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Chapter 34

Studying the immune synapse in HIV-1 infection

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Running Head: Immune synapse in HIV-1 infection

Summary

T cells are the main cellular targets of the human immunodeficiency virus (HIV-1). HIV-1 infection induces pleiotropic effects on the infected T cell that modify the T cell capacity to respond to antigen and facilitates virus replication. HIV-1 infection subverts the formation and function of the immunological synapse altering both actin cytoskeleton remodeling and intracellular vesicle traffic. We describe here our methods to unveil how HIV-1 and in particular its protein Nef modify vesicle traffic to the immunological synapse, perturbing the synapse activation capacity.

1. Introduction

Immunological synapses are characterized by the accumulation and clustering of TCRs, co-signaling receptors, adhesion molecules and signaling effectors. In addition, immunological synapses are characterized by robust and precise rearrangements of the actin and microtubule cytoskeleton, as well as of intracellular vesicle traffic (1, 2) (see also Chapter 7).

HIV-1 infection induces pleiotropic effects in the infected T cells. Among these effects, HIV-1 modulates intracellular vesicle traffic of a variety of plasma membrane proteins, affecting their

subcellular localization, including their cell surface expression. The HIV-1 viral proteins Nef, Vpr and Vpu are responsible for many of these intracellular traffic effects. These proteins target a variety of cell surface molecules, including the virus receptor and co-signaling molecule CD4, the major histocompatibility complex molecules (MHC) class I and II, CD1a, tetraspanins, NK activatory ligands, and others reviewed in (3). In addition, HIV-1 Nef can modify the intracellular traffic of signaling molecules, like the protein tyrosine kinase Lck, the first kinase engaged upon TCR engagement. Thus, Lck accumulates in recycling endosomes, preventing the formation of immunological synapses capable to efficiently transduce TCR signals. The HIV-1 Nef protein is necessary and sufficient to induce these effects (4). In addition, HIV-1 Nef impedes the traffic of vesicles carrying the signaling adapter LAT to the immunological synapse, preventing the local generation of signaling complexes (5). Moreover, it has been reported that Nef-induced Lck accumulation also brings to the Lck intracellular compartment active Erk1/2 serine-threonine kinase. Together, Lck and Erk may enhance IL2 production (6). Under physiological conditions, Lck traffic depends on the transport protein MAL (7), the Unc119 protein (8), and Rab11 GTPase and its effector FIP3 (Bouchet et al, submitted). The mechanism by which HIV-1 subverts Lck intracellular traffic remains poorly understood.

We describe here our recent methods aiming to elucidate how HIV-1 Nef subverts intracellular traffic of signaling molecules and its effects on T cell activation.

2. Materials

2.1. Cells

1. Jurkat T cell leukemia cells, J77 Clone 20 cells and Raji B cell lymphoma cells have been previously described (4). Cells were cultured in RPMI 1640 + GlutaMAX™ + Phenol Red medium (Gibco®) supplemented with 10% fetal calf serum and 10 mM Hepes. We culture Jurkat and Raji cells at a density average of 0.5-1 x 10^6 cells/mL, splitting the cultures every 2-3 days.

2. Peripheral blood mononuclear cells from healthy donors are isolated by centrifugation through Ficoll-Hypaque using Unisep Maxi tubes (Eurobio, No. U-10) (*see* **Note 1**). For HIV-1 infection assays, PBMCs were cultured at 2 x 10^6 cells/mL in RPMI 1640 medium supplemented with 10% FCS, 1% Penicillin-Streptomycin and 5 µg/mL phytohemagglutinin (PHA) for two days. At day 3, PBMCs are washed once in RPMI 1640 medium supplemented with 10% FCS, 1% Penicillin-Streptomycin to get rid of PHA, and resuspended at 2 x 10^6 cells/mL in RPMI 1640 medium supplemented with 10% FCS, 1% Penicillin-Streptomycin. For transfection assays of primary cells, $CD4^+$ T cells are further purified using the $CD4^+$ T cell isolation kit (Miltenyi Biotech, 130-096-533) (*see* **Note 2**). After isolation they are cultured at 2x10^6 cells/mL in RPMI 1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate and 1% MEM Non-essential aminoacids.

