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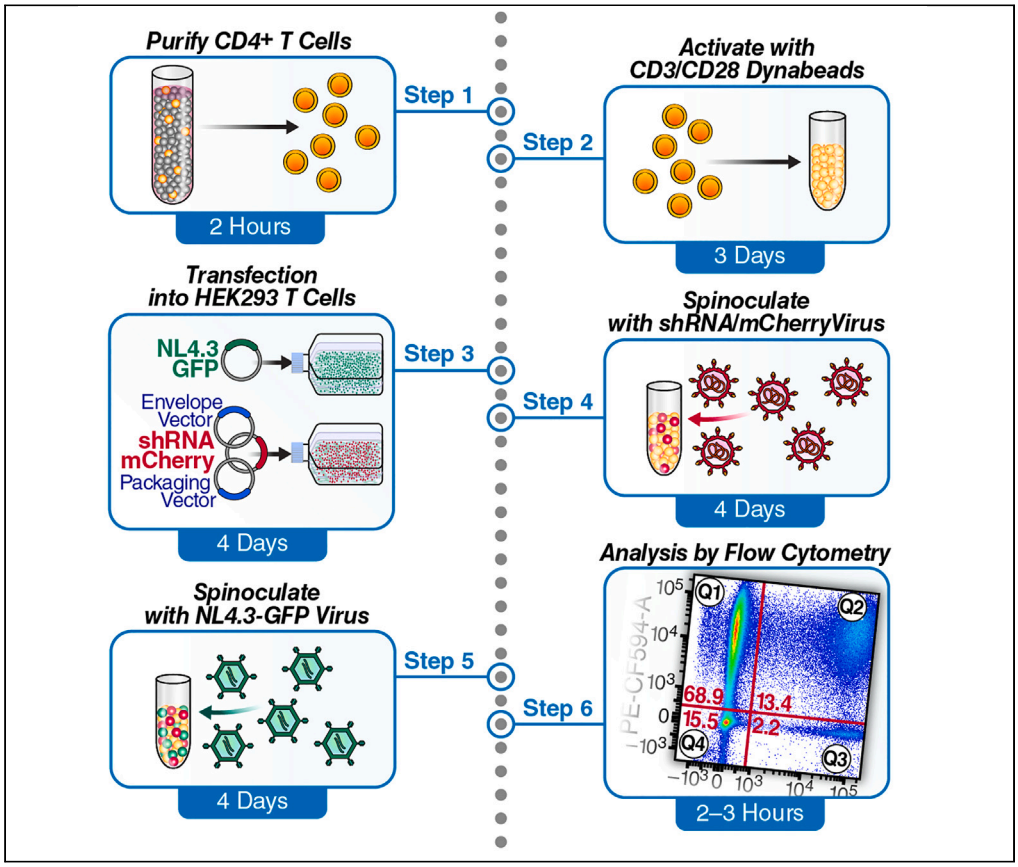
A flow cytometry-based assay to investigate HIV-1 expression in SMYD5 shRNA containing primary CD4$^+$ T cells



Delivering small hairpin RNAs (shRNAs) and the HIV-1 virus to primary CD4+ T cells with high transduction efficiency and high cell viability can be challenging. Here, we present a flow cytometry-based assay to knock down the host protein SMYD5 by shRNA and study the HIV-1 virus specifically in shRNA-containing cells. We describe steps for purifying CD4+ T cells, activating them with CD3/CD28 Dynabeads, transfection of plasmids into HEK293T cells, spinoculation with two different viruses, and analysis by flow cytometry.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for knockdown of the host protein SMYD5 by shRNA in primary CD4+ T cells

Describes approach to study the HIV-1 virus specifically in shRNA-containing cells

Details steps to efficiently double-infect primary CD4+ T cells with two different viruses

Knockdown and flow cytometry analysis details to detect shRNA/HIV-1-infected cells

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A flow cytometry-based assay to investigate HIV-1 expression in SMYD5 shRNA containing primary CD4+ T cells

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SUMMARY

Delivering small hairpin RNAs (shRNAs) and the HIV-1 virus to primary CD4+ T cells with high transduction efficiency and high cell viability can be challenging. Here, we present a flow cytometry-based assay to knock down the host protein SMYD5 by shRNA and study the HIV-1 virus specifically in shRNA-containing cells. We describe steps for purifying CD4+ T cells, activating them with CD3/CD28 Dynabeads, transfection of plasmids into HEK293T cells, spinoculation with two different viruses, and analysis by flow cytometry.

