Video Article

An *In vitro* Co-infection Model to Study *Plasmodium falciparum*-HIV-1 Interactions in Human Primary Monocyte-derived Immune Cells

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Abstract

Plasmodium falciparum, the causative agent of the deadliest form of malaria, and human immunodeficiency virus type-1 (HIV-1) are among the most important health problems worldwide, being responsible for a total of 4 million deaths annually[1]. Due to their extensive overlap in developing regions, especially Sub-Saharan Africa, co-infections with malaria and HIV-1 are common, but the interplay between the two diseases is poorly understood. Epidemiological reports have suggested that malarial infection transiently enhances HIV-1 replication and increases HIV-1 viral load in co-infected individuals[2,3]. Because this viremia stays high for several weeks after treatment with antimalarials, this phenomenon could have an impact on disease progression and transmission.

The cellular immunological mechanisms behind these observations have been studied only scarcely. The few *in vitro* studies investigating the impact of malaria on HIV-1 have demonstrated that exposure to soluble malarial antigens can increase HIV-1 infection and reactivation in immune cells. However, these studies used whole cell extracts of *P. falciparum* schizont stage parasites and peripheral blood mononuclear cells (PBMC), making it hard to decipher which malarial component(s) was responsible for the observed effects and what the target host cells were[4,5]. Recent work has demonstrated that exposure of immature monocyte-derived dendritic cells to the malarial pigment hemozoin increased their ability to transfer HIV-1 to CD4+ T cells[6,7], but that it decreased HIV-1 infection of macrophages[8]. To shed light on this complex process, a systematic analysis of the interactions between the malaria parasite and HIV-1 in different relevant human primary cell populations is critically needed.

Several techniques for investigating the impact of HIV-1 on the phagocytosis of micro-organisms and the effect of such pathogens on HIV-1 replication have been described. We here present a method to investigate the effects of *P. falciparum*-infected erythrocytes on the replication of HIV-1 in human primary monocyte-derived macrophages. The impact of parasite exposure on HIV-1 transcriptional/translational events is monitored by using single cycle pseudotyped viruses in which a luciferase reporter gene has replaced the *Env* gene while the effect on the quantity of virus released by the infected macrophages is determined by measuring the HIV-1 capsid protein p24 by ELISA in cell supernatants.

Video Link

The video component of this article can be found at http://www.jove.com/video/4166/

Protocol

Note: Experiments with HIV-1 and *Plasmodium falciparum* must be performed in the proper biosafety level laboratories (BSL2 for parasites, and BSL3 for HIV-1 and co-infections) and special precaution must be taken when using potentially infected human blood.

1. Peripheral Blood Mononuclear Cells (PBMC) Purification (Based on [8] and [9])

1. Start with fresh human blood from healthy donors (500 ml) treated with anticoagulants (heparin, citrate, acid citrate dextrose or citrate phosphate dextrose). Use endotoxin-free reagents when purifying and isolating PBMC and throughout the rest of the protocol.
2. Disinfect the blood bag, including the tube, with 70% ethanol, cut the tube and carefully transfer the blood into a T150 flask.
3. Prepare 16 tubes with 15 ml Lymphocytes Separation Medium (Ficoll) per 50 ml conical tube (make sure that Ficoll has reached room temperature). Carefully layer 30 ml of fresh blood on top.
4. Centrifuge at 400 x g for 30 min at 20 °C with breaks off.
5. During the centrifugation, prepare 8 x 50 ml conical tubes with 25 ml of room temperature Hank's Balanced Salt Solution (HBSS) per tube.
6. **Carefully** transfer the cloudy cell ring at the interface (contains PBMC) to the 50 ml tube containing the HBSS (pool 2 cell rings per tube). Complete volumes up to 50 ml with HBSS.
7. Centrifuge at 150 x g for 15 min at 20 °C with breaks on.

