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miR-146a controls CXCR4 expression in a pathway that involves PLZF and can be used to inhibit HIV-1 infection of CD4$^+$ T lymphocytes

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ABSTRACT

MicroRNA miR-146a and PLZF are reported as major players in the control of hematopoiesis, immune function and cancer. PLZF is described as a miR-146a repressor, whereas CXCR4 and TRAF6 were identified as miR-146a direct targets in different cell types. CXCR4 is a co-receptor of CD4 molecule that facilitates HIV-1 entry into T lymphocytes and myeloid cells, whereas TRAF6 is involved in immune response. Thus, the role of miR-146a in HIV-1 infection is currently being thoroughly investigated.

In this study, we found that PLZF mediates suppression of miR-146a to control increases of CXCR4 and TRAF6 protein levels in human primary CD4$^+$ T lymphocytes. We show that miR-146a upregulation by AMD3100 treatment or PLZF silencing, decreases CXCR4 protein expression and prevents HIV-1 infection of leukemic monocytic cell line and CD4$^+$ T lymphocytes.

Our findings improve the prospects of developing new therapeutic strategies to prevent HIV-1 entry via CXCR4 by using the PLZF/miR-146a axis.

Introduction

The ability of human immunodeficiency virus type 1 (HIV-1) to bind to and enter into CD4$^+$ T cells depends on optimal expression of CD4$^+$ protein on the surface of these cells (Wilen et al., 2012). However, chemokine receptors, in particular CCR5 and CXCR4, are essential coreceptors of CD4$^+$ molecules on cell membrane of T lymphocytes and other cell types, such as monocytes/macrophages and dendritic cells, to allow HIV-1 entry into these cells (Wilen et al., 2012). Thus, receptor-coreceptors which determine viral entry into various cell types, together with the cellular tropism which defines viral phenotype, are among the major factors influencing HIV pathogenesis (Wu and Yoder, 2009; Naif, 2013).

At the present, the current classification of cellular tropism of HIV-1 relies on the differential expression of CCR5 and CXCR4 in monocytes/macrophages and T-cell lines (Wilen et al., 2012; Naif, 2013). Monocytes/macrophages serve as vehicles for viral dissemination between different tissues of the body (Ryan et al., 2002; Ciborowski and Gendelman, 2006; Carter et al., 2010, 2011), thus acting as reservoirs for HIV-1 in tissues (Van Lint et al., 2013).

Then, CCR5 and CXCR4 are relevant targets for pharmaceutical intervention that aims to block HIV-1 entry into macrophages and primary T lymphocytes and to prevent viral dissemination (Proudfoot, 2002; Henrich and Kuritzkes, 2013).

Coreceptor binding of their natural ligands, such as RANTES/CCL5, MIP-1alpha/CCL3, and MIP-1beta/CCL4 for CCR5 and stromal-derived factor, SDF-1/CXCL12, for CXCR4, inhibits HIV-1 entry into CD4$^+$ T cells, monocytic and CD4$^+$ T cell lines (Naif, 2013). However, any change in co-receptors conformation and surface density on cell membrane impacts the overall susceptibility of a given cell type for HIV infection (Lapham et al., 2002; Sloane et al., 2005; Richardson et al., 2012) and may alter the effectiveness of small drugs targeting HIV co-receptors (Proudfoot, 2002; Choi et al., 2012a, 2012b). Then, the discovery of HIV-1 cellular entry inhibitors remains an important objective in molecular pharmacology.

The first clinical trials with the highly specific CXCR4 antagonist, AMD3100 (Mozobil, plerixafor), were designed for treatment of HIV (Hendrix et al., 2000). Approved by Food and Drug Administration (FDA) as a mobilizer of hematopoietic CD34$^+$ cells from the bone marrow to the circulation (Hendrix et al., 2000; DiPersio et al., 2009), AMD3100 is also used to inhibit growth and metastasis

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of experimental tumors in animal models, as CXCR4 is also involved in metastatic dissemination, growth and survival of cancer cells, including leukemic cells (Domanska et al., 2013).

However AMD3100 is not being further developed for antiretroviral therapy (Hendrix et al., 2004). Highly active antiretroviral therapy (HAART) is currently the only option to control the progression of HIV-1 infection, improve the quality of life of HIV patients and reduce morbidity. Unfortunately, HAART that restores immunological function and helps prevent further diseases transmission, has also developed the phenomenon of drug resistance, a critical factor responsible for failure of this combined therapy (Deeks et al., 2012). In this context, the search for new anti-HIV drugs has been extended to compounds that act against cellular rather than viral target to minimize viral mutagenesis that give rise for drug resistance.

More recently, manipulation of microRNAs (miRNAs) has been proposed as a novel approach to use against HIV-1 infection and for purging the HIV-1 reservoir (Van Lint et al., 2013; Tyagi and Bukrinsky, 2012). miRNAs are small non-coding RNA involved in posttranscriptional gene regulation that participates in RNA interference (RNAi)-mediated gene silencing (Bartel, 2009). RNAi activity is involved in many eukaryotic cellular processes and the deregulated expression of miRNAs and other RNAi components has been described in cancers, metabolic disorders, and infectious diseases (Triboulet et al., 2007; Bivalkar-Mehla et al., 2011; Yeung and Jeang, 2011; Klase et al., 2012). These miRNAs represent a new generation biomarkers for diagnostics and prognostics and, given their stability in vivo (Yeung and Jeang, 2011), new tools to control specific target in disease, including AIDS (Klase et al., 2012).

The role of viral-encoded miRNAs derived from viral genomes (Pfeffer et al., 2004) in virus replication and virus-host interaction is currently under investigation (Ouellet et al., 2013; Zhang et al., 2014), as well as the role of cellular miRNAs in the regulation of HIV-1 viral replication (Lecellier et al., 2005; Huang et al., 2007) and in the complex interactions with HIV-1 (Klase et al., 2012).

miRNA 146a (miR-146a) is considered as a crucial modulator of differentiation and function of cells of the innate and adaptive immunity, acting as a negative feedback regulator of the innate immune response by targeting two adapter proteins, TRAF6 (Tumor necrosis factor receptor -associated factor 6) and IRAK1(IL-1 receptor-associated kinase 1), acting in pro-inflammatory signaling (Taganov et al., 2006; Hou et al., 2009). The latent membrane protein 1 (LMP1), a major oncoprotein of the Epstein-Barr virus (EBV), induces cellular miR-146a expression that may contribute to cellular immortalization and tumorigenesis (Cameron et al., 2008). miR-146a also promotes vesicular stomatitis virus (VSV) replication in macrophages (Hou et al., 2009). The chemokine CCL8/MCP-2, ligand for CCR5 but also potent inhibitor of CD4/CCR5-mediated HIV-1 entry and replication, is also a target of miR-146a in HIV-1 infected microglia (Rom et al., 2010).

Beside its role in immune responses and in disease (Taganov et al., 2006; Boldin et al., 2011; Burger et al., 2014), miR-146a has been also involved in the control of hematopoiesis (Labbaye et al., 2008; Labbaye and Testa, 2012).

We have demonstrated that CXCR4 is a direct target of miR-146a in hematopoietic normal and leukemic cells and identified the transcription factor PLZF (promyelocytic leukemia zinc finger) as a repressor of miR-146a expression in megakaryocytic (Mk) cells (Labbaye et al., 2008).

PLZF was initially identified as a protein whose functions are subverted through chromosomal rearrangements resulting in acute promyelocytic leukemia (Suliman et al., 2012). It is known to regulate progenitor maintenance in multiple tissues (Kelly and Daniel, 2006). Subsequently, PLZF has been found to be a key stem cell maintenance factor in both the hematopoietic system and male germline (Suliman et al., 2012). PLZF was also shown to be a critical regulator of immune system development and function, also implicated in carcinogenesis as a tumor suppressor gene (Suliman et al., 2012). More recently, we have described the regulation of CXCR4 by miR-146a targeting in monocytic (Mo) cells and showed that the enforced expression of miR-146a by AMD3100 treatment, decreases total CXCR4 protein expression and impairs Mo leukemic cell proliferation (Spinello et al., 2011).

In the present study, we showed that PLZF, miR-146a and CXCR4 are expressed and regulated during activation of human primary $CD4^+$ T lymphocytes. TRAF6 expression and regulation was also analyzed in the same cells, as another target gene of miR-146a. In addition, we studied the effects of AMD3100 treatment on miR-146a and CXCR4 expression levels in $CD4^+$ T lymphocytes and during HIV-1 infection of these cells.

We found that PLZF mediates suppression of miR-146a to control increases of CXCR4 and TRAF6 expression levels in $CD4^+$ T lymphocytes, whereas miR-146a upregulation by AMD3100 treatment or PLZF silencing, decreases CXCR4 protein expression level and inhibits HIV-1 infection of $CD4^+$ T lymphocytes.

