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Experimental models to study HIV latency reversal from male genital myeloid cells

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Abstract

HIV reservoirs are poorly understood and their establishment largely depends on the nature of tissues that interact with the virus. In this chapter, we will describe *in vitro* and *ex vivo* models of human urethra mucosal macrophages used in the investigation of the establishment and maintenance of tissue HIV reservoirs. In addition, we will describe how macrophage latent HIV infection was assessed in these models by reverting a nonproductive state of infection back into a productive state. Consequently, infectious particles are released to the macrophage extracellular milieu and detected by adapted viral outgrowth assays. Altogether, these approaches provide invaluable tools for the investigation on tissue-specific pathways that HIV-1 employs to reach host cells and form reservoirs in the genital mucosa, contributing to the development of an efficient and targeted prophylaxis against HIV and of a HIV cure.

1. Introduction

The male genital mucosa is an important entry and establishment site of sexually transmitted infections (STI). The human urethra is rich in immunologically active cells (1) not only implicated in specific and effective immune response against STI pathogens, but also exploited by these pathogens as long-lasting hosts implicated in chronic infections and pathogen persistence. HIV is an emblematic example of STI pathogen capable of establishing long-lasting infected cells, referred to as HIV reservoirs(2), despite the antiretroviral action of combination antiretroviral therapy (cART) that blocks viral replication and propagation but fails in eradicating infection.

In the case of penile mucosa, these HIV reservoirs can be established in the early steps of infection, i.e., during the sexual intercourse, the main transmission route for the virus. HIV-1 gains access to the body by crossing epithelial barriers that compose the ano-rectal and genital mucosa during the sexual intercourse. One pathway of HIV-1 entry at mucosal sites is via transcytosis through epithelial cells after the formation of cell-cell contacts (virological synapses) (3) between epithelial cells and HIV-1 infected cells (4-6) present in all secretions that act as vector for HIV-1 infection (7). Thus, HIV-1 infected cells form viral synapses with the foreskin and urethral tissue, the virus produced at the synapse transcytose, and in turn reaches Langerhans cells in the foreskin or macrophages in urethral mucosa (8-11).

We have been successfully employing models of mucosal macrophage reservoirs to simulate the early steps of HIV transmission during sexual intercourse, to investigate the early establishment and the nature of these reservoirs, and to evaluate strategies to purge HIV-1 from them. Accordingly, the *in vitro* model here described was helpful to demonstrate that mucosal stromal macrophages are infected by HIV-1 upon virological synapse formation between HIV-infected CD4⁺ T-cells and epithelial cells and also to demonstrate that these stromal macrophages reach a latent phase of infection reactivated by Toll-like receptor(TLR)-4 stimulation via bacterial lipopolysaccharide (LPS) (10, 11). Furthermore, using the *ex vivo* model also described in this chapter (urethral tissues obtained from cART-suppressed HIV-infected donors), we have demonstrated that urethral macrophages are the main mucosal HIV-1 reservoir harboring replication-competent viruses (12). These evidences demonstrated that in addition to the main and best characterized reservoir residing in peripheral blood resting memory CD4⁺ T cells, tissue macrophages are persistently infected despite virological suppression by cART and may represent an additional source of viral rebound once cART is interrupted.

As the dynamics of HIV in T cells and macrophages profoundly differs, how such tissue macrophage reservoirs are formed and maintained, remain unclear and need to be elucidated. For instance, during cART, HIV-1 is detected almost exclusively as pro-viral DNA integrated in the genome of latently infected memory CD4⁺ T-cells, while in macrophages of individuals under cART, HIV-1 is detected not only as integrated pro-virus but also as infectious viral particles stored in virus-containing compartments (12, 13).

This chapter describes experimental models that mimic HIV-1 pathogenesis from early infection to reservoir formation in tissue myeloid cells like macrophages. These models, based here on male genital tract tissues but easily transposable to other genital mucosa, are helpful to better understand the tissue-specific mechanisms employed by the virus to access and persist in diverse cell reservoirs, contributing for the development of efficient protective vaccines and antiretroviral therapies against HIV.

2. Materials

In order to execute the presented methodology, the laboratory, must have access, in accordance with ethical rules, to: i) whole penile tissues, easily obtained from men undergoing elective gender reassignment; ii) foreskin tissues, obtained from men undergoing circumcision due to non-infectious pathologies or personal reasons; iii) blood donated from healthy donors.