8. During centrifugation of the cloudy cell ring, collect the yellow upper layer from the Ficoll step, pool the plasma to fill up a 50 ml conical tube. Inactivate complement in plasma by heating the 50 ml tube at 56 °C for 30 min and spin 10 min at 1,800 x g. Keep the supernatant (contains diluted autologous plasma that can be used as medium supplement at later steps).
9. **Carefully** remove the supernatant and resuspend the cell pellet from step 1.7 in 25 ml of HBSS and pool 2 pellets per 50 ml tube. Spin at 300 x g for 8 min at 20 °C.
10. Remove the supernatant carefully and resuspend the pellets in 10 ml HBSS and pool in one 50 ml tube.
11. Take an aliquot, perform trypan blue exclusion to assess mortality and then count PBMC.
12. Complete volume to 50 ml with HBSS and spin 10 min at 300 x g. Remove the supernatant and resuspend the cells in RPMI 1640 at $5x10^6$ cells/ml (one should obtain around 400-500 x 10^6 PBMC from 500 ml of blood).

2. Monocytes-Derived Macrophages (MDM) Differentiation (Based on [8] and [9])

1. Seed approximately 1.25 x 10^8 PBMC in 15 cm dishes with RPMI 1640 (25 ml/dish).
2. Let cells adhere for 1-2 hr at 37 °C with 5% CO_2 atmosphere.
3. Transfer non-adhered cells in a new flask and wash plate with 15 ml endotoxin-free PBS twice (5 min, 300 x g). The adhered cells are isolated monocytes.
4. To allow freshly isolated monocytes to differentiate into MDM, add 20 ml of RPMI 1640 medium supplemented with 5% autologous plasma (from step 1.8). Add human recombinant Macrophage Colony Stimulating Factor (M-CSF, 25 ng/ml) and Penicillin/Streptomycin solution (PS, 100 U/ml Penicillin, 100 µg/ml Streptomycin). Incubate for 2 days, change medium and incubate 2-3 extra days at 37 °C in 5% CO_2 atmosphere.
5. Remove old medium and wash with endotoxin-free PBS once, add the PBS gently and aspirate immediately. To detach fully differentiated MDM, treat cells with ~7 ml Accutase (Accutase, a commercially available mix of proteases and collagenolytic activity that allow the detachment of cells from the plastic dish) for 15-20 min at 37 °C in a 5% CO_2 atmosphere, rock gently at mid-time.
6. Add 3-5 ml autologous plasma (from step 1.8) in each plate (to help protect the cells while scraping).
7. Gently scrape cells making sure that medium covers the cells at all times and transfer/pool in a 50 ml tube.
8. Rinse plates with endotoxin-free PBS to recover as many cells as possible.
9. Spin 5 min at 200 x g, discard supernatant.
10. Resuspend pellet in 5 ml MDM culture medium (RPMI 1640 supplemented with 10% complement-inactivated Fetal Bovine Serum (iFBS), PS, 25 mM HEPES) and count cells (MDM yield usually 2-8% of total PBMC). Note: an aliquot of MDM might be taken in this step to assess cell population purity by flow cytometry; usually 99% of obtained MDM express CD14.
11. Seed $1x10^5$ MDM in 600 µl of MDM culture medium per well in 24-well plates.
12. Incubate overnight at 37 °C in a 5% CO_2 atmosphere before infections.

3. Production and Quantitation of HIV-1 Viral Stocks

1. Produce viral stocks using calcium phosphate transfection in HEK293T cells following the protocol of Fortin *et al.* [10].
2. In order to obtain single cycle viruses containing the luciferase-encoding reporter gene, co-transfect HEK293T cells with either 20 µg of NL4.3*Luc*+*Env*-*R*+ and 10 µg of pHCMV-G, or 15 µg of NL4.3*Luc*+*Env*-*R*+ and 15 µg of pJRFL*env*. Use 30 µg of NL4.3*Luc*+*Env*-*R*+ to generate control glycoprotein-deficient viruses. For the fully replicative virus, use 30 µg of NL4.3Bal*env*. The vectors pHCMV-G and pJRFL*env* encode the envelope glycoproteins of vesicular stomatitis virus (VSV-G) and of a CXCR5-tropic HIV-1 (different than NL4.3Bal*env*), respectively. Additional information on the plasmids can be obtained from [8]. Plasmids can be obtained through the NIH AIDS Research and Reference Program (NIAID, NIH).
3. Quantify all viral stocks using ELISA for the HIV-1 p24 capsid protein[11], and verify their infectious potential prior to use. We assess the infectivity of our virus stocks through the use of TZM-bl indicator cells[12].

