MiRNA-103 downmodulates CCR5 expression reducing human immunodeficiency virus type-1 entry and impacting latency establishment in CD4⁺ T cells

Activated-to-memory transitioning CD4⁺ T cells

| miRNA-103* / CCR5** / HIV latency*** | miRNA-103*** / CCR5* / HIV latency* |
|--------------------------------------|------------------------------------|

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Highlights

- p53-regulated miRNA-103 reduces CCR5 in primary CD4⁺ T cells
- HIV-1 latent infection-prone activated-to-memory transitioning T cells reduce p53
- Stabilizing p53 reduces CCR5 in CD4⁺ T cells via upregulation of miRNA-103
- Decreased CCR5 in CD4⁺ T cells is associated with an increase in miRNA-103 in vivo
MiRNA-103 downmodulates CCR5 expression reducing human immunodeficiency virus type-1 entry and impacting latency establishment in CD4⁺ T cells

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SUMMARY

Activated-to-memory transitioning CD4⁺ T cells display elevated expression of the HIV-1 co-receptor CCR5 and are more prone to HIV-1 latency infection. Here, we show that p53-regulated miRNA-103 downmodulates CCR5 levels in CD4⁺ T lymphocytes. We reveal that miRNA-103 mimics, as well as Nutlin-3, an inhibitor of Mdm2-mediated p53 degradation, decrease CCR5-dependent HIV-1 infection. Using a dual-reporter virus, we subsequently validate that in transitioning CD4⁺ T cells, Nutlin-3 treatment decreases the frequency of both productively and latently infected cells via upregulation of miRNA-103. Importantly, we provide evidence that CD4⁺ T cells from HIV-1 elite controllers express less CCR5 than those from antiretroviral therapy-naïve progressors, an effect linked to a significant increase in miRNA-103 levels. By contributing to the control of CCR5 expression in CD4⁺ T cells, miRNA-103 is likely to play a key role in countering the establishment of latent HIV-1 reservoirs in vivo.

INTRODUCTION

The development of combined antiretroviral therapy (ART) for the suppression of human immunodeficiency virus type-1 (HIV-1) replication represents a remarkable achievement of modern medicine. However, although these treatments improve the health and prolong the life of those infected, they do not eradicate the virus and therefore must be taken for life. HIV-1 can establish latent infection by integrating replication-competent proviral DNA into the genome of specific host target cells, ultimately leading to the formation of a latent reservoir (Chun et al., 1997a; Wong et al., 1997). These HIV-1 latent reservoirs, which escape elimination by the host immune system or antiviral drugs, represent the primary obstacle to an HIV-1 cure as long as these cells are present. Given their lifelong persistence, even when ART is interrupted, these reservoirs represent a significant barrier to achieving a sustained cure for HIV-1.

One of the best characterized latent HIV-1 reservoirs is found in resting memory CD4⁺ T cells (Chomont et al., 2009; Chun et al., 1997a, 1997b; Finzi et al., 1997; Wong et al., 1997), and appears to be seeded at very early stages of infection as was demonstrated in non-human primate models (Whitney et al., 2014, 2018). The mechanisms responsible for the establishment of HIV-1 latency in memory CD4⁺ T cells are not fully elucidated. Latently infected cells are rare in vivo and appear to arise when activated CD4⁺ T cells become infected and survive long enough to revert back to a long-lived resting memory stage, which is nonpermissive for viral gene expression (Sengupta and Siliciano, 2018). An alternative model proposes that HIV-1 latency is directly established in resting CD4⁺ T cells, following interaction with dendritic cells and/or the action of specific chemokines (Chavez et al., 2015; Evans et al., 2013; Saleh et al., 2007). More recently, it was shown that latent infection could also arise from the infection of CD4⁺ T subsets that are transitioning from an activated to a memory stage (Shan et al., 2017). These transitioning CD4⁺ T cells were found to have a unique combination of characteristics: first, they display a transient upregulation of the virus co-receptor C-C chemokine receptor 5 (CCR5) expression, allowing for HIV-1 entry and infection, and second, they exhibit a cellular environment poorly conducive for active HIV-1 provirus transcription. Indeed, ex vivo studies of HIV-1 infection of CD4⁺ T cells transitioning from an activated to a memory stage revealed the establishment of largely latent infection in these cells (Shan et al., 2017). In this context, a comprehensive understanding of host factors and signals that modulate CD4⁺ T cell susceptibility to latent HIV-1 infection is likely to provide important information about cellular pathways that could be countered to limit the establishment of latent infection in these cells.
MicroRNAs (miRNAs) are small single-stranded non-coding RNA molecules (containing about 22 nucleotides) found in plants, mammals, and even viruses, that are involved in RNA silencing and post-transcriptional regulation of gene expression (Hammond, 2015). They can be delivered to neighboring cells via exosomes, which are found to contain large quantities of miRNAs (Nahand et al., 2020). MiRNAs function by base pairing with complementary sequences within target mRNAs, primarily in the 3’ untranslated region (UTR). As a consequence, targeted mRNAs are silenced by one or more mechanisms, including mRNA cleavage and blocking the translation of the mRNA by ribosomes (O’Brien et al., 2018). Increasing evidence indicate that miRNAs play a key role in regulating the susceptibility of target cells to HIV-1 infection and persistence (Baldassarre et al., 2018). Indeed, several miRNAs that target the 3’ UTR of HIV-1 transcripts have been identified, such as miRNA-29a and miRNA-223 (Ahluwalia et al., 2008; Huang et al., 2007). MiRNAs appear also to promote HIV-1 infection by targeting factors that restrict viral replication. For instance, miRNA-181 modulates the expression of SAMHD1, a cellular regulator of deoxynucleoside triphosphate levels and a strong inhibitor of reverse transcription (Riess et al., 2017). Importantly, miRNAs are also found to promote the establishment and maintenance of provirus latency by targeting directly or indirectly host transcription factors that are essential for HIV-1 gene expression (Chiang et al., 2012; Shen et al., 2012; Triboulet et al., 2007). Hence, TRIM32, an E3 ubiquitin ligase that activates NF-κB to promote HIV-1 transcription, is targeted by miRNA-155, therefore limiting long terminal repeat (LTR)-driven gene expression (Ruelas et al., 2015). MiRNAs can also modulate HIV-1 entry into host target cells. Indeed, miRNAs-221/222 inhibit HIV-1 infection by downregulating the HIV-1 receptor CD4 in lymphocytes (Orecchini et al., 2014) as well as in macrophages (Lodge et al., 2017). More recently, we provided evidence that interleukin (IL)-1β induces a downmodulation of CCR5 expression and HIV-1 entry in macrophages through a p53-mediated process involving miRNA-103 (Lodge et al., 2020). Lastly, differences in miRNA expression profiles in HIV-1, hepatitis B virus (HBV), or hepatitis C virus (HCV)-infected individuals, as well as co-infected individuals or HIV-1-infected elite controllers suggest that miRNAs could be used as biomarkers for the risk of disease progression (Moghoofei et al., 2018; Yousefpouran et al., 2020).