2.1 For mucosa reconstructed and infected *in vitro*

2.1.1 Cells and viral strains employed in tissue reconstruction and infection

1. Primary fibroblasts, isolated from foreskin tissues, obtained from healthy adults undergoing circumcision due to non-infectious pathologies or personal reasons via collaboration with appropriate medical services.
2. Peripheral blood mononuclear cells, obtained from healthy volunteers and supplied by the local blood centers.
3. Transformed urethral epithelial cells (THUEC), kindly donated by Professor MA Apicella, University of Iowa, Iowa City, IA).
4. CD4⁺ T-cells: T-cell line Jurkat CD4-CCR5 (ARP094 National Institute for Biological Standards and Control, NIBSC, UK)
5. Plasmids of fluorescent R5 tropic HIV-1 clone (JFRL) carrying a green fluorescent protein (GFP) gene inserted between the viral matrix and capsid genes (gag-iGFP HIV-1, JRFL strain) (14), kindly donated by Dr. Benjamin Cheng (Icahn School of Medicine at Mont Sinai, New York, NY)

2.1.2 Reconstruction media

1. DMEM complete medium: DMEM (41965-039, Gibco), supplemented with 10% v/v fetal calf serum (FCS), 2 mM L-glutamine, 15 mM HEPES, and 100 U/ml of penicillin-100 µg/ml streptomycin (Gibco).
2. RPMI complete medium: RPMI 1640 medium (31870-025, Gibco) supplemented with 10% v/v FCS, 100 U/ml of penicillin-100 µg/ml streptomycin, 2 mM L-glutamin (Gibco), 15 mM HEPES, 25 ng/ml of macrophage colony-stimulating factor (M-CSF, R&D systems, Inc.) and 50 ng/ml of granulocyte macrophage colony-stimulating factor (GM-CSF, R&D systems, Inc.)
3. Extracellular matrix: Phenol-free MatrigelTM (Corning, Inc.).
4. Reconstruction medium: DMEM supplemented with 20% v/v Ham's F-12 Nutrient Mix (Gibco), 10% v/v FCS, 100 U/ml of penicillin-100 µg/ml streptomycin, 5 ng/ml Epithelial Growth Factor (EGF, Thermo Fisher Scientific Inc.), 5 µg/ml insulin (Gibco, Thermo Fisher Scientific Inc.), 400 ng/ml hydrocortisone (Sigma-Aldrich Co. LLC.), 5 µg/ml of transferrin (Sigma-Aldrich Co. LLC.), 0.01 nM of triiodothyronine (Sigma-Aldrich Co. LLC.), 50 µg/ml ascorbic acid (Sigma-Aldrich Co. LLC.), 12.5 ng/ml M-CSF and 25 ng/ml GM-CSF.
5. Macrophage detachment medium: phosphate buffered saline (PBS Ca²⁺/Mg²⁺-free) with 1% w/v of EDTA tetrasodium.

6. THUEC cultivation medium: PrEBMTM medium (CC-3165, Lonza) supplemented with PrEGMTM SingleQuots® (CC-4177, Lonza)

2.1.3 Labware

1. Two-chamber membrane filters (TranswellTM, polycarbonate membrane, 3 µm pore size, 12-mm diameter, Corning, Inc.)
2. Low-adherent plastic petri dishes for bacteriology (Greiner Bio One International, GmbH).

2.1.4 Other reagents using in *in vitro* reconstruction model

1. Enzyme-linked immunosorbent assays (ELISA) for HIV-1 p24-(Gag) protein (p24 Innostest HIV-1 ELISA, InGen, Diaxonhit group, France)
2. Fluorescence-tagging reagents: Quantum dots, a long-term fluorescent cell tracker (Qtracker® 705 Cell Labeling Kit, ThermoFisher Scientific, Inc.). Hoechst 33258 (Sigma-Aldrich Co. LLC.)
3. EasySepTM Human Monocyte Enrichment Kit (STEMCELL Technologies Inc.)

2.2 For *ex vivo* reactivation of latent HIV

2.2.1 Preparation of urethral tissues from HIV-infected cART-suppressed individuals

1. Whole penile tissues were obtained from men undergoing elective gender reassignment. HIV-1 infected individuals were under cART for at least 3 years, with CD4⁺ T-cell count of >400 cells/µl and with undetectable plasma viral loads (<40 RNA copies/ml) as measured few months before surgery. Immediately following surgery, the penile tissues are preserved in phosphate-buffered saline (PBS) supplemented with 100 U/ml penicillin-100 µg/ml streptomycin and transported on ice to a biosecurity level 2+ facility.
2. Dispase solution: PBS Ca²⁺/Mg²⁺-free supplemented with 2.4 U/ml Dispase® II (neutral protease, grade II, ROCHE), 200 U/ml DNase I (ROCHE), and 20% v/v FCS.
3. Trypsin solution: Trypsin-EDTA (0.05%) with phenol red (Gibco)
4. Collagenase solution: PBS Ca²⁺/Mg²⁺-free supplemented with 2 mg/ml Collagenase from *Clostridium histolyticum* (Sigma-Aldrich Co. LLC.), 200 U/ml DNase I (ROCHE), and 20% v/v FCS.