4. Culture of *Plasmodium falciparum* Parasites (Based on [13])

4.1 General culture and maintenance of parasites

1. Maintain *P. falciparum* 3D7 asexual stage parasites in human erythrocytes (blood group O+) at a 4% hematocrit in RPMI 1640 (buffered with 25 mM HEPES and 25 mM $NaHCO_3$) supplemented with 0.5% (w/v) Albumax II, 25 µg/ml Gentamicin and 370µM Hypoxanthine at 37 °C in a gas mixture of 1% oxygen/5% carbon dioxide in nitrogen.
2. Parasitemia can be monitored by making a thin smear of the culture and staining with a 15% Giemsa solution.
3. Always maintain an uninfected red blood cells (uRBC) culture dish in the same conditions as parasites to use as a control.
   To obtain late stage parasites (trophozoites/early schizonts), synchronize the parasites as follows:

4.2 Parasite synchronization

1. In the morning, harvest the parasite culture, spin 5 min at 300 x g and discard the supernatant.
2. Resuspend pellet in 5 packed cell volumes with 5% (w/v) D-Sorbitol and incubate 5 min at 37 °C.
3. Spin 5 min at 300 x *g*, discard supernatant, resuspend pellet in 30 ml culture medium and put back in incubator.
4. Approximately 8 hr later, repeat steps 4.2.1 to 4.2.3 in order to obtain tightly synchronized parasites. Late stage parasites (trophozoites/early schizonts) can then be harvested the following morning to perform the experiment. Prior to purification, perform Giemsa staining to confirm lifecycle stage.

4.3 *Plasmodium falciparum*-infected red blood cells (iRBC) purification

1. Sterilize the VarioMacs separator (stand and magnet) with 70% ethanol and place it under the biological hood.
2. Fix the three-way stopcock to CS column bottom.

3. Cut the end of the needle plastic protector with the Miltenyi special cutter and fix the needle to the stopcock bottom.
4. Lock the column into the magnet carefully.
5. Remove all air bubbles in the column by slowly injecting 10 ml of MDM culture medium with the kit-enclosed syringe screwed on the left side of the stopcock.
6. Wash column with ~20 ml MDM culture medium.
7. Harvest RBC from the culture plate, wash once with 10 ml MDM medium (300 x g, 5 min) and resuspend the RBC pellet in 12 ml MDM culture medium. Slowly add to column and let the suspension flow through (only infected red blood cells containing parasites in the trophozoite or schizont stage will be retained by the magnetic field due to the presence of hemozoin crystals).
8. Wash once with 20 ml medium and stop liquid flow when it reaches the white disc on top of the column.
9. Remove the column from the magnet and elute iRBC in a clean sterile tube with 12 ml MDM culture medium.
10. Smear an aliquot of the recovered iRBC and perform Giemsa staining to confirm lifecycle stage.
11. Count recovered iRBC using a haemocytometer (isolated RBC are 100% iRBC).
12. Pellet cells (300 x g, 5 min), discard supernatant and treat cells with 500 µl fresh AB+ human serum (opsonisation step[14]).
13. Incubate for 30 min at 37 °C in a 5% CO_2 atmosphere.
14. Wash once with 10 ml MDM culture medium (300 x *g*, 5 min), and resuspend iRBC to desired concentration. Usually, a 10% parasitemia 15 cm culture dish roughly gives ~0.5-1x10^8 tightly synchronized iRBC.

5. Exposure of MDM to *Plasmodium falciparum*

1. To expose the MDM to uRBC or iRBC, cells must be resuspended in MDM culture medium in order to obtain a ratio of uRBC/iRBC:MDM of 75:1. In previously prepared 24-well plates containing MDM, add appropriate RBC suspension volume to each well. Perform all experiments in triplicate.
2. Incubate co-cultures for 4 hr at 37 °C in a 5% CO_2 atmosphere.
3. Wash the cells with 600 µl of endotoxin-free PBS 3 times, add the PBS gently and aspirate immediately.
4. To lyse the remaining RBC, wash the wells by adding 200 µl of ice-cold sterile water for 20 sec and aspirate.
5. Add 600 µl MDM culture medium and incubate 24 hr at 37 °C in a 5% CO_2 atmosphere.

