Cellular and Molecular Life Sciences (2020) 77:2859–2870
https://doi.org/10.1007/s00018-019-03334-8

ORIGINAL ARTICLE

Inhibition of HIV replication through siRNA carried by CXCR4-targeted chimeric nanobody

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Received: 15 April 2019 / Revised: 24 September 2019 / Accepted: 3 October 2019 / Published online: 22 October 2019

Abstract

Small interfering RNA (siRNA) application in therapy still faces a major challenge with the lack of an efficient and specific delivery system. Current vehicles are often responsible for poor efficacy, safety concerns, and burden costs of siRNA-based therapeutics. Here, we describe a novel strategy for targeted delivery of siRNA molecules to inhibit human immunodeficiency virus (HIV) infection. Specific membrane translocation of siRNA inhibitor was addressed by an engineered nanobody targeting the HIV co-receptor CXCR4 (NbCXCR4) in fusion with a single-chain variable fragment (4M5.3) that carried the FITC-conjugated siRNA. 4M5.3–NbCXCR4 conjugate (4M5.3X4) efficiently targeted $CXCR4^+$ T lymphocytes, specifically translocating siRNA by receptor-mediated endocytosis. Targeted delivery of siRNA directed to the mRNA of HIV transactivator *tat* silenced Tat-driven viral transcription and inhibited the replication of distinct virus clades. In summary, we have shown that the engineered nanobody chimera developed in this study constitutes an efficient and specific delivery method of siRNAs through CXCR4 receptor.

Keywords Small interfering RNA · CXCR4 · Nanobody · Delivery · HIV

Introduction

Methods of gene modulation have gained tremendous interest for therapeutic purposes, with RNA interference (RNAi) being one of the most well known. This mechanism of post-transcriptional gene silencing is mediated by RNA duplexes of 21–23 nucleotides, termed small interfering RNAs (siRNAs), that trigger the cellular degradation of cognate mRNAs [1]. The silencing potency, target specificity, universal conservation, and broad application—namely to non-druggable targets—of siRNA render it attractive for therapeutics. However, intrinsic characteristics of these small RNA-based molecules such as negative charge and hydrophilicity—responsible for inefficient cellular uptake—settle the need for innovative delivery strategies.

Local administration of siRNAs is sufficient for some clinical applications as reported previously for intraocular and intracerebral approaches in vivo [2]. Nonetheless, and despite the diverse available methods, limitations have been associated with systemic delivery of therapeutic siRNAs, mandatory to target diseases where affected cells/tissues are inaccessible or widespread throughout the human body. Conjugation of siRNA molecules with cell-penetrating peptides results in rapid clearance of the RNA inhibitor due to carrier's size below the threshold of renal filtration [3]. Liposome-based methods are known to trigger diverse inflammatory pathways [4] and to hamper intracellular release of siRNA molecules [5]. In several cases, a high dosage is required to induce a therapeutic benefit [6], which results in burden costs, possible off-target effects, and toxicity.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00018-019-03334-8) contains supplementary material, which is available to authorized users.

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Cell-targeted siRNA delivery through natural cell receptor–ligands or antibody-based formats should address such limitations and overall potentiate the therapeutic benefit.

Despite reported benefit on oncologic patients [7, 8], siRNA therapeutics in non-cancer diseases such as HIV infection remains a hurdle. T lymphocytes targeted by HIV are not only hard-to-transfect cells with conventional lipid-based strategies but also concentrated on densely packed environments/tissues. The high stability and specificity as well as the small format of nanobodies—constituted by the variable domain of camelid heavy-chain-only antibodies [9]—make them excellent candidates as tailored vehicles against therapeutically relevant targets. Extensive studies revealed peculiar chemical and biophysical characteristics of nanobodies. These small antibody fragments can present an array of conformations, namely non-canonical architectures, often improving optimal fitness towards antigen and consequently binding affinity [10]. Nanobodies are also stable and soluble domains presenting no signs of aggregation when produced individually, which contrasts with the early isolated small formats of human antibodies [11]. CXCR4 constitutes a highly attractive target for delivery because this receptor is efficiently and rapidly internalized after interaction with its natural ligand, SDF-1α [12]. Moreover, CXCR4 is overexpressed in CD4-positive ($CD4^+$) T lymphocytes, mainly HIV-infected T cell population. Previous studies have successfully implemented a CXCR4-binding antibody for targeted delivery of cytotoxic drugs for cancer cells treatment [13] and CXCR4-specific peptides for transfection of DNA molecules [14, 15]. Therefore, we explored the potential of CXCR4 targeting to develop a novel nanobody-based delivery of anti-HIV siRNA.

Here, we report the targeted delivery of a siRNA to HIV-susceptible CXCR4-positive ($CXCR4^+$) cells via a scFv (single-chain variable fragment)–nanobody fusion protein, herein termed 4M5.3X4. This chimera is composed of a nanobody portion previously validated for targeting of CXCR4 [16] fused to an anti-fluorescein (FITC) scFv responsible for carrying the FITC-labeled siRNA [17]. We show that 4M5.3X4 selectively binds and releases its siRNA cargo to CXCR4-bearing cells. 4M5.3X4-mediated delivery of siRNA targeting HIV transactivator Tat silences viral transcription and inhibits virus infectivity. To the best of our knowledge, we demonstrate for the first time the potential of a nanobody-based vehicle for efficient delivery of HIV inhibitor molecules. This strategy also supports the concept of using next-generation nanobody-based chimeras for targeted delivery of siRNA inhibitors to treat a wide range of diseases.

Materials and methods

Cells and viruses

The following reagents were obtained through the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH): Jurkat Clone E6-1 T cells from A. Weiss [18], Sup-T1 from James Hoxie [19], TZM-bl from J. C. Kappes, X. Wu and Tranzyme Inc. [20–24], pNL4-3 from M. Martin [25], and human rIL-2 from Dr. Maurice Gately, Hoffmann-La Roche Inc [26]. Human embryonic kidney 293T (HEK293T) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA).

Jurkat Clone E6-1 T cells and Sup-T1 cells were maintained in RPMI-1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo scientific, Waltham, MA), 1% (v/v) penicillin–streptomycin solution (PSA; Thermo scientific, Waltham, MA), and 2 mM L-glutamine (Thermo scientific, Waltham, MA). HEK293T and TZM-bl cells were maintained in DMEM medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) FBS, 1% (v/v) PSA, and 2 mM L-glutamine. Peripheral blood mononuclear cells (PBMCs) from healthy donors were obtained by density gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare, Chicago, IL). Non-stimulated $TCD4^+$ lymphocytes were then isolated and activated as described [27]. Cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

HIV strain 03PTHCC6 was isolated from an HIV-2-infected patient and 01PTHDECJN from an HIV-1 as described elsewhere [28].

siRNAs

siRNA directed against *tat* was designed as previously described [29]:

5′-GCGGAGACAGCGACGAAGAGCTTdTdT-3′ (sense);
5′-GCUCUUCGUCGCUGUCUCCGCdTdT-3′ (antisense).