2.2. Microscopy materials

1. Confocal microscope: LSM 700 confocal microscope (Zeiss) equipped with a Plan-Apochromat 63× objective and ZEN software (Zeiss).

2. Square glass coverslips 20 x 20 mm. Coverslips are coated with 500 µL poly-L-Lysine (0.002% w/v in water) during 20 minutes at room temperature, washed with water and air dried before use (*see* **Note 3**).

3. Round glass coverslips 12 mm diameter, coated as in 2, using 150 µL per round coverslip.

3. Glass Slides 76 x 26 mm

4. ProLong® Gold Antifade mounting medium with DAPI (Molecular Probes®, Life Technologies™, No. P36935).

2.3. Chemicals and biological products.

1. Poly-L-lysine MW: 150-300 kDa, 0.1% (w/v) (Sigma-Aldrich®, No. P8920). Coating solution 0.002% in water.

2. Paraformaldehyde (Electron Microscopy Sciences, No 15714, aqueous stock solution 32%). Paraformaldehyde solution 8% (w/v) in water was prepared from commercial stock at 32% (*see* **Note 4**).

3. Triton X-100 0.1% (v/v) in phosphate buffer pH 7.5, 150 mM NaCl (PBS).

4. Methanol.

5. Bovine serum albumin, 1% (w/v) in PBS (PBS-BSA). Store solution at 4°C.

6. Staphylococcus enterotoxin E superantigen (SEE, Toxin Technology Inc), 10 µg/mL in PBS. Biohazard (*see* **Note 5**).

7. Texas Red-coupled Phalloidin probe from (Invitrogen (1/100 dilution).

8. Phytohemagglutinin PHA-P

9. Primary antibodies for immunofluorescence. Mouse monoclonal IgG2b anti-Lck, clone 3A5 (Santa Cruz Biotechnology) is used at 2 µg/mL. Mouse monoclonal IgG1 anti-CD3ε, clone UCHT1, (BioLegend Inc) is used at 10 µg/mL. Rabbit anti centrin-3, gift of M. Bornens (Institut Curie, France) is used at 1/400 dilution. Mouse IgG2b anti-β-tubulin, clone KMX1 (Millipore) is used at 10 µg/mL. Rabbit anti-phospho-ZAP70 (Y319) (Cell Signaling) is used at 1/100 dilution and mouse monoclonal IgG2a anti-phospho-TCRζ (Y142), clone K25-407.69 (Becton Dickinson), are used at 5 µg/mL. Anti-HIV-1 JR-CSF Nef Monoclonal (6.2) and anti-HIV-1 SF2 p24 polyclonal antibodies can be obtained from NIH AIDS Reagent Program and are used at 1/1000 and 1/50 dilution, respectively.

10. Secondary antibodies for immunofluorescence. Highly cross-adsorbed Cy3-coupled goat anti–mouse IgG2a, anti-mouse IgG2b and anti-rabbit (Jackson Immuno-Research Laboratories) are used at 1/100 dilution. FITC-coupled goat anti-mouse IgG1 (Southern Biotech) is used at 0.7 µg/mL. FITC-coupled goat anti-rabbit (Jackson Immuno-Research Laboratories) is used at 1/100 dilution. Alexa Fluor 488–coupled goat anti-fluorescein (Molecular Probes) is used at 1 µg/mL.

11. Antibody used for pseudo-synapses: mouse IgG1 anti-CD3ε, clone UCHT1 (Bio Legend) is used at 500 ng/mL.

12. Expression vectors: pCGFP and wild type or mutated Nef-hemagglutinin (Nef-HA) and Nef-green fluorescent protein (Nef-GFP), pCNef-GFP (9, 10).

