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▶ **To cite this version:**

Asier Saez-Cirion, So Youn Shin, Pierre Versmisse, Françoise Barré-Sinoussi, Gianfranco Pancino. Ex vivo T cell-based HIV suppression assay to evaluate HIV-specific CD8+ T-cell responses. Nature Protocols, 2010, 5 (6), pp.1033–1041. 10.1038/nprot.2010.73 . pasteur-01420584

HAL Id: pasteur-01420584
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Submitted on 16 May 2017

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

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ABSTRACT

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. Here 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 giving the most reliable results. We also depict the conditions of a rapid assay based on flow cytometry analysis of intracellular HIV-Gag products. These procedures take 14 to 17 days when using the p24 ELISA assay or 6 days 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 play 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 be able to 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 IFNγ Elispot surrogate assay[8]. Actually, 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 most accurately this efficacy, is still a subject of debate [8-10]. Much hope has been deposed 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 over 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 strategies[16]. During the last few years, the analysis of the 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 only revealed upon in vitro activation of the cells with optimal or overlapping HIV-1 peptides, and the 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 super-infection of autologous CD4+ T-cells through cytotoxic activity[20], this being the ultimate function of CD8 T-cells. Actually, the use of ex vivo (non-stimulated) CD8+ T cells, unlike previous assays which used expanded CD8+ T cell lines, CD8+ T cell clones or in vitro stimulated primary CD8+ T cells [5-7], allows assessing the antiviral potential of the whole untouched pool of HIV-specific CD8+ T cells in response to infected relevant target cells, which appears as highly important to accurately evaluate the antiviral efficacy of circulating HIV-specific CD8+ T cells. Using this assay, it is possible to differentiate

between the effective anti-HIV CD8+ T-cell responses in HIC and ineffective responses in patients with persistent viremia[20]. We have shown that the enhanced capacity of HIV-specific CD8+ T cells from HIC to inhibit HIV-1 infection requires contact and 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]. Actually, we only observed an effect of anti-viral factors when working with artificially in vitro activated CD8+ T cells, and this effect was, in any case, very modest [20]. Thus, our 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 showing increase lytic granule loading in HIC's CD8+ T cells upon contact with infected target cells [18,19]. Furthermore, we have found that 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 protein [15]. In fact, the depletion of Gag-specific CD8+ T-cells abrogates HIV suppression [15], 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 the CD4+ T cells [23]. 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 HIV-suppressive capacity of CD8+ T cells is influenced by the antigen sensitivity/functional avidity of the cells [24]. 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 the 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. Based on 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 (Table 1) and it complements assays that quantify the frequency of HIV-specific CD8+ T cells [15], such as the widely used IFN-γ Elispot assay (see Protocol [25]).

EXPERIMENTAL DESIGN

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 (figure 1):

- Isolation of CD4+ and CD8+ T cells and activation of CD4+ T cells
- Infection in vitro with HIV-1 of CD4+ T cells and CD4+:CD8+ T cell co-cultures with HIV-1
- Determination of viral replication in CD4+ T cells cultured alone or in the presence of CD8+ T cells and calculation of the HIV-1 suppressive capacity of CD8+ T cells

Some important points in this schema are depicted

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 PBMC may also be used. However, the level of CD8+ T cell-mediated HIV-1 suppression is then lower than with fresh cells. Therefore, experiments conducted with fresh samples should not be compared with those using frozen samples].* The blood must be kept at ~20^{o}C during transport and until "handling" in the laboratory to avoid haemolysis. If properly stored, the blood samples are workable up to 48h after extraction. Because of the drop in CD4+ T cell counts that is associated to 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 are enough for most healthy individuals. It is extremely important to ensure the purity of the 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 2-3 day activation step with 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 the CD8+ T cells by negative selection, to eliminate other cell fractions, especially 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, TCRγ/δ, Glycophorin A coupled to magnetic beads to eliminate CD4+ T cells (as the prior positive selection does not ensure the total removal of these cells from the negative fraction), B cells, NK cells, dendritic cells, etc. In this way, the CD8+ T cells are recovered untouched in the negative fraction (an alternative approach is to perform a positive selection

of CD8β+ cells, but this may result in an undesirable activation of the cells). We use anti-CD4 magnetic beads and CD8+ T cells enrichment kits from either Miltenyi Biotech or StemCell technologies (both for manual or Robosep assisted separation) with excellent results. We routinely get purities above 95% for CD4+ T cells and CD8+ T cells and yields of around 20% of the initial PBMC 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 the virus depends on the circumstances of the assay (e.g. subtype prevalence within the study population, strain of interest in a vaccine trial, etc.) 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 ([20] and see above) and the results obtained with both HIV-1 are globally comparable.

For the analysis of the HIV-1 suppression capacity based on an HIV-1 p24 ELISA assay, the choice of the infection dose should be one that regularly gives between 100 and 1000 ng/ml 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, an 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 would not allow a good discrimination between high-quality and low-quality CD8+ T cell responses, and infections producing above 1000 ng/ml 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 infection of between 10 and 30% of CD4+ T cells at day 3 post-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 different viral doses in parallel (moi= $10^{-1.15}$, $10^{-1.84}$) to be sure to achieve the desired level of infection.

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 the control cells (activated but non-superinfected, 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 towards infecting viruses than towards "consensus" (lab-adapted) strains, without altering significantly the results ([15] and unpublished). Nevertheless, caution should be applied when analyzing such results.