For complete details on the use and execution of this protocol, please refer to Boehm et al.[1]

BEFORE YOU BEGIN

Detailed timeline

Day 0	Prepare HEK293T cells for transfection
Day 1	Isolate and activate CD4+ T cells; transfect HEK293T to generate shRNA-mCherry containing virus
Day 4	Harvest and concentrate shRNA-mCherry virus; spin infect activated CD4+ T cells; prepare HEK293T cells for transfection
Day 5	Transfect HEK293T to generate HIV-1-$NL_{4.3}$-GFP virus
Day 7	Determine cell survival and the percentage of shRNA-mCherry containing CD4+ T cells by flow cytometry; lyse cells for RNA isolation to confirm knockdown efficiency
Day 8	Harvest and concentrate HIV-1-$NL_{4.3}$-GFP virus; spin infect shRNA containing CD4+ T cells
Day 11 and 12	Analyze samples by flow cytometry; lyse cells for RNA isolation to confirm knockdown efficiency

Institutional permissions

The protocol below describes the specific steps to detect HIV-1 expression in primary CD4+ T cells. HIV-1 virus and blood for the isolation of CD4+ T cells are potentially hazardous biological agents. Prior to proceeding with this protocol, it is necessary to obtain Biological Use Authorization (BUA) by the institution. All tissue culture and virus work need to be performed in biosafety level 2 (BSL2) cabinets. If replication competent HIV-1 is used the experiments need to be performed in a BSL3 laboratory. When working with blood a hepatitis B vaccination should be considered.

CD4+ T lymphocyte isolation from leucocyte reduction chambers (LRC)

Timing: 2 h

This protocol describes the isolation of CD4+ T lymphocytes using the RosetteSep Human CD4+ T Cell Enrichment Cocktail. Determine how many CD4+ T lymphocytes are needed for your experiment. Order LRCs from at least 3 donors. To replicate the experiments shown in Figure 2 of this manuscript,[1] $5*10^7$ CD4+ T cells per donor are sufficient for technical triplicates. All steps should be performed in the biosafety cabinet.

1. Isolation of CD4+ T lymphocytes from LRC.
   a. To avoid coagulation and a reduction in viable cells, have fresh LRCs delivered on the day of the experiment. If this is not possible, LRCs can be stored on a shaker at 4°C overnight for up to 18 h.
   b. Before the isolation, bring all reagents (PBS + 2% FBS, Histopaque-1077 density medium and the RosetteSep Cocktail) to room temperature (20°C–25°C).
   c. Transfer blood into a 50 mL Falcon tube by cutting both ends of the LRC chamber with sterile scissors and letting the blood drop into the tube.
   d. According to the manufacturer's instructions, add 50 μL of RosetteSep Human CD4+ T cell Enrichment Cocktail to 1 mL of blood.
      i. Mix carefully.
      ii. Incubate at 20°C–25°C for 20 min while shaking gently.

   Note: LRCs usually contain ~10 mL of blood, so 500 μL of CD4+ T cell Enrichment Cocktail is needed per donor.

   e. Prepare 15 mL Histopaque-1077 density medium, in a clean 50 mL tube.
   f. After diluting the blood/ Enrichment Cocktail-mix with an equal amount of PBS + 2% FBS (for example 10 mL of blood and 10 mL of PBS + 2% FBS) slowly layer the sample onto the density medium. Avoid mixing the sample with the density medium.
   g. Centrifuge sample for 20 min at 800 x g, and at RT. The brake must be off to avoid mixing the layers.

   Note: Completely closing the brake is recommended by the manufacturer but it will prolong the stopping time. Increasing the deceleration speed to about 2 is possible.

   h. Remove the enriched CD4+ T cell layer which appears as a white ring, below the plasma phase but above the density medium and the erythrocytes and transfer the layer into a new 50 mL Falcon tube.
   i. Fill the tube with PBS + 2% FBS and centrifuge for 3 min at 800 × *g*, with brake on.
   j. To remove contaminating erythrocytes, remove the supernatant, add 15 mL of 1x RBC Lysis Buffer and incubate on ice for 5 min.
   k. Fill the tube up to 50 mL with PBS containing 2% FBS, centrifuge for 5 min at 800 × *g* and remove the supernatant.
   l. If the pellet is still red and erythrocytes are present, repeat steps j and k.
   m. Resuspend the pelleted CD4+ T cells in warm (37°C), fresh complete RPMI medium. Add 20 U/mL of IL-2 to prolong the cells survival.
   n. Count the cells.
      i. Culture cells at a concentration of 5×10^6/mL.
      ii. Change the medium every 3–4 days.
      iii. Place the flasks horizontally to increase the surface area in which the cells are grown.

