

Ex vivo T cell–based HIV suppression assay to evaluate HIV-specific CD8⁺ T-cell responses

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Published online 13 May 2010; doi:10.1038/nprot.2010.73

To advance T cell–based HIV vaccine development, it is necessary to evaluate the immune correlates of a protective CD8⁺ T-cell response. We have developed an assay that assesses the capacity *ex vivo* of HIV-specific CD8⁺ T cells to suppress HIV-1 infection of autologous CD4⁺ T cells. This assay directly reflects the ultimate effector function of CD8⁺ T cells, the elimination of infected cells, and accurately differentiates the effective CD8⁺ T-cell response in spontaneous HIV controllers from ineffective responses in other patients. In this article, we describe all the steps from cell purification to assessment of viral replication by HIV-p24 ELISA and analysis, along with conditions for cell culturing, and how to choose the viral infectious dose that gives the most reliable results. We also depict the conditions of a rapid assay on the basis of flow cytometry analysis of intracellular HIV-Gag products. These procedures take 14–17 d when the p24 ELISA assay is used, or 6 d with the intracellular Gag assay.

INTRODUCTION

Since the identification of HIV-1 as the causative agent of the AIDS pandemic, evidence has been accumulating to support the notion that CD8⁺ T cells have a central role in restraining HIV/SIV infection *in vivo*^{1–4}. These observations have been reinforced by *in vitro* assays showing the capacity of CD8⁺ T cells to inhibit HIV-1 infection of CD4⁺ T cells^{5–7}. This has boosted the efforts of the international scientific community to develop an effective HIV-1 vaccine that would induce T-cell responses that could contain the virus, reducing the rate of transmission and the incidence of HIV disease or contributing to protect against infection. However, the failure of Merck's STEP trial showed the limits of the commonly used IFN- γ Elispot surrogate assay⁸. In fact, in the absence of a successful T cell–based vaccine candidate, the definition of what constitutes an effective T-cell response against HIV, and which assay reflects this efficacy most accurately, is still a subject of debate^{8–10}. Much hope has been placed on identifying new correlates of protection in HIV controllers (HIC), a rare population of HIV-infected individuals who can spontaneously control HIV-1 infection to undetectable levels for more than 10 years^{11–13}.

Most HIC are characterized by a strong and functional CD8⁺ T-cell response that very likely contributes to their long-term control of infection^{14,15}. Thus, the CD8⁺ T-cell response in these HIC currently constitutes one of the most appealing examples of a possible goal for new immune therapies or vaccine strategies¹⁶. During the last few years, the analysis of CD8⁺ T-cell response in HIC has revealed different characteristics of efficient HIV-specific CD8⁺ T cells in HIV infection, including the capacity to proliferate and generate a multifunctional response^{17–20}. However, these characteristics are often revealed only on *in vitro* activation of cells with optimal or overlapping HIV-1 peptides, and their relevance in the control of infection is uncertain^{21,22}. Our group has developed an assay that assesses the *ex vivo* capacity of HIV-specific CD8⁺ T cells to suppress HIV-1 superinfection of autologous CD4⁺ T cells through cytotoxic activity²⁰, this being the ultimate function of CD8 T cells. In fact, the use of *ex vivo* (nonstimulated) CD8⁺ T cells, unlike previous assays that used expanded CD8⁺ T-cell lines, CD8⁺ T-cell clones or *in vitro* stimulated primary CD8⁺

T cells^{5–7}, allows the antiviral potential of the whole untouched pool of HIV-specific CD8⁺ T cells to be assessed in response to infected relevant target cells, which seems to be highly important to evaluate accurately the antiviral efficacy of circulating HIV-specific CD8⁺ T cells. Using this assay, it is possible to differentiate between effective anti-HIV CD8⁺ T-cell responses in HIC and ineffective responses in patients with persistent viremia²⁰. We have shown that the enhanced capacity of HIV-specific CD8⁺ T cells from HIC to inhibit HIV-1 infection requires contact and an MHC matching with target cells, and is caused by the elimination of infected cells rather than by the secretion of soluble antiviral factors (such as β -chemokines or CAF)²⁰. We only observed an effect of antiviral factors when working with artificially *in vitro* activated CD8⁺ T cells, and this effect was, in any case, very modest²⁰. Thus, our study results point to a preponderant role of cytotoxic mechanisms on the highly efficient antiviral response of HIV-specific CD8⁺ T cells from HIC, which is in agreement with reports by Migueles *et al.*^{18,19} showing increased lytic granule loading in CD8⁺ T cells of HIC on contact with infected target cells. Furthermore, we have found that the HIV-suppressive capacity of CD8⁺ T cells is strongly correlated with the frequency of HIV-specific CD8⁺ T cells in HIC (but not in viremic individuals), and in particular with the frequency of CD8⁺ T cells directed against the HIV-1 Gag protein¹⁵. In fact, the depletion of Gag-specific CD8⁺ T cells abrogates HIV suppression¹⁵, suggesting that not all cells have the same anti-HIV potential. This is especially relevant considering that no other differences were observed between CD8⁺ T cells of different antigen specificity in these experiments (either at the phenotypic level or when other parameters were measured, such as the capacity to secrete cytokines or the potential to proliferate, in response to HIV peptides). The impact of antigen specificity on the capacity of CD8⁺ T cells to suppress HIV-1 infection may well be due to the different kinetics of antigen presentation on the surface of CD4⁺ T cells²³. This underlines the convenience of assessing CD8⁺ T-cell function in a context closer to that which the cells encounter *in vivo*, i.e., in response to infected cells rather than to peptide stimulation. In addition, we have shown that the

TABLE 1 | Advantages and disadvantages of using the CD8⁺ T cell-based HIV-1 suppression assay to determine the efficacy of HIV-specific CD8⁺ T-cell responses.