Detection of viral replication: p24 ELISA vs 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 (figure 2A) and to determine the percentage of infected CD4+ T cells by intracellular staining of Gag products (figure 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 co-cultures with highly efficient CD8+ T cells (figure 2). We use a kinetic ELISA assay for HIV-1 p24 because it offers a wider range standard curve than do endpoint 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 days post-infection 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 (Table 2 and figure 3). However, it is not as sensitive/quantitative as the p24 ELISA and it is probably less well-adapted to "categorize" CD8+ T cell responses.

MATERIALS

Reagents

Blood

CAUTION: Blood should be obtained according to an ethical committee approved protocol andfollowing patient consent. When obtained from an HIV-1 infected patient it should be treated as infectious material.

HIV-1 isolate (see Protocol [26])

CAUTION: HIV-1 is a class 3 human pathogen and it should be handled in a BSL3 facility

Dulbecco's PBS 1x (Gibco, cat n° 14190)

Lymphocyte Separation Medium LSM 1077 (PAA, cat n° J15-004)

CAUTION: May cause sensitization by inhalation and skin contact. Wear suitable protective clothing and gloves

RPMI 1640 (Gibco, cat n° 31870-025)

Foetal bovine serum (FBS) (PAA, cat n° A15-073)

L-Glutamine-200 mM (100X) (Gibco, cat n° 25030-081)

Penicillin-Streptomycin (100X) (Gibco, cat n° 15140-122)

CAUTION: May cause sensitization by skin contact; Irritating to eyes, respiratory system and skin. Wear suitable protective clothing, glasses and gloves.

EDTA 500 mM pH 8.0 (Gibco, cat n° 15575-020)

CAUTION: irritating to eyes. Wear suitable protective clothing, glasses and gloves.

Phytohemagglutinin-L (PHA) (Roche, cat n° 11 249 738 001)

Recombinant interleukin-2 (IL-2) (Chiron, Proleukin)

Trypan Blue Stain 0.4% (Invitrogen, cat n° T10282)

Human CD4 microbeads (positive selection) (StemCell, cat n° 18052RF or Miltenyi Biotec, cat n° 130-045-101)

Human CD8+ T Cell Enrichment Cocktail (negative selection) (StemCell, cat n° 19053RF or Miltenyi Biotec, cat n° 130-094-156)

CRITICAL: positive selection of CD4+ cells followed by negative selection of CD8+ T cells optimizes the yield of recovery of the cell fractions, and ensures obtaining untouched CD8+ T cells (see *isolation of cell fractions* on experimental design section).

RoboSep® Buffer (StemCell, cat n° 20104)

CAUTION: This product is a potential irritant to eyes, respiratory system, and skin. Wear suitable protective clothing, glasses and gloves.

HIV-1 P24 Antigen Elisa Bulk (ZeptoMetrix, cat n° 0801200)

HIV-1 P24 Extended range Kit (ZeptoMetrix, cat n° 0801137)

o-Phenylenediamine dihydrochloride tablets (OPD) (Sigma-Aldrich, cat n° P8412)

CAUTION: Harmful by inhalation and in contact with skin. Toxic if swallowed. Irritating 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 of as hazardous waste.

KC57-FITC antibody (Beckman Coulter, cat n° 6604665)

CD4-ECD antibody (Beckman Coulter, cat n° 6604727)

CD8-PC5 antibody (Beckman Coulter, cat n° A07758)

CD3-PE-Cy7 antibody (BD Pharmigen, cat n° 557749)

Fixation and Permeabilization Solution (BD bioscience, cat n° 554722)

CAUTION: Limited evidence of a carcinogenic effect. It may cause sensitization by skin contact. Wear suitable protective clothing and gloves.

Equipment

RoboSep (Stemcell technologies, cat n° 20000)

MidiMACS™ Separator (Miltenyi Biotec, cat n° 130-042-301)

LS Columns (Miltenyi Biotec, cat n° 130-042-301 130-042-401)

25 and 75cm² Cell Culture Flask with Vent Cap

15 and 50 mL Centrifuge Tubes

14 ml Falcon Polystyrene Round-Bottom Tubes (BD Biosciences, cat n° 352057)

1.5 and 2 ml screw cap micro tubes

5 ml round bottom cytometer tube (BD Bioscience, cat n^{o} 352005)

6, 12 and 96-well tissue culture plate. Flat bottom

96-well tissue culture test plates. Round bottom

Sterile 5, 10 and 25 ml disposable pipets

Pipette (0.5-10, 2-20, 20-200, 100-1000 µl)

Multichannel pipette (50-200 µl)

Benchtop centrifuge with sealed buckets and plate carriers

Flow cytometer

Mini vortex

Optical microscope

Microbiological safety cabinet

Plate shaker

Dry Incubator

CO_2 cell incubator

Elisa Microplate reader (with kinetic reading capabilities, suggested)

Countess Automated Cell Counter (Invitrogen, cat n^{o} C10227) or similar

LSR II Flow Cytometer (BD bioscience) or similar

Reagent setup

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

Culture medium: RPMI 1640 supplemented with Glutamine, Pen/Strep 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*) and IL-2 (100 uI/ml*). Should be freshly prepared or stored no longer than 3 days at 4°C.

*these values are indicated as a reference. You need to choose PHA and IL-2 concentrations that induce an optimal activation of CD4+ T cells after three days of culture.

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

Labelling buffer: PBS 1X 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 minutes run time, 30 seconds of time interval to read at 405 nm wavelength.

PROCEDURE

Isolation of PBMC * TIMING 1h30 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 analysed. For healthy individuals 5 ml of blood should be enough.

2- Gently add 20 ml of diluted blood to the top of 10 ml of Lymphocyte Separation medium in a 50-ml tube.

3- Centrifuge at 1200g (2000 rpm/min) for 20 minutes at room temperature

CRITICAL STEP: do not use brake, because a sudden stop will disrupt the density gradient.