Stimulation of CD4+ T lymphocytes

Timing: 3 days

This protocol describes the activation of 5 × 10^7 CD4+ T cells using Dynabeads Human T-Activator CD3/CD28.

2. Stimulation of CD4+ T lymphocytes.
   a. Vortex the vial of Dynabeads Human T-Activator CD3/CD28 and transfer 1.25 mL of Dynabeads (1 bead/cell) to a 15 mL tube.
   b. Wash the Dynabeads once with complete RPMI medium.
   c. Resuspend the washed Dynabeads with 10 mL of the CD4 + T cells to activate (at a concentration of 5 × 10^6/mL).
   d. Cultivate for 3 days at 37°C, in a 5% CO_2 incubator.
   e. Count cells daily to monitor cell growth. Media color change from pink to orange/ yellow and cell aggregates become visible after 2 days of successful activation.

Amplification and isolation of plasmids used in the study

Timing: 3 days

This protocol describes the amplification of the SMYD5 shRNA containing plasmids, the HIV-1 $NL_{4.3}$-GFP lentivirus, the lentiviral packaging construct pCMVdelta R8.91, the VSV-G glycoprotein-expressing vector and the X4-envelope protein-expressing vector used in this study. Since all are lentiviral constructs it is important to use Stbl-3 *Escherichia coli* (*E.coli*) for amplification and perform all growth steps at 30°C to avoid recombination.

3. Amplification and isolation of plasmids used in the study.
   a. Thaw One Shot Stbl-3 Chemically Competent *E.coli* on ice.
   b. Prewarm SOC media and selection agar plates to 30°C in an incubator.
   c. Add 20 μL of *E.coli* to 100–500 ng/μL of plasmid DNA.
      i. Mix by carefully flicking the tube.
      ii. Incubate on ice for 30 min.
   d. Heat shock in a 42°C water bath for 45 s.
   e. Incubate on ice for 2 min then add 200 μL prewarmed SOC medium.
   f. Incubate the tube in the 30°C incubator while shaking at 200 rpm for 30 min.
   g. Plate 20–100 μL of the transfected bacteria onto an agar plate containing a selective antibiotic (ampicillin) and place in a 30°C incubator for 24 h.
   h. Select one colony and inoculate 2 mL liquid broth (LB) medium containing ampicillin antibiotic in a 14 mL culture tube, incubate for 24 h at 30°C.
   i. Transfer the 2 mL bacteria culture to a 1 L flask and inoculate 500 mL liquid broth (LB) medium containing ampicillin antibiotic, incubate for 24 h at 30°C.
   j. In order to obtain transfection-grade plasmid, isolate the plasmids from the transfected cells using a NucleoBond Xtra Maxi kit (Macherey-Nagel) or QIAGEN Plasmid Maxi kit.

Producing SMYD5 shRNA- or HIV-1 $NL_{4.3}$-GFP containing lentivirus in HEK293T cells

Timing: 4 days

This step describes the X-tremeGENE 9 DNA method used for transfection of HEK293T cells with lentiviral vectors. After two to three days of virus production by the HEK293T cells, the virus is harvested and concentrated. On day 4, the stimulated CD4+ T lymphocytes are infected with the freshly produced, concentrated virus.

