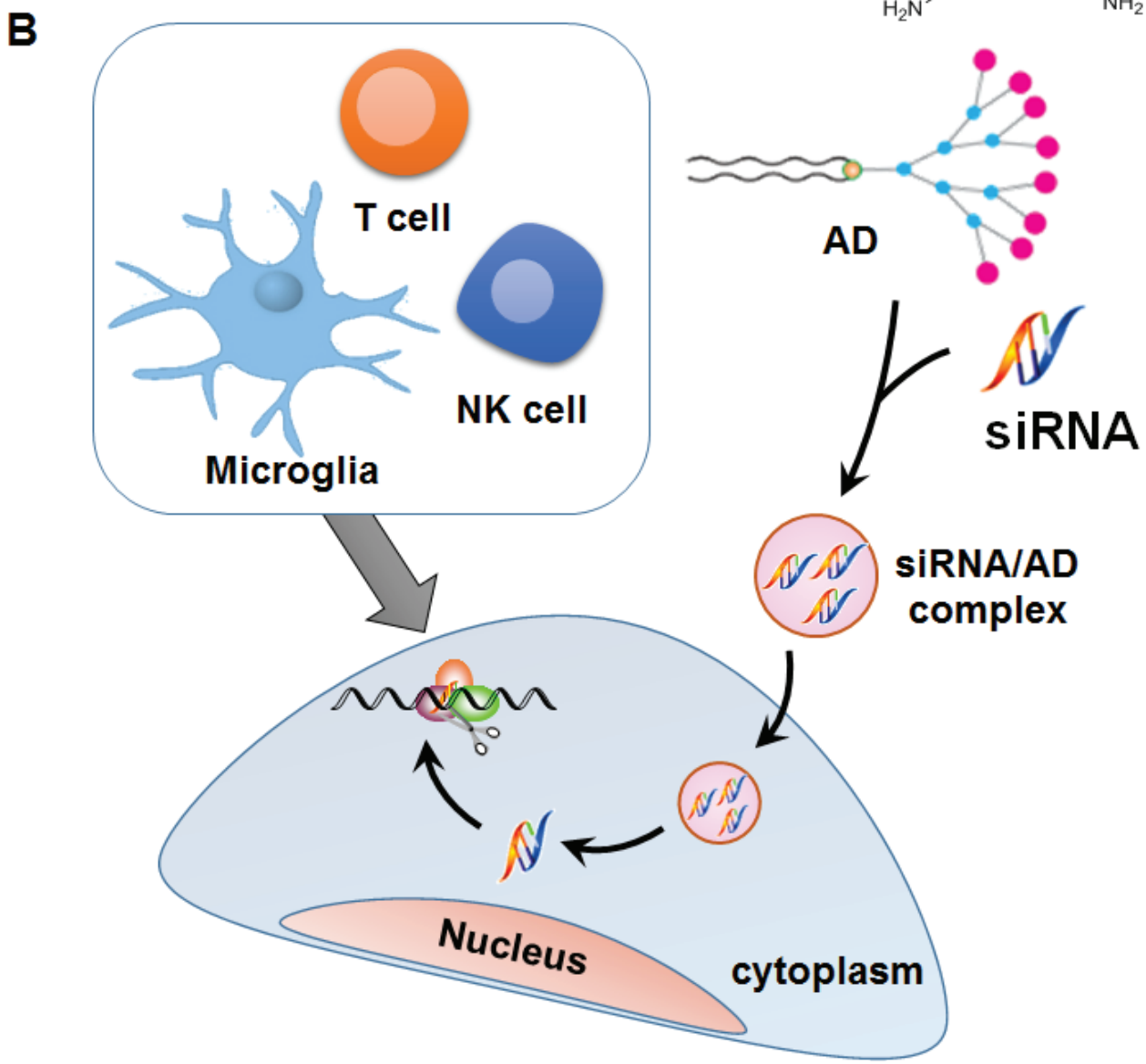
Figure SB2. Cell viability of primary NK-cells after treatment with dendrimer **AD** and klrk1 gene expression using commercial transfection vector Lipofectamine RNAiMAX (lipo). (A) Dendrimer **AD** alone does not affect the number of NK cells. Murine NK cells were cultured alone or incubated with **AD** as indicated. The number of NK (NK1.1-/CD3+) cells after 24 h of stimulation was assessed by FACS. (n = 3; data are expressed as mean ± S.E.M. ns, not significant, one-way ANOVA). (B) Transfection with commercial vector Lipofectamine RNAiMAX (lipo) did not reduce the expression of klrk1 in murine NK cells (n = 4; data are expressed as mean ± S.E.M., one-way ANOVA).