Here, we investigated the role of the p53-regulated miRNA-103 in the regulation of CCR5 expression following the activation of CD4+ T lymphocytes and examined its impact on the susceptibility of activated-to-memory transitioning cells to HIV-1 latent infection. Our findings reveal that miRNA-103 contributes to the downregulation of CCR5 expression in CD4+ T cells progressing to a resting memory phenotype. We also show that the extent of CCR5 control by miRNA-103 is determined by the levels of p53 in CD4+ T cells progressing to a resting memory stage. Indeed, we provide evidence that the pharmacological stabilization of p53 limits both productive and latent infections of CD4+ T lymphocytes by reducing CCR5 expression levels via miRNA-103. Importantly, we show that CD4+ T cells from elite controllers (ECs) display significantly reduced expression of CCR5 compared to ART-naive progressors, a condition that is associated with a statistically meaningful increase in miRNA-103 expression. Overall, these results highlight the role of p53-regulated miRNA-103 in the regulation of CCR5 expression in CD4+ T cells and underscore how the modulation of miRNA-103 could conceivably affect the establishment of HIV-1 latency in these cells.

RESULTS
PS3-modulated miRNA-103 regulates CCR5 mRNA levels in CD4+ T lymphocytes progressing to a resting memory phenotype

Having recently reported that miRNA-103 downregulates CCR5 mRNA levels in macrophages (Lodge et al., 2020), we first sought to directly determine if miRNA-103 mimics could regulate CCR5 mRNA levels in CD4+ T cells. To this end, we used the CD4+ C10/MJ T cell line that expresses low, yet detectable levels of endogenous CCR5 protein at the cell surface (Majka et al., 2000) (Figure 1A). Transfection of miRNA-103 mimics significantly decreased the degree of CCR5 mRNA expression when compared to control (approximately 60%, p = 0.0022), but did not impact CXCR4 mRNA levels (Figure 1B). Consistently, infection of C10/MJ cells with firefly Luciferase (F-Luc)-expressing single-round HIV-1-reporter viruses, either pseudotyped with HIV-1 ADA-Env (CCR5-dependent entry) or vesicular stomatitis virus G (VSV-G) glycoprotein (CCR5-independent entry) revealed that HIV-1 ADA-Env-coated virus infection was significantly reduced (approximately 75%, p = 0.0022) by miRNA-103 mimic treatment as compared to control (Figure 1C). In contrast, no difference in viral infection efficiency was detected with VSV-G-coated viruses, thus excluding potential effects of miRNA-103 on post-entry steps up to provirus expression. These results indicate that miRNA-103 can downregulate CCR5 expression in CD4+ T cells and inhibit CCR5-dependent HIV-1 entry.
To examine the role of miRNA-103 in regulating CCR5 expression during the transition of CD4+ T cells from an activated to a memory phenotype, we first validated if CCR5 expression was modulated during this transition in our culture conditions. Thus, the levels of CCR5 at the surface of either primary resting CD4+ T cells (D0, or “day 0”) or CD4+ T cells activated for 4 days with anti-CD3/anti-CD28 antibodies (day 4) or at different time-points following the removal of antibodies (“relaxation”, day 7-day 21) were measured by flow cytometry (Figures S1A and 2A). As previously shown (Shan et al., 2017), our results reveal that resting CD4+ T cells weakly express CCR5 (with <10% cells being CCR5-positive). However, co-receptor expression increases transiently in activated-to-memory transitioning lymphocytes with a peak of >30% of cells expressing CCR5 after 7 days of “relaxation” post-activation (day 11) (Figures 2A and 2B). These observations were further confirmed at the mRNA level as we detected a parallel transient increase of CCR5 mRNA in activated-to-memory transitioning CD4+ T cells. Following a peak at day 11, a progressive decrease of CCR5 mRNA levels was observed as cells were progressing to a resting phenotype (Figure 2C). This modulation of CCR5 was specific as the levels of cell-surface CXCR4 remained unchanged during this transition despite the progressive decrease of the CD25 activation marker (Figures S1B and S1C).

Recently, we and others reported that miRNA-103 is regulated by the tumor suppressor p53 in Interleukin (IL)-1β treated macrophages as well as in a human colorectal carcinoma cell model (Leslie et al., 2018; Lodge et al., 2020). Therefore, we tested if miRNA-103 and p53 mRNA levels varied in parallel with the changes in CCR5 expression from day 4 (activated) to day 21. Interestingly, we found that the level of miRNA-103 expression varies slightly between day 4 and day 11 (2.5-fold, p = 0.0412 at day 11) but shows a strong increase starting at day 15 (2.9-fold, p = 0.0072 to 4.5-fold, p < 0.00001 between day 15 and day 21) (Figure 2D, left panel). These observations correlate with p53 mRNA levels, which also start to significantly increase at day 15 (2.9-fold, p = 0.0386) (Figure 2D, right panel). These results suggest that miRNA-103 expression levels following activation (day 4) are not sufficient to counter the increase in CCR5 observed, and further indicate that miRNA-103 is implicated in the downregulation of CCR5 that is observed between day 11 and day 21 (Figures 2A-2C). As a decrease in CCR5 expression in transitioning primary CD4+ T cells
Figure 2. MiRNA-103 modulates CCR5 expression in primary activated-to-memory transitioning CD4+ T cells

(A and B) Cell-surface CCR5 expression was measured by flow cytometry and compared in resting, activated, and relaxed CD4+ T cells obtained from healthy blood donors. In (B), data represent results obtained from cells of six different blood donors (2/4_). Error bars represent +/- SEM. Statistical significance was determined by the nonparametric Kruskal Wallis test, values: **p < 0.01, ***p < 0.001.