The siRNA described was labeled with FITC at the 5′ end of the sense strand (A4 grade, Dharmacon Research, Lafayette, CO). Scrambled siRNA (MISSION siRNA Universal Negative Control #1 6-FAM) was purchased from Sigma-Aldrich (St. Louis, MO).

Constructions

FITC-binding scFv (4M5.3) [17] and CXCR4-targeting (282D2) [16] and irrelevant nanobodies were synthetized by Geneart (Regensburg, Germany). Sequence of CXCR4-targeting 282D2 nanobody was obtained from US 2011/0318347 A1 patent [30]. Fragments of 4M5.3 scFv and CXCR4-specific and irrelevant nanobodies were originated by PCR using the primer sequences provided in Supporting Information (Table S1). 4M5.3X4 and 4M5.3I constructions were assembled by ligation and cloned into the NheI and XhoI restriction sites of pET-21a(+) expression vector (Novagen, Madison, WI). CXCR4-targeting nanobody alone was directly cloned into NheI/XhoI sites of pET-21a(+) plasmid. The pCMV-Myc-Tat plasmid was generated by cloning *tat* gene from pNL4-3 into pCMV-Myc vector (Clontech, Takara Bio USA, Inc.). Constructions were verified by DNA sequencing analysis.

Protein expression and purification

Nanobodies for targeted siRNA delivery were produced by soluble protein purification in *Escherichia coli* shuffle cells (NEB, Ipswich, MA). Chemically competent cells were transformed with 4M5.3X4, 4M5.3I, and anti-CXCR4 nanobody alone. Cultures were growth in 1 L of Super Broth (SB) medium supplemented with 100 µg/mL of ampicillin and 20 mM of $MgCl_2$ at 30 °C until OD_{600} of 0.4, induced with 0.4 mM IPTG and further incubated at the same temperature for 4 h. Bacterial cells were collected and resuspended in 40 mL of 20 mM HEPES, pH 7.4, 500 mM NaCl, and 20 mM imidazole before disruption through sonication during 20 min. Proteins were purified by immobilized metal affinity chromatography (IMAC), using His GraviTrap columns (GE Healthcare, Chicago, IL). Purified proteins were buffer exchanged to 20 mM HEPES, pH 7.4, 500 mM NaCl, and 5% glycerol using PD-10 Desalting Columns (GE Healthcare, Chicago, IL).

Purification of all proteins was confirmed by Western blot technique as described previously [31, 32]. Purity was assessed by Coomassie-blue staining and quantification measured by Bradford method according to the manufacturer's instructions.

Cell line construction and virus production

The protocol for construction of Jurkat $CXCR4^-$ cell line through CRISPR/Cas9 gene knockout was adapted from Hou et al. [33]. For the production of CXCR4-CRIPR/Cas9 lentivirus, HEK293T seeded in six-well plate were transfected with 1.3 µg lentiCXCR4-gRNA-Cas9 #6, 1 µg psPAX2, and 0.7 µg pMD2.G plasmids (kindly provided by D. Guo) using lipofectamine 3000 reagent (Invitrogen) according to the improved lentiviral production protocol from the manufacturer. Jurkat T cells (1.0×10^5) seeded in 24-well plate were transduced with 400 ng of p24 lentivirus and incubated for 3 days. Cells lacking surface CXCR4 were sorted by flow cytometry by negative selection of CXCR4-stained cells with APC (allophycocyanin)-conjugated anti-human CXCR4 antibody 12G5 (Biolegend, San Diego, CA).

To produce HIV viral particles, HEK293T seeded in six-well plate were transfected with 3 µg pNL4-3 plasmid using lipofectamine 3000 reagent (Invitrogen, Waltham, MA) according to the improved lentiviral production protocol from the manufacturer.

All viral titers were determined by HIV-1 $p24^{CA}$ antigen capture assay kit (Frederick National Laboratory for Cancer Research—AIDS and Cancer Virus Program, Frederick, MD).

Cell-surface binding and internalization of siRNA inhibitor

FITC-conjugated scramble and *tat* siRNAs were mixed with 4M5.3X4, 4M5.3I control or PBS at a molar ratio 2:1 (4M5.3X4 100 pmol) for 1 h at 37 °C. $CXCR4^+$ and $CXCR4^-$ Jurkat T cells (2.0×10^5 in 100 µl culture medium) or primary T lymphocytes were treated for 2 h at 4 °C and washed twice with PBS or 37 °C and washed twice with trypsin for 3 min. Cells were then analyzed by flow cytometry. For positive control, Jurkat cells were transfected with FITC-siRNA using INTERFERin (Polyplus, Illkirch, France) according to the manufacturer's instructions. The FITC-conjugated tat siRNA was also mixed with increasing amounts of 4M5.3X4 (0 pmol, 6 pmol, 12 pmol, 25 pmol, 50 pmol, 100 pmol or 200 pmol) at a molar ratio of 2:1 for 1 h at 37 °C and then used to treat $CXCR4^+$ Jurkat T cells for 2 h at 37 °C. The cells were washed twice with trypsin for 3 min and analyzed by flow cytometry for the detection of FITC-positive population. For competition assays, *tat* siRNA was mixed with 4M5.3X4 in the presence of crescent quantities of NbCXCR4 (competitor) for 2 h at 37 °C. Cells were washed twice with trypsin for 3 min and analyzed by flow cytometry for detection of FITC-positive population.

Silencing of HIV LTR transcription

For siRNA-mediated LTR shutdown, TZM-bl cells were plated onto 24-well plates at a density of 1.0×10^5 cells per well. At 24 h after plating, cells were transfected with 500 ng of pTat plasmid (kindly provided by M. Simurda) using lipofectamine 3000 (Invitrogen, Waltham, MA) according to the manufacturer's instructions. At 24 h post-transfection, scramble and *tat* siRNAs were mixed with 4M5.3X4, 4M5.3I control or PBS at a molar ratio 2:1 (4M5.3X4 100 pmol) for 1 h at 37 °C. TZM-bl cells

were washed with PBS and then treated with these siRNA carriers for 3 h. At 24 h post-treatment, luciferase activity was measured by OneGlo™ Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized to total amount of protein, as quantified by Bradford method. Cells' viability was assessed by alamarBlue reagent according to the manufacturer's instructions.

tat mRNA and protein degradation

Scramble and *tat* siRNAs were mixed with 4M5.3X4, 4M5.3I control or PBS at a molar ratio 2:1 (4M5.3X4 100 pmol) for 1 h at 37 °C and incubated with HEK293T cells transfected with pCMV-Myc-Tat for 24 h prior to treatment. Cells were then harvested 24 h post-treatment and equally divided into two parts for total RNA and protein extraction.