Advantages	Disadvantages
Reliable quantification of the ultimate function of cytotoxic CD8 T cells, i.e., the killing of infected cells	Labor-intensive handling of sample
Takes into account the infection dynamics: response to infected cells instead of response to peptides	Donor-dependent variability in the susceptibility of CD4 ⁺ T cells to HIV-1 infection
Reflects the magnitude and quality of the HIV-specific CD8 ⁺ T-cell response, the antigen sensitivity of the cells and even the antigenic specificity of the response	Dissection of specificities' contribution requires additional experiments involving sorting of cell subpopulations ^{15,22}
Easy and low-cost adaptability to different HIV-1 strains (tropism, subtype)	

HIV-suppressive capacity of CD8⁺ T cells is influenced by the antigen sensitivity/functional avidity of the cells²⁴. Therefore, the capacity of CD8⁺ T cells to suppress HIV-1 infection *ex vivo*, as measured in our assay, is influenced by the magnitude and quality of the HIV-specific CD8⁺ T-cell response, the antigen sensitivity of the cells and even the specificity of the response. Each of these attributes is thought to be important for the generation of an efficient CD8⁺ T-cell response. On the basis of these observations, we proposed that the HIV-suppressive assay may be highly relevant to assess the efficacy of CD8⁺ T-cell responses generated by vaccine candidates (see **Table 1** for the advantages and disadvantages of this protocol) and it complements assays that quantify the frequency of HIV-specific CD8⁺ T cells¹⁵, such as the widely used IFN- γ Elispot assay (see ref. 25).

Experimental design

Stages involved in the protocol. The technique used to assess the *ex vivo* capacity of CD8⁺ T cells to suppress HIV infection of autologous CD4⁺ T cells consists of three steps (**Fig. 1**):

- Isolation of CD4⁺ and CD8⁺ T cells and activation of CD4⁺ T cells
- Infection *in vitro* of CD4⁺ T cells and of CD4⁺/CD8⁺ T cell cocultures with HIV-1
- Determination of viral replication in CD4⁺ T cells cultured alone or in the presence of CD8⁺ T cells, by either p24 ELISA or intracellular staining for HIV Gag products and detection by flow cytometry, and calculation of the HIV-1- suppressive capacity of CD8⁺ T cells

Some important points in this schema are discussed below.

Isolation of cell fractions. We have standardized this technique with fresh blood samples that were recovered using either lithium heparin or EDTA as anticoagulants (*Note*: frozen peripheral blood mononuclear cell (PBMCs) may also be used. However, the level of CD8⁺ T-cell-mediated HIV-1 suppression is then lower than that with fresh cells. Therefore, experiments conducted with fresh samples should not be compared

with those using frozen samples). Blood must be maintained at ~20 °C during transport and until ‘handling’ in the laboratory to avoid hemolysis. If properly stored, the blood samples are workable up to 48 h after extraction. Because of the drop in CD4⁺ T cell counts that is associated with HIV-1 progression, it is recommended to start with at least 10 ml of blood from HIV-1-infected individuals to obtain enough target cells (higher volumes might be needed if analyzing severely immunodepleted individuals), whereas 5 ml of blood is enough for most healthy individuals. It is extremely important to ensure the purity of CD4⁺ and CD8⁺ T-cell fractions. CD4⁺ cells are selected by positive selection using anti-CD4 antibody-coupled magnetic beads. Although other cells (such as monocytes) are selected along with CD4⁺ T cells during this step, these are subsequently lost during a 2- to 3-d activation step with phytohemagglutinin-L (PHA) and IL-2. The resulting negative fraction (depleted of CD4⁺ cells) is used as a source for the isolation of CD8⁺ T cells. In contrast to CD4⁺ T cells, it is necessary to isolate CD8⁺ T cells by negative selection, to eliminate other cell fractions, especially natural killer (NK) cells, which also express the CD8 molecule and may subsequently interfere with the assay. The cells are incubated with a cocktail of antibodies targeting CD4, CD14, CD16, CD19, CD20, CD56, CD123, TCR γ/δ and glycoporphin A coupled to magnetic beads to eliminate CD4⁺ T cells (as the earlier positive selection does not

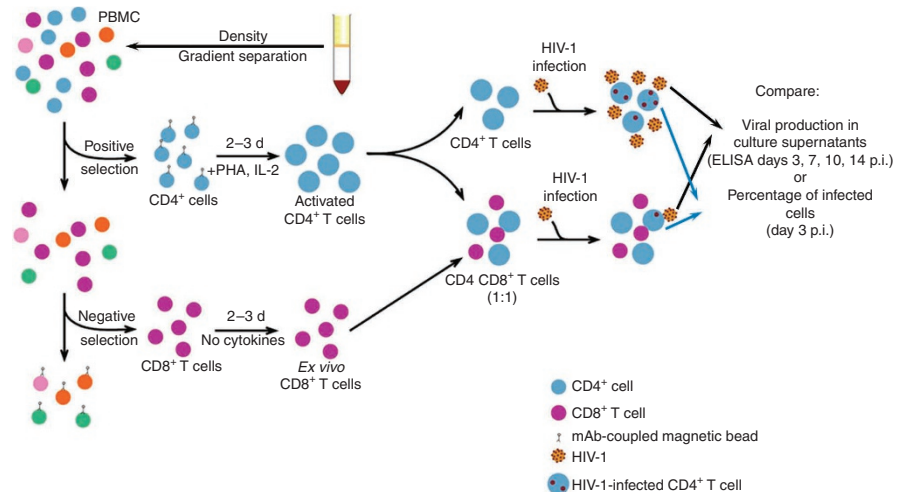
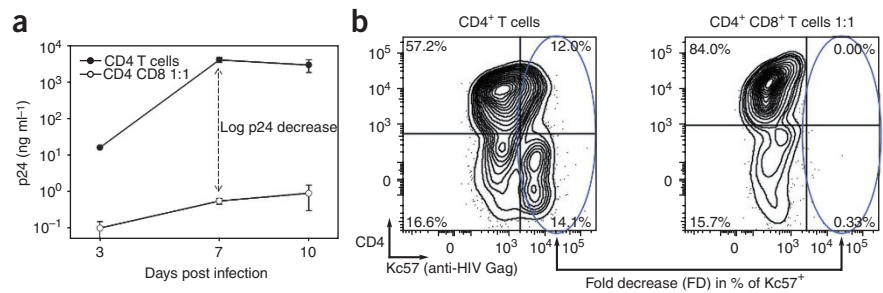


Figure 1 | Schema outlining the *ex vivo* CD8⁺ T cell-based HIV suppression assay.