Altogether, we showed that the PLZF/miR-146a axis that controls CXCR4 expression in $CD4^+$ T lymphocytes, can be used to prevent HIV-1 entry into CD4/CXCR4 expressing target cells in new therapeutic strategies against HIV-1 infection.

Results

PLZF, miR-146a, CXCR4 and TRAF6 expression and regulation in human primary $CD4^+$ T lymphocytes

We previously described in human Mk cells that PLZF suppresses miR-146a transcription and thereby activates CXCR4 translation (Labbaye et al., 2008). To investigate whether this cascade pathway may be present and functional in human primary $CD4^+$ T lymphocytes, we first analyzed PLZF and miR-146a expression and regulation in $CD4^+$ T lymphocytes purified from human peripheral blood (PB), activated by PHA treatment and maintained in culture for few days, as compared to resting $CD4^+$ T lymphocytes (day 0). Then, we examined CXCR4 and TRAF6 expression in the same cells.

Western blot analysis showed that PLZF protein expression, undetectable in resting cells, increases during PHA-activation of $CD4^+$ T lymphocytes (Fig. 1A), whereas miR-146a expression, analyzed by real time PCR, decreases in the same cells (Fig. 1B).

By using real time PCR analysis, we found that CXCR4 mRNA, expressed at very high level in non activated $CD4^+$ T lymphocytes (day 0, Fig. 1C), rapidly decreases during the first hours of activation (0 to 6 hours of culture, Fig. 1C), then remains at a lower but constant level in activated $CD4^+$ T cells (16 to 48 hours of culture, Fig. 1C), in a range of expression quite similar to CXCR4 mRNA levels found in Jurkat and U937 cells (Fig. 1C).

Western blot analysis performed as compared to Jurkat cells, a positive control expressing the 45 kDa isoform of CXCR4 protein, showed that this isoform, almost undetectable in quiescent $CD4^+$ T lymphocytes, increases with the time of culture and activation of $CD4^+$ T lymphocytes (Fig. 1D). Therefore CXCR4 protein expression pathway, inversely correlated to its mRNA expression (Fig. 1C), indicates a posttranscriptional control of CXCR4 mRNA by miR-146a in these cells.

We also examined the expression and regulation of another target of miR-146a, TRAF6, in activated $CD4^+$ T lymphocytes. As observed for CXCR4, TRAF6 mRNA level decreases during the first hours of activation of $CD4^+$ T cells, then remains at a lower level later in culture (from 16 to 24 hours), decreasing slightly at 48 hours (Fig. 1E), whereas the decrease of miR-146a (Fig. 1B) may unblock TRAF6 mRNA translation and then increase TRAF6 protein expression level in these cells (Fig. 1F).

Altogether, our data suggest that the regulation of CXCR4, but also of TRAF6, protein expression level in resting and activated

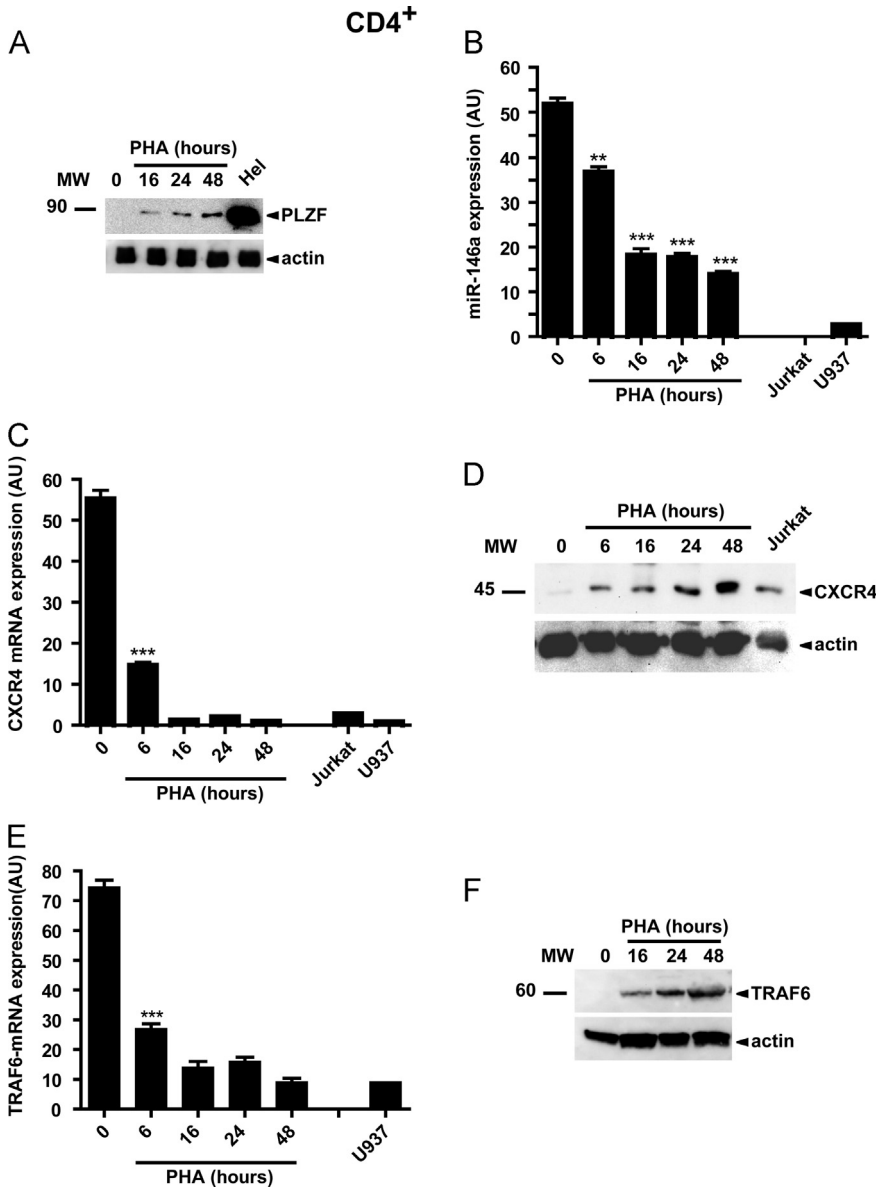

Fig. 1. PLZF, miR-146a and its targets, CXCR4 and TRAF6, are expressed and regulated in CD4$^+$ T lymphocytes. (A) Western blot analysis of PLZF protein expression in quiescent and PHA-activated CD4$^+$ T lymphocytes. Hel cell line is a positive cell line for PLZF expression. (B) Real time PCR analysis of miR-146a expression in quiescent and PHA-activated CD4$^+$ T lymphocytes. Jurkat and U937 cells are, respectively, negative and positive control of miR-146a expression. (C) qRT-PCR analysis of CXCR4 mRNA expression in quiescent (0) CD4$^+$ T lymphocytes and in PHA-activated CD4$^+$ T lymphocytes, maintained in culture for 48 hours and collected at different times. Jurkat and U937 cells are shown as two positive controls for CXCR4 mRNA expression. (D) Western blot analysis of the 45 kDa CXCR4 protein isoform expression in quiescent and PHA-activated CD4$^+$ T lymphocytes maintained in culture. Jurkat cell line is a positive control expressing the 45 kDa isoform of CXCR4 protein (E) qRT-PCR analysis of TRAF6 mRNA expression in quiescent (0) and PHA-activated CD4$^+$ T lymphocytes. U937 cell line is shown as a positive control of TRAF6 expression. (F) Western blot analysis of TRAF6 protein expression in quiescent and PHA-activated CD4$^+$ T lymphocytes. (A, D, F) Actin is an internal control of total protein extracts; one representative experiment of three is shown. (B, C, E) AU is for arbitrary units; Mean ± SEM values from three independent experiments are shown. **, ***: $p < 0.01$, $p < 0.001$, respectively. (B) miR-146a expression levels were normalized to U6 expression levels. (C, E) CXCR4 and TRAF6 mRNAs levels were normalized to GAPDH levels.

CD4$^+$ T lymphocytes is under the posttranscriptional control of miR-146a, whereby an increased PLZF level suppresses transcription of miR-146a, hence activating CXCR4 and TRAF6 mRNA translation.

HIV-1 infection have no effect on PLZF, miR-146a, and CXCR4 expression and regulation in human CD4$^+$ T lymphocytes

To study whether HIV-1 entry and replication into CD4$^+$ T lymphocytes may have an impact on the cascade pathway resulting in the control of CXCR4 protein level in these cells, we analyzed PLZF, miR-146a and CXCR4 expression and regulation in HIV-1 infected CD4$^+$ T lymphocytes.