(C) CCR5 mRNA levels in resting, activated and relaxed CD4+ T cells (n = 6, 2/4_2) were measured by real-time qPCR. Shown are the mean fold changes compared to resting cells (in gray). Error bars represent SEM. Statistical significance was determined by the nonparametric Kruskal Wallis test, values: **p < 0.01, ***p < 0.001.

(D) MiRNA-103 and p53 mRNA levels in activated and relaxed CD4+ T cells (n = 6, 2/4_2) were measured by real-time qPCR. Shown are the mean fold changes compared to activated cells (in gray). Error bars represent SEM. Statistical significance was determined by the nonparametric Kruskal Wallis test, values: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

(E and F) Transitioning primary CD4+ T cells (D11, “day 11”) were transfected with FAM-labeled miRNA-103 inhibitor (purple) or control inhibitor (gray). In (E), mean fluorescence intensity (MFI) of cell surface CCR5 was measured and compared to the control inhibitor-transfected cells 48h after the transfection. Shown are data from a representative
was detectable between day 11 and day 15 (Figures 2A-2C), we assessed the role of miRNA-103 in this down-modulation by transfecting primary CD4+ T cells with an inhibitor (antagomir) of miRNA-103 at the peak of CCR5 expression (day 11). As shown in Figures 2E and 2F, transfection of the miRNA-103 inhibitor abolished the decrease of CCR5 expression with transfected cells displaying a significantly higher level of CCR5 at the surface (approximately 3.4-fold, p = 0.0079) relative to control cells. Overall, these results provide evidence that endogenous miRNA-103 downregulates the expression of CCR5 in primary CD4+ T cells progressing to a resting memory phenotype.

Stabilization of p53 leads to less CCR5 in primary CD4+ T cells through miRNA-103 upregulation

We then investigated the role and the modulation of p53 in CD4+ T cells by comparing the level of p53 mRNA in resting CD4+ T lymphocytes to that in cells at different stages post-activation. We show that p53 mRNA was strongly expressed in resting CD4+ lymphocytes as compared to activated or activated-to-memory transitioning CD4+ T cells (at day 11), which, indeed, displayed reduced p53 mRNA levels by approximately 80% (p = 0.0004) and 60% (p = 0.0479) (Figure 3A).

To investigate whether the low levels of p53 in activated and activated-to-memory transitioning CD4+ T cells impact miRNA-103 expression in these cellular stages, we treated activated primary CD4+ T cells for 24h with different concentrations of Nutlin-3, a Mdm2 inhibitor that stabilizes the p53 protein (Vassilev et al., 2004). Treatment with Nutlin-3 did not significantly affect cell viability, except at higher concentrations (20μM, 66.7% viability, p = 0.0011) (Figure S2A). Interestingly, cell treatment with 15μM of Nutlin-3 significantly increased miRNA-103 (2.7-fold, p = 0.0070), a condition that was linked to a decrease in CCR5 mRNA level by 60% (p = 0.0028) in these cells (Figure 3B). The effectiveness of the Nutlin-3 treatment on p53 stabilization was validated by the detection of a significant increase in expression of the p53-target gene, p21 (Figure 3B). As expected, p53 mRNA levels were not affected by Nutlin-3, which inhibits Mdm2-triggered p53 protein degradation but not p53 transcription (Figure S2B). Consistent with the upregulation of miRNA-103 expression upon Nutlin-3 treatment, we also observed a reduction in the frequency of cell-surface CCR5-positive-cells within 24h, using flow cytometry (12.5% CCR5+ cells compared to 9.9% CCR5+ with 10 and 15μM Nutlin-3, respectively) (Figure 3C). Importantly, we did not observe any significant effect of Nutlin-3 treatment on CXCR4 surface expression (Figure 3C). Together, these results indicate that miRNA-103 is also regulated by p53 in CD4+ T lymphocytes and that Nutlin-3-mediated stabilization of p53 leads to a reduction in the frequency of cells expressing CCR5. They also support the notion that p53 expression (or lack thereof) in activated CD4+ T lymphocytes transitioning to a resting memory phenotype likely impacts the control exerted by miRNA-103 on CCR5 expression.

Activated-to-memory transitioning CD4+ T cells are more susceptible to human immunodeficiency virus type-1 latent infection

Although CD4+ T cells that are transitioning from an activated stage to a resting memory phenotype express CCR5 and are susceptible to HIV-1 infection, their overall cellular environment is reported to not be conducive to HIV-1 gene expression, thus favoring the establishment of latent infection (Shan et al., 2017). To further validate these observations, we used the dual-fluorescent HIV-1 reporter vector, HI.fate, that encodes for the E2-Crimson gene under the control of the HIV-1 LTR, and the ZsGreen gene, under the control of an EF1-z promoter (Figure 4A). This virus reporter system can distinguish infected cells where HIV-1 expression is active (both E2-Crimson and ZsGreen-positive) from those that remain silent (only ZsGreen-positive), thus allowing for the analysis of latently or productively HIV-1 infected cells at a single cell level (Ratnapriya et al., 2021). Indeed, we show that upon the activation of HI.fate infected cells with anti-CD3 and anti-CD28 antibodies, the frequency of latently infected CD4+ T cells was reduced and a concomitant increase in the frequency of productively infected cells was detected (Figures S3A and S3B). Additionally, productively infected cells, which are predicted to express Vpu, were found to display downregulation of its cellular targets, CD4 and BST2, a condition not observed in uninfected or latently infected cells (Figure S3C).
Using this system, we analyzed the establishment of latently and productively HIV-1 infected cell populations upon the infection of activated CD4+ T cells or during their transition to a resting phenotype. Our data reveal that HIV-1 infection was overall comparable on day 11 (with a mean of 5.2% infection, n = 7, “activated-to-memory”), as compared to that on day 4 (mean of 4.3% infection, n = 7, “activated cells”) despite significant differences in the expression levels of CCR5 in these two cell populations (Figures 2, 4B and 4C, left panel). Interestingly, activated-to-memory transitioning cells showed a higher frequency of latent infection (2% of productively infected versus 3.2% of latently infected), when compared to activated infected cells (compare 3.1% productively infected to 1.2% latently infected (Figure 4C, right panel)). In contrast, cells on day 18 were largely refractory to infection despite expressing CCR5 at levels comparable to that on activated CD4+ T cells (day 4) (Figures 2 and 4C). This phenotype most likely reflects the multiple post-entry blocks reported in T cells with a resting phenotype (Baldauf et al., 2012). Taken together (Figure 4D), these results provide additional evidence that CD4+ T cells transitioning from an activated to a memory phenotype are more prone to latent HIV-1 infection.