6. Infection of MDM with a Fully Replicative HIV-1

1. In order to assess the effect of *P. falciparum* on HIV-1 replication in MDM, add, according to the scheme outlined in **Figure 1A**, 10 ng of p24 (in 300 µl of MDM media) of NL4.3Bal*env* viruses in the appropriate wells; add only MDM medium in control wells.
2. Incubate 2 hr at 37 °C in a 5% CO_2 atmosphere.
3. Wash the cells with endotoxin-free PBS 3 times, add the PBS gently and aspirate immediately. Add 600 µl of fresh MDM medium per well.
4. Incubate at 37 °C in a 5% CO_2 atmosphere.
5. At days 3, 6, 9 and 12 following the start of viral infection, harvest 200 µl of each supernatant, adding 200 µl of fresh MDM medium thereafter. Keep the harvested supernatants at -20 °C for quantitation of the major HIV-1 capsid p24 protein by ELISA following Fortin *et al.*[10].

7. Infection of MDM with Single Cycle Virus Encoding Luciferase

1. In order to determine the parasite's impact on HIV-1 gene expression in MDM, add, according to the scheme outlined in **Figure 1B**, 10 ng p24 (in 300 µl of MDM medium) of either NL4.3*Luc*+*Env-R*+(VSV-G), NL4.3*Luc*+*Env-R*+(JRFL*env*) or NL4.3*Luc*+*Env-R*+ viruses in the corresponding wells.
2. Incubate 2 hr at 37 °C in a 5% CO_2 atmosphere.
3. Wash the cells with 600 µl of endotoxin-free PBS 3 times, add the PBS gently and aspirate immediately. Add 600 µl of fresh MDM medium per well.
4. Incubate at 37 °C in a 5% CO_2 atmosphere.
5. Remove the medium 72 hr following infection and add 200 µl of lysis buffer 1X (supplied with the luciferase assay kit). Let the cells lyse at room temperature with gentle shaking. Store at -20 °C.
6. Thaw the plate and transfer 40 µl of the lysate to a 96-well luminometer plate; add 100 µl of luciferase substrate (supplied with the luciferase assay kit) and measure the luciferase (Firefly luciferase) activity in a luminometer following the manufacturer's instructions.

8. Representative Results

Using our co-infection model, we show that exposure of *P. falciparum* to MDM decreases their susceptibility to HIV-1 infection. Indeed, a significant decrease ($p<0.05$; 2 way ANOVA, day 12) in the release of viral particles, as measured by HIV-1 p24 capsid protein in the supernatant, is observed in MDM pretreated with parasites (**Figure 2A**). This observation is confirmed in cells infected by viruses encoding a luciferase-reporter gene. MDM infection with such viruses harboring either exogenous VSV-G or HIV-1 glycoproteins leads to significantly ($p<0.05$, Student's t-test) less luciferase production in cells exposed to *P. falciparum* (**Figure 2B**). It is noteworthy that VSV-G-pseudotyped viruses yielded much greater luciferase activity than their JRFLenv counterparts; this is due to the greater infection efficiency of VSV-G pseudotyped particles[15]. Given that parasite exposition to MDM impacts both types of viruses, this suggests that it influences some step in viral gene expression (**Figure 2B**). It is also important to mention that cell viability was not affected by MDM exposition to iRBC (data not shown), indicating that the inhibition observed is specific and not due to cell mortality.