4- Remove 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 400 g for 10 minutes at room temperature. Discard the supernatant, loosen the pellet and repeat PBS-wash twice.

7- Resuspend PBMC in separation buffer and count them in a haemocytometer.

Cell fractionation and culture * TIMING 2h00 to obtain CD4+ and CD8+ T cell fractions and 2-3 days for the activation of CD4+ T cells

8- Purify CD4+ cells

i. Concentrate PBMC (typically to 10^7 cells/ml) in chilled separation buffer after centrifugation at 400 g for 5 minutes and perform positive selection of CD4+ cells with anti-CD4+ antibody coupled to magnetic beads as recommended by the manufacturer.

ii. Collect positive fraction, count CD4+ cells and centrifuge cells at 400 g for 5 minutes at room temperature.

CRITICAL STEP: also collect negative fraction that will be used in step 9.

iii. Remove supernatant and resuspend cell pellet in activation medium to a final concentration of 10^6 cells/ml.

iv. Place cells into a well of a 12-well plate (or of a 6-well plate if more than $3x10^6$ cells are recovered) and culture them for 2-3 days at 37^oC, under 5% CO_2 with humidified atmosphere.

TROUBLESHOOTING

9- Purify CD8+ T cells

i. Concentrate negative cellular fraction from step 8 (typically to 10^7 cells/ml) in chilled separation buffer after centrifugation at 400 g for 5 minutes, and perform indirect magnetic cell sorting of untouched CD8+ T cells with a CD8+ T cell enrichment kit as recommended by the manufacturer.

ii. Collect negative fraction, count CD8+ T cells and centrifuge them at 400 g for 5 minutes at room temperature.

CRITICAL STEP: in contrast to CD4+ cells, the CD8+ T cells are not magnetically labelled and are recovered in the negative fraction

iii. Remove supernatant and resuspend cell pellet in culture medium to a final concentration of 10^6 cells/ml.

CRITICAL STEP: do not add cytokines to the culture medium of CD8+ T cells to preserve ex vivo activation status

iv. Put cells into a well of a 12-well plate (or of a 6-well plate if more than $3x10^6$ cells are recovered) and culture in a humidified incubator at 37^oC and 5% CO_2during activation of CD4+ T cells.

TROUBLESHOOTING

Evaluation of the capacity of CD8+ T cells to suppress HIV-1 infection of autologous CD4+ T cells * TIMING = 3h for infection and washings; 10 to 14 days for culture (depending on the peak of viral replication (option A)) or 3 days (option B); 4h for p24 ELISA (option A) or 2h30 for intracellular labelling of Gag products (option B).

10- Aspirate CD4+ and CD8+ T cells from wells with 5 ml pipettes and put cells to 15-ml conical tubes. Fill up the tubes with culture medium.

11- Centrifuge at 400 g for 5 minutes 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).

12- Count cells with haemocytometer and adjust concentration to 10^6 cells/ml with suitable medium.

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

13- 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 96-well-round bottom-well plate. Allow three wells for non-infected in vitro control, three wells for HIV-1 infected in vitro CD4+ T cells, three additional wells for co-culture with CD8+ T cells at a 1:1 ratio and, optionally, three wells for each co-culture at additional CD4+:CD8+ T cell ratios (see step ii)

CRITICAL STEP: the use of 96-well-round bottom-well plates is mandatory to ensure that no cells are lost during washing or recovery of supernatants (steps iii, viii and x)

ii. Add 100 µl of CD8+ T cells at 10^6 cells/ml to each of three wells containing 10^5 CD4+ T cells for co-culture at a 1:1 CD4+:CD8+ T cell ratio. Dilute CD8+ T cells 1/3 and 1/10 in culture medium and add (3x) 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 plate at 400 g for 5 minutes at room temperature and remove 100 µl of supernatant from CD4+:CD8+ T cell co-culture-containing wells.

iv. Add 100 µl of IL-2 medium to each of three wells destined as non-infected in vitro controls.

v. Dilute viral stocks to a 2x assay viral dose in IL2 medium and add 100 µl of viral suspension to the three wells containing CD4+ T cells and to wells containing CD4+:CD8+ T cell co-cultures.

CRITICAL STEP: viral dose needs to be determined by the user

vi. Centrifuge plate at 1200 g for 1 hour at 22°C in a refrigerated centrifuge to improve infection efficiency [27].

CAUTION: to ensure biocontainment and to avoid exposure to aerosols, use a centrifuge with sealed plate carriers.

vii. Incubate plate for 1 hour at 37^{o}C in a humidified incubator under 5% CO_2.

viii. Remove 190 µl of supernatants using an 8-channel pipette, add 190 µl of culture medium and centrifuge at 400 g for 5 minutes at room temperature. Repeat twice. Use 190 µl of IL2 medium after the last wash.

CRITICAL STEP: after centrifugation the cells are concentrated at the bottom of the well. Place the tips on the border of the well, well above the cell pellet, to avoid loss of cells.

ix. Incubate plate at 37^{o}C in a humidified incubator under 5% CO_2.

x. At day 3 post-infection, centrifuge the plate at 400 g for 5 minutes at room temperature. Remove 190 µl of supernatants and transfer them to a new 96 well plate and store it. Replenish cell cultures with 190 µl of IL-2 medium and put the 96-well plate containing the cell cultures back to the incubator. Repeat at days 7, 10 and 14.

