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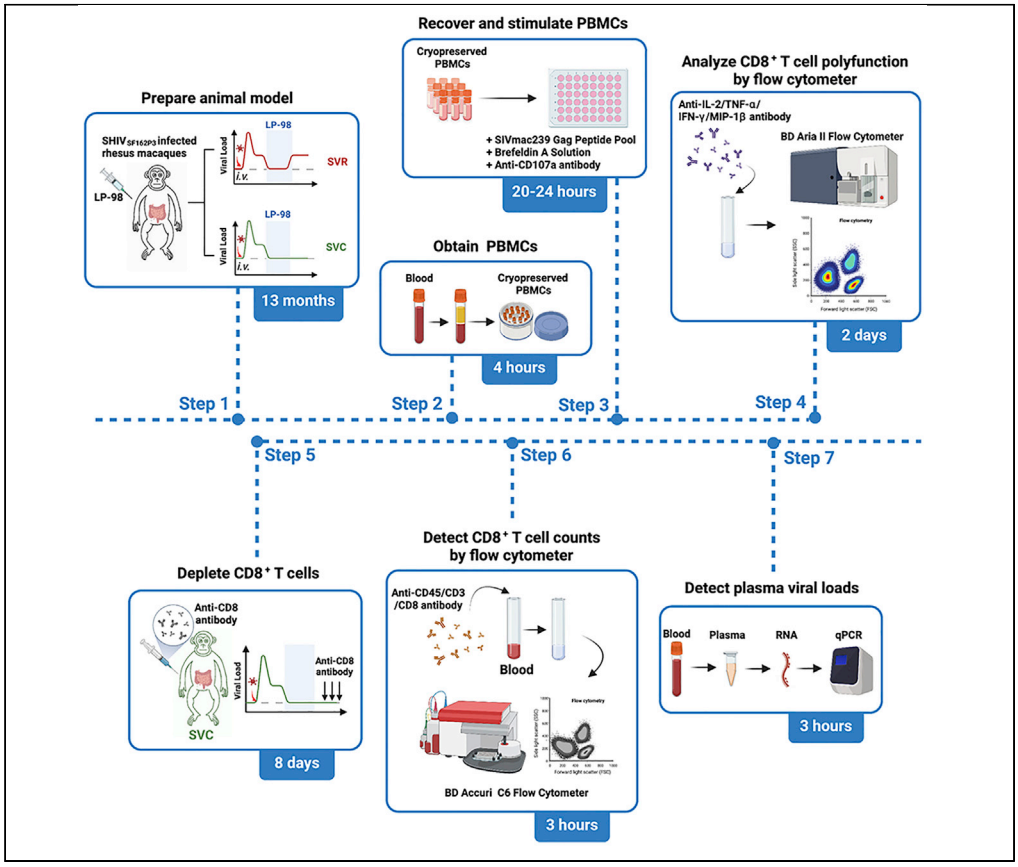
Protocol for evaluating CD8$^+$ T cell-mediated immunity in latently SHIV-infected rhesus macaques with HIV fusion-inhibitory lipopeptide monotherapy



Strong cellular immunity contributes to the control of HIV infection. Here, we describe a step-by-step protocol to assess the simian immunodeficiency virus (SIV)-specific CD8$^+$ T cell responses by quantifying the degranulation, cytokine and chemokine production from SHIV$_{SF162P3}$-infected rhesus macaques with an HIV fusion-inhibitory lipopeptide (LP-98) monotherapy. We also present the steps for adoptive transfer of an anti-CD8 antibody into a stable virologic control (SVC) group of LP-98-treated monkeys, confirming a direct role of CD8$^+$ T cells in SVC macaques.

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

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Highlights

Recover PBMCs from latently SHIV$_{SF162P3}$ infected rhesus macaques treated with LP-98

Evaluation of SIVmac239 Gag-specific CD8$^+$ T cell responses

Adoptive transfer of anti-CD8 antibody to stable virologic control (SVC) macaques

Quantitative detection of CD8$^+$ T cell counts and plasma viral RNA

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Protocol for evaluating CD8$^+$ T cell-mediated immunity in latently SHIV-infected rhesus macaques with HIV fusion-inhibitory lipopeptide monotherapy

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SUMMARY

Strong cellular immunity contributes to the control of HIV infection. Here, we describe a step-by-step protocol to assess the simian immunodeficiency virus (SIV)-specific CD8$^+$ T cell responses by quantifying the degranulation, cytokine and chemokine production from SHIV$_{SF162P3}$-infected rhesus macaques with an HIV fusion-inhibitory lipopeptide (LP-98) monotherapy. We also present the steps for adoptive transfer of an anti-CD8 antibody into a stable virologic control (SVC) group of LP-98-treated monkeys, confirming a direct role of CD8$^+$ T cells in SVC macaques.

For complete details on the use and execution of this protocol, please refer to Xue et al. (2022).

BEFORE YOU BEGIN

Chronic SHIV$_{SF162P3}$ infection of rhesus macaques and HIV fusion-inhibitory lipopeptide (LP-98)

Timing: 13 months

Seven 3- to 5-year-old Chinese rhesus macaques (*Macaca mulatta*) were used in this protocol. All animals were negative for major histocompatibility complex class I (MHC-I) Mamu-A*01, Mamu-A*02, Mamu-B*08, and Mamu-B*17 alleles related to the control of SIV/SHIV replication and negative for SIV, simian type D retrovirus, simian T-lymphotropic virus, and herpes B virus infections. SHIV$_{SF162P3}$ (100 TCID$_{50}$) was administered intravenously (*i.v.*) to seven Chinese rhesus macaques. Begin at 15 weeks post-challenge, all animals were intramuscularly treated with two rounds of low-dose (2 mg/kg) LP-98. Chronically infected macaques treated with LP-98 were divided into two groups based on dynamic changes in plasma viral RNA (vRNA). Five macaques (HB1, HB2, HB5, HB6 and HB7) showed plasma vRNA rebounding after LP-98 cessation and were defined as stable virologic rebound (SVR) group, while the remaining two macaques (HB3 and HB4), with undetectable vRNA, were defined as stable virologic control (SVC) group (Figure 1). Animals were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All animal experiments were conducted in compliance with the guideline (XJ19004) approved by the Institutional Animal Care and Use Committee (IACUC) of the Institute of Laboratory Animal

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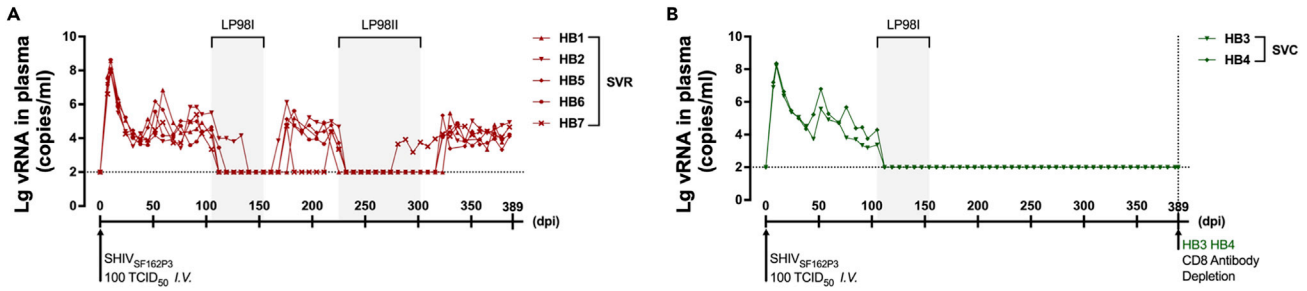

Figure 1. Viral RNA from chronically-infected monkeys treated with LP-98 monotherapy

Plasma viral RNA was detected by TaqMan RT-PCR. Figure reprinted with permission from Xue et al. (2022).

(A) Plasma viral RNA of SVR monkeys with LP-98 monotherapy.

(B) Plasma viral RNA of SVC monkeys with LP-98 monotherapy.