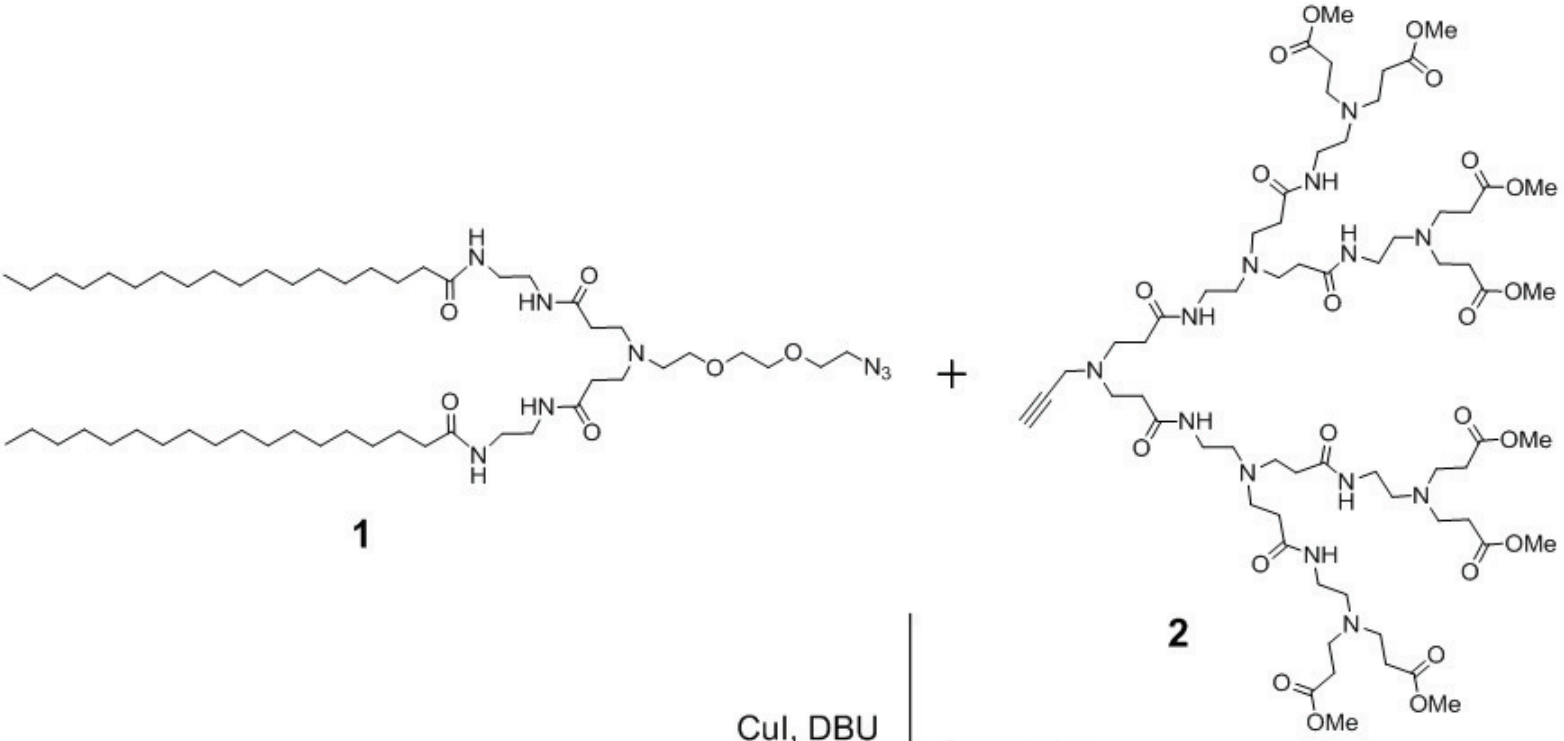
Figure SB3. Assay of cell viability and macrophage polarization upon treatment with siRNA/**AD** complexes as well as siRNA delivery using commercial transfection vector Lipofectamine RNAiMAX (lipo). (A) Cell viability of the primary macrophages was not affected upon treatment with **AD**-mediated delivery of siRNA JAK1 (siJAK1) and siRNA control (siCtrl). Cell viability was measured using CytoTox-ONE™ Homogeneous Membrane Integrity Assay (n = 3, data are expressed as mean ± S.D.); B) The transfection of siRNA JAK1 (siJAK1) and siRNA control (siCtrl) in primary macrophages using lipofectamine RNAiMAX (lipo) was not effective. The protein expression was assessed using western blotting. (C) The polarization of macrophages was assessed by the analyzing the expression of iNOS and arginase1 using western blotting. M0 stands for unpolarized primary macrophages, M1 for LPS polarized macrophages expressing high level of iNOS and M2 for IL-4 polarized macrophages expressing high level of arginase-1. In the protocol, M0 unpolarized