Figure 2 | Examples of experiments conducted using the protocol. **(a)** p24 production (mean \pm s.d., $n = 3$) in the culture supernatants of PHA-activated CD4⁺ T cells from an HIC infected *in vitro* with replicative HIV-1 BaL, in the absence (filled) or presence (white) of autologous unstimulated CD8⁺ T cells (1:1 ratio). HIV-suppressive capacity of CD8⁺ T cells is calculated at the peak of viral replication in CD4⁺ T cells alone (in this case, day 7 p.i.), as the log decrease in p24 production when CD4⁺ T cells are cocultured with CD8⁺ T cells.

(b) PHA-activated CD4⁺ T cells from an HIC infected *in vitro* with HIV-1 BaL and left alone (left) or cocultured with autologous unstimulated CD8⁺ T cells (right). The level of infection was determined by quantifying intracellular HIV-1 Gag products at day 3 p.i. Because surface expression of the CD4 molecule is downregulated on HIV-1 infection of CD4⁺ T cells, plots were gated on CD3⁺CD8⁻ cells to exclude CD8⁺ T cells from analyses and accurately determine the percentage of infected CD4⁺ T cells. The HIV-suppressive capacity of CD8⁺ T cells is calculated as the logarithmic fold decrease in the percentage of infected CD4⁺ T cells when they are cultured in the presence of CD8⁺ T cells.



ensure the total removal of these cells from the negative fraction), B cells, NK cells, dendritic cells and so on. In this way, CD8⁺ T cells are recovered untouched in the negative fraction (an alternative approach is to perform a positive selection of CD8 β ⁺ cells, but this may result in an undesirable activation of the cells). We use anti-CD4 magnetic beads and CD8⁺ T-cell enrichment kits from either Miltenyi Biotec or StemCell Technologies (both for manual or RoboSep-assisted separation) with excellent results. We routinely obtain purities above 95% for CD4⁺ T cells and CD8⁺ T cells and yields of around 20% of the initial PBMCs for CD4⁺ T cells and 10% for CD8⁺ T cells, although these last values vary widely in the case of HIV-1-infected individuals.

Choice of viral isolate and infectious dose. The choice of virus depends on the circumstances of the assay (e.g., subtype prevalence within the study population, strain of interest in a vaccine trial and so on) and both primary and lab-adapted strains can be used. We routinely assay the HIV-1-suppressive capacity of CD8⁺ T cells in parallel against an R5 HIV-1 (BaL) and an X4 HIV-1 (NL4.3). Because the assay is carried out using CD8⁺ T cells *ex vivo*, we have not observed a significant contribution of soluble inhibitory factors to HIV-1 suppression (ref. 20 and see above) and the results obtained with both HIV-1 are globally comparable.

For the analysis of HIV-1 suppression capacity on the basis of an HIV-1 p24 ELISA assay, the choice of the infection dose should be one that regularly yields between 100 and 1,000 ng ml⁻¹ of HIV-1 p24 at the peak of viral replication, using PHA-activated CD4⁺ T cells from healthy donors (in our hands, using the p24 ELISA Kit from Zeptometrix, a multiplicity of infection (MOI) = 10^{-3.14} of HIV-1 BaL results in levels of infection within these values in >95% of the cases). A value below 100 ng ml⁻¹ would not allow a good discrimination between high-quality and low-quality CD8⁺ T-cell responses, and infections producing above 1,000 ng ml⁻¹ of HIV-1 p24 in culture supernatants would be too strong even for most high-quality CD8⁺ T cells.

Similarly, when using intracellular HIV-1 Gag determination, the optimal infectious dose is that which results in between 10 and 30% of infected CD4⁺ T cells at day 3 after infection. However, with this assay, it is more complicated to regularly obtain the desired level of infection using a single viral dose than with the p24 ELISA assay because of a higher case-to-case variability in the levels of infection at day 3. We normally use two viral doses in parallel (MOI = 10^{-1.15}, 10^{-1.84}) to ensure that the desired level of infection is achieved.

When working with cells from HIV-1 patients, the peak level of viral replication that is observed on the CD4⁺ T cells infected *in vitro* is generally much higher than viral replication at the same time point in control cells (activated but nonsuperinfected, see PROCEDURE). However, sometimes it is not possible to distinguish viral replication of the superinfecting virus (the one inoculated *in vitro*) from that of the infecting virus (the one that is carried *ex vivo* by CD4⁺ T cells from the patient). We have observed that the estimated HIV-1-suppressive capacity of CD8⁺ T cells might be slightly stronger toward infecting viruses than toward ‘consensus’ (lab adapted) strains, without significantly altering the results (ref. 15 and unpublished). Nevertheless, caution should be observed when analyzing such results.

Detection of viral replication: p24 ELISA versus intracellular staining for Gag products. Detection of viral replication is one of the key steps in this protocol. Although different approaches may be used to evaluate the level of infection of the cell cultures, we have chosen to monitor the HIV-1 p24 in culture supernatants by ELISA (Fig. 2a) and to determine the percentage of infected CD4⁺ T cells by intracellular staining of Gag products (Fig. 2b). Effectively, these assays are reliable and relatively simple.