Science, Chinese Academy of Medical Sciences. All animal experiments are performed in Animal BioSafety Level 3 (ABSL-3) laboratory.

Sample collection

Timing: 30 min

1. Prepare EDTA blood collection tubes and 1.5 mL microfuge tubes labeled with the animals' codes.
2. To obtain peripheral blood samples during the experimental period:
   a. Sedate monkey with intramuscular injection of 10 mg/kg ketamine hydrochloride.
   b. Collect 2 mL (only used for plasma viral RNA detection) or 10 mL (used for plasma viral RNA detection and PBMC isolation) peripheral blood from vein of upper limb to labeled EDTA blood collection tubes using a multi-sample blood collection needle (BD Biosciences).
3. Clean the surface of the tubes with medical disinfectant wipers.
4. Centrifuge tubes at 500 × *g* for 10 min at 25°C.
5. Pipette supernatant plasma to labeled 1.5 mL microfuge tubes for extraction of viral RNA.
6. Save peripheral blood cells at the bottom of tubes for isolating PBMCs (please see below "obtaining PBMCs and cryopreservation of cells" section).

Note: We recommend collecting 2 mL peripheral blood every week to extract viral RNA for plasma viral RNA level detection and 10 mL peripheral blood every month to extract viral RNA and isolate PBMCs for plasma viral RNA level detection and SIV-specific $CD8^+$ T cell response evaluation.

Determination of plasma viral RNA level

Timing: 3 h

The plasma viral RNA is quantitated to monitor the progression of virus infection. The procedure consists of the following steps:

7. Extract viral RNA from plasma with the QIAmp viral RNA mini kit (QIAGEN) according to manufacturer's instructions:
https://www.qiagen.com/us/resources/resourcedetail?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en.

The viral RNA is extracted and double eluted to 2 × 40 μL Buffer AVE (from the QIAmp viral RNA mini kit, QIAGEN). We use 5 μL viral RNA (typically 0.2–0.35 μg) for following real-time quantitative PCR (RT-qPCR).

8. The TaqMan real-time quantitative PCR (RT-qPCR) assay is carried out on an ABI 7500 real-time PCR system (Applied Biosystems) using the following SIVmac239 gag primers (Hofmann-Lehmann et al., 2000; Liu et al., 2008), qPCR reaction master mix and qPCR cycling protocol:
   a. Primer sequences:
   SIVmac239 gag forward primer: 5′- GCAGAGGAGGAAATTACCCAGTAC- 3′.
   SIVmac239 gag reverse primer: 5′- CAATTTTACCCAGGCATTTAATGTT-3′.
   Probe for viral RNA: 5′-(FAM)-ACCTGCCATTAAGCCCGA-(MGB)-3′.
   b. The viral RNA is quantitated using the TaqMan® RNA-to-CT™ 1-step kit (AB Applied Biosystems) according to manufacturer's instructions:
   https://www.thermofisher.cn/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.cn%2FTFS-Assets%2FLSG%2Fmanuals%2F4393463D.pdf.
   The TaqMan RT-PCR reaction mix is shown in the following table. DEPC (diethyl pyrocarbonate)-treated water and SIVmac239 RNA are included as negative control and positive control, separately.

TaqMan RT-PCR reaction master mix

Reagent	Final concentration	Amount
PCR H_2O	n/a	3.4 μL
2 × TaqMan® RT-PCR Mix	n/a	10 μL
40 × TaqMan® RT Enzyme Mix	n/a	0.3 μL
5′ SIVmac239 gag primer (10 μM)	0.25 μM	0.5 μL
3′ SIVmac239 gag primer (10 μM)	0.25 μM	0.5 μL
Probe for viral RNA	n/a	0.3 μL
Viral RNA	n/a	5 μL
Total		**20 μL**

   c. The TaqMan RT-PCR cycling protocol is shown in the following table.

TaqMan RT-PCR cycling condition

Steps	Temperature	Time	Cycles
RT	48°C	30 min	1
Enzyme Activation	95°C	10 min	1
Denaturation	95°C	15 s	40
Annealing/extension	60°C	1 min	

   d. Virus copy numbers for samples were estimated by comparison with a standard curve of RNA standards (duplicate reactions for half $\log_{10}$ dilutions from 10^2 copies/reaction to 10^7 copies/reaction) for a purified *in vitro* transcript control template and calculated per volume of plasma using input RNA eluent volume (Cline et al., 2005). Calculate the copy number of viral RNA in 1 mL plasma using the following formula, where copiesSample is the copy number of viral RNA per μL in viral RNA elution that TaqMan RT-PCR detects and μLSample elution is the volume of the viral RNA elution and μLPlasma is the volume of plasma for viral RNA extraction:

$$Viral\ RNA\ = copies\ Sample \times \mu L\ Sample\ elution \times \frac{1000}{\mu L\ Plasma}$$

The threshold sensitivity of the assay is 100 copies/mL for viral RNA in plasma.

Obtaining PBMCs and cryopreservation of cells

Timing: 4 h

Timing: 3.5 h for obtaining PBMCs from EDTA blood plasma tubes

Timing: 30 min for cryopreservation of PBMCs

9. Obtain PBMCs from EDTA blood plasma tubes.
   a. Pipet 5 mL of Ficoll-Paque PLUS into 15 mL centrifuge tubes labeled with each animal code.
   b. Dilute peripheral blood cells mentioned in step 6 of the sample collection section with RPMI 1640 medium at an equal volume to that of the peripheral blood cells at 25°C and mix by pipetting gently.
   c. Carefully overlay the diluted peripheral blood cells onto the Ficoll-Paque PLUS in the centrifuge tubes using sterile pipet tips.
   d. Centrifuge tubes at 720 × *g* for 30 min with slow acceleration and deceleration at 25°C. Centrifuge tubes with brake OFF.
   e. After centrifugation, there are four layers, from top to bottom: RPMI, PBMCs, Ficoll-Paque PLUS and the remaining peripheral blood cells. Remove and discard the upper RPMI layer using a sterile plastic pipet being careful not to disturb the PBMC layer.
   f. Recover and wash PBMCs.
      i. Pipet 7 mL RPMI 1640 medium into new 15 mL tubes labeled with each animal code.
      ii. Aspirate the isolated PBMC layers and transfer to the labeled tubes.
      iii. Centrifuge the cells at 400 × *g* for 10 min at 4°C.
      iv. Remove the supernatants and gently tap the bottom of the tubes to resuspend the cells in the small amount of liquid remaining at the bottom of the tubes.
   g. Count PBMCs.
      i. Add 10 mL of RPMI 1640 medium to each tube and gently resuspend PBMCs.
      ii. Pipet 10 μL of cell suspension to stain with trypan blue.
      iii. Transfer the cells into the wells of a Glasstic Slide 10 with counting grids.
      iv. Count and calculate the total number of cells.
   h. Collect PBMCs by centrifuging at 400 × *g* for 10 min at 4°C. Gently tap the bottom of the tubes to resuspend the PBMCs in the small amount of liquid remaining at the bottom of the tubes.

Note: We recommend that the ratio of Ficoll-Paque PLUS volume to diluted peripheral blood cell volume is about 1:2 in step (a).

Note: When pipetting the diluted peripheral blood cells onto the Ficoll-Paque PLUS layer in step (c), go slowly to avoid mixing.

Note: Centrifugation of tubes containing Ficoll-Paque PLUS and peripheral blood cells should be conducted with slow acceleration and deceleration in step (d) to avoid disturbing overlay and improve PBMCs separation. It is important to maintain brake OFF to prevent the centrifuge from slowing down too abruptly, which could lead to mixing of the different layers. However, the centrifuge can be accelerated at max and decelerated at max in other centrifugation steps to spin down PBMCs.

Note: In step (e), the PBMC layer can also be aspirated from below the top RPMI layer without removing it first.

Alternatives: PBS could be used to replace the RPMI 1640 medium for diluting peripheral blood cells for its buffering effect to protect the cells in step (b). However, considering that

RPMI 1640 medium can provide extra nutrients conducive to cell survival, we prefer to dilute peripheral blood cells with RPMI 1640 medium.