PAUSE POINT: Store plates with culture supernatants at 4^{o}C if p24 ELISA is to be performed within 15 days or at -80^{o}C if it will be done later or if further analyses are planned.

xi. If frozen, allow culture supernatants to thaw at room temperature. Dilute supernatants with assay diluent and proceed with HIV-1 p24 antigen ELISA determination.

xii. Allow antibody-coated strips to reach room temperature. Add 300 µl of assay's wash buffer to each well and decant liquid. Repeat this step 6 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), and add 200 µl of culture medium to two other wells, 200 µl of standard dilutions (4 to 0.08 ng/ml) and 50 µl of diluted samples plus 150 µl of culture medium to strips.

xiv. Add 20 µl of lysing buffer to all wells except blank and standard. Cover plate with an adhesive plate sealer and incubate at 37°C for 2 hour 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 decant liquid. Repeat this step 6 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 hour.

xvii. Decant liquid from plate and wash the wells as in step xv.

xviii. Add 100 µl of Streptavidin-Peroxidase solution into each well except the blank, cover plate with adhesive plate sealer and incubate at 37°C for 30 minutes.

xix. Decant liquid from plate and wash the wells as in step xv.

CRITICAL STEP: removal of air bubbles is critical at this point as they interfere with the signal during reading.

xx. Place plate on plate reader and add 100 µl of OPD-substrate solution. Start reading immediately on pre-configured plate reader.

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 µl of CD4+ T cells to wells in 96-well-round bottom-well plate. Allow one well for a non-infected in vitro control, two wells for in vitro HIV-1 infected CD4+ T cells, two additional wells for co-culture with CD8+ T cells at a 1:1 ratio.

CRITICAL STEP: you may want to provide for additional cells for proper monolabelled compensation controls for flow cytometry analysis (step xviii). Alternatively you can use compensation beads for this purpose.

CRITICAL STEP: the use of 96-well-round bottom-well plates is mandatory to ensure that no cells are lost during washing steps (steps iii, viii and ix)

ii. Add 50 µl of CD8+ T cells (10^6 cells/ml) to two wells containing $5x10^4$ CD4+ T cells for co-culture at a 1:1 CD4+:CD8+ T cell ratio.

iii. Centrifuge plate at 400 g for 5 minutes at room temperature and remove 50 µl of supernatant from CD4+:CD8+ T cell co-cultures-containing wells.

iv. Add 50 µl of IL2 medium to well destined to non-infected in vitro control.

v. Prepare two viral dilutions at a 2x assay viral dose in IL-2 medium and for each viral suspension add 50 µl to one well containing CD4+ T cells and 50 µl to one well containing CD4+:CD8+ T cell co-cultures.

CRITICAL STEP: viral dose needs to be determined by the user

vi. Centrifuge plate at 1200 g for 1 hour at 22°C in a refrigerated centrifuge to improve infection efficiency [27].

CAUTION: to ensure biocontainment and to avoid exposure to aerosols, use a centrifuge with sealed plate carriers

vii. Incubate plate for 1 hour at 37°C in a humidified incubator under 5% CO_2.

viii. Remove 90 µl of supernatants using an 8-channel pipette, add 90 µl of culture medium and centrifuge at 400 g for 5 minutes at room temperature. Repeat twice. Use 90 µl of IL2 medium after the last wash.

CRITICAL STEP: after centrifugation the cells are concentrated at the bottom of the well. Place the tips on the border of the well, well above the cell pellet, to avoid loss of cells.

ix. Incubate the plate for three days at 37°C in a humidified incubator under 5% CO_2. Centrifuge the plate at 400 g for 5 minutes at room temperature and discard supernatants.

x. Harvest cell pellets with 100 µl of labelling buffer and transfer to 5 ml cytometry tubes. Add 500 µl of labelling buffer to each tube and centrifuge at 400 g for 5 minutes 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 pre-titered antibodies in a final volume of 50 µl of labelling buffer for each tube. Loosen cell pellet in tubes

and add antibody mixes. Vortex the tubes, wrap in aluminium foil and incubate at room temperature for 20 minutes.

CRITICAL STEP: other than the sample tubes, you need to include a tube with non-labelled cells (cultured in the same condition as the experimental sample) and 4 tubes with single labelled compensation controls (either cells expressing the appropriate molecule or compensation beads).

xii. Add 1 ml of staining buffer to each tube, centrifuge tubes at 400 g for 5 minutes at room temperature. Discard supernatants.

xiii. Loosen pellets and add 250 µl of cytofix/cytoperm solution to all tubes and incubate in the dark at 4^{o}C for 20 minutes.

xiv. Add 1 ml of 1X cytofix/cytoperm wash solution to each tube, centrifuge tubes at 400 g for 5 minutes at room temperature. Discard supernatants.

xv. Prepare anti-Gag KC57-FITC antibody mix (3 µl of antibody and 47 µl of cytofix/cytoperm wash solution for each tube). Loosen pellets, add 50 µl of mix to tubes and incubate at 4°C for 30 minutes in the dark.

xvi. Add 1 ml of 1X cytofix/cytoperm wash solution to each tube, centrifuge tubes at 400 g for 5 minutes at room temperature. Discard supernatants and resuspend cell pellet in 400 µl of staining buffer.

xvii. Keep samples at 4^{o}C in the dark while setting up the flow cytometer with non-stained control and compensation controls.

xviii. Proceed to flow cytometer acquisition of experimental data.

TROUBLESHOOTING

14- Calculate HIV-suppressive capacity of CD8+ T cells from HIV-1 p24-ELISA (A) or intracellular Gag (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) for each sample at the different time points as the mean and standard deviation of triplicate wells (figure 2A).

ii. Calculate 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 the culture at a 1:1 ratio (see figure 2A).