2.2.2 Latency reversal agents and reagents for detection of reactivated HIV

1. Toll-like receptor 4 (TLR-4) agonist: lipopolysaccharide (LPS from *Salmonella enterica* serotype typhimurium, Sigma-Aldrich).
2. Ghost (3) X4/R5 GFP-expressing reporter cells obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH (from Drs. V. N. KewalRamani and D. R. Littman).
3. CD4⁺ T-cell lymphoblasts from healthy donors produced as described (15) and cultivated in RPMI complete medium supplemented with 10 U/ml IL-2 (Sigma-Aldrich Co. LLC.).
4. RPMI complete medium.
5. DMEM complete medium.
6. Paraformaldehyde: Paraformaldehyde (Electron Microscopy Sciences) 4% v/v diluted in PBS Ca²⁺/Mg²⁺-free saline.
7. Anti-GFP antibody reactions: goat anti-GFP antibody (Abcam plc.), anti-goat Cy3 secondary antibody (1:200, Jackson ImmunoResearch Inc.)
8. Blocking/permeabilizing solution: PBS Ca²⁺/Mg²⁺-free supplemented with 0.25% w/v Saponin (Merck Millipore) and 2% w/v Bovine Serum Albumin Fraction V (ROCHE).
9. DAPI solution: 4',6-diamidino-2-phenylindole at 10 µM
10. Primary R5-tropic HIV-1 isolate: primary isolate 93BR029, NIH Reagents program, US
11. TRIzolTM (ThermoFisher Scientific)

2.2.3 Labware

1. Lab-Tek 16-well chamber slide (Nunc Inc.)
2. Low-adherence 96-round-bottom-well plate (Corning Inc.)
3. 70 mm Cell-strainers (Fisher Scientific Inc.)

3. Methods

3.1 HIV latency reversal in male genital mucosa reconstructed and infected *in vitro*

3.1.1 Urethral tissue reconstruction *in vitro*

1. Cultivating and preparing cells used in reconstructions: Isolate primary fibroblasts from foreskin tissues by enzymatic digestion as described (9). Obtain primary macrophages from monocyte-enriched peripheral blood mononuclear cells (PBMC), after isolation from healthy blood donors by a standard Ficoll-Hypaque density gradient centrifugation. Enrich PBMC cell suspension in monocytes using a negative magnetic selection kit for CD14⁺ cells (Stemcell Technologies, Grenoble, France) according to the manufacturer. Cultivate monocyte-enriched PBMCs in RPMI complete medium for 6 days at 37° C, 5% CO₂ environment in low-adherent plastic petri dishes for bacteriology (Greiner Bio One International, GmbH). After this incubation period, collect the peripheral blood monocyte-derived macrophages by treatment with warmed macrophage detachment medium for 10 minutes at 37° C, 5% CO₂ environment, followed by gentle flushing of the cultivation dishes for complete macrophage detachment. (*see Note 1*). Maintain Transformed urethral epithelial cells (THUEC) in culture with THUEC cultivation medium as described (16). Maintain CD4⁺ T-cells (Jurkat CD4-CCR5 cell line) in culture with RPMI complete medium as described (14).
2. Mix primary human foreskin-derived fibroblasts (5×10^5 cells per reconstruction) and macrophages ($2-4 \times 10^4$ cells per reconstruction) in 200 μ l of extracellular matrix and seed them on a two-chamber membrane filter as described previously (8, 17, 18). After 30 minutes for solidification of the matrix, cultivate the system for 48 hours (37°, 5% CO₂) in reconstruction medium. Next, seed transformed urethral epithelial cells (THUEC) on top of the matrix (5×10^5 cells per insert) and cultivate the system overnight at 37°C, 5% CO₂ environment. After seeding of urethral epithelial cells, the system is cultivated for 1 week in a submerged environment (liquid-liquid) with reconstruction medium. An air-liquid system is set for an additional week to induce epithelium polarization before infection experiments (Figure 1A-B).

3.1.2 *In vitro* infection of tissue reconstruction

1. Insert the gag-iGFP HIV-1 DNA plasmid into CD4⁺ T-cells by transfection as described (14) and after 48 hours cultivate 2×10^4 of HIV-1-expressing CD4⁺ T-cells per reconstruction in 37°C, 5% CO₂ environment for 1 hour in 100 μ l RPMI complete medium. Next, centrifuge cells (300g, 10 minutes, room temperature) to separate a cellular fraction of infected cells and the supernatant containing cell-free virus. Collect the supernatant with cell-free virus and resuspend the cellular fraction in RPMI complete medium. (*see Note 2*)
2. Add cell-free or cell-associated viruses to the epithelial, apical side of tissue reconstructions (Figure 1A-B). After 2 hours of incubation at 37°C, 5% CO₂, wash tissue reconstructions to remove cell-free virus or infected CD4⁺ T-cells and incubate the system for up to 1 month in air-liquid system, 37°C, 5% CO₂.
3. Collect tissue reconstruction supernatants in defined time intervals according to the experiment.
4. Measure viral production from macrophages inserted in reconstructions by p24 ELISA using reconstruction supernatants collected after overnight chase periods at low volume (300 μ l in basal chamber) (Figure 1D). Results are expressed as pg of p24 per chase period or as cumulative sum of p24 pg collected. Supernatants can be directly stored at -80°C.

5. Perform a kinetic quantification of viral production throughout the days following infection of reconstructed mucosa until p24 levels reach undetectable levels (below the limit of detection) by the p24 ELISA (indicating viral production arrest and latency) (Figure 1D-F).

