Elevated expression of miR-155 is associated with the differentiation of CD8+ T cells in patients with HIV-1

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Abstract. The differentiation and response ofCD8+ T cells is vital in host defense against human immunodeficiency virus type 1 (HIV-1). MicroRNA (miR)-155 is an important regulator of T cell differentiation. However, the profile of miR-155 in HIV-1 infected individuals and its association with CD8+ T cell differentiation remain to be fully elucidated. The present cross-sectional study was performed involving 63 HIV-1-infected patients undergoing highly active antiretroviral therapy (HAART), 31 HAART-naïve patients and 35 healthy controls. The levels of miR-155 in CD8+ T cells were detected using reverse transcription-quantitative polymerase chain reaction analysis. Subsets of CD8+ T cell differentiation were detected using flow cytometry. The results revealed that the discord controllers and HAART-naïve patients showed higher percentages of effector and effector memory cells, and lower percentages of naïve cells (P<0.05). The levels of miR-155 in CD8+ T cells from the HIV-1-infected patients were higher, particularly in the discord controllers and HAART naïve patients (P<0.01). The expression levels of miR-155 were positively correlated with the percentages of effector and effector memory CD8+ T cells, and negatively correlated with the percentages of naïve and central memory CD8+ T cells (P<0.01). Taken together, these findings suggested that the levels of miR-155 in CD8+ T cells of patients with HIV-1 were increased and associated with CD8+ T cell differentiation.

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Abbreviations: HIV, human immunodeficiency virus; CTLs, cytotoxic T lymphocytes; HAART, highly active antiretroviral therapy; PBMCs, peripheral blood mononuclear cells; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

Key words: human immunodeficiency virus, highly active antiretroviral therapy, microRNA-155, CD8+ T cells, differentiation, subsets

Introduction

Human immunodeficiency virus type 1 (HIV-1) preferentially destroys CD4+ T lymphocytes and leads to disturbed T cell homeostasis, characterized by the depletion of CD4+ T cells (1). The sustained deletion and dysfunction of CD4+ T cells caused by HIV-1 infection can result in opportunistic infections and tumors, diagnosed as acquired immune deficiency syndrome (1-3). The majority of attempts to manage HIV-1 infection have focused on CD4+ T cell recovery, whereas changes in CD8+ T cells have received less attention. However, the overall course of HIV-1 infection is largely shaped by CD8+ T cell responses. The CD4/CD8 ratio has been reported to be a useful marker for clinical outcome, immune dysfunction and viral reservoir size in HIV-1-infected patients (4,5). Cytotoxic T lymphocytes (CTLs), the critical effector CD8+ T cells, are vital in host defense against HIV-1 by impeding viral replication through cytolytic and non-cytolytic pathways (6). The dynamics of effector CD8+ T cell expansion in acute HIV-1 infection is similar to that in other viral infections (7). However, CD8+ T cells in patients with chronic HIV-1 infection exhibit characteristics of exhaustion and immunosenescence (7,8). Although may patients with HIV-1 maintain high circulating CD8+ T cell counts, the suppression of HIV-1 replication is attenuated by the disturbed differentiation and homeostasis of the CD8 T cell compartment (9).

The molecular mechanisms controlling peripheral CD8+ T cell differentiation in humans remain to be fully elucidated. Several microRNAs are critical in the development of hematopoietic cells (10,11). A study by Zhang and Bevan demonstrated that CD8+ T cells with knockout of dicer are defective in cell accumulation and survival (12). Compared with naïve CD8+ T cells, miRNA (miR)-21 and miR-155 were found to be upregulated in effector CD8+ T cells (13). The sustained expression of miR-155 has been associated with effector and effector memory cells, whereas lower expression levels of miR-155 have been associated with central memory cells (14). miR-155 has also been shown to be an important regulator for the

differentiation of T helper cells in HIV-1-infected individuals, particularly Th17 and regulatory T cells (15,16). However, the profile of miR-155 in HIV-1-infected individuals and its association with $CD8^+$ T cell differentiation remain to be fully elucidated.

In the present study, the expression levels of miR-155 were investigated in $CD8^+$ T cells from patients with HIV-1, with or without highly active antiretroviral therapy (HAART), and correlation between the levels of miR-155 and percentages of $CD8^+$ T cell subsets was examined. It was found that the expression of miR-155 in $CD8^+$ T cells of patients with HIV-1 was increased, particularly in the discord controllers and HAART-naïve patients. The level of miR-155 in $CD8^+$ T cells was positively correlated with the percentages of effector and effector memory subsets, and negatively correlated with the percentages of naïve and central memory subsets.

Subjects and methods

Subjects. Patients with HIV-1 were recruited from the First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China) between June 2015 and December 2015. HIV-1 infection was diagnosed on the basis of positive results from serological and HIV-1 RNA detection assays. The subjects were excluded if they had received systemic antibiotics, vaccination or any immunomodulatory drug in the previous 3 months. A total of 35 apparently healthy uninfected control subjects from the community clinics were also recruited. The present study received approval from the Ethics Review Boards of the First Affiliated Hospital, School of Medicine, Zhejiang University (approval no. 2015-06103). All subjects were volunteers and provided written informed consent prior to involvement in the study.