10. Cryopreservation of PBMCs.
    a. Suspend isolated PBMCs at a concentration of 10^7 cells/mL in cryopreservation solution (see below for composition and source).
    b. Aliquot 1 mL of suspended PBMCs into a 2 mL cryovial.
    c. Put the cryovials into the Mr. Frosty (TM) freezing container (Thermo Scientific) and place the container at -80°C for 24 h.
    d. Transfer the cryovials to liquid nitrogen for long-term storage.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC mouse anti-NHP CD45 Antibody (1:10 dilution)	BD Biosciences	Cat#: 561290, RRID: AB_10613814
PerCP mouse anti-human CD3 Antibody (1:2.5 dilution)	BD Biosciences	Cat#: 552851, RRID: AB_394492
PE mouse anti-human CD8 Antibody (1:2.5 dilution)	BD Biosciences	Cat#:555367, RRID: AB_395770
BV605 mouse anti-human CD3 Antibody (1:20 dilution)	BD Biosciences	Cat#:562994, RRID: AB_2737938
FITC mouse anti-human CD8 Antibody (1:5 dilution)	BD Biosciences	Cat#:557085, RRID: AB_396580
BV711 mouse anti-human IFN-γ Antibody (1:20 dilution)	BioLegend	Cat#:502540, RRID: AB_2563506
BV421 mouse anti-human MIP-1β Antibody (1:20 dilution)	BD Biosciences	Cat#:562900, RRID: AB_2737877
BV786 mouse anti-human CD107a Antibody (1:100 dilution)	BD Biosciences	Cat#:563869, RRID: AB_2738458
PE/cyanine7 rat anti-human IL2 Antibody (1:20 dilution)	BioLegend	Cat#: 500326, RRID: AB_2125593
BV650 mouse anti-human TNF-α Antibody (1:20 dilution)	BioLegend	Cat#: 502938, RRID: AB_2125593
Anti-CD8 alpha [MT807R1]	Nonhuman Primate Reagent Resource	Cat#: PR-0817, RRID: AB_2716320
Chemicals, peptides, and recombinant proteins		
Ficoll-Paque PLUS	GE Healthcare	CAT#: 17-1440-03
Phosphate-buffered saline (PBS)	Gibco	CAT#: C0010500BT
Brefeldin A solution (1000 ×)	BioLegend	CAT#: 420601
RPMI 1640 medium	Gibco	CAT#: C22400500BT
Penicillin/streptomycin solution	Cytiva	CAT#: SV30010
Fetal bovine serum (FBS)	Gibco	CAT#:10099-141C
Bovine serum albumin (BSA)	VETEC	CAT#: V900933-100G
Fixation and permeabilization solution	BD Biosciences	CAT#: 554722
Perm/Wash Buffer (10 ×)	BD Biosciences	CAT#: 554723
Zombie NIR Fixable Viability Kit (DMSO)	BioLegend	CAT#: 423106
Brilliant Stain Buffer Plus	BD Biosciences	CAT#: 566385
SIVmac239 Gag Peptide Pool	NIH HIV Reagent Program	CAT#: APR-12364
Cell Stimulation Cocktail (500 ×)	eBioscience	CAT#: 00-4970-93
FACS™ Lysing Solution	BD Biosciences	CAT#: 349202
Staining buffer (BSA)	BD Biosciences	CAT#: 554657
Critical commercial assays		
QIAamp Viral RNA Mini Kit	QIAGEN	CAT#: 52906
TaqMan® RNA-to-C_T™ 1-Step Kit	AB Applied Biosystems	CAT#: 4392938
Experimental models: Organisms/strains		
Rhesus macaques (*Macaca mulatta*), 3–5 years old, 3 males, 4 females	Beijing Institute of Xieerxin Biology Resource	SYXK(Beijing) 2017-0027
$SHIV_{SF162P3}$	NIH HIV Reagent Program	CAT#: APR-6526
Oligonucleotides		
SIVmac239 gag forward Primer: GCAGAGGAGGAAAT TACCCAGTAC	This paper	N/A
SIVmac239 gag reverse Primer: CAATTTTACCCAGGCATTTAATGTT	This paper	N/A
Probe for Viral RNA: ACCTGCCATTAAGCCCGA	This paper	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo V10.0 software	FlowJo	www.flowjo.com
Other		
Glasstic Slide 10 with counting grids	KOVA International	CAT#: 87144E
Trucount™ Tubes	BD Biosciences	CAT#: 340334
Multi-sample blood collection needle	BD Biosciences	CAT#: 360213
Mr. Frosty (TM) Freezing Container	Thermo Scientific	CAT#: 5100-0001
ABI 7500 real-time PCR system	Applied Biosystems Instr.	V7500
BD Aria II Flow Cytometer	BD Biosciences	CAT#: 643180
BD Accuri C6 Flow Cytometer	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Cell cryopreservation solution		
Reagent	Final concentration	Amount
Heat inactivated FBS	90%	9 mL
Dimethyl sulfoxide (DMSO)	10%	1 mL
Total	**n/a**	**10 mL**

Note: The solution should be prepared fresh.

Complete RPMI 1640 medium		
Reagent	Final concentration	Amount
RPMI 1640	n/a	445 mL
Heat inactivated FBS	10%	50 mL
Penicillin/streptomycin solution	Penicillin: 100 U/mL; streptomycin: 100 μg/mL	5 mL
Total	**n/a**	**500 mL**

Note: Complete RPMI 1640 medium should be stored at 4°C for up to one month.

SIVmac239 Gag peptide pool stock solution		
Reagent	Final concentration	Amount
SIVmac239 Gag peptide pool (consists of 125 peptides)	25 mg/mL	12.5 mg (one vial, 100 μg/peptide)
DMSO	n/a	50 μL
PBS	n/a	450 μL
Total	**n/a**	**500 μL**

Note: One vial of SIVmac239 Gag peptide pool composed of 125 peptides (12.5 mg) is dissolved to make up SIVmac239 Gag peptide pool stock solution. The peptide pool is first dissolved in 50 μL DMSO, then 450 μL PBS is added slowly to the solution to avoid precipitation of peptides. The final stock concentration of SIVmac239 Gag peptide pool stock solution is 25 mg/mL. Mix, aliquot and store peptide pool solution at -80°C.

Alternatives: SIVmac239 Gag peptide pool (NIH HIV Reagent Program, CAT#: APR-12364) used in this protocol is a mixture of 125 individual peptides in one vial. The complete set of SIVmac239 Gag peptides (NIH HIV Reagent Program, CAT#: 6204) is comprised of the same 125 peptides, but each peptide is contained in an individual vial, which can be mixed and substituted as need in the stimulation experiments.

SIVmac239 Gag peptide pool working solution		
Reagent	Final concentration	Amount
SIVmac239 Gag peptide pool stock solution	2.5 mg/mL	50 μL
PBS	n/a	450 μL
Total	**n/a**	**500 μL**

Note: When diluting SIVmac239 Gag peptide pool stock solution, add the PBS slowly to avoid precipitation of the peptides. SIVmac239 Gag peptide pool working solution should be prepared fresh.

10 × Cell Stimulation Cocktail solution		
Reagent	Final concentration	Amount
Cell Stimulation Cocktail (500 ×)	10 ×	10 μL
Complete RPMI 1640 medium	n/a	490 μL
Total	**n/a**	**500 μL**

Note: The solution should be prepared fresh.

250 × Brefeldin A solution		
Reagent	Final concentration	Amount
Brefeldin A solution (1000 ×)	250 ×	15 μL
Complete RPMI 1640 medium	n/a	45 μL
Total	**n/a**	**60 μL**

Note: The solution should be prepared fresh.

FACS washing buffer		
Reagent	Final concentration	Amount
PBS	n/a	500 mL
BSA	0.2%	1 g
Sodium azide	0.09%	0.45 g
Total	**n/a**	**500 mL**

Note: The buffer should be prepared fresh or stored at 4°C for up to one week.