3.1.3 LPS-induced HIV latency reversal in infected macrophages from HIV-infected cART-suppressed individuals

1. HIV-1 reactivation from latency in macrophages is evaluated by macrophage activation using LPS, a TLR4 agonist known to reactivate viral production from latency specifically in infected macrophages (19, 20). Once viral production reaches latency, incubate the tissue reconstructions with 300 μ l reconstruction medium for 24 hours at 37°C, 5% CO₂ and collect the medium (pre-reactivation sample). Next, incubate the tissue reconstructions with 300 μ l reconstruction medium containing 1 μ g/ml LPS for 24 hours at same conditions, and collect the medium (post-reactivation) (Figure 1E-F).
2. Measure p24 in pre-reactivation and post-reactivation medium samples to evaluate HIV-1 latency reversal. Consider also the use of non-infected and non-reactivated reconstructions as negative controls.

3.2 *Ex vivo* HIV latency reversal of tissue reservoirs

3.2.1 Preparation of urethral tissues

1. Transfer the penile tissue to petri dish where the urethral mucosa (Figure 2A) can be separated from other tissues by the use of a scalpel. Cut the mucosa in 0.5 x 0.5 cm square pieces and submerge them in PBS Ca²⁺/Mg²⁺-free during the entire procedure.
2. Retrieving epithelial cells: Submerge the tissue square pieces in Dispase solution for 1 hour at 37 °C under agitation (tube on horizontal position, 200 rpm) (Figure 2B). Next, pass the dispase-digested tissue through 70- μ m cell strainer. The solution that passed through the strainer must be collected in 50 ml tubes on ice (collector tube 1). With the aid of tweezers, submerge the tissue retained on the strainer in Trypsin solution for 10 minutes, 37°C, vortex for 30 seconds and pass the sample through a cell strainer again (Figure 2C). The solution that passed through this second strainer is collected again in the same collector tube 1 on ice, containing thus epithelial cells, that are centrifuged (400 g, 10 minutes, room temperature) and resuspended in PBS Ca²⁺/Mg²⁺-free supplemented with 20% FCS.
3. Retrieving stromal cells: Submerge the dispase/trypsin-digested tissue retained on the cell strainer after collection of epithelial cells in Collagenase solution for 1 hour at 37 °C under agitation (tube on horizontal position, 200 rpm) (Figure 2D). Next, pass the collagenase-digested tissue through 70- μ m cell strainer. The solution that passed through the strainer is collected in another 50-ml tube on ice (collector tube 2). (*see Note 3*). Wash the strained with PBS Ca²⁺/Mg²⁺-free saline that will pass through strainer into collector tube 2 on ice. Stromal cells in collector tube 2 are centrifuged (400 g, 10 minutes, room temperature) and resuspended in PBS Ca²⁺/Mg²⁺-free supplemented with 20% FCS (*see Note 4*).
4. Centrifuge the epithelial cell suspension in collector tube 1 and stroma cell suspension in collector tube 2 (400 g, 10 minutes, room temperature) and resuspend cells in RPMI medium (*see Note 5*).

3.2.2 Reactivation of latent HIV from tissue macrophages

1. Seed the tissue cell suspension in RPMI medium cells in a low-adherence round-bottom 96-well plate (1 \times 10⁶ total urethral cells in 200 μ l per well), centrifuge cells (400g, 10 minutes, room temperature), resuspend in RPMI medium supplemented with 1 μ g/ml LPS and cultivate the cells for 48 hours at 37 °C (Figure 3A) (*see Note 6*).
2. Collect the supernatant for further quantification of infectious virus content. Supernatants can be directly stored at -80°C.

3.2.3 Detection of reactivated HIV production from tissue reservoirs using viral outgrowth assays

The amount of latently infected macrophages obtained *in vivo* (12) is limited, and the amount of virus released into the culture supernatant from latently infected macrophages reactivated *ex vitro* is below the limit of detection for most conventional techniques. Therefore, it is necessary to employ highly sensitive detection systems such as microscopic counting of reporter cells (that become green-fluorescent upon HIV infection) or to amplify the production of reactivated HIV in an outgrowth assays using CD4⁺ T-cell lymphoblasts (12, 15) (Figure 3A).