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

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 figure 2B).

$$\text{HIV-suppressive capacity of CD8+ T cells (log fold decrease in \% kc57+CD4+ T cells)} = \text{Log}_{10} \frac{\text{\% of Kc57+ CD3+CD8- cells in culture of CD4+ T cells infected in vitro}}{\text{\% of Kc57+ CD3+CD8- cells in infected CD4+:CD8+ (1:1) co-cultures}}$$

TIMING

Steps 1-9, Isolation of PBMC and cell fractioning: 3-4h; and 2-3d of culture for activation of CD4+ T cells

Steps 10-13.A-B. viii, Infection in vitro with HIV-1 and washings: 3h

Steps 13.A.ix-13.A.x, Culture of infected cells for ELISA assay: 10-14d

Steps 13.A.xi-13.A.xx, Analysis of viral replication by p24 ELISA: 4h

Step 13.B.ix, Culture of infected cells for flow cytometry assay: 3d

Steps 13.B.x-xviii, Analysis of viral replication by intracellular labelling of Gag products: 2h30

TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

ANTICIPATED RESULTS

The peak of viral replication when using the p24 ELISA assay will usually be observed at day 7 or 10 after infection. Ex vivo CD8+ T cells from healthy donor controls should not have any significant effect on viral infection of CD4+ T cells. Highly efficient HIV-specific CD8+ T cells with strong abilities to suppress HIV-1 infection, such as those from HIV controllers, should be able to reduce viral infection by more than 2 logs of p24 when using the ELISA assay, or more than 1 log of infected cells when using the intracellular assay (figure 3). However, it is necessary to keep in mind that HIV-1 infection is also generally inhibited to some extent when CD4+ T cells are cultured in presence of less efficient HIV-specific CD8+ T cells, such as those from HIV-infected viremic individuals [15] (although in less than 1 log of p24 assayed by ELISA or less than 0.5 logs of infected cells with the intracellular assay) (figure 3).

AUTHOR CONTRIBUTION

ASC designed the protocol, performed experiments, analyzed the data and wrote the manuscript; SYS performed experiments and analyzed the data; PV performed experiments and gave technical support; FBS gave conceptual advice; GP designed the protocol and edited the manuscript. All authors discussed the results and commented on the manuscript.

ACKNOWLEDGMENTS

We thank Olivier Lambotte, Alain Venet, Martine Sinet for helpful discussions. We thank the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) HIV Controllers EP36 observatory. We thank Katherine Kean for the English editing of the manuscript. We especially thank the subjects who participate in the studies developed in the laboratory for their cooperation.

This work was financially supported by ANRS. SYS was supported by Korea Science and Engineering Foundation and the Institut Pasteur Korea.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests

REFERENCES

1. Borrow, P., Lewicki, H., Hahn, B.H., Shaw, G.M. & Oldstone, M.B. Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J Virol* **68**, 6103-6110 (1994).
2. Jin, X.*, et al.* Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian immunodeficiency virus-infected macaques. *J Exp Med* **189**, 991-998 (1999).
3. Kiepiela, P.*, et al.* CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nat Med* **13**, 46-53 (2007).
4. Martinez-Picado, J.*, et al.* Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. *J Virol* **80**, 3617-3623 (2006).
5. Mackewicz, C.E., Ortega, H.W. & Levy, J.A. CD8+ cell anti-HIV activity correlates with the clinical state of the infected individual. *The Journal of clinical investigation* **87**, 1462-1466 (1991).
6. Tsubota, H., Lord, C.I., Watkins, D.I., Morimoto, C. & Letvin, N.L. A cytotoxic T lymphocyte inhibits acquired immunodeficiency syndrome virus replication in peripheral blood lymphocytes. *J Exp Med* **169**, 1421-1434 (1989).
7. Yang, O.O.*, et al.* Suppression of human immunodeficiency virus type 1 replication by CD8+ cells: evidence for HLA class I-restricted triggering of cytolytic and noncytolytic mechanisms. *J Virol* **71**, 3120-3128 (1997).
8. Walker, B.D. & Burton, D.R. Toward an AIDS vaccine. *Science* **320**, 760-764 (2008).
9. Appay, V., Douek, D.C. & Price, D.A. CD8+ T cell efficacy in vaccination and disease. *Nat Med* **14**, 623-628 (2008).
10. D'Souza, M.P. & Altfeld, M. Measuring HIV-1-specific T cell immunity: how valid are current assays? *J Infect Dis* **197**, 337-339 (2008).
11. Deeks, S.G. & Walker, B.D. Human immunodeficiency virus controllers: mechanisms of durable virus control in the absence of antiretroviral therapy. *Immunity* **27**, 406-416 (2007).
12. O'Connell, K.A., Bailey, J.R. & Blankson, J.N. Elucidating the elite: mechanisms of control in HIV-1 infection. *Trends in pharmacological sciences* **30**, 631-637 (2009).
13. Saez-Cirion, A., Pancino, G., Sinet, M., Venet, A. & Lambotte, O. HIV controllers: how do they tame the virus? *Trends Immunol* **28**, 532-540 (2007).