1 × Perm/wash buffer		
Reagent	Final concentration	Amount
10 × Perm/wash buffer	1 ×	10 mL
ddH_2O	n/a	90 mL
Total	**n/a**	**100 mL**

Note: The buffer should be prepared fresh for each use.

1 × FACS™ lysis solution		
Reagent	Final concentration	Amount
10 × FACS™ lysis solution	1 ×	1 mL
ddH_2O	n/a	9 mL
Total	**n/a**	**10 mL**

Note: The solution should be prepared fresh for each use.

Extracellular staining solution for evaluation of SIVmac239 Gag-specific $CD8^+$ T cell responses		
Reagent	Final concentration	Amount
BV605 anti-human CD3 antibody	n/a	5 μL
FITC anti-human CD8 antibody	n/a	20 μL
FACS washing buffer	n/a	75 μL
Total	**n/a**	**100 μL**

Note: The volumes of antibodies adopt the volumes recommended by manufacturer's instructions that are tested to be suitable for evaluation of SIVmac239 Gag-specific $CD8^+$ T cell responses in this protocol. The solution should be prepared fresh.

Intracellular staining solution for evaluation of SIVmac239 Gag-specific $CD8^+$ T cell responses		
Reagent	Final concentration	Amount
Brilliant Stain Buffer Plus	n/a	10 μL
BV711 anti-human IFN-γ antibody	n/a	5 μL
BV421 anti-human MIP-1β antibody	n/a	5 μL
PE/cyanine7 anti-human IL-2 antibody	n/a	5 μL
BV650 anti-human TNF-α antibody	n/a	5 μL
1 × Perm/wash buffer	n/a	70 μL
Total	**n/a**	**100 μL**

Note: The volumes of antibodies adopt the volumes recommended by manufacturer's instructions that are tested to be suitable for evaluation of SIVmac239 Gag-specific $CD8^+$ T cell responses in this protocol. The solution should be prepared fresh.

Note: Brilliant Stain Buffer Plus is a buffer formulated for mitigating staining artifacts seen when using two or more Brilliant violet dyes in multicolor immunofluorescent staining.

Extracellular staining solution for detection of $CD8^+$ T cell counts		
Reagent	Final concentration	Amount
APC anti-NHP CD45 antibody	n/a	5 μL
PerCP anti-human CD3 antibody	n/a	20 μL
PE anti-human CD8 antibody	n/a	20 μL
PBS	n/a	5 μL
Total	**n/a**	**50 μL**

Note: The volumes of antibodies adopt the volumes recommended by manufacturer's instructions that are tested to be suitable for detection of $CD8^+$ T cell counts in this protocol. The solution should be prepared fresh.

We use a BD Aria II flow cytometer with three lasers (488 nm, 633 nm and 407 nm) and a BD Accuri C6 flow cytometer with two lasers (488 nm and 640 nm).

Alternatives: Other types of flow cytometers (e. g., BD Fortessa) with appropriate lasers for detection of these fluorophore-tagged antibodies can be used for analysis. The same antibodies with different conjugated fluorophores can be used for instruments with different lasers. It is worth noting that inappropriate combinations of fluorophore-tagged antibodies may give unsatisfactory detection results. We recommend that users get help from technical professionals to determine the best fluorophore-tagged antibody-matching scheme for flow cytometry to minimize signal interference between different detection channels and avoid detecting poor fluorescence signals detection. Every fluorophore-tagged antibody should be tested to work well alone.

STEP-BY-STEP METHOD DETAILS

Evaluation of SIVmac239 gag-specific $CD8^+$ T cell responses after LP-98 withdrawal

Timing: 3 days

Timing: 13-17 h to thaw and recover PBMCs

Timing: 7 h for stimulation of PBMCs

Timing: 4 h for staining with fluorescent antibodies for flow cytometry

Timing: 1 day to acquire flow cytometry data

PBMCs are obtained from blood samples taken from the macaques after LP-98 treatment cessation, to explore the effects of LP-98 therapy on $CD8^+$ T cells and the underlying mechanisms. PBMCs are stimulated with the SIVmac239 Gag peptide pool to evaluate the SIVmac239 Gag-specific $CD8^+$ T cell responses (Xue et al., 2022). Degranulation (CD107a), cytokine (IFN-γ, TNF-α and IL-2) and chemokine production (MIP-1β) were chosen as indicators to determine SIVmac239 Gag-specific $CD8^+$ T cell function (Betts et al., 2006; Nguyen et al., 2019). All steps are performed in biosafety cabinet using sterile reagents and plastic consumables.

1. Thaw and recover PBMCs.
   a. Prepare 15 mL tubes labeled with PBMC IDs.
   b. Transfer 9 mL complete RPMI 1640 medium to 15 mL tubes.
   c. Place cryovials containing cryopreserved PBMCs in a 37°C water bath.
   d. Take cryovials out of the water bath once cells are almost thawed (about 1–2 min) with a small piece of ice remaining.
   e. Transfer 1 mL cell suspensions into 15 mL tubes containing complete RPMI 1640 medium. Mix the cells by pipetting up and down gently.
   f. Count cells.
      i. Pipet 10 μL of resuspended cells to stain with trypan blue.
      ii. Transfer the cells into the wells of a Glasstic Slide 10 with counting grids.
      iii. Count and calculate the total PBMCs for each sample.
   g. Centrifuge 15 mL tubes at 400 × *g* for 10 min at 4°C.
   h. Remove and discard supernatants and gently tap the bottom of the tubes to resuspend the cells in the liquid remaining at the bottom of the tubes.
   i. Suspend PBMCs at a concentration of 2×10^6/mL in complete RPMI 1640 medium.
   j. Prepare 48-well plates labeled with 'negative', 'SIVmac239 Gag peptide pool' and 'positive' for each sample.

k. Seed 500 μL (1×10^6) of PBMCs into each well of 48-well plates.
l. Culture cells at 37°C in a 5% CO_2 incubator for about 12–16 h.

Note: The cell concentration does not have to be 2×10^6/mL in step (i). Users can choose a different cell concentration as long as it is sufficient to ensure seeding 10^6 PBMCs in each well in step (k). The volume of cells and complete RPMI 1640 medium added to each well depends on the cell concentration, and make sure that there are 10^6 PBMCs in 500 μL of complete RPMI 1640 medium.

Note: The thawed PBMCs need to be recovered overnight for the SIVmac239 Gag peptide pool stimulation assay. We recommend that cryopreserved PBMCs be thawed in late afternoon and allowed to recover for about 12–16 h.

Note: Please refer to the troubleshooting section (problem 1) for concerns if the number of viable PBMCs after thawing is too low.

2. Stimulation of PBMCs.
   a. Remove 50 μL solution from each well.
   b. Add 50 μL of SIVmac239 Gag peptide pool working solution (2.5 mg/mL) into each well of the SIVmac239 Gag peptide pool group (at a final concentration of 0.25 mg/mL).
   c. Add 50 μL of complete RPMI 1640 medium per well for the negative group.
   d. Add 50 μL 10 × cell stimulation cocktail solution (at a final concentration of 1 ×) per well for the positive group.
   e. Add 2 μL 250 × brefeldin A solution (at a final concentration of 1 ×) to each well in all experimental groups.
   f. Add 5 μL of BV786 anti-human CD107a antibody per well in all experimental groups.
   g. Mix the reagents and cells by pipetting up and down and culture the cells at 37°C, in a 5% CO_2 incubator for 6 h.

Note: In place of SIVmac239 Gag peptides, the SIVmac239 Env peptides, SIVmac239 Pol peptides, SIVmac239 Nef peptides or SIVmac239 Vif peptides can also be used for antigenic stimulation to measure SIVmac239-specific $CD8^+$ T cell immune response. It may be better to test different peptides to reflect the function of $CD8^+$ T cells more broadly.

Note: Users can choose to add costimulatory antibodies (anti-human CD28 antibody and anti-human CD49d antibody) to both negative, positive and SIVmac239 Gag peptide pool groups to yield clearer results in terms of promoting the production of IFN-γ, MIP-1β, TNF-α, IL-2 and CD107a by SIV Gag-specific $CD8^+$ T cells.