Total cellular RNA was extracted using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA) and eluted in diethyl pyrocarbonate (DPEC)-treated water. Complementary DNA (cDNA) was synthetized using the cDNA synthesis kit (Promega, Madison, WI) and used as template for SYBR green-based quantitative PCR (qPCR) of the *tat* transcription using forward primer: 5′-ATGGAGCCAGTAGATCCTAGACTAGAG-3′ and reverse primer: 5′-CGTCGCTGTCTCCGCTTCTTCCT-3′. The transcription of human β-actin housekeeping gene was detected using the primers forward: 5′-AGGCACCAGGGC GTGAT-3′ and reverse: 5′-GCCCACATAGGAATCCTT CTGAC-3′. HIV-1 Tat relative gene expression was determined using the Delta–Delta Ct method and normalized to *tat*-containing plasmid-transfected cells (non-treated).

Protein extraction was performed by lysing cells with RIPA lysis buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate and 0.1% SDS) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland). Protein was quantified using the BCA Protein Assay Kit (Pierce, Thermo scientific, Waltham, MA). Protein lysate was resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. The membranes were blocked with 1% BSA and 5% non-fat dry milk (Himedia Laboratories) in 1X PBS and washed twice with 0.1% Tween 20 in PBS 1X. Membrane was incubated with 1:1000 of anti-c-myc (Clontech, Takara Bio USA, Inc.) or 1:10,000 of anti-GAPDH (Cell Signaling Technology, Danvers, MA) primary antibodies, washed with 1X PBS containing 0.1% Tween 20 and probed with horseradish peroxide (HRP)-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch, Cambridge, UK). Blots were developed using ECL (enhanced chemiluminescent) reagent.

HIV inhibition assays

For siRNA-mediated viral inhibition, Sup-T1 cells (1.0×10^5) were infected with 10 ng p24 of HIV-1 clone NL4-3 in 24-well plates. At 24 h post-infection, scramble and *tat* siRNAs were mixed with 4M5.3X4, 4M5.3I control or PBS at a molar ratio 2:1 (4M5.3X4 100 pmol) for 1 h at 37 °C and further incubated with cells for 3 h. After 3 days of infection, HIV replication was assessed by p24 capsid quantification on culture supernatants (HIV-1 $p24^{CA}$ antigen capture assay kit; Frederick National Laboratory for Cancer Research—AIDS and Cancer Virus Program, Frederick, MD).

For comparing inhibitory capacity of 4M5.3X4-delivered siRNA among HIV primary isolates, TZM-bl cells (1.0×10^4) were infected with 2.000 $TCID_{50}$/ml of HIV-1 01PTHDECJN or HIV-2 03PTHCC6 strains. At 24 h post-infection, *tat* siRNA was mixed with 4M5.3X4 at a molar ratio 2:1 (4M5.3X4 500 nM) in PBS for 1 h at 37 °C and further incubated with the cell–virus mixture for 3 h. Following 48 h, percent neutralization was determined by calculating the difference in average relative light unit (RLU) between test wells containing 4M5.3X4 + siRNA *tat* and HIV primary isolates and the wells containing the virus controls after the normalization of the results using the average RLU of cell control wells with 4M5.3X4 + siRNA. Results were considered valid if the average RLU of virus control wells was > 10 times the average RLU of cell control wells with 4M5.3X4 + siRNA.

Results

4M5.3X4 scFv–nanobody chimera specifically delivers siRNA inhibitor to $CXCR4^+$ cells

To develop a strategy for targeted delivery of siRNAs, we aimed to design a novel construct capable of carrying inhibitory siRNA molecules to $CXCR4^+$ cells through small antibody formats. We took advantage of a previously validated heavy-chain variable domain from camelid (nanobody) specific for CXCR4 [16] and explored its potential to promote receptor-mediated endocytosis of siRNA. CXCR4-targeted nanobody, herein named NbCXCR4, was fused to the C terminus of an anti-fluorescein (FITC) scFv (4M5.3) through a flexible GGGGS linker to originate the 4M5.3X4 chimera (Fig. 1a, b; Fig. S1). The 4M5.3 scFv was chosen due to its exceptionally high binding affinity for FITC in the femtomolar range (the highest known engineered affinity) [17], optimal to carry fluorochrome-conjugated small molecules such as siRNA inhibitors. As negative controls for targeted delivery, we constructed a chimera composed of 4M5.3 portion fused to an irrelevant nanobody non-targeting