13. Cell free stocks of HIV-1 virions are produced by transient transfection of HEK-293T cells with proviral plasmid as described (11). The concentration of p24 antigen in viral stocks is measured by enzyme-linked immunosorbent assay (ELISA) (PerkinElmer Life Sciences) (*see* **Note 6**). Cell free HIV-1 virions, wild type and ΔNef, are stored at -80°C in 1 mL aliquots at 2 µg/mL p24 in a BSL-3 facility.

14. Human recombinant IL-2.

2.4. Transfection systems

1. Neon™ Transfection System and Neon™ Transfection 100 µL Kit, containing electrolytic buffer E2, resuspension buffer R, 100 µL Neon™ tips and Neon™ Electroporation tubes (Invitrogen™, Life Technologies™).

2. Nucleofector™ 2b Device and Primary Cell Nucleofector™ Kit, containing Nucleofector™ Solution, single use pipette and Amaxa™ 100 µl aluminum electrode cuvettes (Lonza).

3. Methods:

3.1. T cell transfection by electroporation

Cell synapse assays are done 24-48 h after T cell transfection of expression vectors.

3.1.1. Transfection of T cell lines using the Neon™ Transfection System

Transfection is done with 5-10 x 10^6 cells. Use 10 µg of DNA plasmid for 10 x 10^6 cells. Complete with Resuspension Buffer R to equilibrate different volumes for the same amount of DNA when preparing Eppendorf tubes with plasmid DNA.

1. Pre-incubate at 37°C one flask with 10 mL T cell culture medium (RPMI 1640 supplemented with 10% fetal calf serum and 10 mM Hepes) for each transfection. Prepare Eppendorf tubes with DNA plasmid.

2. Harvest the amount of cells required, centrifuge at 290 xg, at 20°C for 4 min. Wash twice in PBS and aspirate supernatant.

3. Insert a new Neon™ tube into the Neon™ Pipette Station and fill it with 3 mL Electrolytic buffer E2. Turn on Neon™ device, and select electroporation protocol (voltage: 1400 V, width: 10 ms, pulses: 3)

4. Resuspend the cell pellet in 100 µL Resuspension buffer R per required transfection.

5. Add 100 µL resuspended cells in each Eppendorf tube with DNA plasmid, and mix gently.

6. Take 100 µL of the cells-plasmid mix using the Neon™ pipette and a 100 µL tip – avoid air bubbles (Change Neon™ tip every transfection with different DNA). Insert the Neon™ pipette with the sample vertically into the Neon® tube and press start.

7. Once electroporation is complete, remove Neon™ pipette and transfer the sample into the pre-warmed culture flask and incubate at 37°C, 5% CO_2 for 24-48 h (*see* **Note 7**).

3.1.2. Transfection of purified primary $CD4^+$ T cells using Nucleofector™ 2b Device

Transfection is done with 5-10 x 10^6 cells. Use 5 µg of DNA plasmid for 10 x 10^6 cells. Complete with Resuspension Buffer R to equilibrate different volumes for the same amount of DNA when preparing Eppendorf tubes with plasmid DNA.

1. Pre-incubate at 37°C one flask with 1 mL Primary $CD4^+$ T cell culture medium (RPMI 1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate and 1% MEM Non-essential aminoacids) per 10 x 10^6 cells transfected. Prepare 1.5 mL Eppendorf tubes with DNA plasmid.

2. Harvest the amount of primary $CD4^+$ T cells required, centrifuge at 453 xg at 20°C for 10 min. Wash twice in PBS.

3. Turn on Nucleofector™ 2b Device and select electroporation protocol (U014).

4. Add corresponding volume of DNA plasmid to the Amaxa cuvette per transfection required.

5. Resuspend the cell pellet in 100 µL Amaxa buffer per 10 x 10^6 $CD4^+$ cells.

6. Take 100 µL of resuspended cells and add them to the cuvette with DNA plasmid. Mix gently, avoid air bubbles.

7. Insert cuvette in the Nucleofector™ 2b Device and electroporate the cells using protocol U014.

8. Use single-use pipettes to recover cells and transfer to the pre-warmed flask with medium. Incubate 10 min at 37°C.

9. Count cells and resuspend to a final concentration of 2 x 10^6 cells/mL in RPMI1640 medium supplemented with 10% FCS, 1 mM sodium pyruvate and 1% MEM Non-essential aminoacids.