Note: Brefeldin A is commonly used to block protein transport processes so that intracellular cytokines accumulate at the Golgi complex/endoplasmic reticulum for better staining.

Note: Users can add GolgiStop to all wells to allow accumulation of intracellular cytokines in the Golgi complex, enhancing the cytokine staining signals for flow cytometric analysis.

Note: We recommend adding anti-CD107a antibody at the start of stimulation because of the transient nature of the surface expression of CD107a on $CD8^+$ T cells. CD107a is located primarily on lysosomal/endosomal membranes, and is rapidly removed via the endocytic pathway in stimulated $CD8^+$ T cells (Betts et al., 2003).

Note: We chose six hours as the optimal stimulation time based on pilot experiments, consistent with that reported in the previous reference (Passaes et al., 2020). Users should perform

pilot experiments to determine the optimal incubation time following SIVmac239 Gag peptide pool stimulation that will vary according to the experimental conditions.

⚠ CRITICAL: Cells will settle to the bottom after 12–16 h of incubation. Users must gently remove the medium at step (a) to avoid cell loss from the bottom of 48-well plates.

3. Staining with fluorescent antibodies for flow cytometry.
   a. Prepare round-bottom polystyrene tubes labeled with 'negative' and 'SIVmac239 Gag stimulated' and 'positive' for each PBMC sample.
   b. After stimulation, transfer cells from 48-well plates to tubes.
   c. Wash cells.
      i. Add 2 mL PBS to wash cells.
      ii. Centrifuge cells at 350 × *g* for 5 min at 25°C.
      iii. Remove and discard supernatants and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
   d. Viability stain.
      i. Resuspend cells in 100 μL PBS.
      ii. Add 1 μL Zombie NIR fixable viability kit (BioLegend) at a final concentration of 1:100.
      iii. Mix cells by vortexing gently and incubate at 25°C for 20 min in the dark.
   e. Wash cells twice.
      i. Add 2 mL FACS washing buffer.
      ii. Centrifuge cells at 350 × *g* for 5 min at 4°C.
      iii. Remove and discard the supernatants and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
      iv. Repeat step (i-iii).
   f. Prepare extracellular staining solution.
      i. Calculate the total volume of extracellular staining solution needed according to number of samples (see 'materials and equipment' section for composition).
      ii. Add required volume of FACS washing buffer to a new tube labeled with extracellular staining solution.
      iii. Add required volume of BV605 anti-human CD3 antibody and FITC anti-human CD8 antibody to the tube.
      iv. Mix extracellular staining solution by vortexing.
   g. Stain extracellular markers.
      i. Resuspend cells in 100 μL extracellular staining solution.
      ii. Mix cells by vortexing gently and incubate at 4°C for 30 min in the dark.
   h. Wash cells twice.
      i. Add 2 mL FACS washing buffer.
      ii. Centrifuge the cells at 350 × *g* for 5 min at 4°C.
      iii. Remove and discard the supernatants and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
      iv. Repeat step (i-iii).
   i. Fix and permeabilize the cells.
      i. Add 250 μL of fixation/permeabilization solution (BD Biosciences) to each tube.
      ii. Mix cells by vortexing gently and incubate at 4°C for 20 min in the dark.
   j. Wash cells twice.
      i. Add 2 mL 1 × Perm/wash buffer (BD Biosciences) to each tube.
      ii. Centrifuge the cells at 350 × *g* for 5 min at 4°C.
      iii. Remove the supernatant and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
      iv. Repeat step (i-iii).
   k. Prepare intracellular staining solution.

i. Calculate the total volume of intracellular staining solution needed according to number of samples (see 'materials and equipment' section for composition).
ii. Add required volume of 1 × Perm/wash buffer to a new tube labeled with intracellular staining solution.
iii. Add required volume of Brilliant Stain Buffer Plus to the tube.
iv. Add required volume of BV711 anti-human IFN-γ antibody, BV421 anti-human MIP-1β antibody, PE/cyanine7 anti-human IL-2 antibody and BV650 anti-human TNF-α antibody to the tube.
v. Mix intracellular staining solution by vortexing.

l. Stain intracellular markers.
i. Resuspend cells in 100 μL intracellular staining solution.
ii. Mix cells by vortexing gently and incubate at 4°C for 30 min in the dark.

m. Wash cells twice.
i. Add 2 mL of 1 × Perm/wash buffer to each tube.
ii. Centrifuge the cells at 350 × *g* for 5 min at 4°C.
iii. Remove and discard the supernatants and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
iv. Repeat step (i-iii).

n. Fix cells.
i. Add 300 μL of 1% paraformaldehyde to each tube.
ii. Mix cells by vortexing gently and incubate at 4°C for 15–20 min in the dark.

o. Wash cells twice.
i. Add 2 mL FACS washing buffer to all tubes.
ii. Centrifuge the cells at 600 × *g* for 3 min at 4°C.
iii. Remove and discard the supernatants and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
iv. Repeat step (i-iii).

p. Resuspend cells in 300 μL PBS.
q. Store the cells at 4°C in the dark and plan to acquire the data from the experimental cells on the BD Aria II flow cytometer within 24 h.

Note: The viability dye and antibody can be conjugated to different fluorophores depending on the lasers available on the type of flow cytometer used.

Note: When removing the supernatant after centrifugation, remove residual solution by pressing the mouth of the tube on a paper towel for 1–2 s.

Note: Dissolve one vial of Zombie NIR fixable viability dye in 100 μL DMSO at 25°C, and dilute at 1:100–1000 in PBS. The optimum concentration is determined before the experiment by measuring the effect of different concentrations on live cell identification.

Note: In consideration of biosafety in handing samples from $SHIV_{SF162P3}$ infected rhesus macaques, we choose to fix samples with 1% paraformaldehyde to inactive the SHIV, protecting operators and BD Aria II flow cytometer against $SHIV_{SF162P3}$ infection. In addition, treating samples with 1% paraformaldehyde can maintain the stability of fluorescence signals among large-sample detection by flow cytometer so that users can acquire enough time to get stable data from cell samples.

Alternatives: FACS washing buffer can be substitute for the commercial cell staining buffer that is useful for the dilution of fluorescent reagents as well as for the suspension, and washing of cells destined for flow cytometric analysis, such as Cell Staining Buffer (Biolegend, Cat#: 420201), Stain Buffer (BD Biosciences, Cat#: 554657), et al.

⚠ CRITICAL: Before staining with the Zombie NIR fixable viability dye, cells must be washed and resuspended in PBS without Tris buffer or proteins like BSA.

4. Acquire flow cytometry data.
   a. Adjust fluorescence detector voltage and compensation appropriately by using a blank tube and positive single fluorescence tubes.
   b. Record data from BD Aria II flow cytometer.
      i. Record data from fluorescence-minus-one (FMO) tubes to define gates for the positive cell populations.
      ii. Acquire data from experimental cells in labeled tubes.
   c. Analyze data using FlowJo v10.0 software as outlined in the quantification and statistical analysis section.

Note: Mix cells by vortexing the tubes briefly before running on the flow cytometer to ensure accuracy and reproducibility of data.

Note: Refer to the troubleshooting section (problem 2) for concerns if the fluorescence of stained intracellular cytokines is not detected.

Note: Refer to the troubleshooting section (problem 3) for concerns regarding inconsistent functional molecule production by SIVmac239 Gag-specific $CD8^+$ T cell in different separate experiments.

Adoptive infusion of anti-CD8 antibody in SVC macaques after cessation of LP-98 treatment

Timing: 8 days

Timing: 8 days to deplete $CD8^+$ T cells

Timing: 3 h to count $CD8^+$ T cells

Timing: 3 h to measure plasma virus RNA

To determine if the fact that the plasma virus of stable virologic control (SVC) macaques remains undetectable after LP-98 treatment cessation was related to a strong $CD8^+$ T cell immune response, we depleted $CD8^+$ T cells by using adoptive infusion of anti-CD8 antibody in SVC macaques. Dynamic changes in $CD8^+$ T cell counts and plasma virus loading were monitored to directly assess the role of $CD8^+$ T cell response (Cardozo et al., 2018).