4. HEK293T cell culture.
   a. HEK293T cells are cultured in a 37°C, 5% CO_2 incubator in complete DMEM medium (supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine) in T175 tissue culture flasks in 25 mL medium. To obtain high-titer virus, HEK293T cells should be thawed at least one week before transfection, split every 2–3 days and kept in culture for no longer than 4 weeks.
5. Plasmid DNA transfection using X-tremeGENE.
   a. Prepare 5 × 10^6 HEK293T cells in a T175 tissue culture flask in 20 mL complete DMEM medium the day before transfection. If using blood from 3 donors prepare 4 flasks per virus.
   b. On the day of transfection, replace the media with fresh complete DMEM. Cells will be adhered to the bottom of the flask. Be careful not to lift them off when you replace the media.
   c. Prepare DNA mix in nuclease-free H_2O to a final volume of 100 µL. For each T175 flask use a total of 40 µg DNA.
   d. For the generation of shRNA containing virus use 20 µg of shRNA-mCherry plasmid, 13 µg lentiviral packaging construct pCMVdelta R8.91, and 7 µg of the VSV-G glycoprotein-expressing plasmid.
   e. For the generation of HIV-1 NL4.3-GFP virus use 20 µg of HIV-1 plasmid, 13 µg lentiviral packaging construct pCMVdelta R8.91, and 7 µg of the VSV-G glycoprotein-expressing plasmid.
   f. Add 840 µL Opti-MEM I reduced-serum media.
   g. Dropwise add 60 µL X-tremeGENE 9 DNA transfection reagent.
      i. Swirl and vortex for 2 s.
      ii. Collect the solution at the bottom of the tube by centrifugation at 500 g for 15 s.
   h. After incubation at 20°C–25°C for 15–30 min, carefully add the prepared DNA solution to the HEK293T cells.
   i. To allow viral production, return the cells to the 37°C, 5% CO_2 incubator for 48–72 h.
6. Harvesting and concentrating the virus.
   a. After two-three days of virus production, collect the HEK293T supernatant containing the lentivirus and pass it through a 0.22 µm syringe filter (for example using a 50 mL Steriflip vacuum filtration tube with Millipore Express PLUS PES membrane).
   b. Use 35 mL UltraClear tubes (Becton Dickinson) for centrifugation.
      i. Completely fill the tubes with viral supernatant or add media or PBS to avoid the collapse of the tube during centrifugation.
      ii. Spin in an ultracentrifuge for 2 h at 100,000 × *g* at 4°C.
   c. Discard supernatant and resuspend the (invisible) pellet with 1 mL of cold complete RPMI medium or FBS.
      i. Pipet up and down on ice for at least 1 min without generating bubbles.
      ii. The concentrated virus will be used immediately for spin infection.

Alternatives: Unused virus can be aliquoted and frozen at -80°C. However, the viral titer will decrease overtime. Frequent freeze/thaw cycles of the virus should be avoided. For concentration of virus commercial kits are available but ultracentrifugation is the preferred method in this protocol.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
One Shot Stbl-3 chemically competent *E. coli*	Thermo Fisher Scientific	Cat# C737303
Biological samples		
Leukocyte reduction chamber (LRC) from Trima apheresis collection	Vitalant Blood Donation, San Francisco	http://www.vitalant.org/

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dynabeads human T-activator CD3/CD28	Life Technologies	Cat# 11131D; Lot# 00979236
AMV reverse transcriptase	Promega	Cat# M5101
Maxima SYBR Green PCR master mix	Thermo Scientific	Cat# K0253
X-tremeGENE 9 DNA transfection reagent	Roche	Cat# XTG9-RO
Critical commercial assays		
Direct-zol RNA MiniPrep Kit	Zymo Research	Cat# 2052
NucleoBond Xtra Maxi Kit	Macherey-Nagel	Cat# 740414.100
eBioscience Fixable Viability Dye eFluor 780	Invitrogen	Cat# 65-0865-14; Lot# 2062571, 2450571
RosetteSep human CD4+ T cell enrichment cocktail	STEMCELL Technologies	Cat# 15062; Lot# 18A86332, 19E101792
Experimental models: Cell lines		
HEK293T, human, female, epithelial kidney cells	ATCC	CRL-3216
Oligonucleotides		
GFP qPCR forward primer: 5′ ATGGTGAGCAAGGGCGAGGAG 3′	Drenan et al.[2]	N/A
GFP qPCR reverse primer: 5′ GTGGTGCAGATGAACTTCAG 3′	Boehm et al.[1]	N/A
SMYD5 qPCR forward primer: AATGCACTTTATCGCTACCGAG	Boehm et al.[1]	PrimerBank ID: 154689857b3; https://pga.mgh.harvard.edu/primerbank/
SMYD5 qPCR reverse primer: CTGCCAACCGACATTCTGC	Boehm et al.[1]	PrimerBank ID: 154689857b3; https://pga.mgh.harvard.edu/primerbank/
RPL13A qPCR forward primer: GCCCTACGACAAGAAAAAGCG	Boehm et al.[1]	PrimerBank ID: 14591905c2; https://pga.mgh.harvard.edu/primerbank/
RPL13A qPCR reverse primer: TACTTCCAGCCAACCTCGTGA	Boehm et al.[1]	PrimerBank ID: 14591905c2; https://pga.mgh.harvard.edu/primerbank/
Recombinant DNA		
Lentiviral packaging construct pCMVdelta R8.91	Naldini et al.[3]	N/A
VSV-G glycoprotein-expressing vector	Naldini et al.[3]	N/A
HIV-1 NL4.3-GFP	Kutsch et al.[4]	HIV-1 NL4.3-GFP
SMYD5 #1 TRC human shRNA	Thermo Scientific	TRCN0000155095
SMYD5 #2 TRC human shRNA	Thermo Scientific	TRCN0000156306
Non-targeting human shRNA	Millipore Sigma	SHC002
pLKO.1 mCherry	Addgene	Cat# 128073; RRID: Addgene_120873
Software and algorithms		
AlphaView – AlphaImager HP 3.5.0.927	ProteinSimple	https://www.proteinsimple.com/resources
FlowJo 9.9–10.8	Tree Star	https://www.flowjo.com/
ImageJ	NIH	https://imagej.nih.gov/ij/
Prism8	GraphPad	https://www.graphpad.com/scientific-software/prism/
SDS 2.4 software	Applied Biosystems	http://home.appliedbiosystems.com
SoftMax Pro 4.7.1–6.5.1	Molecular Devices	https://www.moleculardevices.com/products/microplate-readers/acquisition-and-analysis-software/softmax-pro-software#gref