3.2. Infection

1. Jurkat T cells (5-10 x 10^6) are cultured with 2 µg/mL of cell free HIV-1 virions during 16 h in RPMI 1640 medium supplemented with 10% FCS. Cells are then washed 4 times in RPMI 1640 and resuspended in RPMI 1640 medium supplemented with 10% FCS and cultured during 3 days.

2. After 2 days of PHA stimulation, 5x10^6 PBMCs are resuspended at 2x10^6 cells per mL in a suspension of 2 µg/mL cell free HIV-1 virions (paragraph 2.3.13, above), in RPMI 1640 medium supplemented with 10% FCS, during 16 h. Cells are then washed 4 times in RPMI 1640 and resuspended in RPMI 1640 medium supplemented with 10% FCS and 10 U/mL IL-2 and cultured during 3 days (*see* **Note 8**).

3.3. Immunological synapse formation between T cells and antigen presenting cells

All the procedures involving active viruses have to be performed in a BSL-3 facility by trained personnel.

1. Pulse antigen-presenting cells with superantigen. Harvest Raji cells (5 x 10^6 cells/mL) by centrifuging at 290 xg, 20°C, 4 min. Resuspend in RPMI 1640 (without serum) supplemented with 10 µg/mL *Staphylococcus* enterotoxin E superantigen (SEE) during 30 min at 37°C.

2. Immunological synapse formation. Harvest Jurkat J77Clone20 cells by centrifuging at 290 xg at 20°C for 4 min, or primary CD4 T cells, by centrifuging at 453 xg at 20°C for 6 min and resuspend at 5 x 10^6 cells/mL RPMI 1640 (without serum). T cells are incubated with pulsed-Raji cells at 1:1 ratio, at 37°C during 5, 15 or 30 min in RPMI 1640 medium (without serum) in 1.5 mL Eppendorf tubes at 37°C in a water bath.

3. Plating cells on coverslips. 160 µL of conjugated cells are plated onto poly-L-lysine-coated square coverslips during 3 min at room temperature (*see* **Note 9**).

4. Fixation. 160 µL of 8% paraformaldehyde are mildly dropped onto the coverslips containing cell suspensions (final concentration of paraformaldehyde 4%), and incubated for 20 min at room temperature. After incubation, paraformaldehyde is removed and coverslips washed with PBS-BSA (*see* **Notes 4 and 10**).

5. Saturation. Nonspecific binding is prevented by 15 min incubation in PBS-BSA.

6. First antibody preparation. Primary antibody (or mix of primary antibodies in case of multiple staining), at the recommended dilution is suspended in PBS-BSA, 0.1% Triton X-100. Anti-HIV-1 p24 or anti-HIV-1 Nef antibodies are included to distinguish infected cells.

7. Cell immuno-staining. 80 µL drops of primary antibody solution are deposited on Parafilm® and coverslips with fixed cells are turned over on drops (*see* **Note 11**). Incubate for 1 h at room temperature, protected from light.

8. Washes. After primary antibody incubation, coverslips are washed twice in PBS-BSA by submerging them several times in a beaker using forceps to handle the coverslips.

9. Second antibody preparation: Secondary antibody (or mix of secondary antibodies in case of multiple staining) is resuspended in PBS-BSA.

10. Staining with second antibodies. 80 µL drops of secondary antibody solution are deposited on Parafilm® and coverslips are turned over on drops, as in step 7. Incubate for 45 min–1 h at room temperature, protected from light.

11. Washes. After secondary antibody incubation, coverslips are washed twice in PBS-BSA by submerging in a beaker using forceps to handle the coverslips. Drain the washing solution by setting the edge of the coverslip on absorbent paper, in order to remove excess PBS-BSA before mounting.