Nutlin-3 decreases CD4+ T cell susceptibility to human immunodeficiency virus type-1 infection by inducing miRNA-103-mediated downregulation of CCR5

Given that we observed different levels of latent HIV-1 infection in CD4+ T cells depending on their transitioning phenotype, we investigated the impact of p53-regulated miRNA-103 on the establishment of latent and productive HIV-1 infections in post-activated CD4+ T cells. To this end, we infected Nutlin-3-pre-treated (15μM for 24h) CD4+ T cells with the HI.fate HIV-1 reporter virus pseudotyped with either HIV-1 JRFL-Env glycoproteins (CCR5-dependent HIV-1 entry) or VSV-G (CCR5-independent HIV-1 entry) for 48h (Figure 5A). When compared to control (DMSO), Nutlin-3 decreased the frequency of both
productive and latent populations by 50% in the context of CCR5-dependent HIV-1 infection (p = 0.0286) but not in the case of VSV-G-mediated entry (Figures 5B and 5C). Moreover, real-time qPCR analysis of infected cells revealed that CCR5 mRNA was reduced in Nutlin-3-treated cells which also had higher levels of miRNA-103 (Figure 5D). These results suggest that Nutlin-3 specifically impacts the entry of CCR5-tropic HIV-1 viruses by inducing miRNA-103-mediated CCR5 downregulation. To further establish this link, we took advantage of a highly sensitive Nano-Luciferase (NanoLuc) virus reporter system (Schmidt et al., 2020). Activated CD4+ T cells were treated with Nutlin-3 in the presence of a miRNA-103 inhibitor or a control inhibitor and then infected with JRFL-Env or VSV-G-coated NanoLuc HIV-1 viruses (NL4.3-NanoLuc-).
Figure 5. Nutlin-3 treatment decreases the susceptibility of transitioning CD4+ T cells to HIV-1 infection by downregulating CCR5 expression through miRNA-103 upregulation

(A) Experimental design of Nutlin-3 treatment and HIV-1 infection. (B and C) Primary CD4+ T cells were activated for 4 days, treated with DMSO or Nutlin-3 (15µM) for 24h, and then infected for 2 days with HI fate viruses expressing either the HIV-1 JRFL-Env or VSV-G. The frequencies of productively and latently infected cells were determined by flow cytometry based on E2-Crimson and ZsGreen expression. In (B), shown are data from a representative donor. In (C), data were obtained from cells of four different blood donors (29/24). Shown are the mean fold changes normalized to vehicle (DMSO)-treated cells. Error bars represent SEM. Statistical significance was determined by the nonparametric Kruskal Wallis test, values: *p < 0.05.

(D) Total RNAs from infected cells previously treated with vehicle (DMSO) or Nutlin-3 were extracted and expression of CCR5 mRNA, miRNA-103, and CXCR4 mRNA were analyzed by real-time qPCR. Shown are the mean fold changes compared to vehicle-treated infected cells. Error bars represent SEM. Statistical significance was determined by the nonparametric Kruskal Wallis test, values: *p < 0.05, **p < 0.01.

(E) Primary CD4+ T cells were activated for 4 days, transfected with the miRNA-103 inhibitor or control inhibitor, treated with vehicle (DMSO) or Nutlin-3 (N) at 15µM for 24h, and then infected with NL4.3-NanoLuc+ virus pseudotyped with HIV-1 JRFL-Env or VSV-G. Bald particles (without any viral glycoproteins) were also used as a control. Nano-Luciferase activity was measured in cell lysates after 30h of infection. Shown are the results obtained from four different blood donors (29/28). Error bars represent SEM. Statistical significance was determined by the nonparametric Kruskal Wallis test, values: **p < 0.01.

MiRNA-103 expression is inversely correlated with the levels of CCR5 mRNA in CD4+ T cells in vivo

In view of our data showing that CCR5 expression is regulated by miRNA-103, we investigated whether such a relationship could be observed in HIV-1-infected individuals that control or not HIV-1 infection (Deeks and Walker, 2007; Jiang et al., 2020; Okulicz et al., 2009). We measured the levels of miRNA-103 and CCR5 mRNA in CD4+ T lymphocytes isolated from ART-naïve progressors or in untreated individuals living with HIV-1 who can control the infection by maintaining an undetectable viral load, the so-called elite controllers (ECs) (Table 1). We observed that CD4+ T cells from HIV-1 ECs (n = 12) expressed significantly less CCR5 mRNA (5-fold, p = 0.0008) than those from ART-naïve progressors (n = 6). This decrease in CCR5 mRNA was accompanied by a pronounced increase in miRNA-103 expression in CD4+ T cells obtained from ECs (1.9-fold, p = 0.0047) as compared to those from ART-naïve progressors (Figure 6A). To assess whether viral replication may have impacted the overall expression of miRNAs, we analyzed the levels of miRNA-103 and CCR5 mRNA in healthy individuals. To this end, we found that the inverse relationship between miRNA-103 and CCR5 in HIV-1 infected individuals (Figure 6A) was also observed in healthy persons (r = −0.48, p = 0.0109), further strengthening the notion of the modulation of CCR5 by miRNA-103 (Figure 6B). In contrast, no correlation was observed between small nuclear RNA (snRNA)-U6 and CCR5 mRNA expression (r = −0.03). Overall, these results indicate that the extent of miRNA-103 expression is inversely correlated with the level of CCR5 mRNA in CD4+ T cells of HIV-1-infected individuals and further suggest that miRNA-103 may be a host factor contributing to the differential CCR5 expression observed among HIV-1 ECs and ART-naïve progressors.