Flow cytometry. The subsets of $CD8^+$ T cells were analyzed using four-color flow cytometry with a FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and a commercial flow cytometry assay kit (BD Biosciences). Cell staining was performed at room temperature using a cocktail of the following fluorochrome-conjugated antibodies, at the manufacturer's recommended dilution: Anti-CD3-PerCP-CY5.5 (cat. no: 561478), anti-CD8-APC-Cy7 (cat. no: 561967), anti-CD45RA-PE (cat. no: 560975) and anti-CD62L-FITC (cat. no: 561914) (BD Biosciences). Briefly, 50 μl whole blood was added to the antibody cocktail, mixed and then incubated for 20 min in the dark at room temperature. The red cells in whole blood were lysed with red cell lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China), washed three times with 1 ml phosphate-buffered saline, and subsequently detected using FACScan flow cytometry. The percentages of $CD3^+CD8^+CD45RA^+CD62L^+$, $CD3^+CD8^+CD45RA^-CD62L^-$, $CD3^+CD8^+CD45RA^+CD62L^-$ and $CD3^+CD8^+CD45RA^-CD62L^+$T cells were determined.

Detection of miR-155. Peripheral blood mononuclear cells (PBMCs) were isolated from all patients from 5 ml venous whole blood samples, using a density gradient centrifugation method with Ficoll-Paque PLUS (GE Healthcare Life Sciences, Marlborough, MA, USA) at 2,000 x g for 10 min at room temperature. The $CD8^+$ T lymphocytes were purified from the PBMCs using MACS human CD8 microbeads for positive selection (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of the $CD8^+$ T lymphocytes was >90%. The total RNA was extracted from the $CD8^+$ T lymphocytes using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The quantitative analysis of miR-155 was performed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis with a Bulge-Loop™ miRNA qRT-PCR Starter kit (Guangzhou RiboBio, Co., Ltd., Guangzhou, China) and thehsa-miR-155 qRT-PCR primer set (Guangzhou RiboBio, Co., Ltd.). A U6 small nuclear RNA primer set (Guangzhou RiboBio, Co., Ltd.) was used as the internal control. The experiments were performed according to the protocol provided in the manufacturer of the kit using a 10 μl reaction system. Briefly, the miRNA RT reaction mix included 1 μl RNA template (68 ng/μl), 1 μl miRNA RT primer, 2 μl 5X reverse transcription buffer, 2 μl RTase mix and 4 μl RNase-free water. The mix was incubated at 42˚C for 60 min followed by incubation at 70˚C for10 min. The miRNA qPCR reaction system contained 10 μl SYBR-Green Master mix, 0.8 μl miRNA forward primer, 0.8 μl miRNA reverse primer and 2 μl RT product; the final volume was normalised to 20 μl with DNase-free water. Real-time PCR was performed for 40 cycles of denaturation (95˚C, 45 sec), annealing (62˚C, 30 sec) and extension (72˚C, 30 sec). Double-stranded DNA was measured at 86˚C following each cycle. Each sample was repeated three times. The relative expression levels of miRNA were calculated using the $2^{\Delta\Delta Cq}$ method (17).

Statistical analysis. Statistical analyses were performed using SPSS for Windows version 20.0 (IBM SPSS, Armonk, NY, USA). Student's t-test was used to compare between two groups and one-way analysis of variance was used when comparing more than three groups. χ^2 was used for categorical variables. The correlation was tested using Spearman's correlation test. All tests were two-tailed. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical information of subjects. A total of 94 HIV-1-infected patients were recruited to the present study, including 31 HAART-naïve patients and 63 patients receiving HAART. The patients receiving HAART were divided into two groups according to the recovery of their $CD4^+$ T counts and viral suppression: 34 typical controllers had a $CD4^+$ T count >500 cells/ml and a viral load <400 copies/ml; 29 discord controllers had a $CD4^+$ T count <500 cells/ml or a viral load >400 copies/ml. The three groups of HIV-1-infectedpatientsand normal controls were all appropriately age- and sex-matched. The mean durations of HIV-1 infection for the typical and discord controllers were 6.38±2.51 and 4.96±1.36 years, respectively, which were significantly longer, compared with that for the HAART naïve patients (2.12±1.43 years; $P<0.05$). The durations of HAART for the typical and discord controllers were 4.17±1.13 and 3.84±1.62 years, respectively. The typical controllers had a significantly lower viral load, and a higher $CD4^+$ T cell count and CD4/CD8 ratio, compared with the other HIV-1 patients ($P<0.05$). The regimens for

Table I. Clinical data from the study participants.