5. Deplete $CD8^+$ T cells.

SVC macaques were treated by adoptive infusion of anti-CD8 alpha [MT807R1] to deplete $CD8^+$ T cells after LP-98 withdrawal. All animals were anesthetized with 10 mg/kg intramuscular ketamine hydrochloride before performing the following two steps.

   a. Inject anti-CD8 alpha [MT807R1] subcutaneously into SVC monkeys on day 0.
      i. Shave nape of the neck with hair clipper.
      ii. Lift the skin and inject 10 mg/kg of anti-CD8 alpha [MT807R1] with needle held horizontally.
   b. Inject anti-CD8 alpha [MT807R1] intravenously into SVC monkeys on days 3 and 7 after the initial anti-CD8 antibody infusion.
      i. Shave hind limb with hair clipper.
      ii. Inject 5 mg/kg of anti-CD8 alpha [MT807R1] slowly into the obvious vein on the hind limb (Figure 1).

Note: We strictly recommend injecting anti-CD8alpha [MT807R1] subcutaneously first followed by two intravenous infusions according to previous reports (Amara et al., 2005; Lifson et al., 2001). We recommend injecting anti-CD8 alpha [MT807R1] subcutaneously at multiple spots at the initial infusion for better absorption.

6. Count $CD8^+$ T cells.
   a. Prepare round-bottom polystyrene tubes marked with the animal codes.
   b. Pipet 50 μL of peripheral blood into the bottom of each tube.
   c. Prepare extracellular staining solution.
      i. Calculate the total volume of extracellular staining solution needed for the number of samples (see 'materials and equipment' section for composition).
      ii. Add required volume of PBS to a new tube labeled with extracellular staining solution.
      iii. Add required volume of APC-anti-NHP CD45 antibody, PerCP-anti-human CD3 antibody, and PE-anti-human CD8 antibody to the tube.
      iv. Mix extracellular staining solution by vortexing.
   d. Stain extracellular markers.
      i. Add 50 μL extracellular staining solution to each tube.
      ii. Mix cells by vortexing gently and incubate in the dark at 4°C for 30 min.
   e. Add 1 mL of 1 × FACS™ lysing solution to each tube to lyse red blood cells for about 3 min.
   f. Add 1 mL PBS to each tube to stop lysis once liquid becomes clear.
   g. Centrifuge cells at 1100 × *g* for 5 min at 4°C.
   h. Remove and discard the supernatants and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
   i. Wash cells twice.
      i. Add 2 mL PBS to each tube.
      ii. Centrifuge cells at 1100 × *g* for 5 min at 4°C.
      iii. Remove and discard the supernatants and gently tap the bottom of the tubes to resuspend cells in liquid remaining at the bottom of the tubes.
      iv. Repeat step (i-iii).
   j. Add 1 mL of Staining buffer (BD Biosciences) and resuspend cells.
   k. Prepare Trucount™ Tubes (BD Biosciences) labeled with the animal codes.
   l. Transfer 500 μL of cell suspension to Trucount™ Tubes.
   m. Acquire and analyze the data from experimental cells on BD Accuri C6 flow cytometer in time.

   Note: Mix peripheral blood fully before pipetting it into the tubes in step (b).

   Note: Users should always pay attention to the change of liquid clarity when adding 1 × FACS™ lysing solution to tubes in step (e) and add PBS immediately to prevent excessive lysis and protect lymphocytes in step (f).

   Note: Lysing solutions from different suppliers may have different cell lysis capabilities, therefore the incubation time of different lysing solution before the liquid become clear in step (e) should be determined by pilot experiments.

   Note: After discarding the supernatants after centrifugation, remove residual solution by pressing the mouth of the tube on a paper towel for 1–2 s.

7. Measure plasma virus RNA.

The specific steps are the same as those described in "determination of plasma viral RNA level" in the "before you begin" section.

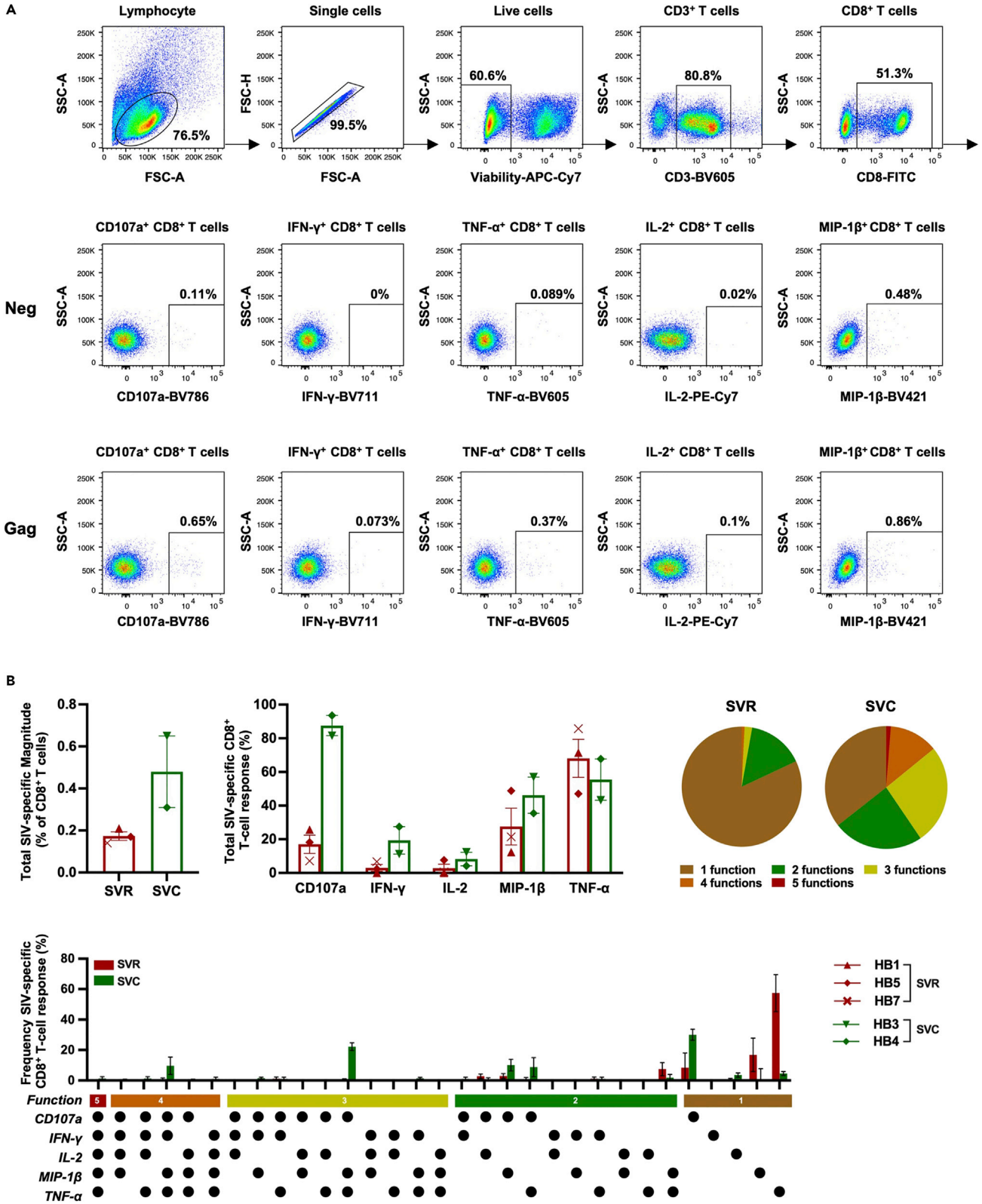
A
Lymphocyte
Single cells
Live cells
CD3+ T cells
CD8+ T cells
SSC-A
FSC-A
76.5%
FSC-H
99.5%
60.6%
Viability-APC-Cy7
80.8%
CD3-BV605
51.3%
CD8-FITC
Neg
CD107a+ CD8+ T cells
IFN-γ+ CD8+ T cells
TNF-α+ CD8+ T cells
IL-2+ CD8+ T cells
MIP-1β+ CD8+ T cells
0.11%
CD107a-BV786
0%
IFN-γ-BV711
0.089%
TNF-α-BV605
0.02%
IL-2-PE-Cy7
0.48%
MIP-1β-BV421
Gag
0.65%
0.073%
0.37%
0.1%
0.86%
B
Total SIV-specific Magnitude (% of CD8+ T cells)
SVR
SVC
Total SIV-specific CD8+ T-cell response (%)
CD107a
IFN-γ
IL-2
MIP-1β
TNF-α
1 function
2 functions
3 functions
4 functions
5 functions
Frequency SIV-specific CD8+ T-cell response (%)
HB1
HB5
HB7
HB3
HB4
Function
CD107a
IFN-γ
IL-2
MIP-1β
TNF-α