DISCUSSION

Levels of CCR5 expression on CD4+ T cells influence not only the susceptibility to HIV-1 infection and, ultimately, the establishment of latent viral reservoirs, but also the preservation of highly functional CD4+ T lymphocyte populations for HIV-1 control (Lederman et al., 2006). Indeed, so far, cases of sterilizing cure have been achieved in patients that received allogeneic hematopoietic stem cell transplantation (HSCT) from donors homozygous for the CCR5-△32 deletion, which impairs surface expression of the CCR5 receptor and therefore confers resistance to infection (Gupta et al., 2019, 2020; Hütter et al., 2009). Furthermore, highly functional HIV-1-specific CD4+ T cells of ECs were found less susceptible to HIV-1 entry owing to a negative regulation of the CCR5 co-receptor, a characteristic that appears to protect these cells from depletion and promote HIV-1 control (Claireaux et al., 2022; Gonzalo-Gil et al., 2019; Meijerink et al., 2014). Thus, understanding how CCR5 is regulated in CD4+ T cells is essential for the development of CCR5-targeted therapies aimed at reducing the establishment of latent viral reservoirs and achieving a functional HIV-1 cure.
In this study, we provide evidence that miRNA-103, a miRNA regulated by p53, exerts a control on CCR5 mRNA levels in CD4+ T cells. We further show that the downregulation of p53 following CD4+ T lymphocyte activation and the resulting downregulation of miRNA-103 contributes to CCR5 upregulation in activated-to-memory transitioning CD4+ T cells, a population that is particularly prone to HIV-1 latent infection. These results extend our previous findings in HIV-1-infected macrophages where we identified miRNA-103 and its paralog miRNA-107 as important p53-regulated effectors of the antiviral response triggered by the proinflammatory cytokine IL-1ß, which downregulates CCR5 and renders macrophages refractory to HIV-1 entry (Lodge et al., 2020). As miRNA abundance relative to target mRNAs as well as the outcome of miRNA-mediated repression can vary in different cell types or under different cellular conditions (Dexheimer and Cochella, 2020), these results highlight a conservation of miRNA-103 targeting and repressing functions toward CCR5 expression in distinct immune cell types. Interestingly, miRNA-103 is not the only miRNA reported to modulate CCR5 mRNA expression. MiRNA-1224 was found to stimulate a /C01 ribosomal frameshift in the CCR5 mRNA sequence by forming a triplex RNA structure, which directs translating ribosomes to slip back one base and encounter a premature termination codon, thus leading to an unstable truncated form of CCR5 (Belew et al., 2014). A long non-coding RNA, CCR5AS, which is associated with HIV-1 disease outcome, also contributes to the post-transcriptional regulation of CCR5 mRNAs by protecting these from degradation induced by Raly proteins (Kulkarni et al., 2019). It has also been shown that the level of CCR5 in CD4+ T cells can be controlled by the cyclic adenosine monophosphate (cAMP)-dependent signaling pathway, and more specifically by the CREB1 transcription factor (Banerjee et al., 2011). Interestingly, our data reveal that although p53 mRNA levels decrease in activated CD4+ T cells (day 4) and activated-to-memory transitioning CD4+ T cells (day 11) as compared to resting T cells (Figure 3A), there is not an important fluctuation in the levels of miRNA-103 and p53 mRNA in the initial period after activation where CCR5 expression levels are increasing. This suggests that the levels of miRNA-103 during this period are not sufficient to counter the increase in CCR5 observed (Figure 2). Therefore, while miRNA-103 appears not directly involved in the upregulation of CCR5 that takes place following activation, the lack of sufficient up-modulation during that period (day 4 to day 11) is likely to facilitate CCR5 upregulation. Indeed, T cell activation has been shown to induce the demethylation of cis regions of CCR5, a process that conceivably contributes to CCR5 expression upregulation (Gornalusse et al., 2015). As CCR5 levels vary widely across T cell subsets, it is more than likely that this regulation is the result of several of these mechanisms acting together. Thus, miRNA-103 is part of the multiple mechanisms regulating CCR5 mRNA levels and variations in its abundance during the transition of CD4+ T cells from an activated stage to a resting memory phenotype influence their susceptibility to HIV-1 infection.

Our results confirm and extend the results of Shan and colleagues (Shan et al., 2017) showing that activated-to-memory transitioning CD4+ T cells are more prone to latent HIV-1 infection. Using a dual-reporter virus that distinguishes between productively and latently infected cells (Battivelli et al., 2018; Lopez et al., 2022; Ratnapriya et al., 2021), we show that the frequency of latent cells increases in activated-to-memory transitioning CD4+ T cells, which, indeed, display a transient increase in CCR5 expression. The mechanism involved in the gradual increase in CCR5 expression following the activation of the TCR with anti-CD3/CD28 antibodies appears partially linked to a decrease in the levels of p53 mRNA. These

Table 1. Clinical data of HIV-1 infected individuals included in the study

|                          | Elite controllers (EC) | ART-naïve progressors |
|--------------------------|------------------------|-----------------------|
| Number of participants   | 12                     | 6                     |
| Number of males/females  | 8/4                    | 5/1                   |
| Age in years (years) a,b | 42 (26–57)             | 35 (24–60)            |
| Duration of infection (year) a,b | 10.5 (2-29)          | <0.5                  |
| CD4 counts (cells/µL of blood) a,b | 735 (260-1080)    | 565 (510–810)        |
| CD8 counts (cells/µL of blood) a,b | 760 (260–1590)    | 1435 (1050–3832)     |
| Viral loads (copies/mL of plasma) a,b | <50                  | 145 557 (34 276-248 677) |
| Years of undetectable viral load (EC) a,b | 6 (1–20)             | N/A                   |

N/A: not applicable.