14. Gea-Banacloche, J.C., *et al.* Maintenance of large numbers of virus-specific CD8+ T cells in HIV-infected progressors and long-term nonprogressors. *J Immunol* **165**, 1082-1092 (2000).
15. Saez-Cirion, A., *et al.* Heterogeneity in HIV suppression by CD8 T cells from HIV controllers: association with Gag-specific CD8 T cell responses. *J Immunol* **182**, 7828-7837 (2009).
16. Baker, B.M., Block, B.L., Rothchild, A.C. & Walker, B.D. Elite control of HIV infection: implications for vaccine design. *Expert opinion on biological therapy* **9**, 55-69 (2009).
17. Betts, M.R., *et al.* HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* **107**, 4781-4789 (2006).
18. Migueles, S.A., *et al.* HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol* **3**, 1061-1068 (2002).
19. Migueles, S.A., *et al.* Lytic granule loading of CD8+ T cells is required for HIV-infected cell elimination associated with immune control. *Immunity* **29**, 1009-1021 (2008).
20. Sáez-Cirión, A., *et al.* HIV controllers exhibit potent CD8 T cell capacity to suppress HIV infection ex vivo and peculiar CTL activation phenotype. *Proc Natl Acad Sci U S A* **104**, 6776-6781 (2007).
21. Migueles, S.A., *et al.* Defective human immunodeficiency virus-specific CD8+ T-cell polyfunctionality, proliferation, and cytotoxicity are not restored by antiretroviral therapy. *J Virol* **83**, 11876-11889 (2009).
22. Vojnov, L., *et al.* Effective simian immunodeficiency virus-specific CD8+ T cells lack an easily detectable, shared characteristic. *J Virol* **84**, 753-764 (2010).
23. Sacha, J.B., *et al.* Gag-specific CD8+ T lymphocytes recognize infected cells before AIDS-virus integration and viral protein expression. *J Immunol* **178**, 2746-2754 (2007).
24. Almeida, J.R., *et al.* Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. *Blood* **113**, 6351-6360 (2009).
25. Streeck, H., Frahm, N. & Walker, B.D. The role of IFN-gamma Elispot assay in HIV vaccine research. *Nature protocols* **4**, 461-469 (2009).
26. van 't Wout, A.B., Schuitemaker, H. & Kootstra, N.A. Isolation and propagation of HIV-1 on peripheral blood mononuclear cells. *Nature protocols* **3**, 363-370 (2008).
27. O'Doherty, U., Swiggard, W.J. & Malim, M.H. Human immunodeficiency virus type 1 spinoculation enhances infection through virus binding. *J Virol* **74**, 10074-10080 (2000).

FIGURE LEGENDS

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

Figure 2. Examples of experiments performed using the protocol. **A**. p24 production (mean ± s.d., n=3) in the culture supernatants of PHA-activated CD4+ T cells from an HIC infected 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 the CD4+ T cells are co-cultured with CD8+ T cells. **B**. PHA-activated CD4+ T cells from an HIC superinfected with HIV-1 BaL and left alone (left panel) or co-cultured with autologous unstimulated CD8+ T cells (right panel). 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 upon 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.

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 the 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).

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	Labour-intensive handling of sample
Takes into account the infection dynamics: response to infected cells instead of 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, heavier, experiments involving sorting of cell subpopulations [15,22]
Easy and low-cost adaptability to different HIV-1 strains (tropism, subtype)	

Table 2. Comparison of p24 ELISA vs intracellular Gag assay.

	HIV-1 p24 ELISA assay	**Intracellular HIV-1 Gag assay**
Time	14-17 days (10-14 days of infection)	6 days (3 days of infection)
Sample required	10 million PBMCs	1-2 million PBMCs
Reproducibility	Good	Good
Range	Excellent	Limited

Table 3. Troubleshooting table

Step	Problem	Possible reason	Solution
8,9	Poor cell recuperation (quantity)	Bad condition of the sample	Pre-filter the PBMC 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 $7.5x10^4$, reducing proportionally the volume of viral inoculum, medium, etc.
13	No or weak viral replication detected in CD4+ T cell cultures	Insufficient activation of CD4+ T cells	Re-titer PHA and IL2 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. Re-purify magnetically-labelled 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
13	“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