Ideally, this protocol should result in a broad range of levels of infection: strong for CD4⁺ T cells and very weak or even undetectable for cocultures with highly efficient CD8⁺ T cells (Fig. 2). We use a kinetic ELISA assay for HIV-1 p24 because it offers a wider range standard curve than do end-point assays, reducing the number of manipulations and dilutions needed to quantify the different culture conditions in a single experiment. Culture supernatants need to be diluted before testing by ELISA (e.g., we often use 1/625 for 7-d postinfection supernatants from CD4⁺ T cells cultured alone, or 1/5 for supernatants from CD4⁺ T cells cultured in the presence of highly inhibitory CD8⁺ T cells). Some experience is required to judge by how much the supernatants should be diluted. The quantification of viral replication by the determination of the number of infected cells (intracellular staining of Gag products) is a convenient method (faster, cheaper and consumes less cells than the p24 ELISA assay) to distinguish CD8⁺ T cells with weak and strong HIV-1-suppressive capacities. However, it is not as sensitive/quantitative as the p24 ELISA and is probably less well adapted to ‘categorize’ CD8⁺ T-cell responses (see Table 2 and Fig. 3 for a comparison of these methods).

MATERIALS

REAGENTS

- Blood **! CAUTION** Blood should be obtained according to a protocol approved by ethical committee and after obtaining consent from patients. When obtained from an HIV-1-infected patient, it should be treated as infectious material.
- HIV-1 isolate (see protocol²⁶) **! CAUTION** HIV-1 is a class 3 human pathogen and it should be handled in a BSL3 facility
- Dulbecco's PBS 1× (Gibco, cat. no. 14190)
- LSM 1077 Lymphocyte Separation Medium (PAA, cat. no. J15-004) **! CAUTION** May cause sensitization if inhaled and if it comes in contact with skin. Wear suitable protective clothing and gloves
- RPMI 1640 (Gibco, cat. no. 31870-025)
- Fetal bovine serum (FBS; PAA, cat. no. A15-073)
- L-Glutamine-200 mM (100×; Gibco, cat. no. 25030-081)
- Penicillin-streptomycin (100×; Gibco, cat. no. 15140-122) **! CAUTION** May cause sensitization if it comes in contact with skin; irritant to eyes, respiratory system and skin. Wear suitable protective clothing, glasses and gloves.
- EDTA, 500 mM (pH 8.0; Gibco, cat. no. 15575-020) **! CAUTION** Irritant to eyes. Wear suitable protective clothing, glasses and gloves.
- Phytohemagglutinin-L (PHA) (Roche, cat. no. 11 249 738 001)
- Recombinant interleukin-2 (IL-2, Proleukin; Chiron)
- Trypan Blue Stain 0.4% (Invitrogen, cat. no. T10282)
- Human CD4 microbeads (positive selection; StemCell Technologies, cat. no. 18052RF or Miltenyi Biotec, cat. no. 130-045-101)
- Human CD8⁺ T Cell Enrichment Cocktail (negative selection; StemCell Technologies, cat. no. 19053RF or Miltenyi Biotec, cat. no. 130-094-156) **▲ CRITICAL** Positive selection of CD4⁺ cells followed by negative selection of CD8⁺ T cells optimizes the yield of recovery of cell fractions, and ensures obtaining untouched CD8⁺ T cells (see Isolation of cell fractions in Experimental design section).
- RoboSep Buffer (StemCell Technologies, cat. no. 20104) **! CAUTION** Potential irritant to eyes, respiratory system and skin. Wear suitable protective clothing, glasses and gloves.
- HIV-1 P24 Antigen ELISA Bulk (ZeptoMetrix, cat. no. 0801200)
- HIV-1 P24 Extended Range Kit (ZeptoMetrix, cat. no. 0801137)
- o-Phenylenediamine dihydrochloride tablets (OPD; Sigma-Aldrich, cat. no. P8412) **! CAUTION** Harmful if inhaled or comes in contact with skin. Toxic if swallowed. Irritant to eyes. Limited evidence of a carcinogenic effect. Very toxic to aquatic organisms, may cause long-term adverse effects in the aquatic environment. Possible risk of irreversible effects. Wear suitable protective clothing, glasses and gloves. This material and its container must be disposed off as hazardous waste.
- KC57-FITC antibody (Beckman Coulter, cat. no. 6604665)
- CD4-ECD antibody (Beckman Coulter, cat. no. 6604727)
- CD8-PC5 antibody (Beckman Coulter, cat. no. A07758)
- CD3-PE-Cy7 antibody (BD Pharmingen, cat. no. 557749)
- Fixation and Permeabilization Solution (BD Biosciences, cat. no. 554722) **! CAUTION** Limited evidence of a carcinogenic effect. May cause sensitization if it comes in contact with skin. Wear suitable protective clothing and gloves.

EQUIPMENT

- RoboSep (StemCell Technologies, cat. no. 20000)
- MidiMACS Separator (Miltenyi Biotec, cat. no. 130-042-301)

TABLE 2 | Comparison of p24 ELISA versus intracellular Gag assay.

	HIV-1 p24 ELISA assay	Intracellular HIV-1 Gag assay
Time	14–17 d (10–14 d of infection)	6 d (3 d of infection)
Sample required	10 million PBMCs	1–2 million PBMCs
Reproducibility	Good	Good
Linearity of results	Excellent	Limited

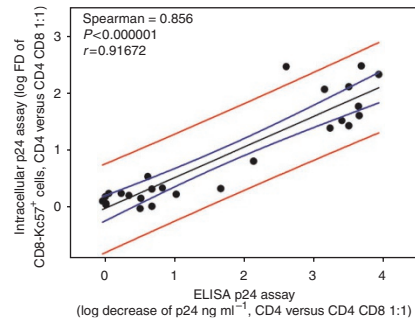


Figure 3 | Correlation between the values of HIV-suppressive capacity of CD8⁺ T cells as obtained by ELISA monitoring of HIV-1 p24 in culture supernatants and those obtained by flow cytometry analysis of the percentage of infected CD4⁺ T cells. Each circle represents experiments performed in parallel with cells from a single individual. The black line represents linear regression. Blue lines and red lines are the 95% confidence intervals and 95% prediction intervals, respectively. Statistics were calculated by SigmaPlot with SigmaStat software (Systat Software Inc.).