3.2.3.1 Reporter cell assay

1. Reporter cell preparation: Seed the GFP-expressing indicator cells maintained as described (21) into a Lab-Tek 16-well chamber slide (Nunc) (2.5×10^3 reporter cells per well), and incubate overnight at 37°C 5% CO₂ in DMEM complete medium.
2. After LPS-mediated reactivation of tissue macrophages in the tissue cell suspension, add tissue cell supernatants directly to reporter cell cultures (40 µl of supernatant per well) in the presence of 20 µg/ml polybrene (Sigma). Cell-free HIV-1 R5-tropic virus at different concentrations (in terms of p24 ng/ml) are added to reporter cells for a standard curve. Negative controls include DMEM complete medium only and DMEM complete medium supplemented with the latency reversal agent used to reactivate HIV-1 production from the tissue cell suspension. After 2 hours at 37°C to favor contact of HIV with indicator cells, add 200 µl/well of DMEM complete medium to cells and cultivate overnight, 37°C 5% CO₂. Wash cells with PBS Ca²⁺/Mg²⁺-free and cultivate cells for additional 24 hours for detectable GFP expression.
3. To improve detection of green fluorescence protein (GFP) expressed by GFP-indicator cells, fix samples with paraformaldehyde, treat them with blocking/permeabilizing solution for 15 minutes, room temperature, incubate overnight at 4°C with 5 µg/ml of anti-GFP antibody followed by 1 hour, room temperature, with anti-goat Cy3-coupled secondary antibody (1:200 v/v), both antibodies diluted in blocking/permeabilizing solution. Next, stain reporter cell nuclei with DAPI for 15 minutes, room temperature, before proceed to cell blind count under epifluorescence microscope.
4. Blind count of reporter cells: Score GFP⁺ reporter cells in the entire cultivation well and count the number of GFP⁺ cell nuclei without reference of the sample used. Subtract the count per well by the maximum number of GFP⁺ cells nuclei counted in the negative controls (Figure 3B-C).
5. The total number of reporter cells per well is assessed by automatic counting of DAPI-stained nuclei in images acquired under an epifluorescence or confocal microscope system, using low magnification objectives capable to acquire images of the entire well. Nuclei are counted by image segmentation using isospots algorithms built on Imaris software (Imaris version 9.0.2, Oxford Instruments) following software tutorials. Infectious units were calculated from GFP-indicator cell assay as previously described (55) (see **Note 7**).

2.2.3.2 Adapted viral outgrowth assay using CD4⁺ T-cell lymphoblasts

1. After LPS-mediated reactivation of tissue macrophages in the tissue cell suspension, add tissue cell supernatants directly to 5×10^5 CD4⁺ T-cell lymphoblasts from healthy donors and incubate them for 7 days supplemented with 10 U/ml IL-2 (Sigma-Aldrich) in round-bottom 96-well plates. Centrifuge lymphoblasts at 300g, 10 min, room temperature, collect the supernatant (7-day outgrowth supernatant) and resuspend lymphoblasts in fresh RPMI complete medium containing 10 U/ml IL-2 and additional, non-infected 5×10^5 lymphoblasts for additional 7 days. Centrifuge again lymphoblasts at same speed, time and temperature, collect the supernatant (14-day outgrowth supernatant) and resuspend cells in 1 ml TRIzol™.
2. Measure p24 production in 7-day and 14-day outgrowth supernatants by p24 ELISA (see **Note 8**).

3. Measure the amount of HIV-1 RNA detected in lymphoblasts by RT-qPCR of HIV-1 long terminal repeats as described (22).

4. Notes

1. Previously to their detachment and insertion into tissue reconstructions, macrophages can be fluorescence-tagged by cultivation in presence of 10 nM of far-red-emitting Quantum dots, a long-term fluorescent cell tracker. Quantum dot staining on macrophages can be detected for several days in tissue reconstructions, helping to keep track on macrophages during cultivation of tissue reconstructions.
2. The gag-iGFP HIV-1 DNA plasmid will produce infectious fluorescent virus detected by epifluorescence or confocal microscopy, helping in the visualization of virological synapses formed between infected CD4⁺ T-cells and the epithelial cells and in tracking viral particles toward the mucosal stroma. Unfortunately, although the gag-iGFP HIV-1 productively infects macrophages until latency as measured by p24 ELISA in reconstruction supernatants, only the single-round viral production by the original transfected CD4⁺ T-cells yields GFP-fluorescent HIV-1. Labelling of HIV components by secondary immunostaining or *in situ* hybridization in macrophages latently infected by CD4⁺ T-cell-derived gag-iGFP HIV-1 is required.
3. Tissue retained on strainer is squeezed by the aid of 10-ml syringe rubber plunger head, to improve tissue dissociation.
4. Epithelial cells and stroma cells are used separately or mixed depending on the downstream experiments.
5. Specific cell types can be isolated from the tissue cell suspension using cell sorting protocols to be standardized.
6. Other latency reversal agents can be employed to check cell type-specific reactivation of latent HIV.
7. Using this technique, infectious HIV is detected at viral concentrations as low as 0.1 pg HIV-1 p24 per 30 μ l sample test corresponding to 3.3 pg/ml p24. Blind counts should be performed in microscope sessions of no longer than 1 hour, as fatigue of the observer interfere in blind counting (Figure 3B-C).
8. We recommend measure p24 in this system by Single Molecule Array technology (SIMOA, Quanterix HD-1 system), a p24 immunoassay 1000x more sensitive as compared to current automated immunoassays instruments (23), following manufacturer instructions.