*aMedian with range.

bAt sample acquisition.
findings are consistent with results in mice showing that TCR signaling results in early termination of p53 protein expression by decreasing p53 mRNA as well as strong transcriptional induction of the p53-regulating E3 ubiquitin ligase, Mdm2. Down modulation of p53 in response to antigen stimulation is, indeed, a critical step for the proliferation of antigen-specific T cells in mice (Watanabe et al., 2014). This decrease in p53 mRNA levels following CD4+ T cell activation and its sustained low levels in activated-to-memory transitioning CD4+ T cells as compared to resting cells support the notion that the control exerted by p53-regulated miRNA-103 on CCR5 mRNA levels is drastically reduced and not effective after CD4+ T cell activation. Interestingly, however, miRNA-103 levels and control over CCR5 expression are gradually re-established as CD4+ T cells further progress to a resting memory phenotype. In line with the reported p53 mRNA decrease and Mdm2 upregulation after TCR signaling (Watanabe et al., 2014), pharmacological stabilization of the p53 protein by Nutlin-3, an inhibitor of Mdm2, resulted in an increase of miRNA-103 and a decrease of CCR5, which reduced HIV-1 entry and in consequence, viral infection. Importantly, the effect of Nutlin-3 was alleviated by the presence of miRNA-103 inhibitors demonstrating the involvement of this miRNA. Taken together, our results indicate that upregulating miRNA-103 by stabilizing p53 limits the levels of CCR5 expression after CD4+ T cell activation, a condition that dampens viral entry and the establishment of HIV-1 latent infection. These findings afford a sound scientific rationale for considering p53-activating drugs, which are currently tested in cancer clinical trials (Khurana and Shafer, 2019), as agents to limit the establishment of viral reservoirs in both T cells and macrophages in HIV-1 cure strategies.

Several studies have shown that PBMCs from ECs have a miRNA repertoire that is distinct from that of HIV-1 ART-naïve progressors, and such a difference may in part explain the control of viral infection in

Figure 6. Analyses of miRNA-103 and CCR5 mRNA expression levels in CD4+ T cells isolated from HIV-1-infected and healthy individuals

(A) CD4+ T cells from ART-naïve progressors (P) and elite controllers (EC) were isolated from PBMCs by negative selection. Following total RNA extraction, the expression of miRNA-103 and CCR5 mRNA in CD4+ T lymphocytes from EC (n = 12) and P (n = 6) were determined by real-time qPCR. Shown are expression levels (in fold change) relative to the mean of ART-naïve progressors (which was set to 1). Error bars represent +/- SEM. Statistical significance was determined by the nonparametric Mann-Whitney test, values: **p < 0.01, ***p < 0.001. See also Table 1.

(B) The inclusion of healthy individuals (n = 6, 3/3, purple dots) in the analysis as shown in (A) demonstrates a negative correlation between miRNA-103 expression and CCR5 mRNA levels in CD4+ T cells, as determined by Pearson r test, values: *p < 0.05. As controls, snRNA-U6 and CCR5 mRNA levels in these cells were compared by the Pearson r test.
ECs (Witwer et al., 2012; Reynoso et al., 2014; Ayala-Suárez et al., 2020). In this context, we assessed the levels of miRNA-103 and CCR5 mRNA in CD4+ T cells from ECs and ART-naïve progressors and found that CCR5 mRNA in CD4+ T cells obtained from ECs was significantly decreased when compared to that in CD4+ T cells from ART-naïve progressors, in line with recently published results (Claireaux et al., 2022; Gonzalo-Gil et al., 2019; Meijerink et al., 2014). Thus, in addition to displaying specific immune signatures (Krishnan et al., 2014; Nguyen et al., 2019), ECs could also control HIV-1 infection by limiting CCR5 expression levels, particularly in HIV-1-specific CD4+ T cells, via multiple mechanisms, a characteristic that results in decreased susceptibility to viral infection and depletion (Claireaux et al., 2022). Protection of highly functional CD4+ T cells in HIV-1 controllers may be sufficient to sustain a fully adaptive antiviral response and thus viral control. The upregulation in miRNA-103 expression that we observe in CD4+ T cells from ECs suggests that this miRNA contributes to CCR5 downregulation in HIV-1 controllers although the mechanism is not exclusive given that multiple transcriptional and post-transcriptional mechanisms regulate CCR5 expression and accumulation at the cell surface (Brelot and Chakrabarti, 2018). Nevertheless, pathways that enhance miRNA-103 have been reported to be upregulated in CD4+ T cells from ECs as compared to ART-naïve progressors. Indeed, increased p21 mRNA and protein in CD4+ T cells from the EC group suggest that the p53 pathway is more active in CD4+ T cells from these individuals and may explain the enhancement of p53-regulated miRNA-103 that we observe (Chen et al., 2011). Thus, the analysis of miRNA-103 and p53 in a larger cohort of ECs is, therefore, warranted to further assess the role of p53-regulated miRNA-103 in the modulation of CCR5 expression in these patients. Previous meta-analyses of genetic studies showed a genome-wide significant association between the CCR5 locus and viral load, highlighting the impact of CCR5 expression limitation on HIV-1 replication and control (McLaren et al., 2015). Indeed, it has been shown that PBMCs from ART-naïve progressors may have less total miRNAs than cells from ECs (Ayala-Suárez et al., 2020). As well, viral infection can have an impact on miRNA expression (Bennasser et al., 2005; Triboulet et al., 2007) as viral proteins such as Tat and Vpr have been reported to act as RNA silencing suppressors (Casey Klockow et al., 2013; Cojo et al., 2017). However, the fact that the negative correlation between miRNA-103 and CCR5 was also observed with healthy individuals supports the idea that this relationship is independent of plasma viral loads.

In conclusion, our study identifies miRNA-103 as a post-transcriptional negative regulator of CCR5 expression and HIV-1 entry in CD4+ T cells. We also provide evidence that the upregulation of this miRNA may limit the establishment of latent HIV-1 infection in CD4+ T cells. Lastly, we document that low-level CCR5 mRNA in HIV-1 ECs is associated with an enhancement of miRNA-103 expression. Given the central role played by CCR5 inactivation or downregulation in the natural control of HIV-1 infection (Tebas et al., 2021; Zeidan et al., 2021), strategies aimed at enhancing the expression of miRNA-103 might represent a promising complementary approach toward achieving a functional HIV-1 cure.