Following 6 hours of activation, cells were infected by using NL4-3 HIV-1 virus (Adachi et al., 1986), and harvested at 16, 24 and 48 hours post-infection for analysis.

Similarly to what we observed in CD4$^+$ T lymphocytes (Fig. 1), western blot analysis showed that PLZF protein expression is activated (Fig. 2A), whereas miR-146a expression level is decreased in PHA activated CD4$^+$(HIV-1) T lymphocytes (Fig. 2B), as compared to quiescent CD4$^+$ T lymphocytes (0, Fig. 2B) and activated CD4$^+$ T lymphocytes of control (Fig. 1B).

Real time PCR analysis showed that CXCR4 mRNA expression level decreases in activated CD4$^+$(HIV-1) cells (Fig. 2C), whereas the 45 kDa isoform of CXCR4 protein increases in these cells from 16 to 48 hours of culture, as also found in uninfected CD4$^+$ T lymphocytes

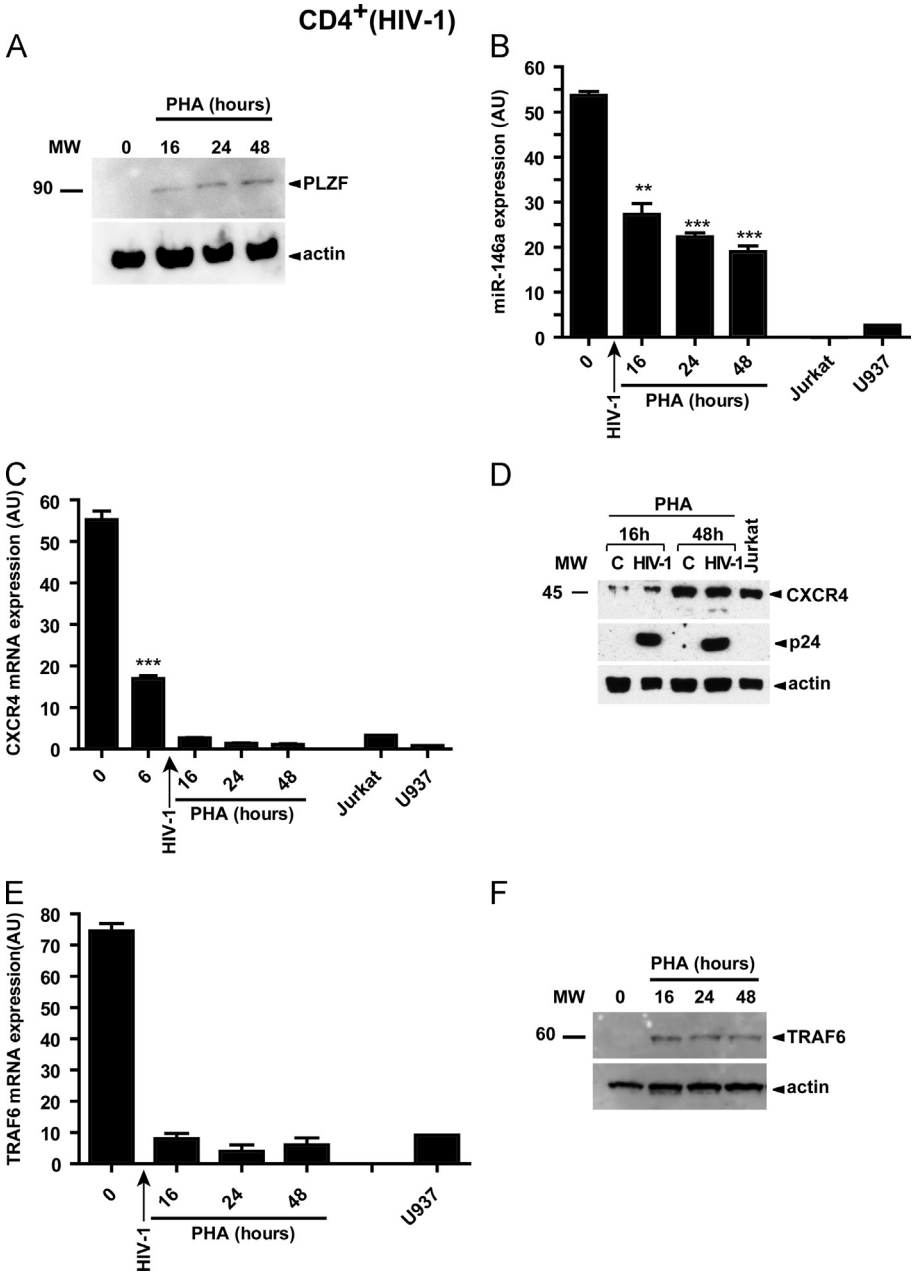

Fig. 2. HIV-1 infection of activated CD4$^+$ T lymphocytes does not modify PLZF, miR-146a, CXCR4 and TRAF6 expression patterns. (A) Western blot analysis of PLZF protein expression in PHA-activated and infected CD4$^+$(HIV-1) T lymphocytes maintained in culture from 16 to 48 hours, as compared to quiescent (0) CD4$^+$ control T lymphocytes. (B) Real time PCR analysis of miR-146a expression in PHA-activated- (from 0 to 3 hours) and HIV-1-infected- (arrow) CD4$^+$ T lymphocytes, maintained in culture for 48 hours, as compared to quiescent (0) CD4$^+$ T lymphocytes. Jurkat and U937 cells are, respectively, negative and positive control for miR-146a expression. (C) qRT-PCR analysis of CXCR4 mRNA expression in CD4$^+$(HIV-1) T lymphocytes, PHA-activated (from 0 to 3 hours) first, then HIV-1 infected (arrow) and maintained in culture for 48 hours, as compared to quiescent (0) CD4$^+$ T lymphocytes. Jurkat and U937 cells are positive controls for CXCR4 mRNA expression. (D) Western blot analysis of the 45 kDa CXCR4 protein isoform expression in PHA-activated (PHA 16 and 48 hours) and HIV-1-infected (HIV-1) CD4$^+$ T lymphocytes, as compared to 16 and 48 h PHA-activated CD4$^+$ control (C) T lymphocytes. Jurkat cells are shown as positive control of 45 kDa isoform of CXCR4 protein. Western blot analysis of viral p24 protein expression is shown as a control of HIV-1 infection of PHA-activated CD4$^+$(HIV-1) T lymphocytes. (E) qRT-PCR analysis of TRAF6 mRNA expression in CD4$^+$(HIV-1) T lymphocytes, PHA-activated (from 0 to 3 h) first, then HIV-1 infected (arrow) and maintained in culture for 48 h, as compared to quiescent (0) CD4$^+$ T lymphocytes. U937 cells are positive control for TRAF6 mRNA expression. (F) Western blot analysis of TRAF6 protein expression in PHA-activated and infected CD4$^+$(HIV-1) T lymphocytes maintained in culture from 16 to 48 h, as compared to quiescent (0) CD4$^+$ control T lymphocytes. (B, C, E) Mean ± SEM values from three independent experiments are shown. **, ***: $p < 0.01$, $p < 0.001$, respectively. (A, D, F) Actin is shown as an internal control of total protein extracts; one representative experiment of three is shown.

(Fig. 1D). Western blot analysis of p24 viral protein was also performed to control HIV-1 infection of PHA activated CD4$^+$ (HIV-1) T lymphocytes (Fig. 2D).

Furthermore, HIV-1 infection seems without significant effect on TRAF6 mRNA and protein expression and regulation in CD4$^+$ (HIV-1) T lymphocytes (Fig. 2E and F), as the increase of TRAF6 protein expression from resting to PHA activated CD4$^+$ T cells (Fig. 2F) is probably due to the decrease of miR-146a level observed in PHA activated CD4$^+$(HIV-1) T lymphocytes (Fig. 2B), whereas TRAF6 mRNA expression is quite constant in the same cells (Fig. 2E).

To exclude that the HIV-negative population can mask any changes in infected sub-population,we have replicated the experiments and performed real time PCR assays by using RNA from infected and uninfected cells purified with a monoclonal antibody anti-gp120 (mAb 4G10 from NIH AIDS Research and Reference Program) and separated with anti-mouse magnetic beads and gp120 negative cells. Our results

confirmed that miR-146a, CXCR4 and TRAF-6 mRNAs expression are not modified between infected and uninfected cells (data not shown).

Overall, our data show that HIV-1 entry and replication into activated CD4$^+$ T lymphocytes is without any significant effect on PLZF, miR-146a and, consequently, on CXCR4 or TRAF6 expression and regulation in these cells.

AMD3100 that impairs HIV-1 entry via CXCR4 into CD4$^+$ T lymphocytes induces miR-146a expression in both CD4$^+$ and CD4$^+$(HIV-1) T lymphocytes

AMD3100, as an inhibitor of CXCR4, is able to block HIV-1 entry via CXCR4 into CD4$^+$ T lymphocytes.

