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Modulation of epigenetic factors during the early stages of HIV-1 infection in CD4$^+$ T cells *in vitro*

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ABSTRACT

Several studies have related epigenetic mechanisms to HIV-1 latency. However, the epigenetic modifications of the host cell genome involved in the early stages of HIV-1 infection remain unclear. This study aimed to investigate epigenetic factors that are regulated at the beginning of HIV-1 infection in activated and resting CD4$^+$ T cells. We analyzed the gene expression of 84 epigenetic targets, global DNA methylation, and HIV-1 replication kinetics for 36 h after infecting CD4$^+$ T cells obtained from the blood of twelve healthy donors. The epigenetic targets aurora kinase B (*AURKB*), aurora kinase C (*AURKC*) and DNA methyltransferase 3B (*DNMT3B*), and the global DNA methylation profile are regulated during HIV-1 replication in CD4$^+$ T cells, and this regulation can be influenced by the activation state of the cell at the time of infection. Approaches that affect the expression of these epigenetic targets could help current strategies to suppress HIV-1 replication.

1. Introduction

Although antiretroviral therapy decreases the human immunodeficiency virus 1 (HIV-1) viral load, the virus may establish latent infections in cells, and the latent state of the virus is refractory to the host's immune response and antiretroviral therapy. Thus, HIV-1 latency has been considered the greatest barrier in eradicating the virus from infected patients (Blazkova et al., 2009; Churchill et al., 2015; Deeks et al., 2016; Kauder et al., 2009).

Resting CD4$^+$ T cells are the most well-defined reservoirs of latent HIV-1. The establishment of HIV-1 latency stems primarily from the transition of activated CD4$^+$ T cells into resting memory T cells, and the mechanism underlying this transition is not yet fully understood. The integration of HIV-1 can also occur directly in resting cells *in vitro*, even upon exposure to a small viral inoculum (Agosto et al., 2007; Chun, 2013; Siliciano et al., 2003).

Several studies have focused on strategies for the reactivation of latent viruses with the objective of eradicating HIV-1 in infected patients (Archin et al., 2009; Blazkova et al., 2009; Matalon et al., 2011). Specifically, viral latency is strongly associated with epigenetic mechanisms, and the epigenetics of HIV-host interactions are a potential target for understanding the mechanisms involved in the infection and replication cycle of HIV-1.

Epigenetics refers to a set of alterations in gene expression as a response to environmental, behavioral, physiological, and pathological signals that do not involve modifications of the DNA sequence (Bird, 2007; Bollati and Baccarelli, 2010). Moreover, the epigenome – the set of epigenetic alterations – is characteristic of each cell type, and it is inherited and stable over many cell division cycles.

Epigenetic mechanisms include several molecular alterations and may occur on three levels: *i)* directly on DNA, such as the methylation of CpG islands found in eukaryotic DNA; *ii)* at the transcriptional/translational level, such as the regulation of protein expression or pathways responsible for performing epigenetic changes directly to DNA; or *iii)* at the post-translational level, such as the acetylation, deacetylation, and methylation of histones or other DNA-associated proteins (Bird, 2007; Egger et al., 2004). These modifications define the structure and transcriptional activity of chromatin (Delcuve et al.,

Abbreviations: AIDS, Acquired Immunodeficiency Syndrome; AURKB, Aurora kinase B; AURKC, Aurora kinase C; CCR5, C-C chemokine receptor type 5; DNMT3B, DNA methyltransferase 3 beta; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; HIV-1, Human Immunodeficiency Virus type 1; LTR, Long terminal repeats; MOI, Multiplicity of infection (ratio of virions per cell); PBMC, Peripheral blood mononuclear cells

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2009; Jirtle and Skinner, 2011).

The epigenome enables cell diversity and differentiation by regulating the accessibility of the cellular machinery to genetic information. Indeed, errors in epigenetic mechanisms may result in the inadequate activation or inhibition of several genes and alter normal cellular physiology (Egger et al., 2004). Epigenetic alterations can also be mediated by pathogens to influence the host's immune and inflammatory response, which can lead to the development of various diseases (Paschos and Allday, 2010).

Current research trends in HIV-1/AIDS and epigenetic phenomena have focused on elucidating how the viral genome, or its protein products, may be affected by the differential methylation or modification of histones and how this modification affects the virus's ability to infect $CD4^+$ T cells, replicate, and produce effective progeny or, alternatively, remain latent to infect cells at a later stage (Archin et al., 2009; Blazkova et al., 2009; Kauder et al., 2009; Matalon et al., 2011).

The entry of a virus can trigger a number of changes in the infected cell, either as a response to the stress brought upon by the infection or from an unknown and strategic action by the virus on the host genome (Israël and Gougerot-Pocidalo, 1997; Paschos and Allday, 2010).

Thus, after HIV-1-infected cells recognize an internalized viral particle, their epigenetic patterns likely change to protect the cells from a potentially lethal infection. However, the virus may also be able to change the epigenetic configuration of the cell to maximize its chances of integration, such as the activation of as yet unknown signaling pathways, which are mediated by its entry (Blazkova et al., 2009; Maricato et al., 2015).

Several studies have related epigenetic mechanisms to HIV-1 latency, a late event in the viral life cycle that occurs post-integration. However, the epigenetic mechanisms involved in the early stages of HIV-1 infection remain unclear.