macrophages, which were transfected with siRNA control (siCtrl) complexed with **AD** at N/P 5, remained unpolarized.

Figure SB4. The effect of **AD** on viability and polarization of primary microglia and siRNA delivery using a commercial transfection reagent Viromer. (A) Viability of rat microglial cells was not affected by **AD** alone or **AD** complexed with non-targeting siRNA (siCtrl) at N/P=10, 12.5 nM siRNA. Number of proliferating cells was evaluated using BrdU incorporation assay at 72 h post-treatment (n = 3, mean ± SD). (B) **AD**-mediated siRNA delivery did not upregulate the inflammatory response in rat microglia in contrast to a commercial vector Viromer. Primary rat microglia cultures were transfected with 25 nM of the control non-targeting siRNA (siCtrl) using **AD** at N/P 10 or commercial vector Viromer. The mRNA level of the inflammatory gene *irf7* was measured using qPCR 24 h post-transfection and presented as a fold change relative to untreated cells (n = 3, mean ± SD). (C) There was no significant change in the expression of genes related to pro-inflammatory (nos2, cox2 and irf7) or pro-invasive (arg1, cmyc and id1) phenotype in microglia upon treatment with **AD** alone or **AD** complexed with non-targeting siRNA (siCtrl) at N/P=10, 12.5 nM siRNA. The mRNA levels were measured using qPCR 48 h post-transfection and presented as a fold change relative to untreated cells (n = 3, mean ± SD). Groups were compared using two-way ANOVA with a post-hoc Tukey test for multiple comparisons. *** $p<0.001$.


A
Amphiphilic Dendrimer (AD)