12. Mounting. Drops of 20 µL of ProLong Gold Antifade mounting medium with DAPI are deposited on 76 x 26 mm slides and coverslips are turned over on the drops. Let mounted slides harden overnight at room temperature, protected from light. Slides may be then stored at 4°C and used during 2-4 weeks (*see* **Note 12**).

13. Microscopy analysis. Cells are observed under a LSM 700 confocal microscope (Zeiss) equipped with an oil-immersion Plan-Apochromat 63× objective. Z-stack optical sections are acquired at different increments depending on desired image analysis: 0.2 µm depth increments for deconvolution and colocalization analysis and 1 µm depth increments for fluorescence intensity analysis are used. Green and red laser excitations are intercalated to minimize fluorescence spill over different channels. Image acquisition is done with ZEN software (Zeiss).

3.4. Pseudosynapse formation on anti-CD3-coated coverslips.

1. Coating coverslips with anti-CD3 antibodies. Prepare 10 µg/mL anti-CD3 (UCHT-1) in PBS. Round poly-L-Lysine coated coverslips are turned over 50 µL drops of anti-CD3 solution and incubated 2 h at 37°C in an humidified chamber or overnight at 4°C (*see* **Note 13**). Coverslips are washed once in PBS and saturated with 200 µL RPMI 1640 with serum to prevent nonspecific binding to poly-L-Lysine.

2. Cell resuspension. Jurkat J77 Clone 20 cells or primary CD4 T cells are washed twice by centrifugation at 290 xg, 20°C, 4 min and resuspended at 2 x 10^6 cells/mL in RPMI 1640 medium without serum.

3. Plating cells on coverslips. 100 µL of cells are plated onto anti-CD3-coated coverslips during 3, 5 or 15 minutes 37°C in a humidified chamber kept at 37°C during the assay.

4. Fixation. 100 µL of 8% paraformaldehyde are mildly dropped onto the coverslips containing cell suspensions (final concentration of paraformaldehyde 4%) and incubated for 20 min at room temperature. After incubation PFA is removed and coverslips washed with PBS-BSA (*see* **Notes 4 and 10**).

5. Saturation. Nonspecific binding is prevented by 15 min incubation in PBS-BSA.

6. First antibody preparation. Primary antibody (or mix of primary antibodies in case of multiple staining), at the recommended dilution is suspended in PBS-BSA, 0.1% Triton X-100.

7. Staining of coverslips. 30 µL drops of primary antibody solution are deposited on Parafilm® and coverslips with fixed cells are turned over on drops (*see* **Note 11**). Incubate for 1 h at room temperature, protected from light.

8. Washes. After primary antibody incubation, coverslips are washed twice in PBS-BSA by submerging several times in a beaker using forceps to handle the coverslips.

9. Second antibody preparation. Secondary antibody (or mix of secondary antibodies in case of multiple staining) is resuspended in PBS supplemented with 1% (w/v) BSA.

10. Staining of coverslips: 30 µL drops of second antibody solution are deposited on Parafilm® and coverslips are turned over on drops, as done in step 7. Incubate for 45 min-1 h, at room temperature, protected from light.

11. Washes. After second antibody incubation, coverslips are washed twice in PBS-BSA by submerging in a beaker using forceps to handle the coverslips. Drain the washing solution by setting the edge of the coverslip on absorbent paper, in order to remove excess PBS-BSA before mounting.

12. Mounting. Drops of 20 µL of ProLong Gold Antifade mounting medium with DAPI are deposited on 76 x 26 mm slides and coverslips are turned on the drops. Let mounted slides harden overnight at room temperature, protected from light. Slides may be then stored at 4°C and used during 2-4 weeks (*see* **Note 12**).

13. Microscopy analysis. Cells are observed under a LSM 700 confocal microscope (Zeiss) equipped with an oil-immersion Plan-Apochromat 63× objective.). Z-stack optical sections are acquired at different increments depending on image analysis performed after: 0.2 µm depth increments for deconvolution and colocalization analysis and 1 µm depth increments for fluorescence intensity analysis. Green and red laser excitation are intercalated to minimize cross talk between the acquired fluorescence channels. Image acquisition is done with ZEN software (Zeiss).