STEP-BY-STEP METHOD DETAILS

Spin infection of stimulated CD4+ T lymphocytes with shRNA-mCherry lentivirus

Timing: 3 h

This section provides step-by-step instructions on how to spin infect stimulated CD4+ T lymphocytes with shRNA containing lentivirus on Day 4.

1. Spin infection of CD4+ T cells.
   a. Three days post-activation, resuspend cells and transfer to a 15 mL Falcon tube or any other suitable tube to remove the Dynabeads.

Table 1. cDNA synthesis reaction 1

Components	Final concentration	Amount
RNA	10 pg–5 μg	Up to 11 μL
Oligo dT primer (0.5 μg/ μL)	0.25 μg primer/ μg RNA	Up to 2.5 μL
Random Hexamer primer (0.5 μg/ μL)	0.25 μg primer/ μg RNA	Up to 2.5 μL
dNTP mix (10 mM)	1 mM	2 μL
ddH_2O		Up to 13 μL total volume

Prepare fresh at 20°C–25°C. Discard unused reaction mix.

b. For best results, infect stimulated CD4+ T cells immediately with the shRNA-mCherry virus produced in steps 4–6.
c. Add 1 × 10^6 cells to 1.7 mL microcentrifuge tubes and pellet them by centrifuging at 800 x g for 3 min at 20°C–25°C.
d. Remove supernatant without disturbing the pellet and add 500 μL of shRNA virus (10,000 ng of p24 Gag/mL), 5 μL 1 M HEPES and 0.5 μL of polybrene (4 μg/mL).
e. Spinoculate by centrifuging at 800 x g and 32°C for 1.5 h.
f. Resuspend the spinoculated cells and transfer them into one well of a 6-well Flat-bottom plate.
g. Add 3 mL of fresh complete RPMI medium + 20 U/mL of IL-2 to each well.

Note: The viral solution can be removed after spin-infection or can be left on the cells to allow a slight increase in infection over time. Dynabeads can be added back to the spin infected cells to keep activating the CD4+ T cells before the 2nd spin infection with HIV-1-$NL_{4.3}$-GFP virus. This will slightly increase the spin-infection efficiency. However, the second spin-infection will also work without this continuous activation.

h. Culture for 4 days at 37°C in a 5% CO_2 in an incubator.
i. Replace the medium with fresh complete RPMI medium + 20 U/mL of IL-2 every other day.

Validation of knockdown efficiency by mRNA expression

Timing: 2 h for RNA extraction; 2.5 h to generate cDNA from RNA; 3 h for RT-qPCR

Here, we provide step-by-step instructions to validate the knockdown efficiency of the shRNA by analysis of SMYD5 mRNA expression on Day 7.

2. Validation of knockdown efficiency by mRNA expression.
   a. Extract total RNA from CD4+ T cells from step 1 using the Direct-zol RNA Miniprep Kit following the manufacturer's instructions or any other RNA extraction methodology. Elute RNA in 50 μL of RNase free water.
   b. Keep RNA on ice to avoid degradation and quantify total RNA using a NanoDrop spectrophotometer.
   c. Prepare cDNA using Promega AMV Reverse Transcriptase following the manufacturer's instructions. Prepare the cDNA synthesis reaction mix according to Tables 1 and 2.