We have previously demonstrated that AMD3100 treatment of U937 cells increases miR-146a expression, thus decreasing CXCR4 protein expression level in these cells (Spinello et al., 2011). Here we analyzed whether AMD3100 may have a similar effect in CD4$^+$ T lymphocytes, HIV-1 infected or not (Fig. 3).

First, we evaluated CXCR4 membrane protein expression level in PHA activated CD4$^+$ T lymphocytes maintained in culture for 48 hours in presence, or not, of AMD3100 (Fig. 3A). Flow cytometry analysis showed that CXCR4 membrane protein expression is upregulated on CD4$^+$ T lymphocytes after 48 hours of activation, as compared to resting (d0) CD4$^+$ T lymphocytes, while the blockade of CXCR4 by AMD3100 treatment (Hatse et al., 2003) decreases CXCR4 membrane protein level detected by flow cytometry analysis (Fig. 3A).

Next, we infected activated CD4$^+$ T lymphocytes, treated or not with AMD3100, with NL4-3 HIV-1 strain (Adachi et al.,1986) and analyzed these cells at 24- and 48-h postinfection by flow cytometry to examine the infection efficiencies (Fig. 3B). Levels of intracytoplasmic HIV-CAp24 expression increase with the time of culture, as indicated by the number of PE-p24-CD4$^+$ positive cells detected by flow cytometry analysis from 8.9% to 25%. Moreover, AMD3100 treatment inhibits HIV-1 entry via CXCR4 in CD4$^+$ T lymphocytes, as shown by the low level of PE-p24-CD4$^+$ positive cells detected by flow cytometry analysis in these cultures (Fig. 3B).

Then, we analyzed miR-146a expression in activated CD4$^+$ and CD4$^+$(HIV-1) T lymphocytes, AMD3100 treated or not, harvested following 48 hours of culture.

We found that AMD3100 treatment of CD4$^+$ and CD4$^+$(HIV-1) T lymphocytes increases miR-146a expression (Fig. 3C) that, in turn, decreases protein expression levels of both 45 kDa CXCR4 protein isoform (Fig. 3D) and TRAF6 (Fig. 3E), without significant modulation of CXCR4 and TRAF6 mRNAs expression level (data not shown). However, we did not detect any significant effect of AMD3100 treatment on PLZF protein expression in AMD3100 treated -CD4$^+$ and -CD4$^+$(HIV-1) cells (not shown), as compared to untreated cells (Figs. 1A and 2A).

Altogether, our data showed that other that an inhibitor of CXCR4 that blocks HIV-1 entry into CD4$^+$ T lymphocytes, AMD3100 treatment can also be used to increase miR-146a expression level that controls two pathways, the one involving CXCR4 and HIV-1 entry in CD4$^+$ T lymphocytes, and the other involving TRAF6 to further investigate in HIV-1 infection of CD4$^+$ T lymphocytes.

miR-146a enforced expression or treatment with AMD3100, decreases CXCR4 protein level and inhibits HIV-1 infection of U937(miR-146a) cells

We have previously demonstrated that miR-146a decreases CXCR4 protein expression level in U937(miR-146a) cells, as compared to CXCR4 expressing U937(E) control cells (Spinello et al., 2011). Here, we used these cell lines to verify whether miR-146a overexpression in U937(miR-146a) cells may inhibit HIV-1 infection of these cells.

We used the HIV-1 chronically infected U937 cell line (U937$_{HIV-1}$) (Muratori et al., 2007) to infect U937(E) control cells, as compared to U937(miR-146a) cells.

By co-cultivating U937$_{HIV-1}$ cells with U937(E), as compared to U937(miR-146a) cells, we monitored HIV-1 infection at different times of co-cultures (6, 24 and 48 hours) analyzing intracellular p24 viral protein and green fluorescence protein (GFP) expression by flow cytometry (FC) analysis (Fig. 4).

FC analysis showed U937$_{HIV-1}$ cells as PE labeled-p24 positive cells, U937(miR-146a) and U937(E) uninfected cells as GFP positive cells, whereas U937(E) and U937(miR-146a) infected cells are PE labeled-p24/ GFP double positive cells (Fig. 4).

In co-cultures of U937(E)/U937$_{HIV-1}$ cells, we observed an increase of the percentage of PE-p24/GFP double positive cells from 15.6% to 33% corresponding to the increase of U937(E) infected cells in co-culture (Fig. 4A), whereas the percentage of PE-p24/GFP double positive cells detected in co-cultures of U937(miR-146a)/ U937$_{HIV-1}$ cells is lower (Fig. 4B).

After 48 hours of co-culture, 15% U937(miR-146a) infected cells are detected by FC analysis (Fig. 4B), whereas 33% of U937 (E) infected cells are found (Fig. 4A), indicating that U937(miR-146a) cells are less susceptible to HIV-1 infection, as compared to U937(E) cells.

Then we showed that a treatment of 48 hours with AMD3100 inhibits HIV-1 infection of CXCR4 expressing U937(E) cells, as indicated by the low percentage (5,1%) of PE-p24/GFP double positive cells detected in co-cultures (Fig. 4A, 48 h + AMD3100), as compared to the high percentage (34%) of PE-p24/GFP double positive cells detected in untreated U937(E)/U937$_{HIV-1}$ co-cultures (Fig. 4A, 48 h – AMD3100). Because U937(miR-146a) cells are negative for CXCR4 membrane expression, AMD3100 treatment has no significant effect on HIV-1 infection of these cells (not shown) that are quite resistant to HIV-1 entry (Fig. 4B).

Overall, these data showed the ability of HIV-1 virus produced by U937$_{HIV-1}$ cells to infect U937(E), but not U937(miR-146a) cells, thus indicating that HIV-1 entry occurs via CXCR4 coreceptor in these cells.

Altogether, our data demonstrated that miR-146a enforced expression and AMD3100, acting through different mechanism to down-modulate CXCR4 protein levels (Spinello et al., 2011), inhibits HIV-1 entry into U937(miR-146a) and U937(E) cells, as also observed in CD4$^+$ T lymphocytes (Fig. 3).

Knockdown of PLZF increases miR-146a that, in turn, decreases CXCR4 protein level and impairs HIV-1 infection via CXCR4 of CD4$^+$ T lymphocytes

We investigated whether PLZF, as a repressor of miR-146a identified in Mk cells (Labbaye et al., 2008), may control CXCR4 protein expression in CD4$^+$ T lymphocytes and play a role in HIV-1 infection of these cells.

First, we transfected activated CD4$^+$ cells with siRNAs that specifically blocked PLZF expression (siR-PLZF) (Labbaye et al., 2008), or non-targeting control siRNA (siR-C), and examined PLZF, miR-146a and CXCR4 protein expression in CD4$^+$(siR-PLZF) cells, as compared to control CD4$^+$(siR-C) cells.

Our data show that silencing of PLZF mRNA that induces a decrease of PLZF protein expression level (Fig. 5A, upper panel), up-regulates miR-146a expression in CD4$^+$(siR-PLZF) cells (Fig. 5B) that, in turn, decreases the 45 kDa CXCR4 protein isoform in these cells, as compared to control CD4$^+$(siR-C) cells (Fig. 5A, middle panel).

Next, we performed HIV-1 infection of CD4$^+$(siR-PLZF) cells and CD4$^+$(siR-C) cells, by using NL4-3 HIV-1 virus to analyze susceptibility of these cells to viral infection.