Figure 1

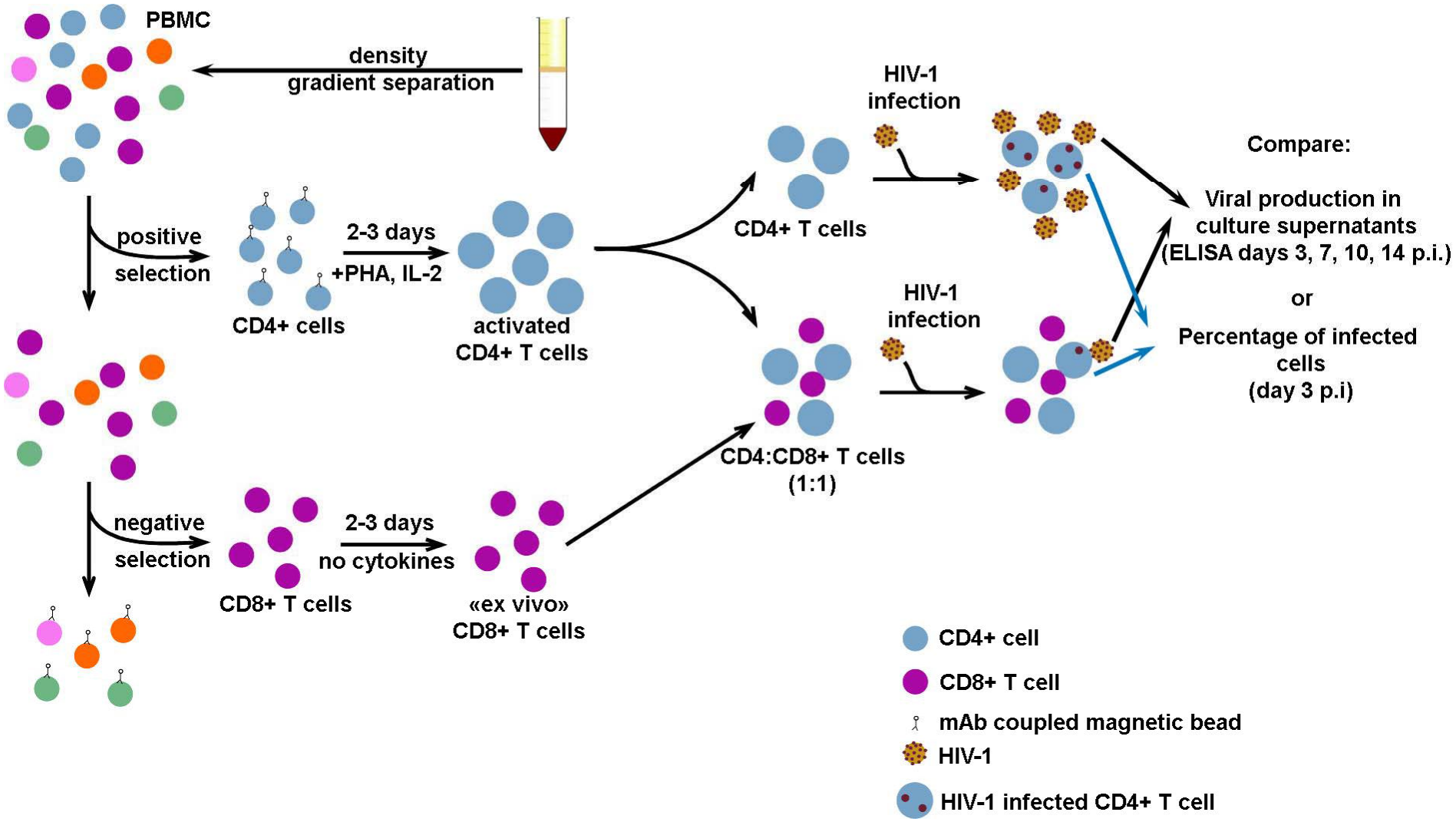
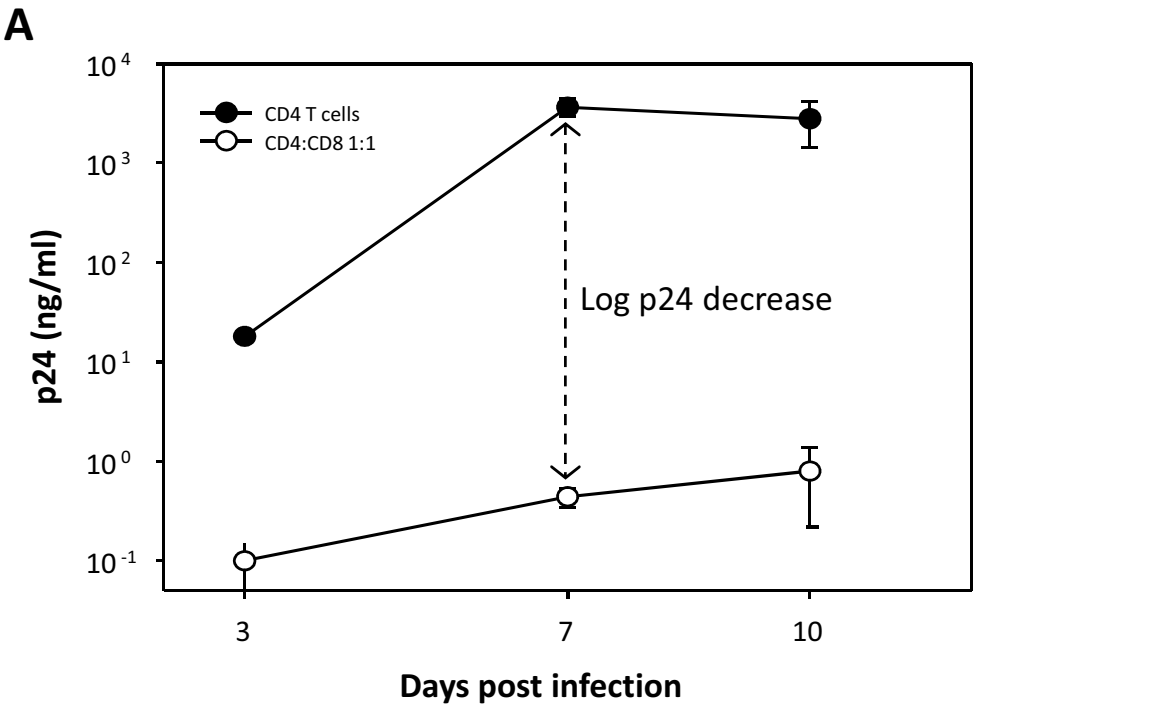

PBMC
density
gradient separation
positive
selection
CD4+ cells
2-3 days
+PHA, IL-2
activated
CD4+ T cells
CD4+ T cells
HIV-1
infection
Compare:
Viral production in
culture supernatants
(ELISA days 3, 7, 10, 14 p.i.)
or
Percentage of infected
cells
(day 3 p.i)
HIV-1
infection
CD4:CD8+ T cells
(1:1)
negative
selection
CD8+ T cells
2-3 days
no cytokines
«ex vivo»
CD8+ T cells
CD4+ cell
CD8+ T cell
mAb coupled magnetic bead
HIV-1
HIV-1 infected CD4+ T cell

Figure 2


A
CD4 T cells
CD4:CD8 1:1
Log p24 decrease
p24 (ng/ml)
Days post infection
3
7
10