Note: Any other suitable cDNA synthesis kit can be used here.

   d. Incubate the components of Table 1 for 5 min at 65°C and place it on ice for 1 min.
      i. Then add the components of Table 2 and incubate at 42°C for 60 min.
      ii. Inactivate the enzyme at 95°C for 5 min.
      iii. Dilute the synthesized cDNA 1:5 before qPCR analysis.
   e. Prepare qPCR reactions using Maxima SYBR Green qPCR Master Mix (Thermo Scientific) following the manufacturer's instructions. Prepare the qPCR reaction according to Table 3.

Table 2. cDNA synthesis reaction 2

Components	Final concentration	Amount
5x AMV Reaction Buffer	1×	4 µL
RNasin Ribonuclease Inhibitor	40 units	0.5 µL
AMV Reverse Transcriptase	30 units	1 µL
ddH_2O		1.5 µL

Prepare on ice. Discard unused reaction mix.

f. Run the qPCR reaction on a 7900HT Fast Real-Time PCR System (Applied Biosystems) or other suitable thermocycler using the cycling protocol shown in Table 4.
g. Analyze the results using the 2^{-ddCt} method.
   i. To quantify the percentage of SMYD5 knockdown, the expression of SMYD5 is first normalized in all samples to the expression level of the housekeeping gene RPL13A.
   ii. Next, compare the mRNA expression of shSMYD5 samples sh-non-targeting control samples.

Analysis of SMYD5-mCherry expressing CD4+ T cells by flow cytometry

Timing: 2 h

In this paragraph we describe of how to analyze SMYD5-mCherry expressing CD4+ T cells by flow cytometry on Day 7.

3. Analysis of SMYD5-mCherry infection efficacy by flow cytometry.
   a. 3 days after spin infection, wash cells twice with cold PBS.
   b. Spin down cells at 800 x g at 20°C–25°C for 3 min.
      i. Discard supernatant and stain cells with live/dead eBioscience Fixable Viability Dye eFluor 780.
      ii. Prepare a 1:1,000 dilution of the dye in cold PBS buffer.
      iii. Resuspend the cells in 100 µL/well in a 96-well plate.
      iv. Incubate for 15 min on ice in the dark.

   Note: Live/dead eBioscience Fixable Viability Dye eFluor 780 works best since it is detected in the APC-Cy7 channel (red/637 nm) and does not overlap with FITC (blue/488 nm) and PE (yellow/green/561 nm) channels. Therefore, no compensation is needed.

   c. Wash cells 1x with cold PBS buffer and proceed directly to flow cytometry analysis (see Figure 2 for gating strategy).

   Note: Avoid fixing samples with Paraformaldehyde (PFA) since it decreases fluorescence intensity. Cells can also be resuspended in PBS + 2% FBS to keep cell survival stable. For flow cytometry, a Fortessa X-20 cell analyzer was used.

Table 3. RT-qPCR reaction

Components	Final concentration	Amount
cDNA (diluted 1:5)	Maximum 10% of the final qPCR Reaction set-up volume	4 µL
Maxima SYBR Green qPCR Master Mix (2×)	1×	6 µL
Forward Primer (10 µM stock)	0.5 µM	0.6 µL
Reverse Primer (10 µM stock)	0.5 µM	0.6 µL
ddH_2O		Up to 12 µL

Prepare fresh at 20°C–25°C. Discard unused reaction mix.

Table 4. RT-qPCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	50°C	2 min	1
Initial Denaturation	95°C	10 min	1
Denaturation	95°C	5 s	40 cycles
Annealing/Extension	60°C	30 s	
Dissociation Curve	95°C	15 s	1
Dissociation Curve	60°C	15 s	1
Hold	4°C	forever	

Infection of shRNA containing CD4+ lymphocytes with HIV-1-$NL_{4.3}$-GFP lentivirus

Timing: 3 h

This section provides step-by-step instructions for the 2nd spin infection of stimulated, shRNA containing CD4+ T lymphocytes with HIV-1-$NL_{4.3}$-GFP lentivirus on Day 8.