Limitations of the study
We acknowledge several limitations to our study. We show that the HIV-1 infection of activated-to-memory transitioning CD4+ T cells, which transiently express higher levels of CCR5, leads preferentially to latent infection. However, we are mindful to term these cells “activated-to-memory transitioning CD4+ T cells,” although no single phenotypic marker might faithfully identify these cells exclusively. It is also important to mention that the levels of CCR5, p53, and miRNA-103 in CD4+ T lymphocytes, as well as the efficiency of infection by a CCR5-tropic HIV-1 strain, are very variable in cells from one blood donor to another, which can lead to variations in the results.

Also, owing to the rarity of ECs and ART-naïve samples, the number of individuals included in this study from these two groups is low. We conclude that miRNA-103 might participate in the decrease in CCR5 observed in CD4+ T cells from ECs as compared to ART-naïve individuals; however, we cannot exclude that a different activation profile between the cells of the two groups plays an important role in this decrease. The number of cells per sample did not allow us to verify the activation profile of CD4+ T cells, but this variable could be included in future studies.

STAR+METHODS
Detailed methods are provided in the online version of this paper and include the following:
SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105234.

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AUTHOR CONTRIBUTIONS

N.B and E.A.C designed the study. N.B performed the experiments. N.B and E.A.C analyzed data. T.N.Q.P and R.L assisted with data analyses. J.J and T.T.M assisted with the characterization of the dual-fluorescent HIV-1 vector system, which was provided by A.H. J.P.R provided access to the cohort of ART-naïve progressors, whereas C.L.T and N.F.B provided samples from the cohort of slow progressors. N.B, R.L, T.N.Q.P, and E.A.C wrote the article. E.A.C secured funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Mouse anti-human CD3| BioLegend | Cat #300438, RRID: AB_11146991 |
| Mouse anti-human CD28| BioLegend | Cat #302934, RRID: AB_11148949 |
| Mouse anti-human CCR5 (BV421) | BioLegend | Cat #359118, RRID: AB_2563577 |
| Mouse anti-human CD4 (APC) | BioLegend | Cat #317416, RRID: AB_571959 |
| Mouse anti-human CD4 (PE/Cy7) | BioLegend | Cat #317414, RRID: AB_571945 |
| Mouse anti-human BST2 (PE) | BioLegend | Cat #384806, RRID: AB_10564402 |
| Mouse anti-human CD3 (Pacific Blue) | BioLegend | Cat #300330, RRID: AB_10551436 |
| Mouse anti-human CD25 (PE) | BioLegend | Cat #302066, RRID: AB_314276 |
| Mouse anti-human CD69 (PE) | BioLegend | Cat #310906, RRID: AB_314841 |
| Mouse anti-human HLA-DR (PE) | BioLegend | Cat #307606, RRID: AB_314684 |
| Mouse anti-human CXCR4 (PerCP/Cy5.5) | BioLegend | Cat #306516, RRID: AB_10642818 |
| **Bacterial and virus strains** | | |
| HIV: Hi.fate | Obtained from Dr. A. Herschhorn (Ratnapriya et al., 2021) | N/A |
| HIV: pNL4.3-Env^Vpr^-Luc^+ | Obtained from Dr. N.R. Landau (Connor et al., 1995) | N/A |
| HIV: pNL4.3-Env^NanoLuc^+ | Obtained from Dr. P. Bieniasz (Schmidt et al., 2020) | N/A |
| **Biological samples** | | |
| Healthy individuals (PBMCs) | Informed volunteers | N/A |
| Elite controllers (PBMCs) | Informed volunteers | N/A |
| ART-naïve progressors (PBMCs) | Informed volunteers | N/A |
| **Chemicals, peptides, and recombinant proteins** | | |
| Interleukin-2 | Peprotech | Cat #200-02-100UG |
| Interleukin-7 | Abcam | Cat #73201 |
| Dimethyl Sulfoxide (DMSO) | MP Biomedicals | Cat #191418 |
| Nutlin-3 | Sigma Aldrich | Cat #N6287 |
| Raltegravir | Santa Cruz Biotechnology | Cat # sc-364600 |
| **Critical commercial assays** | | |
| Fixable violet dead cell stain kit | ThermoFisher | Cat #L34963 |
| SuperScript II Reverse Transcriptase | ThermoFisher | Cat #18064-014 |
| RNaseOUT Recombinant Ribonuclease Inhibitor | ThermoFisher | Cat #10777-019 |
| RNase Mini plus kit | Qiagen Sciences | Cat #74136 |
| Human CD4+ isolation kit | Mitenyi Biotec | Cat #130-096-533 |
| Dead Cell Removal kit | Mitenyi Biotec | Cat #130-090-101 |
| Powerup Sybergreen Mastermix | Applied Biosystems | Cat #A25741 |
| Lipofectamine™ 3000 | ThermoFisher | Cat #L30000001 |
| Human T Cell Nucleofector Kit | Lonza | Cat #VPA-1002 |
| Luciferase Assay System | Promega | Cat #E1501 |
| Nano-Glo® Luciferase Assay System | Promega | Cat #N1110 |

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**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Éric A. Cohen (eric.cohen@ircm.qc.ca).

**Materials availability**
This study did not generate new unique reagents.

**Data and code availability**
- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Human subjects**
Peripheral blood samples and leukaphereses were obtained from HIV and HCV seronegative adults (of either sex). ART-naïve progressors (n = 6) were treatment-naïve adults with viral load (VL) > 30,000 copies of HIV RNA per mL (c/mL) of plasma. Elite controllers (n = 12) were treatment-naïve adults, tested for HIV-1 viral load every 6 months and having VL < 50 copies of HIV RNA per mL (c/mL) of plasma (Deeks and Walker,
ECs were enrolled in the Canadian Cohort of HIV Infected Slow Progressors. ART-naïve progressor PBMCs were obtained from the primary infection cohort of the FRQ-S AIDS and Infectious Diseases Research Network. The sex, age, and all clinical information of these individuals are summarized in Table 1.

All participants had given written informed consent in accordance with the Declaration of Helsinki under research protocols approved by the research ethics review board of the Institut de recherches cliniques de Montréal (IRCM).