5. Conclusions

These *in vitro* reconstruction and *ex vivo* explant models are complementary. The *in vitro* model allows for controlling the cell type content in the reconstruction and is easily modulated. Furthermore, mucosal barrier function of the reconstruction is quite stable allowing envisioning experiments in the long-term range (days/weeks). The *ex vivo* model has the advantage to mimic better to the *in vivo* situation but the integrity and tissue function of the explant are not maintained longer than hours after surgery. Finally, although we describe the technique based on male urethral tissues, the same protocol can be easily adapted to other mucosal tissues, covered by either mono or pluristratified epithelia. Combination of these two complementary models will help in the search for eradication of tissue HIV reservoirs and the establishment of an HIV cure.

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Figures and figure legends

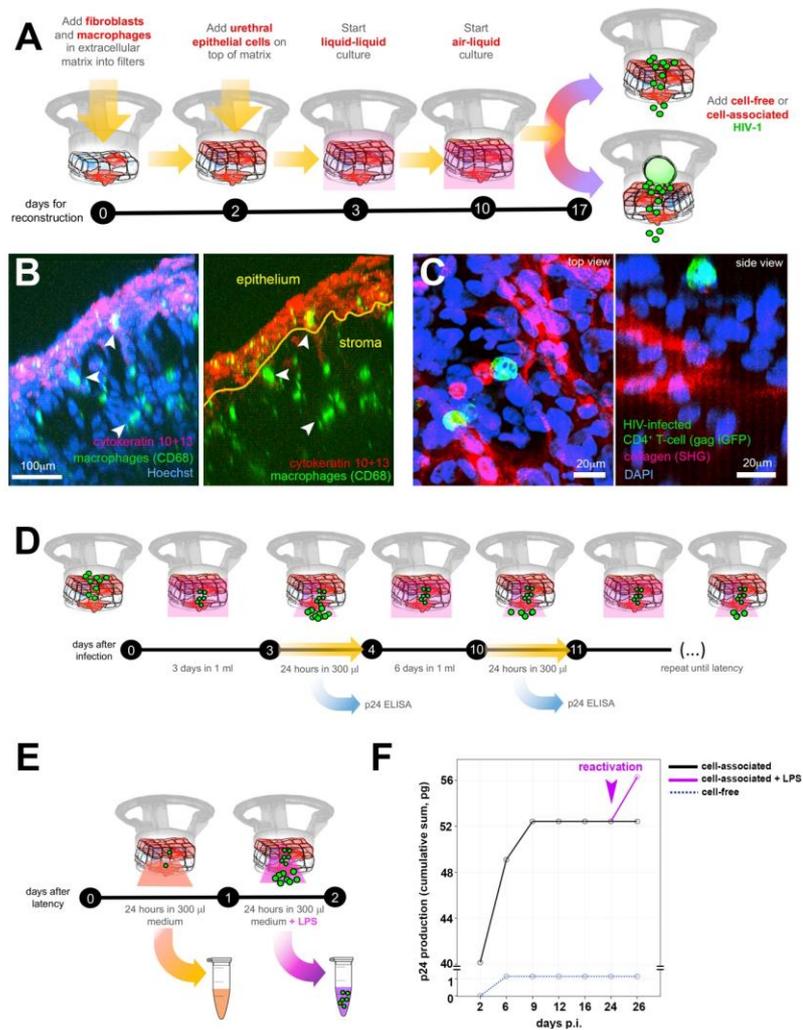


Figure 1: *In vitro* model of reversible HIV-1 proviral latency in mucosal macrophages.

A) Sequential steps for reconstructing urethral mucosa *in vitro*, followed by HIV infection using cell-free or cell-associated virus.

B) Polarization of the reconstructed mucosal epithelium and maintenance of stromal macrophages is verified by immunolabelling of cytokeratins 10+13, epithelial polarization markers, and of CD68, a macrophage marker.

C) Top view and side view of the reconstructed mucosa interacting with a CD4⁺ T-cell infected and expressing fluorescent virus. Collagen expressed by the reconstructed mucosa in red (observed by Second-harmonic Generation, SHG), fluorescent virus (HIV-1 gag-iGFP) in green.

D) Serial collection of reconstruction culture medium for p24 evaluation by ELISA in the days following cell-free or cell-associated HIV-1 infection of mucosal cells. Reconstructions are maintained in culture using 1 ml of reconstruction medium in the basal chamber of filters. Once a week, the 1 ml cultivation medium is replaced by fresh 300µl reconstruction medium for a 24-hour period, allowing to collect concentrated virus produced by the cells in the mucosal reconstruction at this time point. At the end of the 24hrs, this “24-hour” supernatant of 300 µl is collected from each reconstruction, and its content in p24 measured by ELISA. Such test is performed weekly until p24 levels become undetectable by the technique.

E) When mucosal infection reaches latency, reconstructions are cultivated for 24 hours in 300µl reconstruction medium (pre-reactivation 24-hour supernatant, orange) followed by adding fresh 300µl reconstruction medium containing 1mg/ml LPS (post-reactivation medium, purple). Both pre- and post-reactivation supernatants are collected and measured for p24.