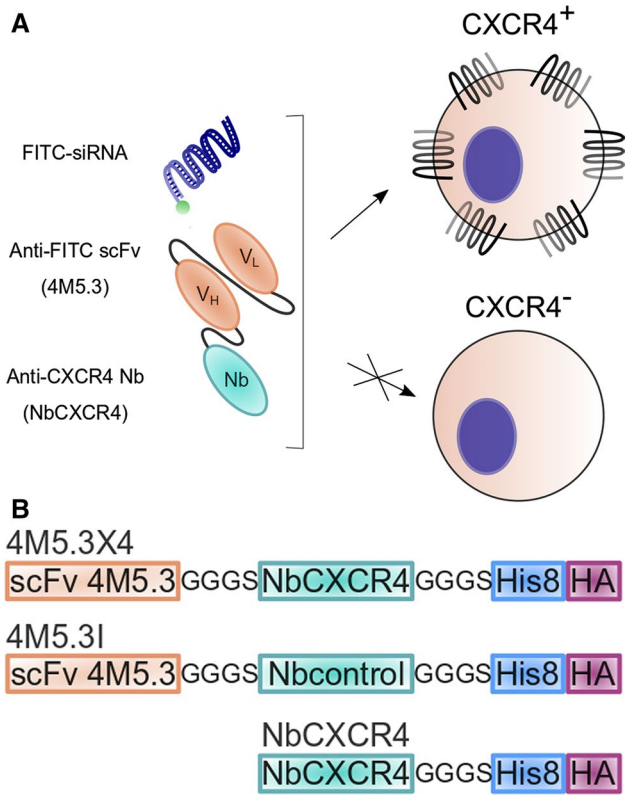


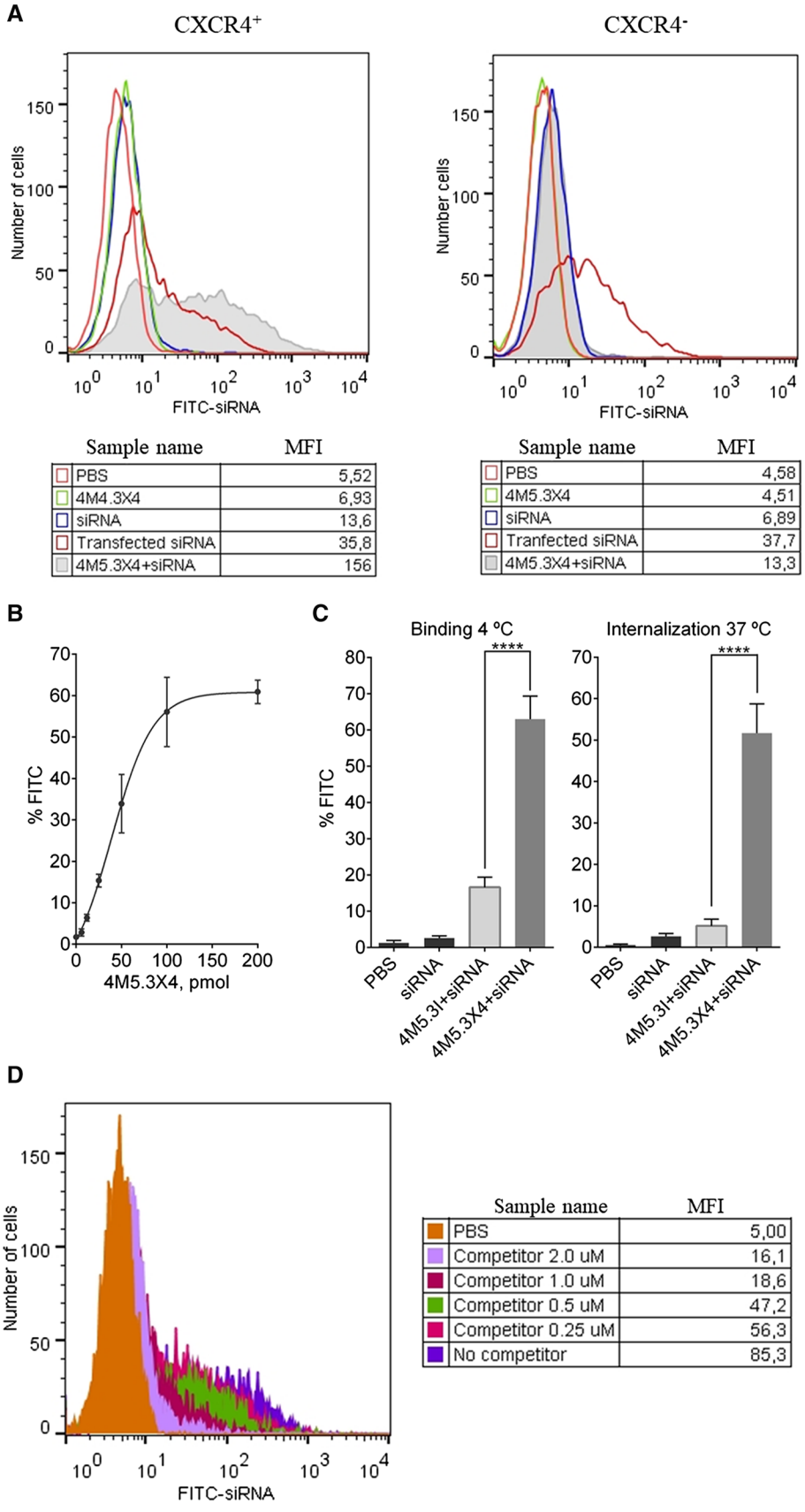
Fig. 1 Design of CXCR4-targeted scFv–nanobody chimera. **a** Representation of CXCR4-targeted strategy for siRNA delivery through scFv–nanobody chimera. The 4M5.3 scFv is constituted by the variable domains of the homonym antibody specific for the FITC fluorochrome connected by a flexible linker [17]. This part of the chimera carries the fluorochrome-tagged siRNA, whereas the nanobody fragment (NbCXCR4) targets CXCR4-positive cells. The nanobody part is constituted by the heavy-chain variable domain of an anti-CXCR4 camelid antibody. Only cells displaying cell-surface CXCR4 receptor should internalize the scFv–nanobody chimera tagged with siRNA cargo. *siRNA* small interfering RNA, *scFv* single-chain variable fragment, V_L light-chain variable domain, V_H heavy-chain variable domain, *Nb* nanobody. **b** Schematic representation of siRNA delivery construct and controls. Anti-FITC scFv (4M5.3) is positioned at the N-terminal of CXCR4-targeted (NbCXCR4) or irrelevant (Nbcontrol) nanobody to generate 4M5.3X4 or 4M5.3I, respectively. NbCXCR4 control construct is devoid of anti-FITC scFv fragment. GGGS linkers were placed between the scFv fragment and the nanobody portion or histidine (His) and hemagglutinin A (HA) tags, respectively, for protein purification or detection

mammalian cells (4M5.3I) and a construct composed of NbCXCR4 alone (Fig. 1b). All constructions were expressed and purified from *E. coli*, with purity assessed as > 90% by SDS-PAGE (Fig. S2).

Receptor-specific internalization of siRNA was assessed in Jurkat T cells either with ($CXCR4^+$) or without ($CXCR4^-$) CXCR4 expression at cell surface. $CXCR4^-$ knockout (CXCR4 KO) cell line was generated through CRISPR/Cas9 technology for disruption of *CXCR4* gene using a protocol adapted from Hou et al. [33]. CXCR4 KO population presents no detectable levels of surface CXCR4 (Fig. S3). $CXCR4^+$ and CXCR4 KO cell lines were incubated with FITC-siRNA alone or in complex with 4M5.3X4 (2:1 molar ratio). Trypsin washing was performed to eliminate FITC signal from CXCR4-surface-bound molecules. Transfection of FITC-siRNA was tested as positive control. High levels of FITC fluorescence derived from siRNA internalization were detected only in $CXCR4^+$ Jurkat T cells when incubated with 4M5.3X4 + siRNA conjugate (Fig. 2a), even surpassing transfection control (fold-increase of mean fluorescence intensity was ~ 4 for 4M5.3X4 + siRNA comparing with transfected siRNA; Fig. 2a). From flow cytometry analysis, 4M5.3X4-mediated internalization of CXCR4 appears also to have no deleterious effect on cell viability (Table S2). We observed that increase of FITC fluorescence in $CXCR4^+$ Jurkat T cells is also dependent of 4M5.3X4 + siRNA quantity, reaching up to 60% of cell population internalized with siRNA (Fig. 2b). To further exclude unspecific cell-permeability to 4M5.3X4, we compared FITC-siRNA binding (4 °C) and internalization (37 °C) between CXCR4-targeted 4M5.3X4 and irrelevant 4M5.3I control. In this case, trypsin washing to eliminate FITC signal from CXCR4 surface binding was only performed at 37 °C conditions. As shown in Fig. 2c, 4M5.3X4 construct efficiently delivered FITC-tagged siRNA to $CXCR4^+$ Jurkat T cells at 37 °C. A similar effect was observed with CXCR4 binding at cell surface at 4 °C, further verifying 4M5.3X4 specificity to CXCR4. Non-specific 4M5.3I binding should explain the background FITC signal observed at 4 °C, which is removed following trypsin treatment during the internalization assay. To further evidence 4M5.3X4 specificity towards target receptor, we incubated $CXCR4^+$ Jurkat T cells with FITC-siRNA/4M5.3X4 complexes in the presence of increasing amounts of NbCXCR4 alone as competitor for CXCR4 binding (Fig. 2d). We observed that detection of FITC decreased as the concentration of NbCXCR4 competitor increased, demonstrating that blocking CXCR4 receptor abolishes siRNA internalization by 4M5.3X4. Overall, these results indicate that engineered 4M5.3X4 can efficiently deliver FITC-conjugated siRNA through CXCR4-endocytosis.