4. Spin infection of CD4+ T cells.
   a. Three days post-activation, resuspend cells and transfer everything to a suitable tube to remove the Dynabeads.
   b. For best results, immediately infect stimulated CD4+ T cells with the HIV-1-$NL_{4.3}$-GFP virus produced in steps 4–6.
   c. Add 1 × 10^6 cells to 1.7 mL microcentrifuge tubes and pellet cells by centrifugation at 800 × *g* at 20°C–25°C for 3 min.
   d. Remove supernatant without disturbing the pellet and add 500 μL of shRNA virus (10,000 ng of p24 Gag/mL), 5 μL 1 M HEPES and 0.5 μL of polybrene (4 μg/mL).
   e. Centrifuge at 800 × *g* at 32°C for 1.5 h.
   f. Resuspend cells with the virus containing supernatant and transfer it to one well of a 6 well Flat-bottom plate.
   g. Add 3 mL of fresh complete RPMI medium + 20 U/mL of IL-2 in each well.

   Note: The viral solution can be removed after spin-infection or remain with the cells to slightly increase infection over time.

   h. Culture for 4 days in a 5% CO_2 incubator at 37°C.
   i. Replace the medium with fresh complete RPMI medium + 20 U/mL of IL-2 every other day.

Analysis of shRNA-mCherry and HIV-1-$NL_{4.3}$-GFP containing CD4+ lymphocytes by flow cytometry

Timing: 2–4 h

In this paragraph we describe how to analyze SMYD5-mCherry and HIV-1-$NL_{4.3}$-GFP expressing CD4+ T cells by flow cytometry on Days 11 and 12.

5. Analysis of HIV-1-NL4.3-GFP expressing shSMYD5-mCherry containing cells by flow cytometry.
   a. 3 days after spin infection, wash cells twice with cold PBS.
   b. Spin down cells at 800 x g at 20°C–25°C for 3 min.
   c. Discard supernatant and stain cells with live/dead eBioscience Fixable Viability Dye eFluor 780.
      i. Prepare a 1:1,000 dilution of the dye in cold PBS buffer.
      ii. Resuspend the cells in 100 μL/well in a 96-well plate.

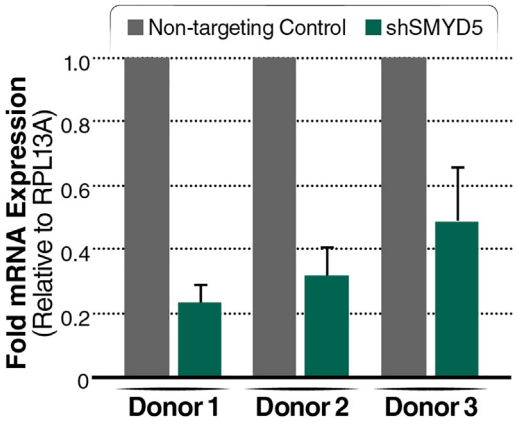

Figure 1. The level of SMYD5 mRNA, as determined by RT-PCR normalized to RPL13A mRNA

Average (mean ± SEM) from three technical replicates performed with three different donors is shown. For complete details, please refer to Figure 2 of Boehm et al.[1]

iii. Incubate for 15 min on ice in the dark.

Note: Live/dead eBioscience Fixable Viability Dye eFluor 780 works best since it is detected in the APC-Cy7 channel (red/ 637 nm) and does not overlap with FITC (blue/ 488 nm) and PE (yellow/ green/ 561 nm) channels. Therefore, no compensation is needed.

d. Wash cells 1x with cold PBS buffer and proceed directly to flow cytometry analysis (see Figure 2 for gating strategy).

Note: Avoid fixing samples with Paraformaldehyde (PFA) since it decreases fluorescence intensity. Cells can also be resuspended in PBS + 2% FBS to keep cell survival stable. For flow cytometry, a Fortessa X-20 cell analyzer was used.

EXPECTED OUTCOMES

Human CD4+ T lymphocytes isolated from three different donors were transduced with lentivirus expressing shRNA targeting SMYD5 or a non-targeting control. Knockout efficiency was assessed by RT-qPCR. SMYD5 expression levels should be reduced by 50%–80% (Figure 1).

Transduced $CD4^+$ T cell cultures were subsequently infected with a molecular clone of the viral isolate HIV-$1_{NL4.3}$. This virus contains the GFP open reading frame in place of *nef*, allowing identification of infected cells by flow cytometry.[5] After analysis by flow cytometry, populations were sub-gated to identify the percentage of HIV-1-$NL_{4.3}$-GFP CD4+ T lymphocytes in shRNA-mCherry containing cells (Figure 2).

Knockdown of SMYD5, as marked by mCherry expression, in all donors caused a reduction in GFP expression as compared to cells expressing the non-targeting shRNA control (Figure 3A), confirming that SMYD5 activates HIV-1 transcription in primary T cells. Cell viability was measured by flow cytometry with live/dead eBioscience Fixable Viability Dye eFluor 780 and was not affected by SMYD5 knockdown (Figure 3B).