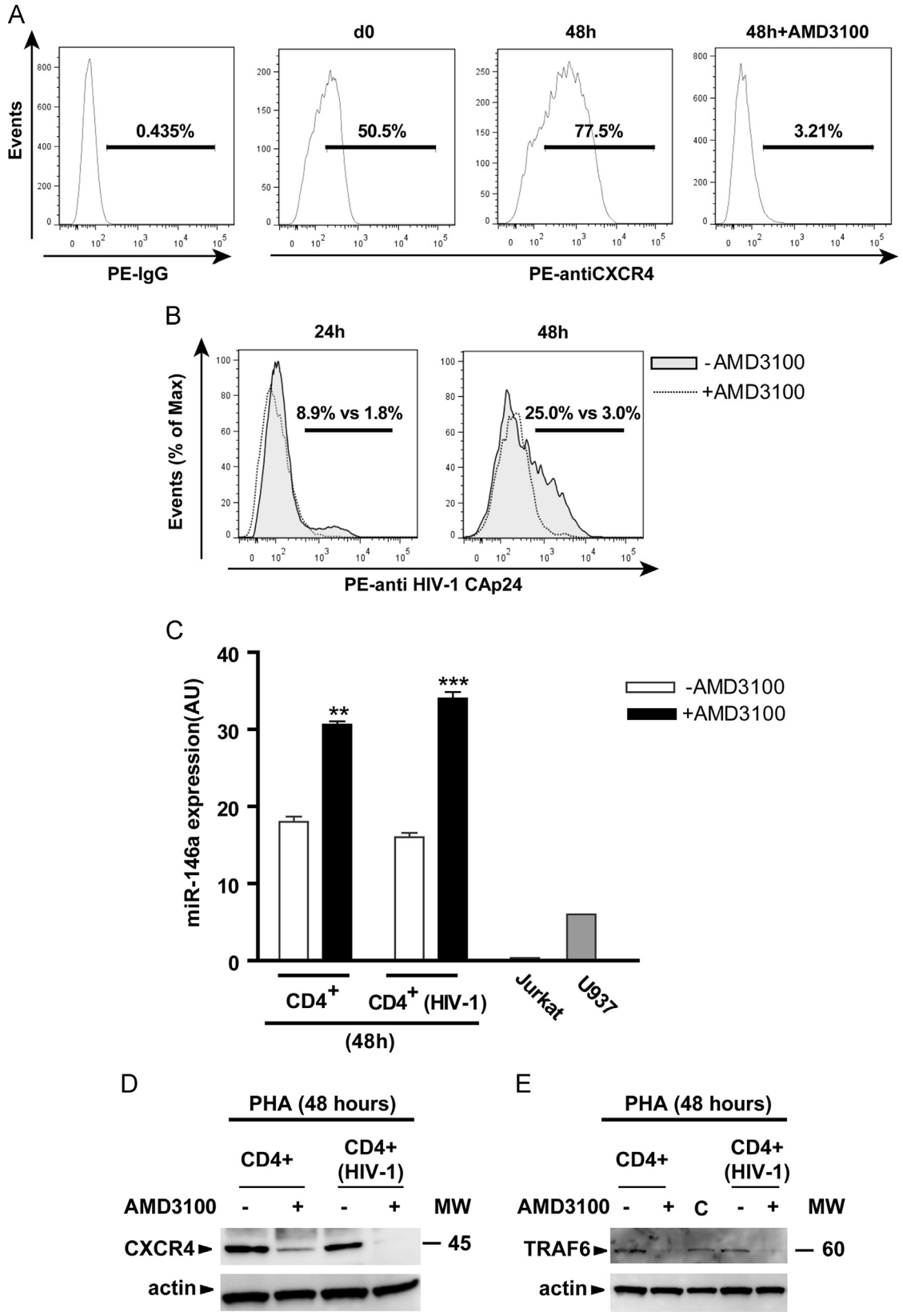

Fig. 3. AMD3100 increases miR-146a expression that, in turn, decreases CXCR4 and TRAF6 protein levels, thus preventing HIV-1 entry into $CD4^+$ T lymphocytes. (A) Representative histograms of $CD4^+$ T lymphocytes analyzed by flow cytometry (FC) for CXCR4 membrane protein expression in resting (d0), PHA-activated (48 h) and PHA-activated-AMD3100 treated (48 h + AMD3100) $CD4^+$ T lymphocytes. The relative percentage of CXCR4 membrane protein detected is indicated as compare to IgG-PE control $CD4^+$ cells. One representative experiment of three is shown. (B) FC analysis for the expression of HIV-1 CAp24 in activated $CD4^+$ T lymphocytes 24 and 48 h after challenge. The cells, treated or not, with AMD3100 and infected with 150 ng/10^5 cells of HIV-1 NL4-3 strain, are labeled with a PE-conjugated anti Cap24 HIV-1 mAb and analyzed by FC analysis. In the histograms, dotted line and solid gray histogram represent respectively, AMD3100 treated cells and not. The scale of Y axis is linear and represents events as % of max. One representative experiment of three is shown. (C) qRT-PCR of miR-146a expression shows that AMD3100 upregulates miR-146a expression level in, both, $CD4^+$ T lymphocytes and infected $CD4^+$(HIV-1) T lymphocytes maintained in culture 48 hours, as compared to untreated cells (-AMD3100). Mean ± SEM values from three independent experiments are shown. **, ***: $p < 0.01$, $p < 0.001$, respectively. Jurkat and U937 cells are respectively used as negative and positive control of miR-146a expression. (D) Western blot analysis of the 45 kDa CXCR4 isoform expression in PHA (48 h) activated -$CD4^+$ and infected -$CD4^+$(HIV-1) T lymphocytes, AMD3100 treated (+) or not (−). (E) Western blot analysis of TRAF6 protein expression in PHA (48 h) activated -$CD4^+$ and -$CD4^+$(HIV-1) T cells, AMD3100 treated (+) or not (−). C, is U937 cells used as a TRAF6 expressing positive control. (D, E) Actin is shown as an internal control of total protein extracts; one representative experiment of three is shown.