4M5.3X4 delivers siRNA to primary $CD4^+$ T lymphocytes

Following validation of 4M5.3X4 as a vehicle for targeted siRNA delivery into a $CXCR4^+$ T cell line, we evaluated chimera efficiency in primary CD4-positive ($CD4^+$) T lymphocytes—the main cellular targets of HIV infection. Total $CD4^+$ T lymphocytes isolated from blood of healthy individuals were incubated with FITC-siRNA alone or in complex with CXCR4-specific 4M5.3X4 or irrelevant 4M5.3I. We compared siRNA delivery between non-stimulated and stimulated $CD4^+$ T


A
CXCR4+
CXCR4-
Number of cells
FITC-siRNA
Sample name MFI
PBS 5,52
4M4.3X4 6,93
siRNA 13,6
Transfected siRNA 35,8
4M5.3X4+siRNA 156
Sample name MFI
PBS 4,58
4M5.3X4 4,51
siRNA 6,89
Tranfected siRNA 37,7
4M5.3X4+siRNA 13,3
B
% FITC
4M5.3X4, pmol
C
Binding 4 °C
Internalization 37 °C
****
% FITC
PBS
siRNA
4M5.3I+siRNA
4M5.3X4+siRNA
D
Number of cells
FITC-siRNA
Sample name MFI
PBS 5,00
Competitor 2.0 uM 16,1
Competitor 1.0 uM 18,6
Competitor 0.5 uM 47,2
Competitor 0.25 uM 56,3
No competitor 85,3

◂**Fig. 2** CXCR4-targeted delivery of FITC-conjugated siRNA through 4M5.3X4 chimera. **a** Histograms illustrating internalization of FITC-conjugated siRNA into CXCR4-positive ($CXCR4^+$) or CXCR4-negative ($CXCR4^-$) cells treated with 4M5.3X4 or siRNA alone, or 4M5.3X4–siRNA complex (2:1 ratio) for 2 h at 37 ºC (Upper). Cells were washed with trypsin to eliminate the CXCR4-surface-bound molecules before FITC detection by flow cytometry. "PBS" represents untreated cells. "Transfected siRNA" represents siRNA transfection, the positive control for delivery. Schematic tables represent mean fluorescence intensity (MFI) values for all samples (lower). **b** Percentage of FITC-positive cells following analysis by flow cytometry of CXCR4-positive Jurkat T cells treated with increasing amounts of siRNA conjugated with 4M5.3X4 (2:1 molar ratio) for 2 h at 37 °C. Solid line represents the fitted nonlinear regression curve. 4M5.3X4 + siRNA vs 4M5.3I + siRNA; $****p \leq 0.001$ (Student's *t* test). **c** Percentage of FITC-positive cells following flow cytometry analysis of CXCR4-positive Jurkat T cells treated with CXCR4-targeted 4M5.3X4-siRNA or irrelevant 4M5.3I–siRNA conjugations for 2 h at 4 °C (binding) or 37 °C (internalization). Only cells incubated at 37 °C were washed with trypsin. "PBS" represents untreated cells. "siRNA" indicates cells incubated with FITC-siRNA alone. **d** Histogram illustrating FITC-siRNA internalization in CXCR4-positive Jurkat T cells treated with 4M5.3X4-siRNA in the absence (No competitor) or presence of increasing concentrations of NbCXCR4 competitor. "PBS" represents the untreated cells. For **a** and **d**, histograms represent one of at least six independent assays. For **b**, values represent mean ± SEM of at least six independent assays. For **c**, values represent mean ± SD of at least ten independent assays

cells due to possible discrepancies in cell-surface receptor levels between these populations [34]. As shown in Fig. 3a, only 4M5.3X4 was able to promote CXCR4-targeted delivery of FITC-labeled siRNA to ~ 15% ($p < 0.01$) of non-stimulated and ~ 34% ($p < 0.01$) of stimulated $TCD4^+$ cells. The 4M5.3X4-mediated delivery was also concentration-dependent as shown by successive increments in FITC fluorescence detection as the presence of 4M5.3X4 + siRNA conjugate increased (Fig. 3b). When we compare siRNA internalization to previously tested Jurkat cell line, we observe similar delivery efficiency in stimulated primary T lymphocytes but reduced internalization in non-stimulated $TCD4^+$ cells (Fig. 3c). To provide a reasonable explanation for the lower 4M5.3X4-mediated siRNA delivery into non-stimulated $TCD4^+$, we assessed receptor levels for both T-cell populations using an anti-CXCR4 antibody conjugated with APC (Fig. 3c). In fact, whilst the non-stimulated $TCD4^+$ presented approximately ten times less cell-surface concentration of CXCR4 compared with Jurkat cell line, stimulated $TCD4^+$ only presented a two times reduction. These results indicate that siRNA internalization efficacy correlates with levels of CXCR4 receptor displayed at the target population, further supporting that 4M5.3X4 mediates CXCR4-targeted siRNA delivery. Overall, these data demonstrate that 4M5.3X4 capacity to deliver inhibitor siRNA to $CXCR4^+$ cells extends to human primary T cells.

4M5.3X4-mediated siRNA delivery silences Tat-driven HIV transcription

During the early phase of HIV replication cycle, viral transactivator Tat is expressed and promotes a positive feedback by stimulating virus transcription by more than two orders of magnitude [35]. To evaluate whether 4M5.3X4-delivered siRNA could silence target gene expression, we employed a siRNA targeting HIV *tat* transcripts [29]. Our strategy was tested in $CXCR4^+$ TZM-bl cells, an HIV-reporter cell line that encodes the *luciferase* reporter gene under control of the long-terminal repeat (LTR)—HIV promoter—which in turn is transactivated by Tat. TZM-bl cells were transfected with Tat-encoding plasmid (pTat) and afterwards incubated with 4M5.3X4 fusion protein carrying the *tat* siRNA. Transfection of *tat* siRNA was used as positive control for silencing of LTR expression. LTR-driven HIV transcription was normalized to TZM-bl cells transfected with pTat only. As demonstrated in Fig. 4a, *tat* siRNA delivered by 4M5.3X4 reduced LTR transcription by ~ 85% ($p \leq 0.01$). In contrast, the presence of non-targeting 4M5.3I + siRNA *tat* or 4M5.3X4 + scramble siRNA did not significantly affect *luciferase* transcription from the LTR promoter. For the latter, we confirmed that 4M5.3X4-mediated internalization of scramble siRNA is similar to that of *tat* siRNA (Fig. S4). Furthermore, silencing of LTR expression in the presence of 4M5.3X4 + siRNA *tat* was not due to a loss in cell viability as demonstrated in Fig. 4b. Inhibition of Tat expression by 4M5.3X4 was confirmed in HEK293T cells transfected with a Tat-expression plasmid (Fig. 4c). Reduction in the levels of *tat* mRNA or Tat protein only occurred in cells treated with 4M5.3X4 + siRNA *tat* and was similar to those observed in the positive control for *tat* inhibition. Taken together, these data indicate that 4M5.3X4-mediated delivery of siRNA *tat* promotes Tat knockdown, specifically silencing Tat-driven transcription from the HIV LTR promoter.