QUANTIFICATION AND STATISTICAL ANALYSIS

All values are depicted as mean ± SD. Statistical analyses were performed using Prism Software (GraphPad). The average of three independent experiments analyzed in triplicate ± SEM is shown and compared with control samples by ANOVA: *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. For statistical analysis of T-cell activation experiments 1-way ANOVA with Dunnett's multiple comparison test $p < 0.01$, $n = 4$ was employed.

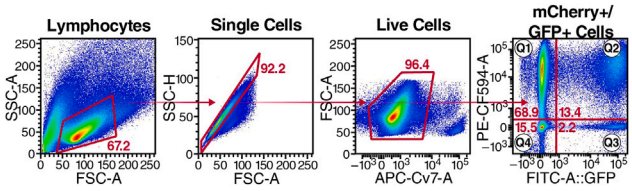

Figure 2. Representative gating strategy illustrating how the lymphocyte population from one of the three blood donors was sub-gated to the level of shRNA-mCherry+/ HIV- NL4.3-GFP+ CD4+ T lymphocytes

Red numbers represent the percentage of cells in each population. For complete details, please refer to Figure S2C of Boehm et al.[1]

LIMITATIONS

This protocol explains how to culture and transfect primary CD4+ T cells with shRNAs against the lysine methyltransferase SMYD5. This will knock-down but not eliminate this protein.

An alternative approach would be the use of gRNA/Cas9.

TROUBLESHOOTING

Problem 1

Low CD4+ T cell numbers or CD4+ T cells contaminated with other cells obtained from LRC (before you begin steps 1a-1n).

Potential solution

If possible, order blood from younger donors as CD4+ T cell count declines with age.

Instead of RosetteSep Human CD4+ T Cell Enrichment Cocktail try isolating first all peripheral blood mononuclear cells (PBMCs) using EasySep Direct Human PBMC Isolation Kit and then enrich CD4+ cells using the EasySep Human CD4+ T Cell Isolation Kit. It takes longer but often yields purer CD4+ cells cultures.

Problem 2

Transduction efficiency is too low (before you begin step 6a).

Potential solution

Make sure you are using the right filter material. For filtration, the virus supernatant should be filtered using filters made of polyethersulfone (PES), while other materials such as nitrocellulose (NC) membranes may adsorb the virus.

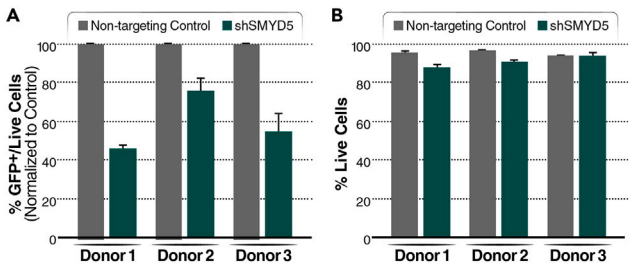

Figure 3. Knockdown of SMYD5 in primary CD4+ T cells inhibits HIV-1 expression

(A) Impact of non-targeting or SMYD5-targeting shRNAs on the percentage of HIV-1 expressing (GFP+) cells and (B) the percentage of live cells as measured by forward scatter analysis and viability stain. Average (mean ± SEM) from three technical replicates performed with three different donors is shown. For complete details, please refer to Figure 2 and S2C of Boehm et al.[1]

Problem 3

Virus concentration is too low (before you begin steps 6b and 6c).

Potential solution

Ultra-high speed centrifugation may have damaged the virus particles or the invisible pellet was not resuspended properly. A commercial concentration kit can be used to obtain high-titer virus.

Problem 4

CD4+ T cell number and activation of CD4+ T cells is too low (before you begin step 2).

Potential solution

Consider increasing the amounts of beads used and increase the time of activation with Dynabeads. CD4+ T cells can be activated for about 10 days, during this time they will expand.

Problem 5

The final cell number of shRNA-mCherry+/HIV- NL4.3-GFP+ CD4+ T lymphocytes is too low (Step 5).

Potential solution

Increase the number of cells used for the experiment and the amount of virus used for spin-infection or consider cell sorting.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Melanie Ott (mott@gladstone.ucsf.edu).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

Data reported in this paper will be shared by the lead contact upon request. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

D.B. designed the protocols and prepared the manuscript. M.O. supervised the studies and edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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