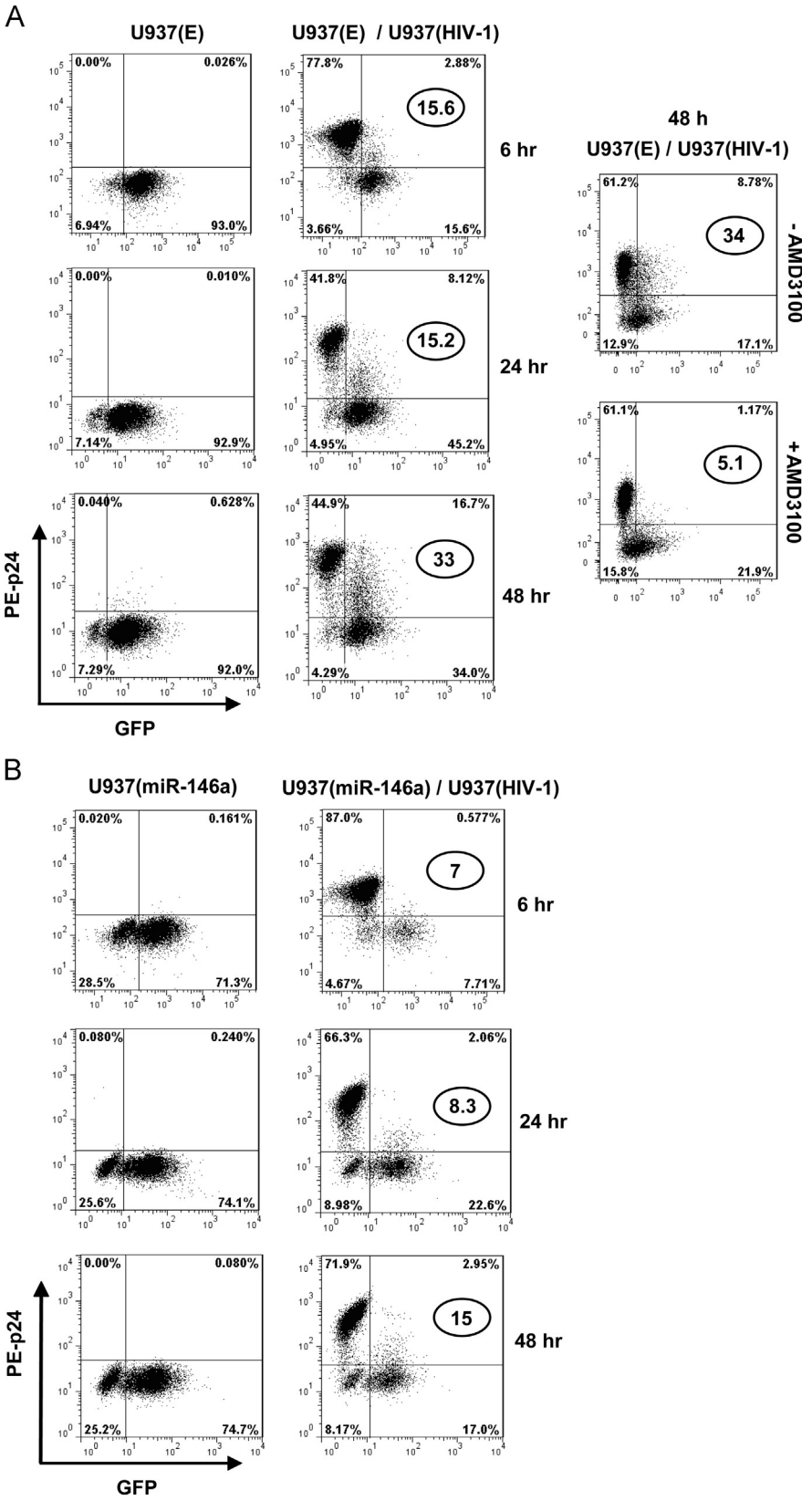

Fig. 4. MiR-146a enforced expression and AMD3100 treatment, that decrease CXCR4 protein level through different mechanisms, are able to prevent HIV-1 entry into U937 (miR-146a) and U937(E) cells, respectively. (A, B) The infection efficiency is evaluated by estimating the percentage of GFP positive cells that are also p24 positive by flow cytometry (FC) analysis. (A) U937(E) -GFP positive cells, maintained from 6 to 48 hours in co-culture with $U937_{HIV-1}$ cells, show an increase, of double PE-p24-/ GFP-labeled U937(E) cells. AMD3100 treatment inhibits HIV-1 infection of CXCR4-expressing U937(E) cells, as compared to non-treated co-cultures, as shown by FC analysis of HIV-1 CAp24 viral p24 (PE-p24) and GFP protein expression in U937(E) -GFP positive cells, treated (+AMD3100), or not (-AMD3100), with AMD3100 and maintained 48 hours in co-culture with $U937_{HIV-1}$ cells. (B) U937(miR-146a)-GFP positive cells co-cultivated with $U937_{HIV-1}$ cells show a percentage of double PE-p24-/GFP- labeled U937(miR-146a) cells that indicates a lower infection, as compared to infection of U937(E) cells. The percentages of events for each quadrant are reported. The circled numbers represent the percentage of the GFP positive cells that are also p24 positive. One representative experiment of three is shown.

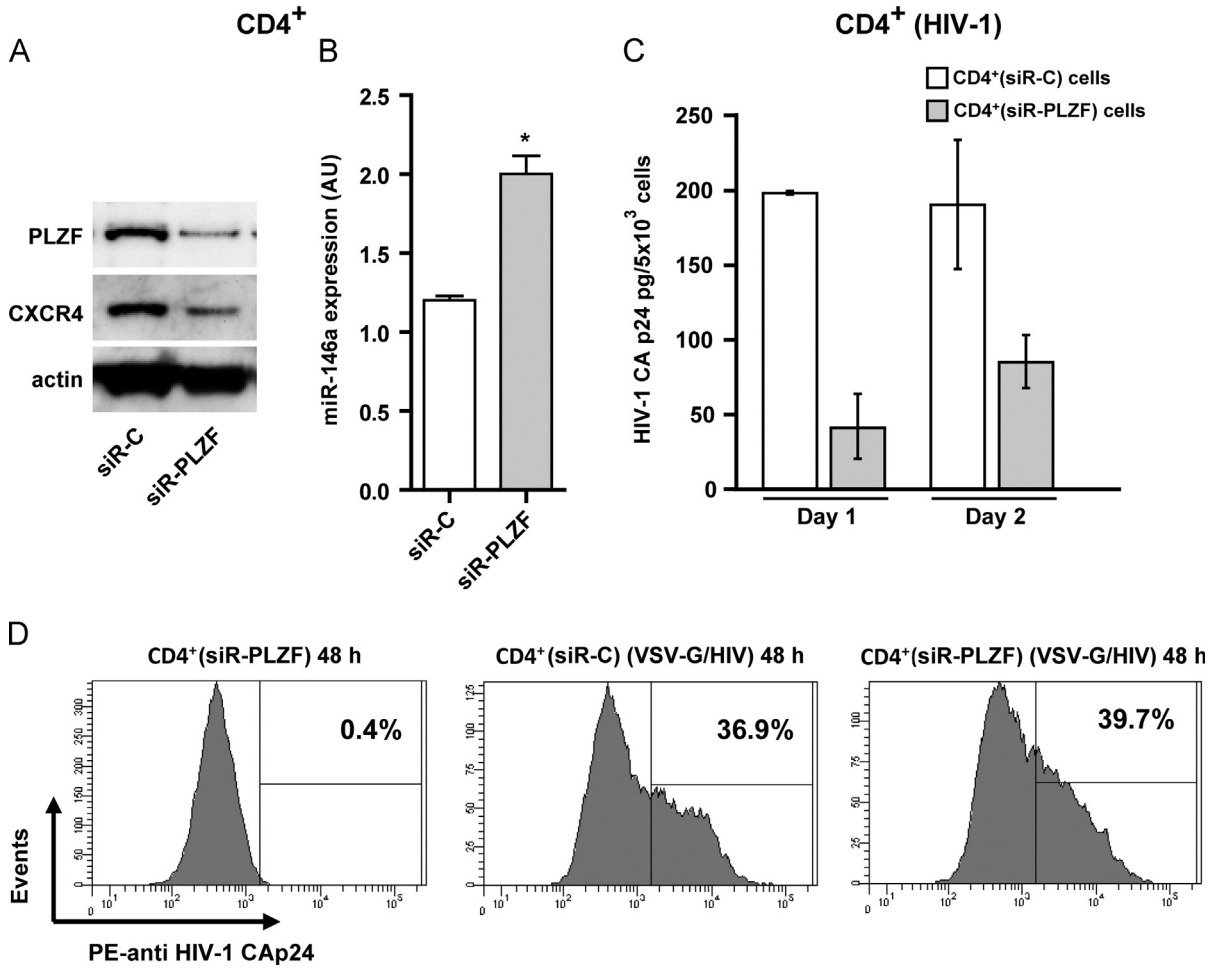

Fig. 5. PLZF silencing increases miR-146a expression that in turn, decreases CXCR4 protein level, and impairs HIV-1 infection of $CD4^+$ T lymphocytes. (A) Western blot analysis shows that inhibition of PLZF by siRNA-PLZF treatment of $CD4^+$ T lymphocytes (siR-PLZF, *upper panel*), also decreases the 45 kDa CXCR4 isoform level in these cells (siR-PLZF, *middle panel*), as compared to siRNA control-transfected $CD4^+$(siR-C) T cells. Actin is shown as an internal control of total protein extracts. One representative experiment of three is shown. (B) qRT-PCR shows that PLZF silencing in activated $CD4^+$ T lymphocytes increases miR-146a expression in $CD4^+$(siR-PLZF) T cells, as compared to $CD4^+$(siR-C) T lymphocytes. Mean ± SEM values from three independent experiments are shown. *: $p < 0.05$. (C) $CD4^+$(siR-PLZF) and $CD4^+$(siR-C) T lymphocytes infected with NL4-3 HIV-1 are analyzed by ELISA assay for intracellular HIV-1 CA p24 expression. (D) FC analysis of HIV-1 CAp24 viral p24 (PE-p24) on $CD4^+$(siR-PLZF) and $CD4^+$(siR-C) T lymphocytes infected with 150 ng/10^5 cells of VSV-G NL4-3 HIV-1, and maintained in culture for 48h. p24 expression level is the same in both infected $CD4^+$(siR-PLZF) (VSV-G/HIV) and $CD4^+$(siR-C) (VSV-G/HIV) T lymphocytes. (C, D) The data shown are representative of three independent experiments with similar results.

$CD4^+$(siR-PLZF) and $CD4^+$(siR-C) transfected cells are maintained in cultured for 24 hours and then infected with NL4-3 HIV-1. ELISA assay for intracellular viral p24 protein expression performed 1 and 2 days after HIV-1 infection shows about a 30% of inhibition of HIV-1 infection in $CD4^+$(siR-PLZF) cells, as compared to $CD4^+$(siR-C) cells (Fig. 5C), thus indicating that the decrease of CXCR4 protein level found in $CD4^+$(siR-PLZF) cells impairs HIV-1 entry into these cells, as compared to HIV-1 entry into $CD4^+$(siR-C) cells.

Then, we performed supplementary infection experiments by using a VSV-G pseudotyped HIV-1 NL4-3 strain that enters cells bypassing CXCR4 receptor. We infected both $CD4^+$(siR-PLZF) and $CD4^+$(siR-C) transfected cells with 150 ng/10^5 of virus and collected 48 h thereafter. FC analysis for the expression of p24 shows there is no effect of the siRNA-PLZF on infection (Fig. 5D).

Altogether, our results show that the miR-146a/CXCR4 pathway is controlled by the PLZF suppressor in $CD4^+$ cells and may be used to inhibit HIV-1 entry via CXCR4 into these cells.

Discussion

CXCR4/SDF-1α interactions are central to hematopoiesis, immune responsiveness and angiogenesis (Campbell et al., 2001; Busillo and Benovic; 2007). Implicated in a variety of pathological conditions, including inflammation and the metastasis of a variety of cancers (Ratajczak et al; 2006; Burger and Kipps, 2006), CXCR4 provides a receptor binding complex for the HIV envelope glycoprotein (gp120), which facilitates HIV-1 cell fusion and viral entry by CXCR4 utilizing X4 strains of HIV-1 (Wilen et al., 2012).