The objective of the present study was to identify epigenetic factors that are regulated at the beginning of HIV-1 infection of $CD4^+$ T cells *in vitro* and determine the effect of the activation state of the cell on this regulation. For this purpose, we analyzed global DNA methylation and the gene and protein expression of epigenetic targets in activated and resting $CD4^+$ T cells upon HIV-1 infection.

2. Materials and methods

2.1. Obtaining peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained from the blood bags of twelve healthy subjects provided by the Blood Collection Center (*Associação Beneficente de Coleta de Sangue* – Colsan). Only blood bags that tested negative for all serologic screenings were used; the tests conducted by the blood bank included tests for hepatitis B using anti-hepatitis B core total antibodies (Anti-HBc) and hepatitis B surface antigen (HBS-Ag), a test for hepatitis C using anti-hepatitis C antibody (Anti-HCV), tests for HIV-1 and HIV-2 using Anti-HIV-1 and Anti-HIV-2 antibodies, a test for human T cell lymphotropic virus (HTLV) using Anti-HTLV-1 and Anti-HTLV-2 antibodies, the venereal disease research laboratories (VDRL) test for syphilis, and a test for Chagas disease. The samples were processed at the Biosafety Level 3 Laboratory (BSL-3) Facility of the Retrovirology Laboratory - Discipline of Infectious Diseases - *Universidade Federal de São Paulo* - UNIFESP. PBMCs were isolated using the density gradient cell sorting technique with Ficoll-Paque (GE Healthcare, #17-1440-02).

2.2. Purification and cultivation of $CD4^+$ T cells

$CD4^+$ T cells were purified from isolated PBMCs using the EasySep® Human CD4 Positive Selection kit (STEMCELL Technologies, #18052).

In order to synchronize the cell cycle, the cells were initially cultured in suspension in serum-free RPMI 1640 medium (Invitrogen) containing 0.1% bovine serum albumin (BSA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) for 24 h at 37 °C in a humidified incubator containing 5% CO_2. Thus, the cells are expected to remain in a basal state.

After this incubation step, the culture medium was replaced with RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 U/mL penicillin, and 100 mg/mL streptomycin. At this stage, the cells were divided into two different groups: i) one received activation stimulus by adding Dynabeads T-activator CD3/CD28 from the T Cell Activation/Expansion Human Kit (Gibco, Life Technologies, #130-091-441) at a 1:1 ratio plus 30 U/mL interleukin (IL)-2; and ii) the other was maintained in culture without activation stimulus. The cultures were incubated for 72 h at 37 °C under a 5% CO_2 atmosphere. The cells that received activation stimulus were designated activated cells, whereas the unstimulated cells were defined as resting cells.

2.3. Flow cytometry and cell activation analysis

The cell activation status was analyzed with a FACSCANTO II flow cytometer (BD Biosciences, USA) using the anti-CD25 FITC (#340694) and anti-CD69 PerCp (#344508) markers (BD Biosciences). In addition, the cells were labeled with anti-CD3 APC (#17-0037-42) and anti-CD4 PE (#12-0048-42) antibodies (eBioscience) to identify the $CD4^+$ T cell populations (Vatakis et al., 2009). The obtained events were analyzed using the FlowJo software (Tree Star, Inc., Oregon, USA).

2.4. HIV-1 infection of $CD4^+$ T cells

After determining the activation status, cells were maintained in contact with HIV-1 in culture medium under the previously described conditions for 2 h at an MOI of 0.2. After this period, the cultures were washed and incubated for periods of 6, 12, 24, and 36 h from initial contact to the virus. The viruses used in this study had been previously expanded from the pNL4-3 viral clone (catalog. 114; AIDS Reagent Program, National Institutes of Health (NIH), USA).

A total of 16 experimental conditions were assayed in the present study. The two cell groups – activated or resting $CD4^+$ T cells – were divided into two subgroups, infected cells and uninfected control cells. The four subgroups were analyzed for incubation periods post-viral infection of 6, 12, 24, and 36 h.

The cells were washed three times with RPMI 1640 medium to remove free viruses after the respective incubation times of each experimental condition. The cells were immediately lysed to obtain DNA, RNA, and protein extract, and the culture supernatant was saved. For each time point, 1×10^6 cells/mL in a final volume of 10 mL were obtained.

2.5. Extraction of nucleic acids and proteins

DNA, RNA, and proteins were extracted using the column purification method with the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, #80004). A total of 5×10^6 cells were used per extraction for each experimental condition.

DNA and RNA were spectrophotometrically quantified using a NanoDrop 3300 fluorospectrometer (Thermo Scientific, USA). Proteins were quantified with the bicinchoninic acid (BCA) method using the Pierce™ BCA Protein Assay kit (Thermo Scientific, #23227).

To increase the detection power of our assays, each experimental condition was analyzed using equimolar pools of DNA, RNA, and proteins obtained from four blood bags. Thus, the blood bags of the twelve healthy donors were used to form three biological replicates, each consisting of four different blood bags. We believe this strategy avoids a possible individual bias because it minimizes the individual characteristics of each donor.

2.6. Gene expression profile evaluation by PCR Array

Epigenetic alteration-related changes in gene expression were