3.5. Quantitative image analysis

1. Deconvolution of confocal images is used to improve image rendering, especially for 3D reconstruction and prior to colocalization analysis. Deconvolution is performed on Z-stacks of confocal optical sections obtained at 0.2 µm depth increments, using Huygens Professional software (Scientific Volume Imaging).

2. Colocalization analysis is performed with FiJi software (open platform for scientific image analysis). JaCoP plugin or Colocalization Threshold option of Fiji are used.

Pearson's correlation coefficient corresponds to the linear relationship between intensity of pixels in the two analyzed channels. Mander's coefficient is defined as the ratio of the summed intensities of pixels from one image for which the intensity in the second channel is above the threshold (12).

Costes Automatic Threshold allows us to consider only the pixels in each channel showing statistical correlation, as explained in Costes et al., 2004 (13).

3. Fluorescence intensity analysis. Using FiJi, in a chosen channel, create a Z-projection of the image (whole cell, or a number of optical sections corresponding to an intracellular compartment) and select a region of interest (i.e. immunological synapse, or an intracellular compartment), depending on experimental requirements. Then, in the other channel, measure fluorescence intensity of the selected area of interest (**Figure 1**).

4. Notes:

1. Density-Gradient centrifugation to isolate peripheral blood mononuclear cells (PBMC). Ficoll separation: spin Ficoll tubes 5 min at 453 xg at room temperature. Lay 28 mL of blood per tube of 15 mL of Ficoll-Hypaque-Lymphoprep tubes. Centrifuge 30 min 805 xg at room temperature with no brake. Aspirate plasma layer containing PBMCs (**Figure 2**) and add it to new empty falcon tubes (15 mL recovered PBMCs per 50 mL falcon tube). Add 35 mL RPMI no FCS per 15 mL of recovered PBMCs per tube. Spin 10 min 453 xg, room temperature. Discard supernatants and pool pellets together to one tube. Wash twice with 50 RPMI no FCS (10 min 453 xg. Resuspend pellet in 50 mL of RPMI no FCS and count cells using cell counter, or Trypan blue vital staining and Malassez chambers

2. Purification of $CD4^+$ T cells using MACS CD4 Cell Isolation Kit II: follow manufacturer's instructions.

3. Coverslip coating with poly-lysine: prepare a 1:50 dilution of 0.01% poly-L-Lysine solution in water. Cover a flat surface with Parafilm, and place round or square coverslips over it. Add 500 µL for square coverslips or 150 µL for round coverslips of the 1:50 poly-L-Lysine dilution over the coverslips, covering the surface completely. Incubate 20 min at room temperature and remove excess. Wash once with water and dry the coverslips before use.

4. Paraformaldehyde. We recommend purchasing a stock commercial solution, avoiding manipulation of paraformaldehyde powder, in order to prevent toxicity. Paraformaldehyde solution manipulation should be carried out under a chemical hood.

5. *Staphylococcus* enterotoxins are a biohazard and should be manipulated as such. Inactivation of all solutions and materials in contact with the toxin should be performed at the end of experiments using an excess of 0.5% (w/v) sodium hypochlorite for at least 1 h. Dispose as biohazard.

6. All the procedures involving active viruses have to be performed in a BSL-3 facility by trained personnel. For the detection and quantification of HIV-1 p24 antigen in cell culture supernatant, follow manufacturer's instructions.

7. Different plasmids might need different expression times in order to reach enough protein levels. Expression may be different in Jurkat and in primary T cells.

8. Schematic representation of infection time line (**Figure 3**). All the procedures involving active viruses have to be performed in a BSL-3 facility by trained personnel.

9. Cover with parafilm a flat surface and place the poly-L-Lysine coated coverslips over it (poly-L-Lysine side of the coverslips facing up). Add cell suspension over the coverslip, trying to distribute it over the whole coverslip surface.