F) p24 cumulative production detected in reconstructions supernatants in the days following cell-free or cell-associated HIV infection. Productive infection of reconstructed is only achieved when cell-associated virus is added to apical side of the reconstructed mucosa. In the experiment shown, latency is reached at 9 days post-infection (p.i.) and 24-hour supernatants at 12, 16 and 24 days p.i. were surveyed for p24 production to confirm latency. At day 24 p.i., pre- and post-reactivation supernatants are collected and measured for p24; reactivation of p24 production was detected at day 26 p.i. in the 24-hours post-reactivation supernatant.

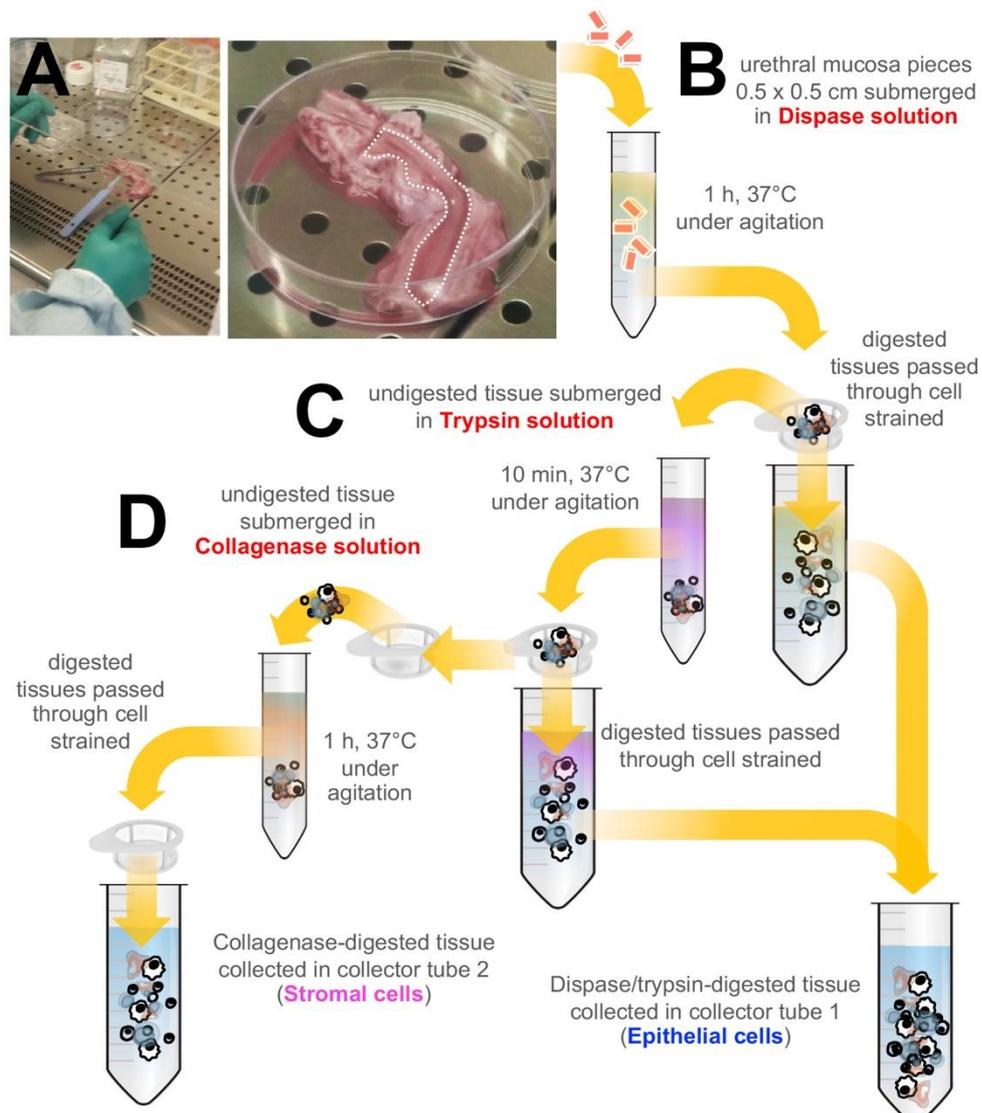


Figure 2: Schematized protocol for isolation of urethral mucosal cells.

A) Penile tissue in petri dish inside a laminar flow hood, 2 hours after gender reassignment surgery. Dotted white line indicate the urethral mucosa.

B) Pieces of urethral mucosa are submerged in Dispase solution and digested solution containing the first batch of dispase-digested epithelial cells is collected in collector tube 1.

C) Undigested tissue is submerged in Trypsin solution for a second batch of epithelial cells, collected again in the same collector tube 1 after trypsin digestion. Collector tube 1 will contain mucosal epithelium cells.

D) After Dispase/Trypsin treatment, undigested tissue is submerged in Collagenase solution. Digested tissue is collected in collector tube 2 that will contain cells from the stroma of the urethral mucosa.