4M5.3X4-mediated siRNA delivery inhibits HIV replication

For the siRNA to exert its antiviral activity, it is essential that 4M5.3X4 allows CXCR4-mediated internalization into HIV-infected cells. To evaluate the inhibitory effect of CXCR4-targeted delivery of siRNA *tat*, the T-lymphotropic cell line SupT1 was infected with HIV-1 NL4-3 strain. Given that this HIV-1 strain leads to CXCR4 internalization (NL4-3 strain requires CXCR4 as co-receptor for infection), we allowed a 24-h period to allow re-expression of this receptor at cell surface before incubating infected cells with 4M5.3X4 + siRNA *tat* conjugate. 4M5.3X4 loaded with *tat* siRNA reduced HIV replication on ~ 80% in T-lymphotropic cell line as measured by the amount of viral capsid p24 released into the cell supernatant ($p \leq 0.05$; Fig. 5a). We

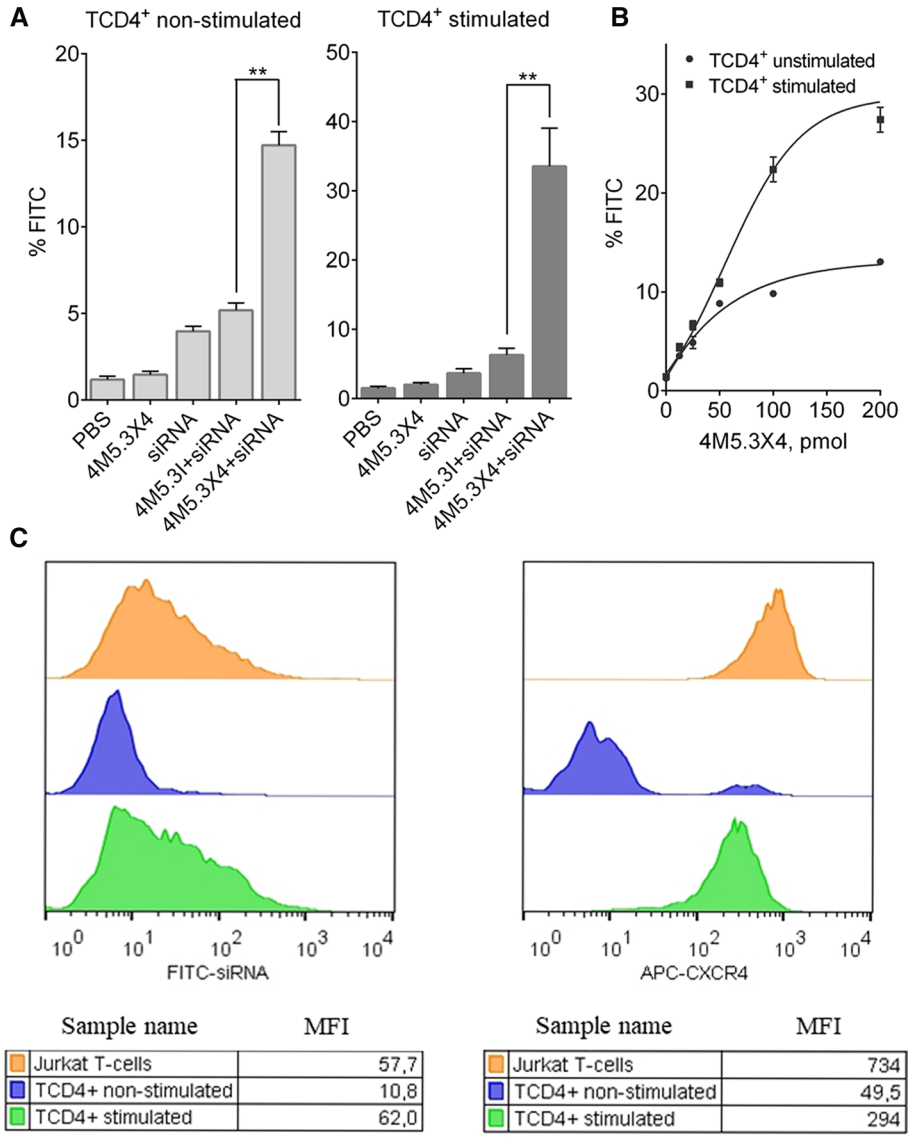


Fig. 3 4M5.3X4-mediated delivery of *tat* siRNA into primary human T lymphocytes. **a** Percentage of FITC-positive cells following analysis by flow cytometry of CXCR4-positive non-stimulated or stimulated T cells treated with 4M5.3X4 or siRNA alone, or CXCR4-targeted 4M5.3X4–siRNA or irrelevant 4M5.3I–siRNA complexes for 2 h at 37 °C. "PBS" represents untreated cells. **b** Percentage of FITC-positive cells following flow cytometry analysis of CXCR4-positive non-stimulated or stimulated T cells treated with increasing amounts of 4M5.3X4–siRNA conjugation for 2 h at 37 °C. Solid lines represent the fitted nonlinear regression curves. 4M5.3X4 + siRNA vs 4M5.3I + siRNA; $^{**}p \leq 0.01$ (Mann–Whitney test). **c** Comparison of 4M5.3X4-mediated delivery in CXCR4-positive non-stimulated or stimulated T cells with Jurkat cell line. (Left) Histogram represents the FITC-positive cells by flow cytometry analysis after treatment with CXCR4 targeted 4M5.3X4-siRNA. (Right) Histogram represents the CXCR4 levels detected at surface of T cells comparing with Jurkat cell line after staining with CXCR4-specific antibody tagged with APC fluorochrome. For **a** and **b**, cells were washed with trypsin to eliminate the CXCR4-surface-bound molecules before FITC detection by flow cytometry. For **a** and **b**, values represent mean ± SEM of six healthy donors. For **c**, histograms represent one of six healthy donors

observed partial HIV inhibition with 4M5.3X4 alone, possibly due to NbCXCR4 reported viral neutralization activity as a fusion inhibitor [16]. Nevertheless, HIV infection was dramatically decreased only when this construct was conjugated with siRNA *tat*. Neither 4M5.3X4 conjugated with scramble siRNA nor irrelevant 4M4.3I conjugated with *tat* siRNA was able to inhibit NL4-3 infection. Importantly, 4M5.3X4-delivered *tat* siRNA also inhibited the replication of a primary isolate of HIV-1 (strain 01PTHDECJN, circulating recombinant form CRF02_AG) by more than 90% and a primary isolate of HIV-2 (strain 03PTHCC6, Group A) by approximately 65% (Fig. 5b). In contrast with HIV-1 strain NL4-3 tested previously, both primary HIV strains are R5 tropic—require CCR5 as co-receptor for virus entry—which