B
T cell
NK cell
Microglia
AD
siRNA
siRNA/AD
complex
Nucleus
cytoplasm



1

2

CuI, DBU
DMF, 60 °C

Step 1-24

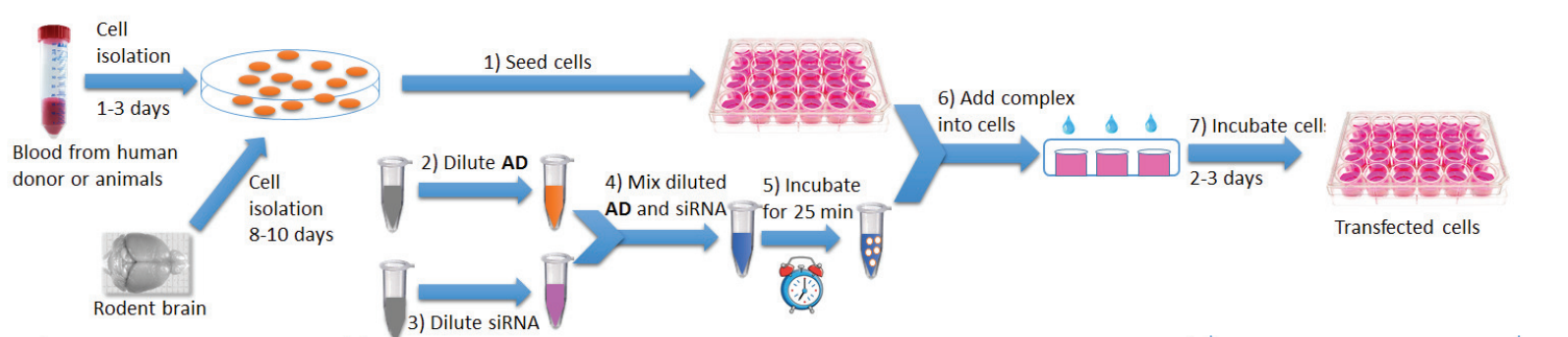
3

H_2N NH_2

MeOH, 30 °C

Step 25-29

AD



Days before assay –
Isolation of cells
(expansion/activation if need)

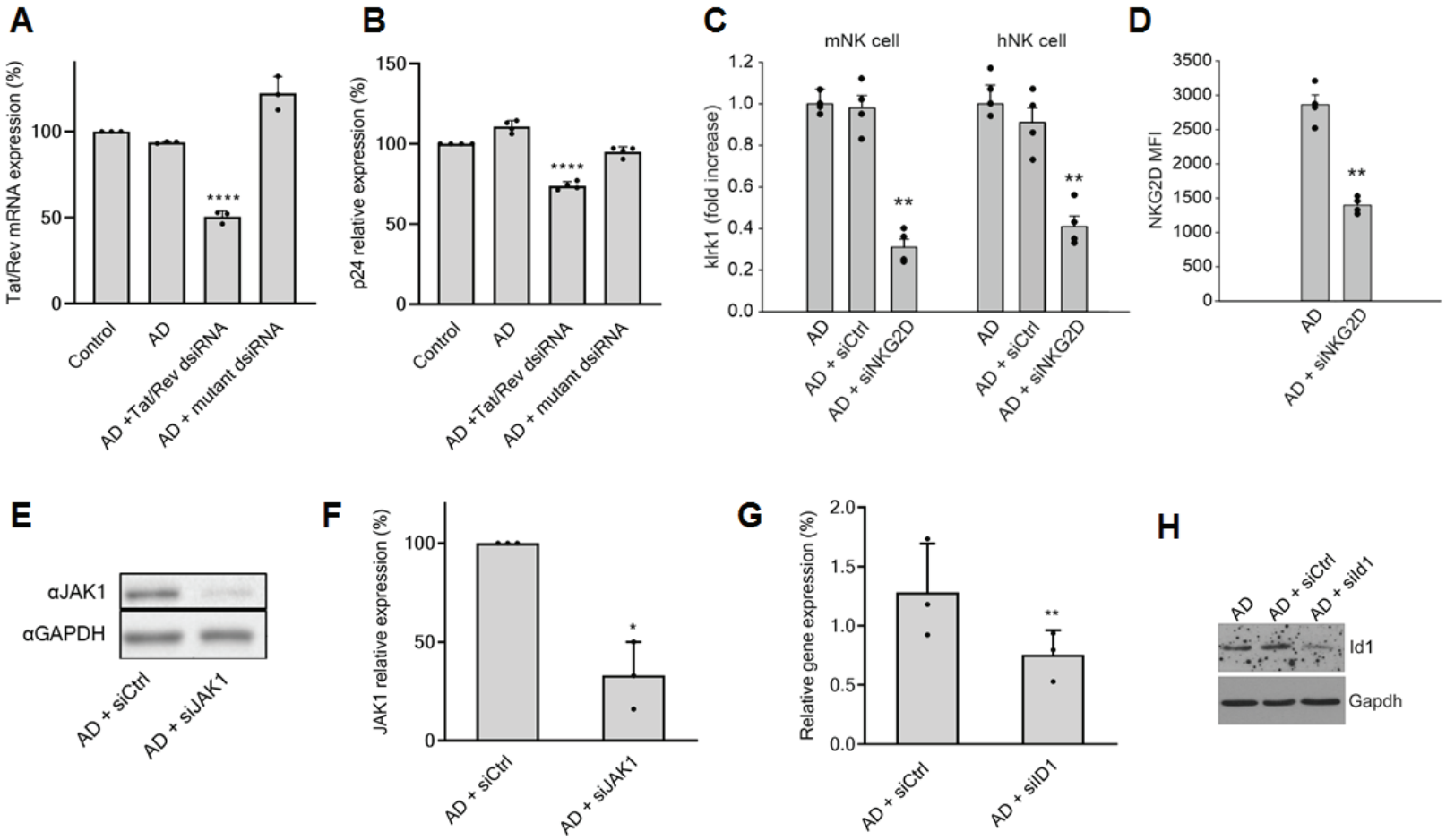
- B-lymphocytes
- T-lymphocytes: CD4+ T, CD8+ T cells
- Natural killer cells
- Monocytes: macrophages, microglia, dendritic cells
- Granulocytes: neutrophils, eosinophil, basophil