In this study, we showed that activation of $CD4^+$ T lymphocytes increases CXCR4 protein level while miR-146a decreases, unblocking CXCR4 mRNA translation. This effect results in an increase expression of the 45 kDa CXCR4 protein isoform that may support the turn-over of CXCR4 protein surface expression, thus enhancing the susceptibility for HIV-1 entry via CXCR4 into $CD4^+$ T lymphocytes.

Because TRAF6 is one component of the IFN signaling pathway that controls viral replication (Sirois et al., 2011; Khan et al., 2013), but also a target of miR-146a in immune cells (Taganov et al., 2006; Boldin et al., 2011), we analyzed TRAF6 expression at both mRNA and protein level in resting and activated $CD4^+$ T lymphocytes, to examine miR-146a activity in these cells. Our data are significant of a posttranscriptional control of TRAF6 mRNA translation by miR-146a targeting, as the decrease of miR-146a results in an increase of TRAF6 protein level during activation of $CD4^+$ T lymphocytes, independently of HIV-1 infection, as also observed for CXCR4 in these cells.

miR-146a expression is downregulated by the activation of CD4$^+$ T lymphocytes, without any significant alteration of expression during HIV-1 replication in HIV-1 infected CD4$^+$ T lymphocytes, thus increasing both CXCR4 and TRAF6 protein expression level in these cells. Upregulation of CXCR4 and TRAF6, through different pathways, promotes HIV-1 entry and replication in activated CD4$^+$ T lymphocytes. During viral infection, the expression of miRNAs that target viral sequences or host genes that influence the course of viral replication and pathogenesis may be altered (Houzet and Jeang, 2011; Klase et al., 2012). Here we found that miR-146a is expressed in human primary resting CD4$^+$ T lymphocytes, downregulated during their activation, but its influence on HIV-1 replication should be further investigated.

Altogether, our findings indicate that miR-146a is implicated in two regulatory pathways during activation of CD4$^+$ T lymphocytes, the one that involved CXCR4 and HIV-1 entry into CD4$^+$ cells, the other involving TRAF6 possibly correlated with HIV-1 replication in CD4$^+$ cells, indicating the possibility for miR-146a to be used as a tool to knockdown its targets and control HIV-1 entry/replication in human CD4$^+$ T lymphocytes.

Interestingly, as we previously downmodulated CXCR4 at both surface and intracytoplasmic levels by enforced miR-146a expression in monocytic leukemic cells (Spinello et al., 2011), here we showed that miR-146a overexpression prevents HIV-1 entry into U937(miR-146a) cells, as compared to the CXCR4 expressing U937(E) cells of control. TRAF6 protein is also downmodulated in U937(miR-146a) cells (our unpublished data) and may be involved in resistance to HIV-1 infection that we observed with these cells. Therefore, as a recent study performed on human macrophages has shown that the knockdown of TRAF6, but also TRAF2 and 5, results in decreased NF-kB activation and reduces HIV-1 replication in monocyte-derived macrophages (Khan et al., 2013), understanding the role of the miR-146a/TRAF6 axis in HIV-1 infection of Mo leukemic cells and CD4$^+$ T lymphocytes, in the context of the complex TRAF signaling, requires future dedicated studies.

The critical role of CXCR4 in HIV-1 infection, together with the cell types expressing CXCR4, encouraged the design of new anti-HIV drugs aimed at blocking CXCR4 function (Steen et al., 2009; Murakami and Yamamoto, 2010; Choi et al., 2012a, 2012b). In fact, whether the use of specific inhibitors of CCR5 has been approved for clinical use (Boesecke and Pett, 2012), the necessity to first identify which co-receptor is used by the strains infecting each patient has emerged, in part due to the risk on selecting the more pathogenic strains that use the CXCR4 co-receptor. Viruses that use CXCR4 (X4) or both, CCR5 (R5) and CXCR4, rare in early disease, emerge over time and are associated with disease progression, as the prevalence of X4 variants increases with decreasing CD4$^+$ T cell count observed in patients with chronic disease (Fiser et al., 2010; Duquenne et al., 2014).

Whether preliminary studies with AMD3100, used as an antiretroviral agent antagonist of CXCR4, have not been satisfactory (Hendrix et al., 2000, 2004), exposure of CD4$^+$ T lymphocytes to this agent determines the rapid and massive downmodulation of surface CXCR4 due to receptor internalization, while the total receptor expression remains virtually unmodified. In our study, we showed that prolonged exposure of activated CD4$^+$ T lymphocytes to AMD3100 induces a decrease of total CXCR4, a phenomenon possibly related to intracellular receptor degradation and also to reduced receptor translation, but also associated to an increased expression of miR-146a in CD4$^+$ T lymphocytes, these effects being associated to an inhibition of HIV-1 infection of these cells.

Resting CD4$^+$ T cells and monocytes are non-permissive for HIV-1 infection. Also, the resting state and the cause behind viral restriction in resting cells are currently under discussion (Chiang and Rice, 2012). It is of interest to note that miR-146a is highly expressed in resting CD4$^+$ T lymphocytes and inhibits the expression of both CXCR4 (i.e., the 45 kDa isoform) and TRAF6 cellular proteins that are not viral co-factors, without excluding some other target genes of miR-146a directly involved in viral replication to identify. However, if miR-146a is, currently, not an anti-HIV miRNAs (Chiang and Rice, 2012), following activation miR-146a exhibits decreased expression, while CXCR4 and TRAF6 are up-regulated contributing to a favorable milieu for virus entry and/or replication.

Here, we found that the transcription factor PLZF is a repressor of miR-146a expression in activated CD4$^+$ T lymphocytes. Undetectable in resting CD4$^+$ T lymphocytes, following activation, PLZF increases and downregulates miR-146a, thus increasing CXCR4 protein level in activated CD4$^+$ T lymphocytes. PLZF knockdown induced the opposite effects and impaired HIV-1 infection of these cells.

Whether PLZF expression was found to be restricted to invariant NKT (iNKT) cells, a subgroup of NK T-lymphocyte (NKT) cells with distinctive properties, (Savage et al., 2008) and more recently, PLZF was shown to direct the developmental acquisition of the innate effector program of NK T cells (Michael et al., 2014), in human the role of PLZF should be reconsidered in the adaptive immune response and in the pathogenesis of the HIV-1 infection.

Altogether, we identify a cascade pathway, in which PLZF suppresses miR-146a transcription and thereby activates CXCR4 translation during activation of CD4$^+$ cells, that may be used to inhibit HIV-1 infection. Our findings also suggest that an optimal therapeutic effect potentially deriving from inhibition of the CXCR4/SDF-1α axis may require a combined effect both at the level of CXCR4 content (i.e., decreasing it by miR-146a overexpression) and of SDF-1α/CXCR4 interaction (i.e., inhibiting this interaction using AMD3100 or similar agents). miR-146a overexpression may be achieved by using the miRNA mimic technology, an innovative approach for gene silencing to use in miRNA-based cancer therapy (Yeung and Jeang, 2011), but also by blocking the active recruitment of histone deacetylases (HDACs) with which PLZF interacts to achieve its inhibitory effect through chromatin remodeling (Suliman et al., 2012). In this context, it is of interest to note that a study has previously reported the synergistic antileukemic activity of AMD3100 and a histone deacetylase inhibitor that markedly upmodulates miR-146a levels and decreases CXCR4 level (Mandawat et al, 2010).

Materials and methods

Cell cultures

Human adult peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors after written informed consent and processed under approval of the ethical committee of Istituto Superiore di Sanità (ISS), in accordance with the Declaration of Helsinki and prepared by ficoll (Ficoll-Paque GE Healthcare) gradient centrifugation.

Primary CD4$^+$ T lymphocytes were isolated untouched from PBMCs by negative selection using magnetic beads (CD4$^+$ T cell isolation kit II, Miltenyi Biotech, Germany) following the manufacturer's recommendations. The purity of recovered cell populations was assayed by FACS analysis by means of PE-conjugated anti-CD4 mAb (BD Biosciences, Mountain View, CA) labeling. Cell preparations staining below 95% positive for CD4 were discarded.

For activation, primary CD4$^+$ T lymphocytes were treated with phytohemagglutinin (PHA) 5 μg/ml, (Sigma-Aldrich Co., St. Louis, MO) for 24 h at 37 °C and cultured in interleukin (IL-2) containing medium (50 U/ml, R&D Systems, Minneapolis, MN). Non-activated T cells were kept in RPMI 1640 (Life Technologies, Italy) supplemented with 10% FBS (Gibco, Carlsbad, CA, USA) without exogenous cytokines. Cultures of CD4$^+$ cells were maintained few days, after

addition (PHA) for activation of these cells for the duration of experimentation (Gowda et al., 1989a, 1989b).