10. Once fixed and washed, round or square coverslips containing cells may be stored in 6- or 24-well plates, respectively, in an excess of PBS-BSA, for 1-2 weeks at 4°C. Staining does not require to be performed immediately after fixation, except for protein phosphorylation staining, which requires freshly prepared cells for optimum results.

11. Cover with parafilm a flat surface and label areas for different staining solutions planned. Deposit 80 µL drops of primary antibody mix on the parafilm. Take the coverslips with fixed cells and drain the excess of PBS-BSA by setting the edge of the coverslip on absorbent paper. This prevents dilution of antibody staining solutions. Place coverslips over primary antibody drops on parafilm, cells facing the drops. Create a humidified chamber for the incubation by placing wet pieces of paper around the parafilm, and cover everything with a lid or opaque surface, in order to protect from light.

12. Once slides have been analyzed, it is recommended to store them at -20°C, to prevent loss of fluorescence. While fluorochrome fluorescence tends to be stable, GFP fluorescence is much faster lost.

13. Anti-CD3 coating of coverslips. Cover with parafilm a flat surface and deposit 50 µL drops of anti-CD3 antibody mix on the parafilm. Create a humidified chamber for the incubation by placing wet pieces of paper around the parafilm, and cover everything with a lid.

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Figure legends

Figure 1 : Quantification of fluorescence accumulation at the immunological synapse

Immunological synapse of a Jurkat (J77cl20) lymphocyte with Raji B lymphocyte as antigen presenting cell (APC) pulsed with SEE superantigen. Confocal image was post-treated by deconvolution. A 4 µm Z-projection is shown. **A:** Non-transfected cells. **B:** Nef-GFP transfected cells. From left to right: Nef-GFP (green), Lck (red) intracellular, CD3 (far red) surface staining, merge and

phase contrast image. Rectangles show the region of interest in which quantification of the intensity of fluorescence is performed and is referred to the total fluorescence of the cell. Scale bar, 5µm.

Figure 2: Scheme of a Ficoll gradient centrifugation tube after centrifugation

Figure 3. Schematic representation of infection time line.