- LS Columns (Miltenyi Biotec, cat. no. 130-042-301 130-042-401)
- 25 and 75 cm² cell culture flask with vent cap
- 15 and 50 ml centrifuge tubes
- 14 ml Falcon Polystyrene Round-Bottom Tubes (BD Biosciences, cat. no. 352057)
- 1.5 and 2 ml screw cap microtubes
- 5 ml round-bottom cytometer tube (BD Biosciences, cat. no. 352005)
- Flat-bottom 6-, 12- and 96-well tissue culture plates
- Round-bottom 96-well tissue culture test plates
- Sterile 5, 10 and 25 ml disposable pipettes
- Pipettes (0.5–10, 2–20, 20–200, 100–1,000 µl)
- Multichannel pipette (50–200 µl)
- Benchtop centrifuge with sealed buckets and plate carriers
- Mini vortex
- Optical microscope
- Microbiological safety cabinet
- Plate shaker
- Dry incubator
- CO₂ cell incubator
- ELISA microplate reader (with kinetic reading capabilities, suggested)
- Countess Automated Cell Counter (Invitrogen, cat. no. C10227) or similar
- LSR II Flow Cytometer (BD Biosciences) or similar

REAGENT SETUP

Separation buffer PBS 1× supplemented with 2 mM EDTA and FBS 0.5% (vol/vol). Can be stored at 4 °C for up to 3 weeks.

Culture medium RPMI 1640 supplemented with glutamine, penicillin-streptomycin and FBS 10% (vol/vol). Can be stored at 4 °C for up to 3 weeks.

Activation medium Culture medium supplemented with PHA (1 µg ml⁻¹) and IL-2 (100 µl ml⁻¹) (Values in parentheses are indicated as a reference. You need to choose PHA and IL-2 concentrations that induce an optimal activation of CD4⁺ T cells after 3 d of culture). Should be freshly prepared or stored for no longer than 3 d at 4 °C.

IL-2 medium Culture medium supplemented with IL-2. Can be stored at 4 °C for up to 2 weeks.

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

EQUIPMENT SETUP

Configure ELISA microplate reader as follows: 15 min run time, 30 s of time interval to read at 405 nm wavelength.

Perform quality control of flow cytometer (a detailed guideline can be found in ref. 27).



PROCEDURE

Isolation of PBMCs ● TIMING 1 h 30 min for Ficoll gradient separation

- 1| Transfer fresh blood from peripheral vein into a sterile 50 ml plastic tube and dilute with an equal volume of PBS.
▲ **CRITICAL STEP** It is recommended to start with at least 10 ml of blood to ensure that enough CD4⁺ T cells are recovered, especially when blood from HIV-1-infected individuals is analyzed. For healthy individuals, 5 ml of blood should be enough.
- 2| Gently add 20 ml diluted blood to 10 ml lymphocyte separation medium in a 50 ml tube.
- 3| Centrifuge at 1,200g for 20 min at room temperature (*T* = 22 °C)
▲ **CRITICAL STEP** Do not use brake, because a sudden stop will disrupt the density gradient.
- 4| Remove the top plasma phase (yellow) through gentle aspiration with a 10 ml pipette and discard (or store for further analysis)
- 5| Recover the mononuclear cell-containing interface (white) with a 5 ml pipette and transfer to a new 50 ml tube.
- 6| Add PBS to fill up the tube, and centrifuge at 400g for 10 min at room temperature. Discard the supernatant, loosen the pellet and repeat PBS wash twice.
- 7| Resuspend PBMCs in separation buffer and count them in a hemocytometer.

Isolation and activation of CD4⁺ T cells ● TIMING 45 min to obtain CD4⁺, and 2–3 d for the activation of CD4⁺ T cells

- 8| Concentrate PBMCs (typically to 10⁷ cells per ml) in chilled separation buffer after centrifugation at 400g for 5 min and perform positive selection of CD4⁺ cells with an anti-CD4⁺ antibody coupled to magnetic beads as recommended by the manufacturer (see Experimental design for further discussion).
- 9| Collect positive fraction from Step 8, count CD4⁺ cells and centrifuge cells at 400g for 5 min at room temperature.
▲ **CRITICAL STEP** Also collect negative fraction for use in Step 12 to purify CD8⁺ T cells.
- 10| Remove supernatant and resuspend cell pellet in activation medium to a final concentration of 10⁶ cells per ml.

? TROUBLESHOOTING

- 11| Place cells into a well of a 12-well plate (or of a six-well plate if more than 3 × 10⁶ cells are recovered) and culture them for 2–3 d at 37 °C, under 5% CO₂ in a humidified atmosphere.

Isolation and culture of CD8⁺ T cells ● TIMING 50 min to obtain CD8⁺ T cells and 2–3 d of cell culture

- 12| Concentrate the negative cellular fraction from Step 9 (typically to 10⁷ cells per ml) in chilled separation buffer after centrifugation at 400g for 5 min, and perform indirect magnetic cell sorting of untouched CD8⁺ T cells with a CD8⁺ T-cell enrichment kit as recommended by the manufacturer (see Experimental design for further discussion).
- 13| Collect negative fraction, count CD8⁺ T cells and centrifuge them at 400g for 5 min at room temperature.
▲ **CRITICAL STEP** In contrast to CD4⁺ cells, CD8⁺ T cells are not magnetically labeled and are recovered in the negative fraction.
- 14| Remove supernatant and resuspend cell pellet in culture medium to a final concentration of 10⁶ cells per ml.
▲ **CRITICAL STEP** Do not add cytokines to the culture medium of CD8⁺ T cells to preserve *ex vivo* activation status.
? TROUBLESHOOTING
- 15| Place cells into a well of a 12-well plate (or of a six-well plate if more than 3 × 10⁶ cells are recovered) and culture in a humidified incubator at 37 °C, 5% CO₂, during the activation of CD4⁺ T cells (Step 11).