Co-cultivations were typically set up in 1ml of RPMI-10% Fetal Bovine Serum in 12-well plates by seeding 5×10^5 target cells with 10^6 donor cells.

U937 cells, an human cell line displaying many monocyte/macrophage features, Jurkat cells, an immortalized line of human T lymphocyte cells and HEL cells, a human erythroleukemic cell line were cultured using standard methods.

$U937_{HIV\text{-}1}$ is a cell line derived from U937 cells recovered upon chronic infection with the human T-lymphotropic virus IIIB HIV-1 isolated, as previously described (Muratori et al., 2007).

U937(E) and U937(miR-146a) cell lines were previously prepared upon infection of target U937 cells respectively with, an empty pCDH/Green Fluorescent Protein expression lentivector (pCDH/GFP-E) used for internal control and with the human miR-146a precursor cloned into the pCDH/GFP-miR-146a expression lentivector (PMIRH146aPA-1, System Biosciences, Mountain View, CA, USA) (Spinello et al, 2011).

$U937_{HIV\text{-}1}$, U937(E) and U937(miR-146a) cells are grown in RPMI 1640 culture medium supplemented with 10% heat-inactivated FBS. GFP expression was evaluated by flow cytometry analysis. Their co-cultivations were also set up in 1 ml of RPMI-10% FBS in 12-well plates by seeding 5×10^5 target cells with 10^6 donor cells.

HIV-1 preparation and infection

Supernatants from transiently transfected 293T cells were used as the source of both NL4-3 HIV-1 and NL4-3 HIV-1 pseudotyped with the envelope glycoprotein of the vesicular stomatitis virus (VSV-G) as described (Adachi et al., 1986). For the infection experiments of $CD4^+$ cells, supernatants were concentrated by ultracentrifugation and titrated by measuring the HIV-1 CAp24 content by quantitative enzyme-linked immunosorbent assays (ELISA, Innogenetics, Gent, Belgium) and by reverse transcriptase assays (Olivetta et al., 2000). $CD4^+$ cells were infected by spinoculation at $400 \times g$ for 30 min at room temperature using 150 ng/10^5 cells of viruses. Virus adsorption was prolonged for an additional 2 h at 37 °C, and finally, the cells were washed and reefed with the complete medium.

Flow cytometry analysis (FC)

2×10^4 cells aliquots were labeled on the membrane using the phycoerythrin (PE)-conjugated anti-CXCR4 monoclonal antibody (clone 12G5, BD/Pharmingen, SanDiego,CA, USA) or a control PE-conjugated mAb of the same isotype (Spinello et al., 2011). All experiments were performed with the same batch of anti-CXCR4 conjugated antibody.

The percentages of cells expressing intracytoplasmic HIV-1 Gag-related products were evaluated by FC analysis after permeabilization with Cytofix/Cytoperm solutions (BD biosciences) for 20 min at 4 °C and labeling with 1/100 dilution of KC57-RD1 phycoerythrin (PE)-conjugated anti HIV-1 Gag CAp24 KC-57 (PE-p24) MAb (Coulter Corp., Hialeah, FL, USA). The % of positive cells were calculated comparing samples stained with matched isotype used as control of non-specific fluorescence signals.

Dead cells were excluded by Sytox Blue staining (1 μM, Molecular Probes, Carlsband, CA, USA).

Stained cells were analyzed by using a BD FACSAria (BD Biosciences), equipped with three lasers (488 nm, 635 nm and 407 nm), and the results were analyzed by BD FACSDiva Software version 6.1.3 (BD Biosciences) or FlowJo Software version 7.6.1 (Tree Star, Inc., Ashland, OR, USA).

Quantitative real time RT-Polymerase Chain Reaction (PCR)

Total RNAs were extracted using Trizol reagent and reverse transcribed (RT-) by Moloney murine Leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with random primers.

Gene expression analysis of CXCR4 and TRAF6 mRNAs was detected by real time quantitative RT-PCR analysis (qRT-PCR) using commercial ready-to-use primers/probe mixes (Applied Biosystems, Foster City, CA, USA) for CXCR4 (Hs00607978_s1), TRAF6 (Hs00270336) and normalized with GAPDH (assay 20X, 4328317E), according to the manufacturer's procedure and by using TaqMan technology and an ABI Prism 7900 Sequence Detector (Applied Biosystems, Foster City, CA, USA) (Spinello et al., 2011).

Real time PCR for miR-146a was performed using TaqMan*H* MiRNA Assays protocol (assay ID 000468, Applied Biosystems, Foster City, CA, USA). Reverse transcriptase reaction was performed using 50 ng of total RNA and 50 nM miRNA specific stemloop miR-146a-RT primers. Real-time quantitative RT-PCR was performed using standard protocol (Spinello et al., 2011). All reactions were run in duplicate. Normalization was performed by using U6 snRNA primer kit (ID 001973, Applied Biosystems). Relative expression was calculated with relative standard curves for both the miRNA of interest and the endogenous control, as previously described (Spinello et al., 2011).

Western blotting

Total protein extract and Western blot analysis on 7, 5% SDS-PAGE gel were performed according standard procedures for CXCR4, PLZF, TRAF6 and p24 proteins expression, by using polyclonal antibodies (pAb) anti-CXCR4 and anti-PLZF (respectively, ab2074 and ab39354, Abcam, Cambridge, UK) and monoclonal antibodies (mAb) anti-TRAF6 (D-10) (sc-8409, Santa Cruz Biotechnology,) and anti-HIV p24 (ab9071, Abcam, Cambridge, UK). As an internal control of total proteins, we used the anti-Actin mAb (clone JLA20, Calbiochem, San Diego, CA, USA).

AMD3100 drug treatment cells and HIV-1 infection

$CD4^+$ T lymphocytes were first activated few hours by addition of PHA in culture and then treated, or not, with (1 μM) AMD3100 (Sigma-Aldric, St Louis, MO) 16 hours. Infection of AMD3100 treated $CD4^+$ T lymphocytes was then performed as described above and as compared to untreated HIV-1 infected or not $CD4^+$ T lymphocytes. FC analysis of viral p24 expression was performed after 24 and 48 hours of co-culture with, or without, AMD3100.

U937(miR-146a) and U937(E) cells (5×10^4 cells/ml) were treated or not few hours (3 to 6 hours) with AMD3100 (10 μM) (Sigma-Aldric, St Louis, MO) as previously described (Spinello et al., 2011), before to perform infection in co-culture experiments with $U937_{HIV\text{-}1}$ cells, maintained for 12 to 24 hours, always in presence of AMD3100. Cellular populations present in co-cultures were then analyzed by FC for GFP, p24 and double GFP/p24 expression.

Silencing of PLZF expression by siRNAs transfection

Two double-stranded PLZF siRNA (siR-PLZF) sequences (NM_001018011) and the control siRNA sequence (siR-C) with no homology to the human genome (Dharmacon), were previously used to knockdown PLZF mRNA and protein expression (Labbaye et al., 2008). Activated $CD4^+$ T lymphocytes (5×10^5 cells/ml) were transfected with 90 nM siR-PLZF or siR-C by using Lipofectamine 3000 according to the manufacturer's instructions and as previously described (Labbaye et al., 2008). After 24 hours, transfected $CD4^+$(siR-PLZF) and $CD4^+$(siR-C) T lymphocytes were

infected or not by spinoculation with 150 ng CAp24 equivalent of HIV-1 NL4-3 strain/10^5 cells and maintained in culture 24 to 48 hours and then collected to analyze PLZF and CXCR4 protein expression by Western blotting and miR-146a by real-time PCR. At the same time infection was carried out on $CD8^+$ T cell purified from the same donor, in order to normalize the amount of virus entry. Infection of $CD4^+$(siR-PLZF), $CD4^+$(siR-C) and $CD8^+$ T cells was then evaluated by intracellular assay of p24 viral protein expression in these cells.

Viral intracellular p24 assay

Purified $CD4^+$ and $CD8^+$ T cells (10^5 cells/well in 96-well plates) were infected or not by spinoculation at 400 *g* for 30' at room temperature using 150 ng CAp24 equivalent of NL4-3 HIV-1/10^5 cells, followed by virus adsorption for 3 hours at 37 °C and addition of complete medium. At different times, cells were washed and treated with trypsin for 5 min to remove uninternalized viruses from their surface. Then, the cells were disrupted in lysis buffer (PBS, 0.05% Tween 20, 2.5% Triton X-100) and infection efficiency was evaluated 24 and 48 h after infection by intracellular p24 ELISA. The experiment was performed in duplicate, and the amount of intracellular p24 was normalized for number cells after background subtraction.

Statistical analysis

Statistical significance of differences observed between different experimental groups was determined using a Student's *t* test. The minimal level of significance was a P value below 0.05.

Competing interests

The authors reported no potential competing interest.

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