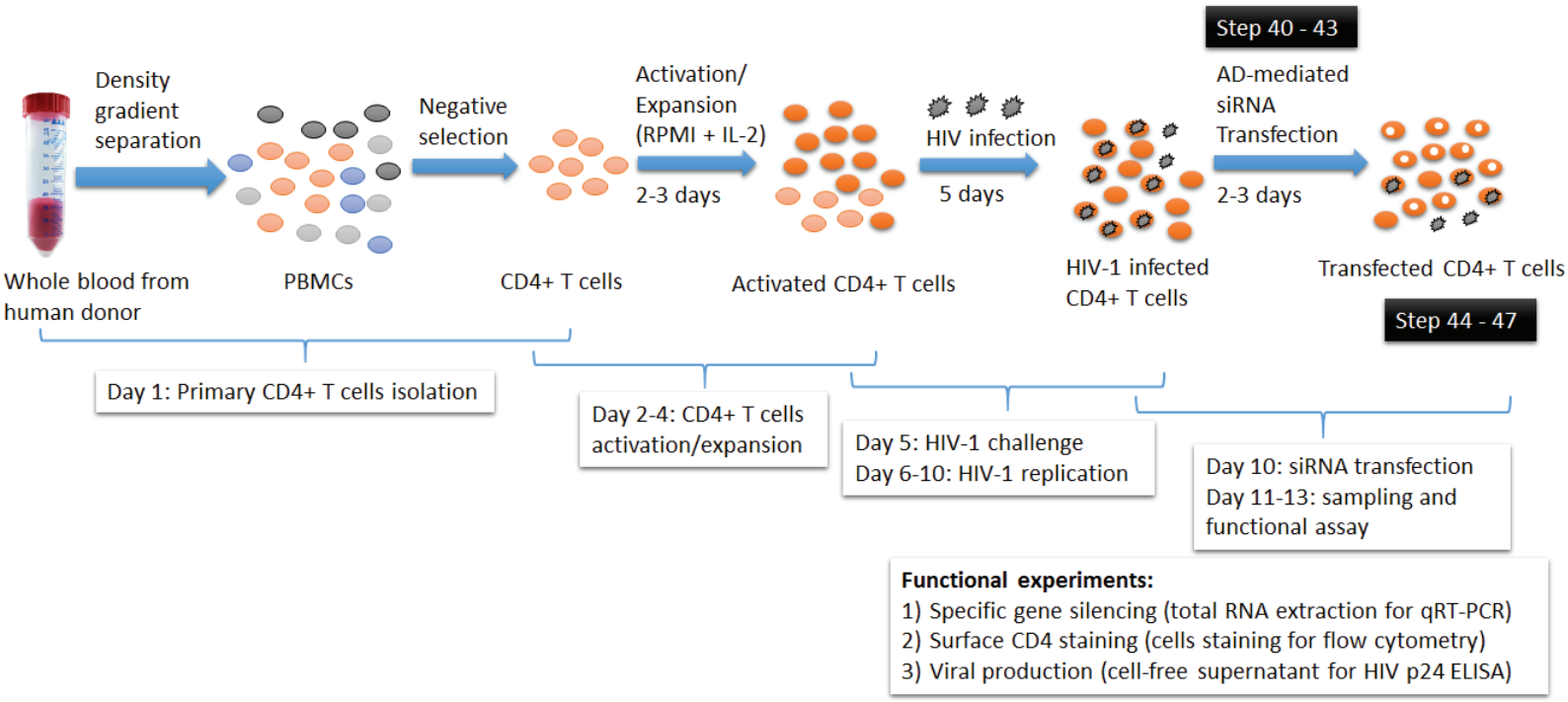
Day 1 – cell preparation and transfection