Figure 2. Functional analysis of SIVmac239 Gag-specific CD8$^+$ T cells after cessation of LP-98 therapy

FACS analysis of SIV-specific CD8$^+$ T cells stimulated with SIVmac239 Gag peptide pool from PBMCs of monkeys after stopping LP-98 treatment. Figure reprinted with permission from Xue et al. (2022).

(A) FACS gating strategy of CD8$^+$ T cells producing CD107a, IFN-α, TNF-γ, IL-2, and MIP-1β.

(B) Quantification of cytokine production by SIVmac239 Gag-specific CD8$^+$ T cells. The percentages of SIVmac239 Gag-specific CD8$^+$ T cells were calculated after subtracting the negative group background. The total and all combinations of functions of SIVmac239 Gag-specific CD8$^+$ T cells were determined by using the Boolean gate platform after background subtraction. Error bars represent the mean ± SEM.

Note: Refer to the troubleshooting section (problem 4) for concerns regarding lack of fluctuation in plasma vRNA after adoptive infusion of anti-CD8 alpha [MT807R1].

EXPECTED OUTCOMES

This protocol is based on the observation that CD8$^+$ T cells play a key immune role in viral control after LP-98 treatment cessation. The rhesus macaque model for the evaluation of LP-98 treatment was successfully established, and we showed that plasma vRNA could be suppressed by LP-98 therapy in chronic infectors. Stable virologic control (SVC) and stable virologic rebound (SVR) were two typical outcomes observed after LP-98 withdrawal (Figure 1).

We directly measured the immune responses of SIVmac239 Gag-specific CD8$^+$ T cells in SVC and SVR monkeys and found greater numbers of SIVmac239 Gag-specific CD8$^+$ T cells in SVC monkeys compared to SVR monkeys. Notably, the percentages of SIVmac239 Gag-specific CD8$^+$ T cells producing CD107a, IFN-γ, IL-2 and MIP-1β were increased in SVC monkeys. More polyfunctional SIVmac239 Gag-specific CD8$^+$ T cells were observed in SVC monkeys than SVR monkeys (Figure 2). These results indicated LP-98 therapy produced stronger SIVmac239 Gag-specific CD8$^+$ T cell responses to drive virus control in SVC monkeys.

Additionally, we also conducted a CD8$^+$ T cell depletion assay in SVC monkeys to confirm that the CD8$^+$ T cell response was the predominant mechanism restraining virus rebound after LP-98 therapy interruption. Plasma vRNA rebound was observed along with the loss of CD8$^+$ T cells after carrying out adoptive infusion of anti-CD8 antibody, implying that the reactivation of virus latency was associated with the CD8$^+$ T cell depletion. Correspondingly, plasma vRNA was depressed again when CD8$^+$ T cells recovered (Figure 3).

In brief, our SIVmac239 Gag peptide pool stimulation assay and CD8$^+$ T cell depletion assay were highly effective in assessing the potential role of CD8$^+$ T cells in virus control after LP-98 treatment cessation. The methods can be applied to other rhesus macaque models with different drug treatments to determine the contribution of CD8$^+$ T cells to potential efficacy.

QUANTIFICATION AND STATISTICAL ANALYSIS

The FlowJo v10.0 software was used to analyze flow cytometry data. IFN-γ$^+$ CD8$^+$ T cells, MIP-1β$^+$ CD8$^+$ T cells, IL-2$^+$ CD8$^+$ T cells, TNF-α$^+$ CD8$^+$ T cells and CD107a$^+$ CD8$^+$ T cells were first gated using FMO. The percentages of SIVmac239 Gag-specific IFN-γ$^+$ CD8$^+$ T cells, MIP-1β$^+$ CD8$^+$ T cells, IL-2$^+$ CD8$^+$ T cells, TNF-α$^+$ CD8$^+$ T cells and CD107a$^+$ CD8$^+$ T cells were calculated after subtracting the negative control background. The percentage of total SIVmac239 Gag specific CD8$^+$ T cells was calculated with a Boolean gate platform that summarized the percentage of SIVmac239 Gag specific CD8$^+$ T cells producing five cytokines after background subtraction. All combinations of 5, 4, 3, 2, and 1 functions of SIVmac239-specific CD8$^+$ T cells were also determined by using the Boolean gate platform after background subtraction (Figure 2).

Statistical evaluation included using nonparametric Mann-Whitney *U*-tests between the two groups. All data were analyzed using GraphPad Prism 9.0.2 software.

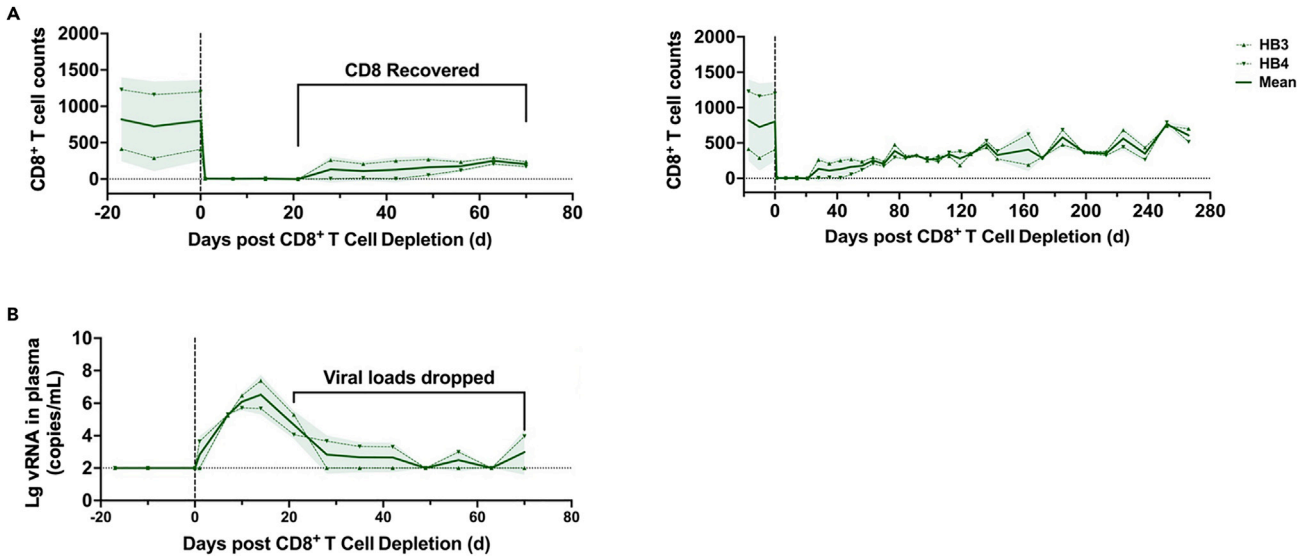

Figure 3. Depletion of CD8$^+$ T cells and viral rebound for SVC monkeys

CD8$^+$ T cell counts and plasma viral RNA were monitored by flow cytometry and TaqMan RT-PCR separately after injecting depleting anti-CD8 antibody into SVC monkeys. Shaded areas are displayed as the SD. Figure reprinted with permission from Xue et al. (2022).