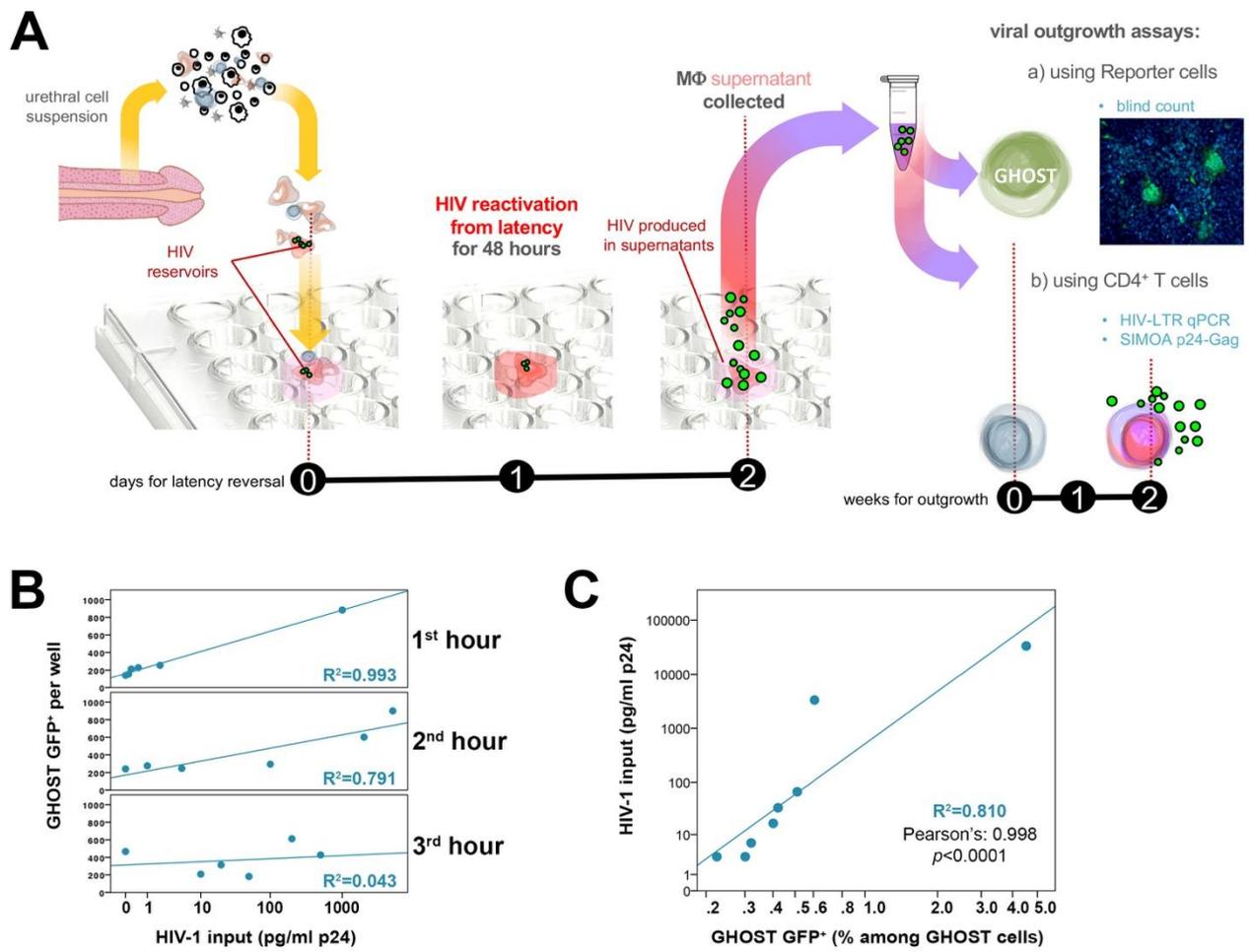


Figure 3: *Ex vivo* model of reversible HIV-1 proviral latency in mucosal macrophages.

A) Urethral tissue cell suspensions are placed in 96-well plates in the presence of latency reversal agents for 48 hours. The tissue cell suspension supernatants containing virus from reactivated viral production are collected and employed for viral outgrowth assays using a) reporter cells (GHOST) which express GFP once infectious virus replicates in the reporter cell; or b) CD4⁺ T-cells lymphoblasts which will propagate and amplify infectious viruses to levels detectable by HIV LTR qPCR (on CD4⁺ T-cell fraction at 2 weeks of outgrowth) or by p24 Single Molecule Array (on CD4⁺ T-cell supernatants collected at 1 and 2 weeks of outgrowth).

B) Blind count assay showing the number of GHOST GFP⁺ cells counted per well containing GHOST cells infected using different amounts of HIV-1 (in terms of p24 pg/ml). The same investigator performed counting throughout a counting session of 3 hours. Correlation between the number of GHOST GFP⁺ cells per well and the amount of HIV-1 added to the test well is stronger in the first hour of blind counting session and multiple counting sessions of one hour each should be thus preferred.

C) Standard curve of Reporter cell assays, showing the amount of HIV-1 added to test wells (in terms of p24 pg/ml) and the percentage of GHOST GFP⁺ cells among total GHOST cells counted in the well.