1) Seed cells to be 70-90% confluent at transfection
2) Dilute **AD** in Opti-MEM® Medium – Mix well
3) Prepare master mix of siRNA by diluting siRNA in Opti-MEM® Medium at desired concentration
4) Add diluted siRNA to each diluted **AD** solution (1:1 ratio)
5) Incubate at room temperature (RT) for 25 min to allow nanoparticles to form
6) Add **AD**-siRNA complex dropwise to cells
7) Incubate cells in a humidified 5% CO_2 incubator at 37 °C

Day 2-3: Sampling and functional experiments

1) Specific gene silencing (total RNA extraction for qRT-PCR)
2) Target protein downregulation (protein extraction for western blotting, surface protein staining for flow cytometry)
3) Internalization study (confocal microscopy)
4) Toxicity study (MTS assay)
5) Other assays to determine immune cell function


A
Tat/Rev mRNA expression (%)
100
50
0
****
Control
AD
AD +Tat/Rev dsiRNA
AD + mutant dsiRNA
B
p24 relative expression (%)
150
100
50
0
****
Control
AD
AD +Tat/Rev dsiRNA
AD + mutant dsiRNA
C
mNK cell
hNK cell
klrk1 (fold increase)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
AD
AD + siCtrl
AD + siNKG2D
AD
AD + siCtrl
AD + siNKG2D
D
NKG2D MFI
3500
3000
2500
2000
1500
1000
500
0
**
AD
AD + siNKG2D
E
αJAK1
αGAPDH
AD + siCtrl
AD + siJAK1
F
JAK1 relative expression (%)
100
50
0
*
AD + siCtrl
AD + siJAK1
G
Relative gene expression (%)
2.0
1.5
1.0
0.5
0.0
**
AD + siCtrl
AD + siId1
H
AD
AD + siCtrl
AD + siId1
Id1
Gapdh


Step 40 - 43
Density gradient separation
Negative selection
Activation/ Expansion (RPMI + IL-2)
2-3 days
HIV infection
5 days
AD-mediated siRNA Transfection
2-3 days
Whole blood from human donor
PBMCs
CD4+ T cells
Activated CD4+ T cells
HIV-1 infected CD4+ T cells
Transfected CD4+ T cells
Step 44 - 47
Day 1: Primary CD4+ T cells isolation
Day 2-4: CD4+ T cells activation/expansion
Day 5: HIV-1 challenge
Day 6-10: HIV-1 replication
Day 10: siRNA transfection
Day 11-13: sampling and functional assay
Functional experiments:
1) Specific gene silencing (total RNA extraction for qRT-PCR)
2) Surface CD4 staining (cells staining for flow cytometry)
3) Viral production (cell-free supernatant for HIV p24 ELISA)