(A) CD8$^+$ T cell counts of SVC monkeys following anti-CD8 antibody adoptive infusion.

(B) Plasma viral RNA of SVC monkeys following anti-CD8 antibody adoptive infusion.

LIMITATIONS

SIV-specific CD8$^+$ T cells usually play a predominant role in controlling SIV infection. Superior, polyfunctional SIV-specific CD8$^+$ T cell responses could suppress SIV replication and prevent disease recurrence. Production of cytokines by CD8$^+$ T cells stimulated with SIV-peptides was detected by flow cytometry in conjunction with intracellular cytokine-staining (ICS), thus permitting assessment of SIV-specific CD8$^+$ T cell responses (Petrovas et al., 2007). Our SIVmac239 peptide pool stimulation assay only measured SIVmac239 specific CD8$^+$ T cell responses against SIVmac239 Gag peptides after LP-98 therapy. Other SIVmac239 peptides, such as env, pol, nef and vif, were not used for stimulation, thus this protocol may not reflect the comprehensive SIVmac239 specific CD8$^+$ T cell immune response against a variety of antigens.

CD8$^+$ T cell-mediated cytolytic and non-cytolytic effector functions play major roles in effective immune control of the virus. In this protocol, we measured the level of CD107a to assess the SIVmac239 Gag-specific cytolytic effector function of CD8$^+$ T cell. CD107a, a marker of degranulation, mediates cytolytic function by regulating perforin and granzyme B secretion by CD8$^+$ T cell (Betts et al., 2003). The expression of perforin and granzyme B in CD8$^+$ T cells, which defines the frequency of cytolytic SIVmac239 Gag-specific CD8$^+$ T cells, can also be used as indicators for cytolytic CD8$^+$ T cell effector function. The antiviral cytokine, IFN-γ, the pro-inflammatory cytokine, TNF-α, the antiviral cytokine, IL-2, and CCR5-binding chemokine MIP-1β were included as indicators to evaluate SIVmac239 Gag-specific non-cytolytic effector function. Nevertheless, there still exist some other functional molecules such as CCR5-binding chemokine MIP-1α, homeostatic cytokine IL-7, and others, that are capable of maintaining CD8$^+$ T cell-mediated non-cytolytic effector functions. In future, more representative functional indicators should be measured to elucidate the SIVmac239-specific CD8$^+$ T cell response.

The anti-CD8 alpha [MT807R1] used in this protocol can be capable to deplete cells expressing the CD8 alpha protein, including CD8$^+$ T cells, but also CD8$^+$ NK cells in rhesus macaques. In this study, our follow-up observation on tracking of the recovery of CD8$^+$ T cell counts in SVC monkeys found that the CD8$^+$ T cell counts were gradually recovered and returned to the normal level before anti-CD8 antibody adoptive infusion about 260 days (Figure 3A, right panel). Additionally, new

anti-CD8 beta [CD8b255R1] (NIH Nonhuman Primate Reagent Resource, Cat#: PR-2557, RRID: AB_2716321) is also developed recently, which should be more specific for $CD8^+$ T cell depletion. Therefore, users can replace the anti-CD8 alpha [MT807R1] with anti-CD8 beta [CD8b255R1] to exhaust $CD8^+$ T cells in this protocol, which can more specifically reveal the effect of $CD8^+$ T cells in controlling $SHIV_{SF162P3}$ infection after LP-98 withdrawal.

TROUBLESHOOTING

Problem 1

Inadequate cell numbers after recovering PBMCs.

Potential solution

Cell cryopreservation solution should be prepared fresh for each cell cryopreservation. Users must ensure that the concentration of frozen PBMCs is at least 10^7 cells/mL in the cell cryopreservation solution. The cell density should not be too high, otherwise the cell cryopreservation solution will not be fully protective and the survival rate of recovered cells will be reduced. Users should replace the isopropyl alcohol in the cryopreservation box after freezing cells 5–7 times to ensure the proper cooling rate for maintaining cell viability. The cryovials containing cryopreserved PBMCs should be transferred from the -80°C freezer after 24 h to liquid nitrogen for preservation as soon as possible. Additionally, it's critical to thaw and dilute PBMCs relatively quickly to avoid prolonged exposure of thawed PBMCs to the DMSO from the cryopreservation solution.

Problem 2

Intracellular cytokine staining not detected.

Potential solution

Fresh PBMCs yield better results because they produce more functional molecules than cryopreserved PBMCs following antigen stimulation. Because cryopreservation may reduce $CD8^+$ T cells' response to SIV peptides, this protocol recommended that thawed cryopreserved PBMCs should be recovered overnight (about 12–16 h) before use. Costimulatory antibodies and protein transport inhibitors should be added to all experimental wells at the beginning of peptide stimulation to increase production of functional molecules. Anti-human CD28 antibody (clone CD28.2) and anti-human CD49d (clone 9F10) antibody are two common costimulatory antibodies used to stimulate T cells in rhesus macaques. The binding of anti-human CD28 antibody and CD28 initiates and regulates a separate and distinct signal transduction pathway from those stimulated by the TCR complex. Anti-human CD49d (clone 9F10) antibody reacts with the integrin α4 chain, which is expressed as a heterodimer with either of two β integrin subunits, β1 or β7, and can cause a cascade reaction of signal amplification in T cells by mediating binding to VCAM-1 (CD106) and fibronectin. GolgiStop, which is used as a protein transport inhibitor acts differently from brefeldin A and allows the accumulation of intracellular proteins for staining in the Golgi complex to improve the detectability of cytokine-producing cells by flow cytometry. Moreover, it's necessary to stimulate PBMCs with cell stimulation cocktail as positive control to verify the feasibility of detecting intracellular cytokines from $CD8^+$ T cell by this protocol.

Additionally, we also recommended prolonging the time of fixation and permeabilization of cells to 30–40 min to enhance the recognition and binding between fluorescent antibodies and corresponding cytokines for better fluorescence detection.

Problem 3

The measurement of functional molecules produced by SIVmac239 Gag-specific $CD8^+$ T cells is not consistent from experiment to experiment.

Potential solution

The SIVmac239 Gag peptide pool solution is stored at -80°C, and long storage times may possibly affect its ability to stimulate T cells. Therefore, we recommend using the same reagents and

equipment at the same time during a given set of experiments as far as possible. Sufficient PBMCs should be collected and cryopreserved to cover the experiment plans for carrying out peptide stimulation assays under the same conditions.

Problem 4

No fluctuation in plasma vRNA after adoptive infusion of anti-CD8 antibody.

Potential solution

We used anti-CD8 alpha [MT807R1] as the depleting anti-CD8 antibody to exhaust $CD8^+$ T cells to study the immune effect of $CD8^+$ T cells on viral dynamics in SVC macaques. Anti-CD8 alpha [MT807R1] should be stored at 4°C instead of frozen, to maintain the antibody's effectiveness. Users should always monitor the storage temperature and check the expiration date for anti-CD8 alpha [MT807R1] before use. To ensure the effectiveness of this anti-CD8 alpha antibody, users should assess the dynamic changes in $CD8^+$ T cell counts by flow cytometry. We recommend that users simultaneously monitor the $CD8^+$ T cell counts along with plasma vRNA measurements to exclude the possibility of anti-CD8 antibody failure.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jing Xue (xuejing@cnilas.org).

Materials availability

This study did not generate any new or unique reagents.

Data and code availability

This study did not generate or analyze any datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, Z.C. and J.X.; Methodology, Y.S., Z.C., and J.X.; Investigation, Y.S., J.Z., L.T., J.L., and T.C.; Writing – Original Draft, Y.S., Z.C., and J.X.; Writing – Review & Editing, Z.C. and J.X.; Funding Acquisition, J.X. and Y.H.; Supervision, Q.W. and J.X.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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