Evaluation of the capacity of CD8⁺ T cells to suppress HIV-1 infection of autologous CD4⁺ T cells

- **TIMING** 3 h for infection and washings; 10–14 d for culture (depending on the peak of viral replication (option A) or 3 d (option B); 4 h for p24 ELISA (option A) or 2 h 30 min for intracellular labeling of Gag products (option B)
- 16| Aspirate CD4⁺ and CD8⁺ T cells from wells with 5 ml pipettes and insert cells into 15 ml conical tubes. Fill up the tubes with culture medium.

PROTOCOL

17| Centrifuge at 400g for 5 min at room temperature. Aspirate medium and resuspend cell pellets in 1 ml of IL-2 medium (for CD4⁺ T cells) or culture medium (for CD8⁺ T cells).

18| Count cells with a hemocytometer and adjust concentration to 10⁶ cells per ml with suitable medium.

▲ CRITICAL STEP It is important to count cells accurately to allow different experiments to be compared. An automated cell counter is preferable.

19| Evaluate HIV-1 suppression by HIV-1 p24 ELISA (option A) or by quantification of intracellular HIV-1 Gag products (option B).

(A) Determination of the capacity of CD8⁺ T cells to suppress HIV-1 infection of autologous CD4⁺ T cells by ELISA monitoring of HIV-1 p24 in culture supernatants

- (i) Add 100 µl of CD4⁺ T cells to wells in a 96-well round-bottom plate. Allow three wells for noninfected *in vitro* control, three wells for HIV-1-infected *in vitro* CD4⁺ T cells, three more wells for coculture with CD8⁺ T cells at a 1:1 ratio and, optionally, three wells for each coculture at additional CD4⁺/CD8⁺ T cell ratios (see Step 19A(ii)).
▲ CRITICAL STEP The use of 96-well round-bottom plates is mandatory to ensure that no cells are lost during washing or recovery of supernatants (Step 19A(iii, viii and x)).
- (ii) Add 100 µl of CD8⁺ T cells at 10⁶ cells per ml to each of three wells containing 10⁵ CD4⁺ T cells for coculture at a 1:1 CD4⁺/CD8⁺ T cell ratio. Dilute CD8⁺ T cells 1/3 and 1/10 in culture medium and add (3×) 100 µl of the different dilutions to additional CD4⁺ T cell-containing wells for 1:0.3 and 1:0.1 CD4⁺/CD8⁺ T-cell ratios (optional).
- (iii) Centrifuge the plate at 400g for 5 min at room temperature and remove 100 µl of the supernatant from CD4⁺/CD8⁺ T cell coculture-containing wells.
- (iv) Add 100 µl IL-2 medium to each of three wells destined as noninfected *in vitro* controls.
- (v) Dilute viral stocks to a 2× assay viral dose in IL-2 medium and add 100 µl of viral suspension to the three wells containing CD4⁺ T cells and to wells containing CD4⁺/CD8⁺ T cell cocultures.
▲ CRITICAL STEP The viral dose needs to be determined by the user (see Experimental design for further discussion).
- (vi) Centrifuge plate at 1,200g for 1 h at 22 °C in a refrigerated centrifuge to improve infection efficiency²⁸.
! CAUTION To ensure biocontainment and to avoid exposure to aerosols, it is recommended that a centrifuge with sealed plate carriers should be used.
- (vii) Incubate plate for 1 h at 37 °C in a humidified incubator under 5% CO₂.
- (viii) Remove 190 µl of supernatants using an eight-channel pipette, add 190 µl of culture medium and centrifuge at 400g for 5 min at room temperature. Repeat twice. Use 190 µl of IL-2 medium after the last wash.
▲ CRITICAL STEP After centrifugation, the cells are concentrated at the bottom of the well. For aspiration, place the tips on the border of the well, well above the cell pellet, to avoid loss of cells.
- (ix) Incubate plate at 37 °C in a humidified incubator under 5% CO₂.
- (x) At day 3 after infection, centrifuge the plate at 400g for 5 min at room temperature. Remove 190 µl of supernatants and transfer them to a new 96-well plate and store. Replenish cell cultures with 190 µl of IL-2 medium and place the 96-well plate containing cell cultures back into the incubator. Repeat on days 7, 10 and 14.
■ PAUSE POINT Store plates with culture supernatants at 4 °C if p24 ELISA is to be performed within 15 days or at -80 °C if it will be carried out later or if further analyses are planned.
- (xi) If frozen, allow culture supernatants to thaw at room temperature. Dilute supernatants with assay diluent (see Experimental design for further discussion).
- (xii) Allow antibody-coated strips to reach room temperature. Add 300 µl of assay wash buffer to each well and then remove liquid. Repeat this step six times. Tap inverted plate gently but firmly on a pad of absorbent towels to remove liquid and bubbles.
▲ CRITICAL STEP Do not allow washed plates to dry completely. Proceed immediately to sample addition.
- (xiii) Leave one well empty (substrate blank); add 200 µl of culture medium to two wells; 200 µl of each standard dilution (4 to 0.08 ng ml⁻¹) to different wells; and 200 µl of diluted samples to remaining wells, as necessary (see **Supplementary Fig. 1** for suggested plate layout and a summary of treatment for each well in the following steps).
- (xiv) Add 20 µl of lysing buffer to all wells except blank, medium and standard. Cover plate with an adhesive plate sealer and incubate at 37 °C for 2 h in a dry incubator.
■ PAUSE POINT You can also incubate the plate overnight at this point.
- (xv) Decant liquid and add 300 µl of assay wash buffer to each well and remove liquid. Repeat this step six times. Tap inverted plate gently but firmly on a pad of absorbent towels to remove all liquid and bubbles.
- (xvi) Add 100 µl of HIV-1 p24 detector antibody (anti-p24 biotin reagent) to each well except the blank, cover plate with adhesive plate sealer and incubate at 37 °C for 1 h.