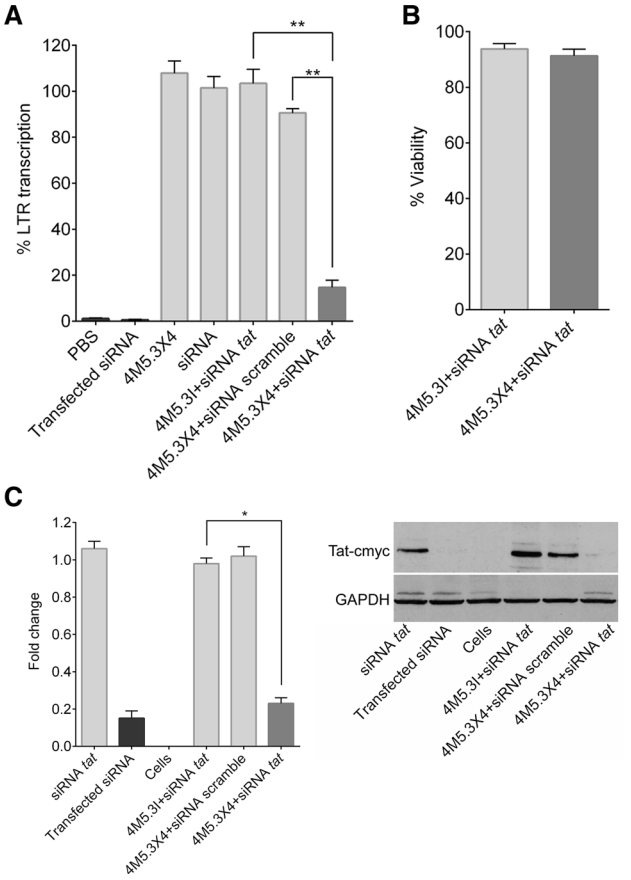


Fig. 4 4M5.3X4-delivered tat siRNA silences Tat-driven LTR transcription in HIV reporter cells. **a** Evaluation of Tat-driven LTR transcription assessed by luciferase activity in TZM-bl reporter cell line transfected with pTat plasmid followed by treatment with FITC-conjugated *tat* siRNA (siRNA), 4M5.3X4 alone or 4M5.3X4-siRNA *tat* conjugation. Cells were transfected with pTat 24 h previously to protein treatment. Cells were also incubated with FITC-tagged siRNA scramble conjugated with CXCR4-targeted 4M5.3X4 construct (4M5.3X4 + siRNA scramble) or irrelevant 4M5.3I control conjugated with tat siRNA (4M5.3I + siRNA tat). LTR-driven HIV transcription is normalized to TZM-bl cells transfected with pTat only. "PBS" represents untreated cells. "Transfected siRNA" represents siRNA transfection for positive control of LTR transcription silencing. Values represent mean ± SEM of at least three independent assays and are normalized to pTat-transfected cells. 4M5.3X4 + siRNA vs 4M5.3I + siRNA or vs 4M5.3X4 + siRNA scramble $^{**}p \leq 0.01$ (Mann–Whitney test). **b** Viability of TZM-bl cells in the presence of tat siRNA-conjugated 4M5.3X4 and 4M5.3I constructs. Cell viability was assessed by alamarBlue viability assay 3 h after treatment. Values represent mean ± SEM of at least three independent assays and are normalized to non-treated cells. **c** Evaluation of Tat knockdown in HEK293T cells transfected with pCMV-Myc-Tat followed by treatment with siRNA alone, or CXCR4-targeted 4M5.3X4-siRNA tat complexes for 2 h at 37 °C. Cells were also incubated with siRNA scramble conjugated with CXCR4-targeted 4M5.3X4 construct (4M5.3X4 + siRNA scramble) or irrelevant 4M5.3I control conjugated with tat siRNA (4M5.3I + siRNA tat). "Cells" sample represents the non-transfected cells. (Left) Fold change in tat transcription assessed by qPCR and normalized to transfected cells (non-treated). Values represent mean ± SD of three independent assays, being calculated from 2dCt and plotted. 4M5.3X4 + siRNA tat vs 4M5.3I + siRNA tat $^{*}p \leq 0.05$ (two-tailed *t* test). (Right) Western blot analysis of Tat protein expression. Samples were probed with anti-Tat and anti-GAPDH (loading control) antibodies

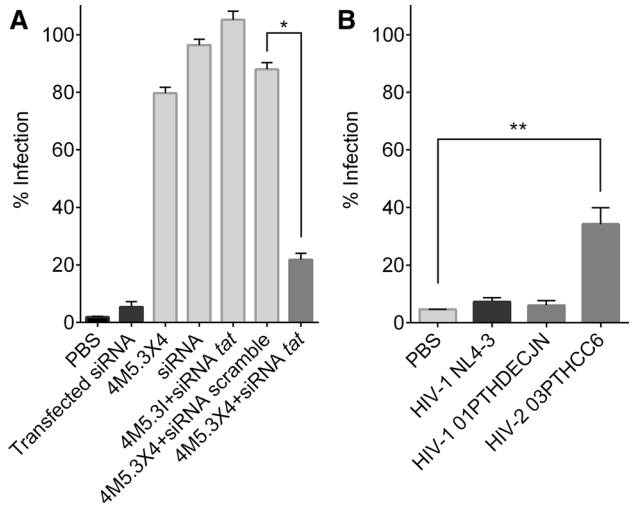


Fig. 5 4M5.3X4-delivered tat siRNA inhibits HIV replication. **a** Percentage of virus inhibition in T-lymphocytic SupT1 cell line infected with HIV-1 NL4-3 strain followed by treatment with 4M5.3X4–siRNA tat conjugation. After 1 day of NL4-3 infection, SupT1 cells were incubated in the presence of tat siRNA (siRNA) or 4M5.3X4 alone, or 4M5.3X4 + siRNA conjugation. "PBS" represents the non-infected cells. "Transfected siRNA" represents siRNA transfection for positive control of HIV inhibition. Percentage of HIV inhibition of NL4-3-infected SupT1 cells treated with CXCR4-targeted 4M5.3X4 construct conjugated with tat siRNA (4M5.3X4 + siRNA tat) or scramble siRNA (4M5.3X4 + siRNA scramble), or irrelevant 4M5.3I control conjugated with tat siRNA (4M5.3I + siRNA tat). HIV replication was measured by viral p24 capsid quantification 72 h after treatment. Values represent mean ± SEM of four independent assays and are normalized to HIV-1 NL4-3 infection. 4M5.3X4 + siRNA tat vs 4M5.3I + siRNA or vs 4M5.3X4 + siRNA scramble; $^{*}p \leq 0.05$ (Mann–Whitney test). **b** Evaluation of luciferase activity in TZM-bl reporter cell line infected with distinct HIV clades followed by treatment with 4M5.3X4–siRNA tat conjugation. Cells were infected with HIV virions previously to protein treatment. Values represent ± SEM of at least two independent assays in triplicate and are normalized to HIV-1 infection. "PBS" represents untreated cells. HIV-1 01PTHDECJN vs HIV-1 NL4-3 or vs HIV-2 03PTHCC6; $^{**}p \leq 0.01$ (Mann–Whitney test)

demonstrates the efficacy of 4M5.3X4-delivered siRNA in inhibiting virus replication regardless of its co-receptor tropism. This result was expected since the used siRNA inhibitor is specific for *tat* transcripts, highly conserved among HIV strains (HIV Sequence Compendium 2018; http://www.hiv.lanl.gov/). Thus, our results demonstrate that CXCR4-targeted delivery of *tat* siRNA by 4M5.3X4 construct is able to directly inhibit HIV-1 and HIV-2 infection.