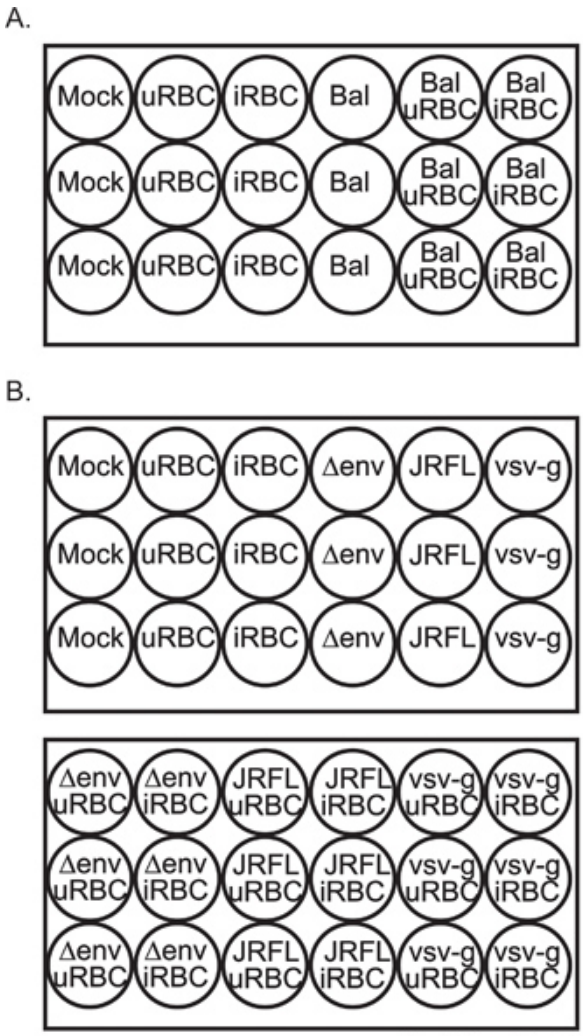

Fig. 1 Andreani et al, 2012

Figure 1. A) Plate scheme for MDM infection with a fully replicative virus. Mock: uninfected cells. uRBC: cells exposed to uninfected red blood cells. iRBC: cells exposed to *Plasmodium falciparum*-infected red blood cells. Bal: cells infected with NL4.3Bal*env.* Bal/uRBC: cells exposed to uRBC and infected with NL4.3Bal*env.* Bal/iRBC: cells exposed to iRBC and infected with NL4.3Bal*env.* **B) Plate scheme for MDM infection with single cycle luciferase-encoding virus.** Mock: uninfected cells. uRBC: cells exposed to uninfected red blood cells. iRBC: cells exposed to *Plasmodium falciparum*-infected red blood cells. Δenv: cells infected with NL4.3*Luc*+*Env-R+*. JRFL: cells infected with NL4.3*Luc*+*Env-R*+(JRFL*env*). vsv-g: cells infected with NL4.3*Luc*+*Env-R*+(vsv-g). Δenv/uRBC: cells exposed to uRBC and infected with NL4.3*Luc*+*Env-R+*. Δenv/iRBC: cells exposed to iRBC and infected with NL4.3*Luc*+*Env-R+*. JRFL/uRBC: cells exposed to uRBC and infected with NL4.3*Luc*+*Env-R*+(JRFL*env*). JRFL/iRBC: cells exposed to iRBC and infected with NL4.3*Luc*+*Env-R*+(JRFL*env*). vsv-g/uRBC: cells exposed to uRBC and infected with NL4.3*Luc*+*Env-R*+(vsv-g). vsv-g/iRBC: cells exposed to iRBC and infected with NL4.3*Luc*+*Env-R*+(vsv-g).