- (xvii) Decant liquid from the plate and wash the wells as in Step 19A(xv).
- (xviii) Add 100 μ l streptavidin–peroxidase solution into each well except the blank, cover plate with adhesive plate sealer and incubate at 37 °C for 30 min.
- (xix) Decant liquid from the plate and wash the wells as in Step 19A(xv).
 - ▲ **CRITICAL STEP** Removal of air bubbles is critical at this point as they interfere with the signal during reading.
- (xx) Place the plate on plate reader and add 100 μ l of OPD substrate solution. Start reading immediately on preconfigured plate reader (see EQUIPMENT SETUP).

? TROUBLESHOOTING

(B) Determination of the capacity of CD8⁺ T cells to suppress HIV-1 infection of autologous CD4⁺ T cells by analysis of the percentage of infected CD4⁺ T cells using flow cytometry

- (i) Add 50 μ l of CD4⁺ T cells to wells in a 96-well round-bottom plate. Allow one well for a noninfected *in vitro* control, two wells for *in vitro* HIV-1-infected CD4⁺ T cells and two additional wells for coculture with CD8⁺ T cells at a 1:1 ratio.
 - ▲ **CRITICAL STEP** You may want to provide for additional cells for proper monolabeled compensation controls for flow cytometry analysis (Step 19B(xviii)). Alternatively, you can use compensation beads for this purpose.
 - ▲ **CRITICAL STEP** The use of 96-well round-bottom plates is mandatory to ensure that no cells are lost during washing (Step 19B(iii, viii and ix)).
- (ii) Add 50 μ l of CD8⁺ T cells (10^6 cells per ml) to two wells containing 5×10^4 CD4⁺ T cells for coculture at a 1:1 CD4⁺/CD8⁺ T-cell ratio.
- (iii) Centrifuge plate at 400g for 5 min at room temperature and remove 50 μ l of supernatant from CD4⁺/CD8⁺ T-cell coculture-containing wells.
- (iv) Add 50 μ l IL-2 medium to the well destined for noninfected *in vitro* control.
- (v) Prepare two viral dilutions at a 2 \times assay viral dose in IL-2 medium and, for each viral suspension, add 50 μ l to one well containing CD4⁺ T cells and 50 μ l to one well containing CD4⁺/CD8⁺ T-cell cocultures.
 - ▲ **CRITICAL STEP** Viral dose needs to be determined by the user (see Experimental design for further discussion).
- (vi) Centrifuge plate at 1,200g for 1 h at 22 °C in a refrigerated centrifuge to improve infection efficiency²⁸.
 - ! **CAUTION** To ensure biocontainment and to avoid exposure to aerosols, use a centrifuge with sealed plate carriers.
- (vii) Incubate plate for 1 h at 37 °C in a humidified incubator under 5% CO₂.
- (viii) Remove 90 μ l of supernatants using an eight-channel pipette, add 90 μ l of culture medium and centrifuge at 400g for 5 min at room temperature. Repeat twice. Use 90 μ l IL-2 medium after the last wash.
 - ▲ **CRITICAL STEP** After centrifugation, the cells are concentrated at the bottom of the well. For aspiration, place the tips on the border of the well, well above the cell pellet, to avoid loss of cells.
- (ix) Incubate the plate for 3 d at 37 °C in a humidified incubator under 5% CO₂. Centrifuge the plate at 400g for 5 min at room temperature and discard the supernatants.
- (x) Harvest cell pellets with 100 μ l of labeling buffer and transfer to 5 ml cytometry tubes. Add 500 μ l labeling buffer to each tube and centrifuge at 400g for 5 min at room temperature. Discard supernatants.
- (xi) Prepare a mix of anti-CD4-ECD, anti-CD8-PE-Cy5 and anti-CD3-PE-Cy7 antibodies for cell-surface staining. Dilute pretitered antibodies in a final volume of 50 μ l of labeling buffer for each tube. Tap tubes to loosen cell pellets and add antibody mixes. Vortex the tubes, wrap in aluminum foil and incubate at room temperature for 20 min.
 - ▲ **CRITICAL STEP** Other than sample tubes, you need to include a tube with nonlabeled cells (cultured in the same condition as the experimental sample) and four tubes with single-labeled compensation controls (either cells expressing the appropriate molecule or compensation beads).
- (xii) Add 1 ml of staining buffer to each tube, centrifuge tubes at 400g for 5 min at room temperature. Discard supernatants.
- (xiii) Loosen pellets and add 250 μ l of cytofix/cytoperm solution to all tubes and incubate in the dark at 4 °C for 20 min.
- (xiv) Add 1 ml of 1 \times cytofix/cytoperm wash solution to each tube, centrifuge tubes at 400g for 5 min at room temperature. Discard supernatants.
- (xv) Prepare anti-Gag KC57-FITC antibody mix (3 μ l of antibody and 47 μ l of cytofix/cytoperm wash solution for each tube). Loosen pellets, add 50 μ l of mix to tubes and incubate at 4 °C for 30 min in the dark.
- (xvi) Add 1 ml of 1 \times cytofix/cytoperm wash solution to each tube, centrifuge tubes at 400g for 5 min at room temperature. Discard supernatants and resuspend cell pellet in 400 μ l of staining buffer.
- (xvii) Store samples at 4 °C in the dark while setting up the flow cytometer with nonstained control and compensation controls.
- (xviii) Proceed to flow cytometer acquisition of experimental data (see ref. 29 for a standard procedure).

? TROUBLESHOOTING

20| Calculate HIV-suppressive capacity of CD8⁺ T cells from HIV-1 p24-ELISA (option A) or intracellular Gag (option B) results.

(A) Calculate HIV-suppressive capacity of CD8⁺ T cells using HIV-1 p24 ELISA assay

- (i) Express the level of secreted p24 (ng ml⁻¹) for each sample at different time points as the mean and standard deviation of triplicate wells (**Fig. 2a**).