Discussion

Potency and breath of siRNA inhibitors hold promise for the treatment of numerous diseases. Nevertheless, the lack of efficient and specific delivery methods continues to postpone the application of these technologies for human therapeutics.

Despite the proof-of-principle for treatment of solid tumors [36, 37], delivery of therapeutic siRNAs in non-cancer applications is limited to disorders with simplified target-site accessibility such as ocular conditions or propensity to uptake circulating molecules, as occurs with liver-located diseases [36–38]. Inhibitors targeting HIV expression require a systemic delivery system due to the widespread distribution of virus-susceptible cells through human body. Here, we present a novel targeted approach to deliver siRNA inhibitors of HIV expression by developing a nanobody-based engineered chimera. In this construct, we conjugated a nanobody for membrane translocation through CXCR4-mediated endocytosis with a scFv for promoting efficient uptake of FITC-conjugated siRNA. Exceptional high stability and increased tissue penetration of nanobodies [9] make them a suitable option for targeting HIV-infected cells primarily located onto densely packed tissues. Furthermore, the absence of an effector region (Fc) in these antibody formats constitutes an additional safety feature to prevent undesirable immune activation.

In this study, we demonstrate 4M5.3X4-mediated delivery of *tat* siRNA to $CXCR4^+$ cells. Engineered 4M5.3X4 chimera delivered FITC-tagged siRNA to a T-lymphocytic cell line only when CXCR4 receptor was present, assuring specificity of the designed strategy. Similar levels of CXCR4-targeted siRNA were internalized in human primary T cells, anticipating the potential of this CXCR4-mediated delivery for in vivo purposes. Levels of CXCR4 receptor are crucial to mediate effective siRNA delivery, as observed by the limited effect of 4M5.3X4 in non-stimulated primary $CD4^+$ T cells that are scarce for the presence of this receptor [34]. Delivery of 4M5.3X4 conjugated with *tat* siRNA abolished Tat-driven transcription from HIV LTR promoter and impaired viral replication in T cells, ensuring full activity of 4M5.3X4-internalized siRNA. Overall, our work validates the 4M5.3X4 chimera as a vehicle for delivery of anti-HIV siRNAs, further demonstrating the potential of ligand-dependent CXCR4 endocytosis as an entry route for therapeutic molecules as others already described [13–15, 39]. While few studies have reported targeted delivery of anti-HIV siRNAs [40–42], particularly through antibody-based vehicles [4], our work is the first to describe a nanobody format as a targeting moiety for the delivery of this class of antiviral inhibitors. In contrast to others [43], our study was also able to demonstrate functionality of a therapeutic siRNA when delivered by an antibody-based construct alone, without any encapsulation method.

The use of a cellular receptor as a delivery target constitutes an advantage relative to other approaches directed at HIV-1 envelope glycoprotein [40, 44, 45], given this is prone to mutational modifications that could halt siRNA delivery. Also, this viral glycoprotein is only present in the surface of actively replicating (non-latent) cells. On the other hand, CXCR4 should pose an optimal target for broad inhibition of HIV-1 infection independently of the virus tropism, given the presence of this receptor across all HIV-susceptible cells [46, 47]. Despite the current predominance of antiviral strategies aimed at disrupting CCR5 expression [48–50], evidence of viral tropism shift to CXCR4 [51, 52] further highlights the importance of targeting CXCR4-positive-infected cells. Related to this, the reported ability of NbCXCR4 to inhibit HIV-1 fusion [16] should enable the use of this chimera system as a dual-inhibitory approach to also prevent de novo CXCR4-tropic viral infections by competing for this viral co-receptor [53]. Opposed to "shock and kill' strategies that aim to reactivate HIV latent expression [54], 4M5.3X4-mediated siRNA delivery strategy could potentially be tested against latently infected cells as a "lock out" strategy to prevent long-term reactivation of hidden viral reservoirs [53]. Still, studies on latent HIV models will be necessary to investigate whether 4M5.3X4 can target HIV reservoirs. Previous reports already demonstrated that the inhibition of Tat activity is alone sufficient to prevent reactivation of HIV reservoirs from patients receiving antiretroviral treatment, providing basis for a lock-out strategy based on Tat impairment as a HIV-1 treatment [55, 56].

Future studies should validate our nanobody-based strategy for CXCR4-mediated systemic delivery of anti-HIV siRNAs within in vivo models of infection [57]. We should also address long-term therapeutic benefits by evaluating the capacity of 4M5.3X4 to mask the conjugated siRNA from host immune system or provide a shield against serum nuclease-mediated degradation. The latter can be also further improved by chemical siRNA modifications [4]. Pharmacokinetic studies should confirm the extension of siRNA half-life considering that the molecular size of the 4M5.3X4 + siRNA complex (~ 52 kDa) is well above the renal filtration cut-off (30 kDa). Despite preliminary results demonstrating 4M5.3X4 potential, further modifications to this construct may improve efficacy and specificity of siRNA delivery by exploring alternative targeting nanobodies and distinct RNA-binding molecules. The versatility of 4M5.3X4 conjugation to any FITC-conjugated siRNA enables a multiplex approach to direct numerous antivirals towards multiple HIV genes to provide a broad inhibition of viral replication and overcome potential gain-of-resistance towards a single inhibitor [58, 59]. Moreover, 4M5.3X4-mediated siRNA therapeutic could be extended to other relevant diseases. In particular, cancer diseases should constitute an attractive target owing to the overexpression of CXCR4 on malignant cells [16].

In conclusion, the present study provides a new paradigm for CXCR4-targeted delivery of HIV siRNA repressors by a scFv–nanobody chimera. We expect that this method could pave the way for novel and more efficient delivery systems of HIV inhibitors.

Acknowledgements This work was supported by the HIVERA—Harmonizing, Integrating and Vitalizing European Research on AIDS/HIV [Grant number HIVERA/0002/2013], and the Fundação para a Ciência e a Tecnologia—Ministério da Educação e Ciência (FCT-MEC), Portugal [Grant numbers UTAP-ICDT/DTP-FTO/0016/2014, VIH/SAU/0013/2011, VIH/SAU/0020/2011, VIH/SAU/0029/2011, PTDC/SAU-EPI/122400/2010]. CC-S acknowledges FCT-MEC for PhD fellowship SFRH/BD/73838/2010. PRLP acknowledges FCT-MEC for PhD fellowship SFRH/BD/81941/2011. FM acknowledges FCT-MEC for PhD fellowship SFRH/BD/87488/2012.

Author contributions CC-S, NT, and JG conceived and designed the experiments. CC-S, PRLP, FM, JGO, MC, and AM performed the experiments and analyzed the data. CC-S drafted the manuscript. All authors read and approved the final manuscript.

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