**Method Details**

**Preparation of activated, activated-to-memory transitioning, and resting memory CD4+ T cells**

CD4+ T cells were isolated from peripheral blood mononuclear cells (PBMCs) by negative depletion using the human CD4+ Isolation Kit II (Miltenyi) according to manufacturer’s instructions. Cells were cultured in RPMI-1640 medium containing 10% heat inactivated fetal bovine serum (FBS) at 37°C, 5% CO2.

Resting CD4+ T cells were isolated by flow cytometry according to the following phenotypic markers: CD4+, CD3+, CD25, HLA-DR-, CD69-. Activated CD4+ T cells were generated by co-stimulation of resting cells with anti-CD3 (10 μg/mL) and anti-CD28 (2 μg/mL) and cultured in the presence of IL-2 (100U/mL) and IL-7 (100U/mL) for 4 days. To obtain activated-to-memory transitioning lymphocytes, activated cells were cultured in basal medium (RPMI-1640) with IL-2/IL-7 (20U/mL) without anti-CD3 and anti-CD28 antibodies.

**Flow cytometry**

Briefly, fluorochrome-labeled antibodies were added directly to the CD4+ T cells (1:150), incubated 45min on ice, washed twice in FACS buffer (PBS, 1% of FBS) and fixed with 4% paraformaldehyde in PBS. Finally, cells were resuspended in PBS-EDTA for analyses. Flow cytometry analyses and cell sorting were performed on a FACSAria III (BD), LSR Fortessa (BD) or CyAn (Beckman) equipped with appropriate lasers. Acquired data were analyzed with FlowJo 10 software (TreeStar).

**RNA extraction, reverse-transcription, and real-time qPCR analyses**

Total cellular RNAs were extracted using RNeasy RNA extraction columns (Qiagen) according to the manufacturer’s instructions and conserved at −80°C. Total RNAs (100 to 200 ng) were reverse transcribed using SuperScript II reverse transcriptase (Invitrogen) with poly(dT) and specific loop primers for the appropriate microRNAs using the two-tailed real-time qPCR method (Androvic et al., 2017). For real-time qPCR, cDNA and appropriate primers (see Table S1) were added to SYBR green select master mix (Applied Biosystems) in 96-well plates and run on a Viia96 thermocycler (Thermo Fisher Scientific) with the following cycling conditions: 50°C for 2 min, 95°C for 5 min, and 40 cycles of 95°C for 20 sec and 60°C for 40 sec. Dissociation curve analysis after the end of the PCR confirmed the presence of a single and specific product. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or small nuclear RNA (snRNA) U6 were used as endogenous controls. Control conditions, as described in each figure legend, were set at 1 to determine the fold change.

**Transfection of C10/MJ cells and primary CD4+ T cells**

Primary CD4+ T cells or C10/MJ cells were transfected with either control RNA, mimics of miRNA-103, or inhibitors of miRNA-103 by nucleofection (Lonza) according to the manufacturer’s instructions and cultured for 48h in 48-well plates in basal medium (RPMI-1640) supplemented with IL-2/IL-7 (100U/mL). The miRNA-103 and negative control miRCURY locked nucleic acid (LNA) mimics, or the miRNA-103 and negative control inhibitors were FAM-labeled (see key resources table).
Virus production
Pseudotyped double fluorescent HI.fate (JRFL-Env or VSV-G), NL4.3-Vpr’Luc’-(ADA-Env or VSV-G) and NL4.3-NanoLuc’-(JRFL-Env or VSV-G) were generated by co-transfecting the corresponding proviral constructs with HIV-1 Env or VSV-G expression vectors in 5 x 10^6 HEK 293T cells using lipofectamine 3000. In all cases, HEK 293T cells were maintained in DMEM medium supplemented with 10% heat inactivated FBS at 37°C in a 5% CO_2 incubator. Virus-containing supernatants were recovered 48h post-transfection. Supernatants were cleared of cells by centrifugation at low speed, filtered and virus pellets recovered following ultracentrifugation on a 20% sucrose cushion. Viruses were resuspended in DMEM, and aliquots kept at −80°C. The multiplicity of infection (MOI) was determined using the CEM.NKR-CCR5 cell line.

Infection of primary CD4+ T cells
Primary CD4+ T cells were infected with NL4.3-based viruses at MOI of 1 (VSV-G) or 2 (HIV-1 CCR5 tropic strains) by spinoculation (centrifugation for 2h at 1200g, 22°C). In the case of HI.fate-based viruses, treatment with Raltegravir (15µM) was used as a negative control. Cells were pre-treated 2 hours before infection and thereafter, the drug was retained throughout the infection. Infection rates were determined 48h post-infection by measurement of E2-Crimson-positive (Crim+), and GFP-positive (GFP+) cells by flow cytometry.

Cells infected with NL4.3-Vpr’Luc’ or NL4.3-NanoLuc’ virus were washed 6h after viral adsorption and lysed 48h (F-Luc) or 30h (NanoLuc) post-infection. The levels of viral entry were determined by measuring Luciferase activity using either the F-Luc or the Nano-Glo Luciferase Assay Systems on a GloMax luminometer (Promega).

Nutlin-3 treatment
Primary CD4+ T cells previously activated with anti-CD3 (10 µg/mL) and anti-CD28 (2 µg/mL) and cultured in the presence of IL-2/IL-7 for 4 days were treated with the Mdm2 inhibitor Nutlin-3 (Sigma) at 15µM, unless mentioned otherwise, for 24h in RPMI-1640 supplemented with IL-2/IL-7 (100U/mL). Cell death was determined using the live/dead fixable violet dead cell stain kit (ThermoFisher).

QUANTIFICATION AND STATISTICAL ANALYSES
Statistical analyses were performed using GraphPad Prism (Version 8). Non-parametric Kruskal Wallis tests were used to compare ranks between more than two groups. Non-parametric Mann-Whitney’s U-tests (two-tailed) were used to compare ranks between two groups. A p value of less than 0.05 was considered statistically significant. *, **, ***, **** signify <0.05, <0.01, <0.001, <0.0001, respectively; ns, not significant.