PROTOCOL

- (ii) Calculate the HIV-1-suppressive capacity of CD8⁺ T cells at the peak of viral replication in the CD4⁺ T-cell cultures infected *in vitro* with HIV-1 (typically day 7 or 10) as the log of p24 production lost when CD8⁺ T cells are present in culture at a 1:1 ratio (see **Fig. 2a**).

HIV - suppressive capacity of CD8⁺ T cells(log p24 decrease)=

$$\text{Log}_{10} \frac{\text{mean p24 ng/ml in cultures of HIV - 1 infected } in vitro \text{ CD4}^+ \text{ T cells at peak of viral replication}}{\text{mean p24 ng/ml in infected } in vitro \text{ - CD4}^+ : \text{CD8}^+ (1 : 1) \text{ co - cultures at the same time point}}$$

(B) Calculate HIV-suppressive capacity of CD8⁺ T cells using intracellular HIV-1 Gag assay

- (i) Because HIV-1 infection downregulates surface expression of CD4⁺ molecules, gate on CD3⁺CD8⁻ cells to calculate the percentage of infected CD4⁺ T cells.
- (ii) Calculate HIV-1-suppressive capacity of CD8⁺ T cells as the logarithmic fold decrease in the percentage of HIV-infected CD4⁺ T cells when CD8⁺ T cells are present in the culture at a 1:1 ratio (see **Fig. 2b**).

HIV - suppressive capacity of CD8⁺ T cells (log fold decrease in % Kc57⁺CD4⁺ T cells)=

$$\text{Log}_{10} \frac{\% \text{ of Kc57}^+ \text{CD3}^+ \text{CD8}^- \text{ cells in culture of CD4}^+ \text{ T cells infected } in vitro}{\% \text{ of Kc57}^+ \text{CD3}^+ \text{CD8}^- \text{ cells in infected CD4}^+ : \text{CD8}^+ (1 : 1) \text{ co - cultures}}$$

● TIMING

Steps 1–15, isolation of PBMCs and cell fractioning: 3–4 h; and 2–3 d of culture for activation of CD4⁺ T cells
Steps 16–19, 3 h for infection and washings; 10–14 d for culture (depending on the peak of viral replication (option A)) or 3 d (option B); 4 h for p24 ELISA (option A) or 2 h 30 min for intracellular labeling of Gag products (option B)
Step 20, 10 min for calculation

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10, 14	Poor cell recuperation (quantity)	Bad condition of the sample	Pre-filter the PBMCs to avoid interference by cell aggregates during staining with antibody-coupled magnetic beads and/or the clogging of separation column (when used)
		Low CD4 ⁺ T cell counts in HIV-infected patients	Reduce the number of CD4 ⁺ and/or CD8 ⁺ T cells per well to 7.5×10^4 , reducing proportionally the volume of viral inoculum, medium, etc.
19A(xx), 19B(xviii)	No or weak viral replication detected in CD4 ⁺ T-cell cultures	Insufficient activation of CD4 ⁺ T cells	Retiter PHA and IL-2 concentrations to obtain a maximum of CD25 ⁺ CD4 ⁺ T cells at day 3
		Incomplete depletion of CD8 ⁺ T cells	Check purity of the cell fractions after isolation. Repurify magnetically labeled CD4 ⁺ T cells
		Loss of cells during washing	Check homogeneity of cell pellets at the bottom of the wells after removing supernatants. Be careful to always place tips on the border of the well, well above cell pellets, and remove less than 90% of the volume of the supernatant
		Culture supernatant used in p24 ELISA determination too diluted	Negative results need to be corroborated with less diluted (1:5) supernatants
19A(xx)	'Jumping' curves during kinetic reading of p24 ELISA plate	Air bubbles in wells interfere with optical density	Tap inverted plate firmly on a pad of absorbent towels to remove all liquid and bubbles before adding the substrate

ANTICIPATED RESULTS

The peak of viral replication when using the p24 ELISA assay is usually observed at day 7 or 10 after infection. *Ex vivo* CD8⁺ T cells from healthy donor controls should not have any significant effect on the viral infection of CD4⁺ T cells (see **Supplementary Fig. 2**

for an example). In contrast, CD8⁺ T cells from HIV-infected individuals can be readily classified by measuring their capacity to suppress HIV infection either by ELISA monitoring of HIV-1 p24 in culture supernatants or by flow cytometry analysis of the percentage of infected CD4⁺ T cells. The results obtained using these techniques are strongly correlated (**Fig. 3**). Highly efficient HIV-specific CD8⁺ T cells with strong abilities to suppress HIV-1 infection, such as those from HIC, should be able to reduce viral infection by more than 2 logs of p24 when using the ELISA assay, or by more than 1 log of infected cells when using the intracellular assay (**Fig. 3**, top right corner of the distribution). However, it is necessary to bear in mind that HIV-1 infection is also generally inhibited to some extent when CD4⁺ T cells are cultured in the presence of less efficient HIV-specific CD8⁺ T cells, such as those from HIV-infected viremic individuals¹⁵ (although by less than 1 log of p24 assayed by ELISA or by less than 0.5 log of infected cells with the intracellular assay; **Fig. 3**, bottom left corner of the distribution).

Note: Supplementary information is available via the HTML version of this article.

ACKNOWLEDGMENTS We thank Olivier Lambotte, Alain Venet and Martine Sinet for helpful discussions. We thank the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) HIV Controllers EP36 observatory. We also thank Katherine Kean for the English language editing of the article. We especially thank the subjects who participated in the studies developed in the laboratory for their cooperation. This work was financially supported by ANRS. S.Y.S. was supported by the Korea Science and Engineering Foundation and by the Institut Pasteur Korea.

AUTHOR CONTRIBUTIONS A.S.-C. designed the protocol, conducted experiments, analyzed the data and wrote the article; S.Y.S. conducted experiments and analyzed the data; P.V. conducted experiments and gave technical support; F.B.-S. gave conceptual advice; G.P. designed the protocol and edited the article. All authors discussed the results and commented on the article.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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