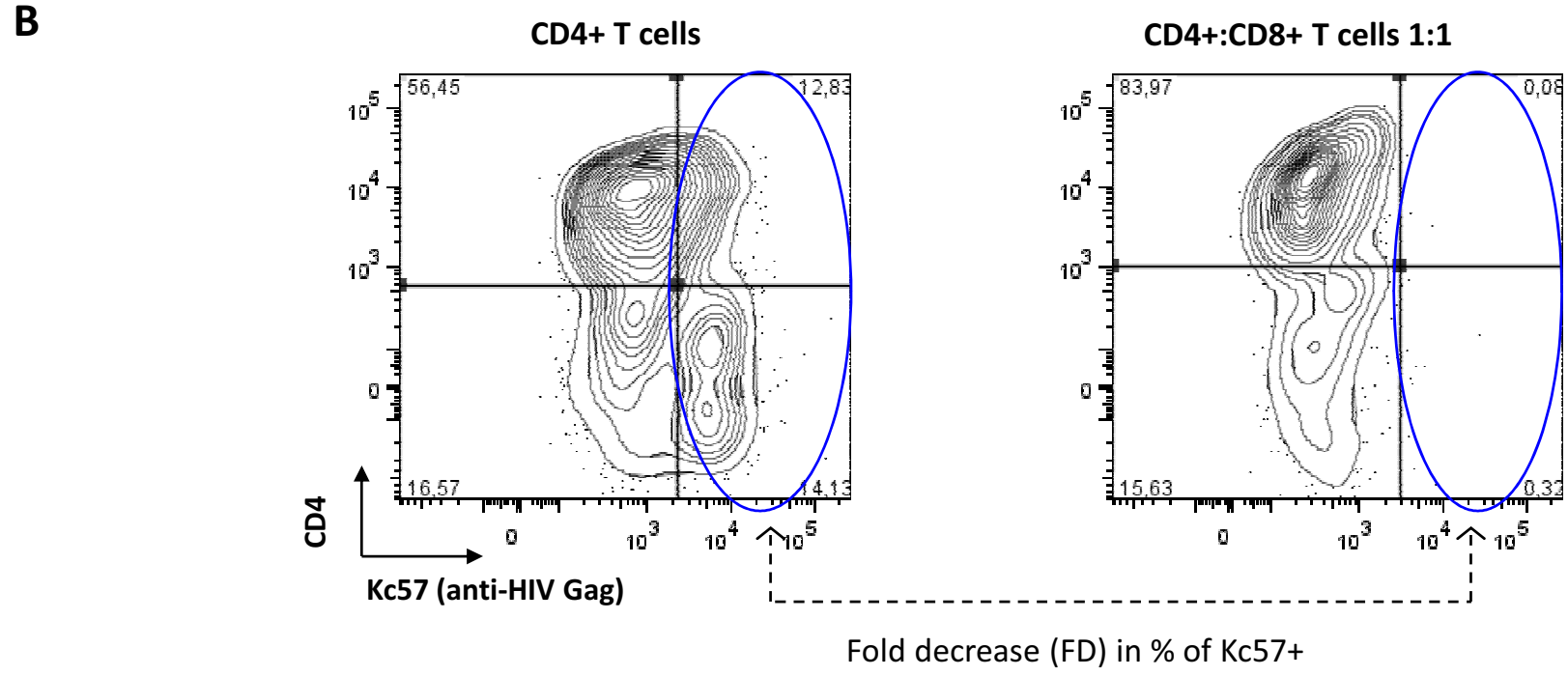
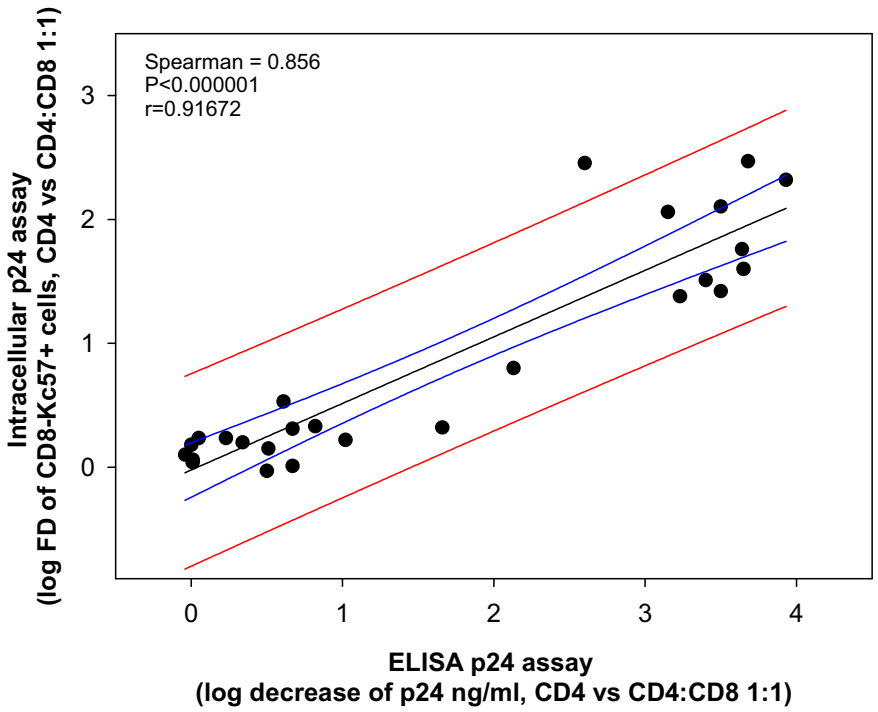

B
CD4+ T cells
CD4+:CD8+ T cells 1:1
56,45
12,83
16,57
14,13
83,97
0,08
15,63
0,32
CD4
Kc57 (anti-HIV Gag)
Fold decrease (FD) in % of Kc57+

Figure 3


Spearman = 0.856
P<0.000001
r=0.91672
Intracellular p24 assay
(log FD of CD8-Kc57+ cells, CD4 vs CD4:CD8 1:1)
ELISA p24 assay
(log decrease of p24 ng/ml, CD4 vs CD4:CD8 1:1)
0
1
2
3
4