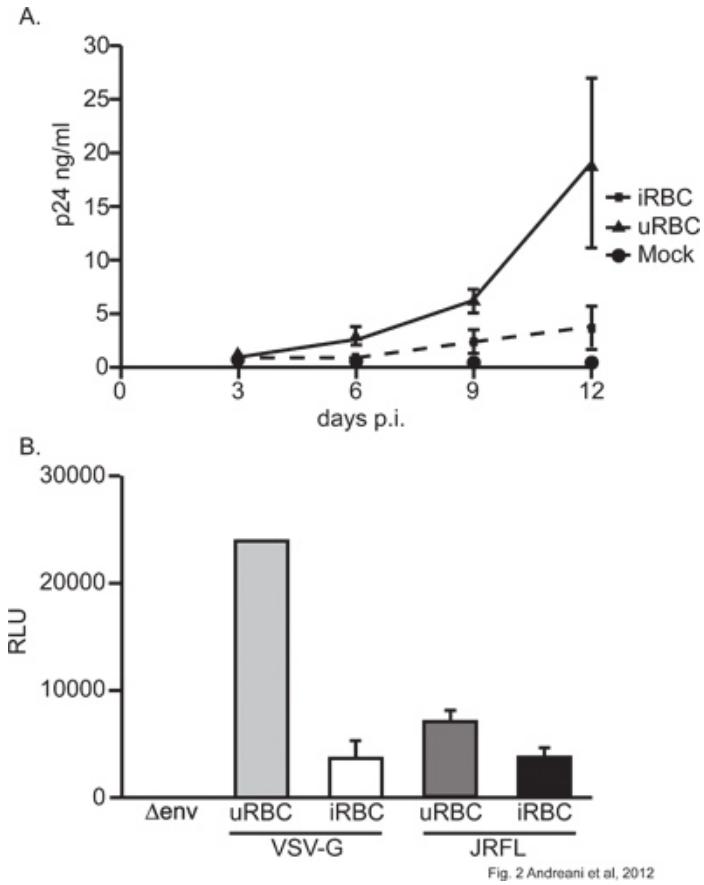

Figure 2. A) Effect of *Plasmodium falciparum* on HIV-1 viral production in MDM. MDM were exposed to uRBC or iRBC at a ratio 75:1 (uRBC/iRBC:MDM) for 4 hr and extensively washed. Cells were infected with 10ng of NL4.3Bal*env* p24 for 2 hr. Viral production was monitored by ELISA for HIV-1 p24 in cell-free supernatant at different time points following initial viral infection. A representative experiment is shown. **B) Effect of *Plasmodium falciparum* on HIV-1 viral transcription in MDM.** MDM were infected with uRBC or iRBC at a ratio 75:1 (uRBC/iRBC:MDM). Cells were then infected with 10ng of p24 of single cycle virus (either NL4.3*Luc*+*Env*-*R*+, NL4.3*Luc*+*Env*-*R*+(JRFL*env*) or NL4.3*Luc*+*Env*-*R*+(vsv-g)). Luciferase expression was evaluated in cell lysates 72 hr following initial virus infection. A representative experiment is shown.

Discussion

We have illustrated here two different approaches to analyze the impact of the malaria parasite on the HIV-1 viral cycle, *i.e.* by analyzing either viral gene expression or progeny virus production and replication in monocyte-derived macrophages. Similar approaches have been used for other HIV-1-parasite co-infections[16]. However, these new data are a step forward in the investigation of malaria-HIV-1 co-infections. Indeed, Diou *et al.*[8] studied the effect of hemozoin, not live parasites, on HIV-1 replication; in agreement with our results, they observed that hemozoin was itself sufficient to inhibit viral production by MDM, and not MDMs.

Using the described experimental layout, we observed that *P. falciparum* exerts a clear detrimental effect on the HIV-1 replicative cycle in macrophages: a significant inhibition of viral production is observed in macrophages pre-exposed to parasites (**Figure 2A**) and a specific impact of the parasite on viral transcription is illustrated in **Figure 2B**. However, we cannot leave out any additional effects of the parasite on viral entry or fusion (decapsidation), or on post-integration mechanisms, such as protein synthesis or viral particle assembly and budding. Furthermore, it is possible that a different impact on HIV-1 replication would be obtained if the parasite were added either at the same time or following MDM infection with the virus.

Our *in vitro* co-infection model provides a powerful tool to perform detailed investigations of HIV-1/*P. falciparum* interactions in the host cell. For example, the combination of this experimental layout with other techniques such as quantitative real time PCR, which can target and quantify specific steps in viral retrotranscription and viral genome integration to the host cell genome, are quite feasible and should yield further insights into the mechanisms involved in co-infections. Moreover, specific assays to evaluate early steps of the viral cycle (viral fusion, decapsidation, entry quantitation) can be applied to this basic protocol to further analyze the effect on viral replication. These modifications show how flexible this protocol is concerning HIV-1 quantitation and detection: indeed, even standard HIV-1 reverse transcriptase quantitation assays using tritium-labeled nucleotides could conceivably replace the ELISA for HIV-1 p24. In addition to MDM, we expect our system to be adaptable to other cell types relevant for HIV-1-malaria interactions, such as monocytes and dendritic cells. The fact that MDM are adherent cells facilitates their manipulation, allowing for easy washing out of iRBC that have not been taken in, or that are in contact with MDM, without eliminating any macrophages. Such an advantage would not apply to dendritic cells and monocytes, which are cultured in suspension, complicating the separation of uRBC and free iRBC with such cells and we are currently addressing this issue. Our system could also be useful to study more complex interactions such as the effects of *Plasmodium*-exposed monocyte-derived cells on the HIV-1 viral cycle in other immune cells such as

CD4-positive T cells in co-culture experiments. Finally, our protocol could be suitable for experiments looking at the effect of *P. falciparum* iRBCs on HIV-1 reactivation in PBMC for HIV-1 infected individuals.

Ethics statement

Human PBMC were obtained from the blood of healthy donors, in accordance with the guidelines of the Bioethics Committee of the Centre Hospitalier de l'Université Laval Research Center. A written consent was obtained from all blood donors.

Disclosures

No conflicts of interest declared.

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