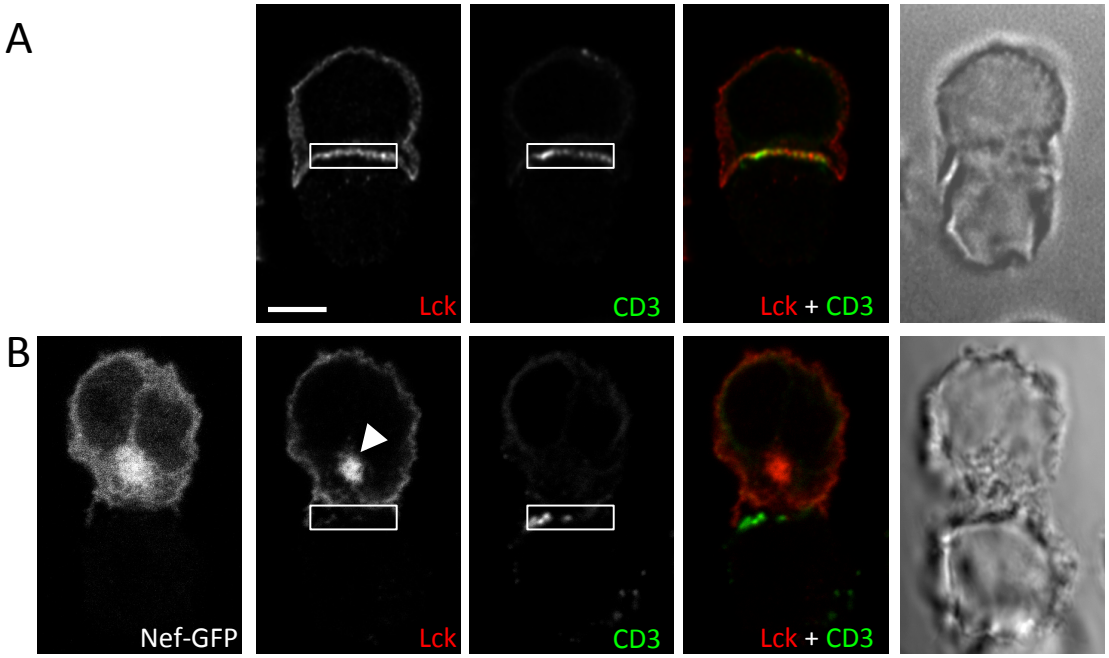


Figure 1

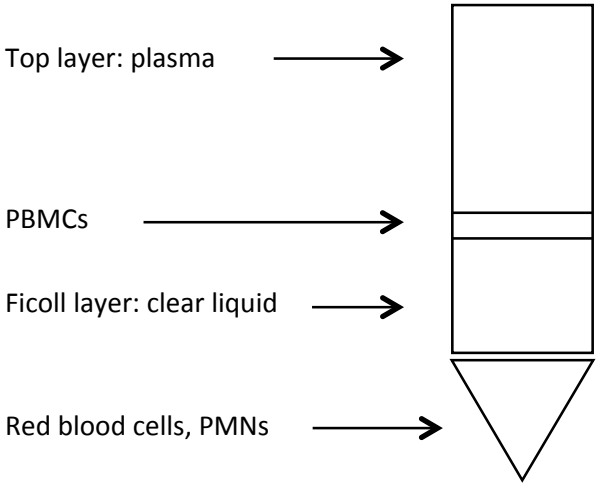


Figure 2

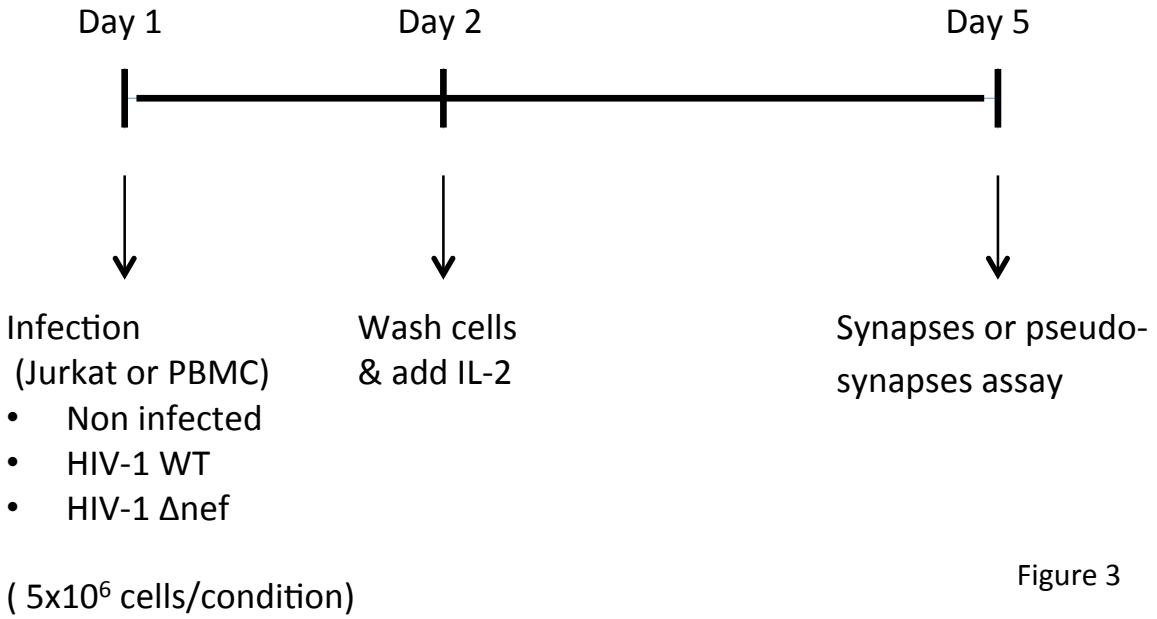


Figure 3