Characteristic	Typical controller (n=34)	Discord controller (n=29)	HAART-naïve (n=31)	Control (n=35)	P-value
Males, n (%)	21 (62%)	17 (59%)	19 (61%)	27 (77%)	0.317
Age (years)	41.57±10.01	38.35±15.36	36.41±13.47	39.63±11.79	0.254
Years with HIV-1	6.38±2.51	4.96±1.36	2.12±1.43	NA	0.027
Years on HAART	4.17±1.13	3.84±1.62	NA	NA	0.184
Viral load (log 10)	1.631±0.75	3.91±0.49	4.32±1.54	NA	<0.001
$CD4^+$ cells (/μl)	655.45±263.81	353.68±187.95	431.77±284.56	798.32±261.19	0.001
$CD8^+$ cells (/μl)	618.76±330.17	785.35±296.54	801.52±272.21	518.23±241.36	0.008
CD4/CD8 ratio	1.21±0.42	0. 54±0.22	0.57±0.30	1.63±0.41	0.017
HAART regimen, n (%)					0.116
d4T+3TC+NVP	11 (32%)	9 (31%)	NA	NA	-
AZT+3TC+NVP	17 (50%)	12 (41%)	NA	NA	-
Other	6 (18%)	8 (28%)	NA	NA	-

Data are presented as the mean ± standard deviation. HIV-1, human immunodeficiency virus; HAART, highly active antiretroviral therapy; NA, not applicable.

patients receiving HAART were primarilyd 4T+3TC+NVP and AZT+3TC+NVP (49 cases; 78%). The detailed participant data is presented in Table I.

Subsets of circulating $CD8^+$ T cells are altered in patients with HIV-1. Based on the expression of CD45RA and CD62L, human $CD8^+$ T cells can be divided into four subsets with distinct homing and functional properties: Naïve ($CD45RA^+CD62L^+$), central memory ($CD45RA^-CD62L^+$), effector memory ($CD45RA^-CD62L^-$) and effector ($CD45RA^+CD62L^-$) cells (18,19). In the present study, the $CD8^+$ T cell subsets were determined in patients with HIV-1. Compared with the normal controls and typical controllers, the discord controllers and HAART naïve patients showed higher percentages of effector and effector memory $CD8^+$ T cells, and a lower percentage of naïve $CD8^+$ T cells (P<0.05; Fig. 1). The typical controllers had a similar composition of $CD8^+$ T cell subsets to the normal controls, but had a higher percentage of central memory $CD8^+$ T cells (P<0.05; Fig. 1).

Expression of miR-155 is elevated in $CD8^+$ T cells of patients with HIV-1. The present study compared the expression levels ofmiR-155 in $CD8^+$ T cells of typical and discord controllers with HAART and HAART-naïve patients with normal levels. It was found that the levels of miR-155 in $CD8^+$ T cells of all three groups of HIV-1 patients were significantly higher, compared with that in the normal controls (P<0.05; Fig. 2). Although increased, the expression levels of miR-155 in the $CD8^+$ T cells of typical controllers were almost normal, and were significantly lower, compared with the levels in the discord controllers and HAART-naïve patients, in which viremia was not suppressed (P<0.01; Fig. 2).

Correlation between levels of miR-155 and percentages of $CD8^+$ T cell subsets in patients with HIV-1. To determine whether miR-155 is associated with $CD8^+$ T cell differentiation in HIV-1-infected individuals, the present study analyzed the correlation between levels of miR-155 and percentages of $CD8^+$ T cell subsets. In all patients with HIV-1, the expression of miR-155 in $CD8^+$ T cells was positively correlated with the percentages of effector and effector memory $CD8^+$ T cells (r=0.692 and 0.803 respectively; P<0.01; Fig. 3), and was negatively correlated with the percentage of naïve and central memory $CD8^+$ T cells (r=-0.457 and -0.522 respectively; P<0.01; Fig. 3).

Discussion

The cellular immune response is critical in controlling the viral replication of HIV-1 (6-8). The majority of circulating $CD8^+$ T cells in healthy individuals are naïve and central memory cells. When combined with antigens, $CD8^+$ T cells become activated and undergo a program of clonal expansion (20). The majority of effector cells die through apoptosis whereas certain surviving CD8 T cells become long-lasting virus-specific memory $CD8^+$ T cells, forming a protective mechanism upon antigen re-exposure (21,22). In the present study, higher percentages of effector and effector memory cells, and a lower percentage of naïve $CD8^+$ T cells were found in discord controllers and HAART-naïve patients, in which viral replication was not suppressed. In typical controllers with higher $CD4^+$ T counts and suppressed viremia, the $CD8^+$ T cell subsets were restored almost to normal levels. These results are consistent with previous studies, which reported that the majority of circulating $CD8^+$ T cells in individuals with chronic HIV-1 infection were mature effector and effector memory $CD8^+$ T cells (23,24). However, in a cross sectional study by Groves *et al* (19), discord controllers and typical controllers had higher numbers of naive $CD8^+$ T cells and reduced $CD8^+$ T cell activation, compared with the patients with rapidly progressing disease. Notably, Groves *et al* defined discord controllers as patients with

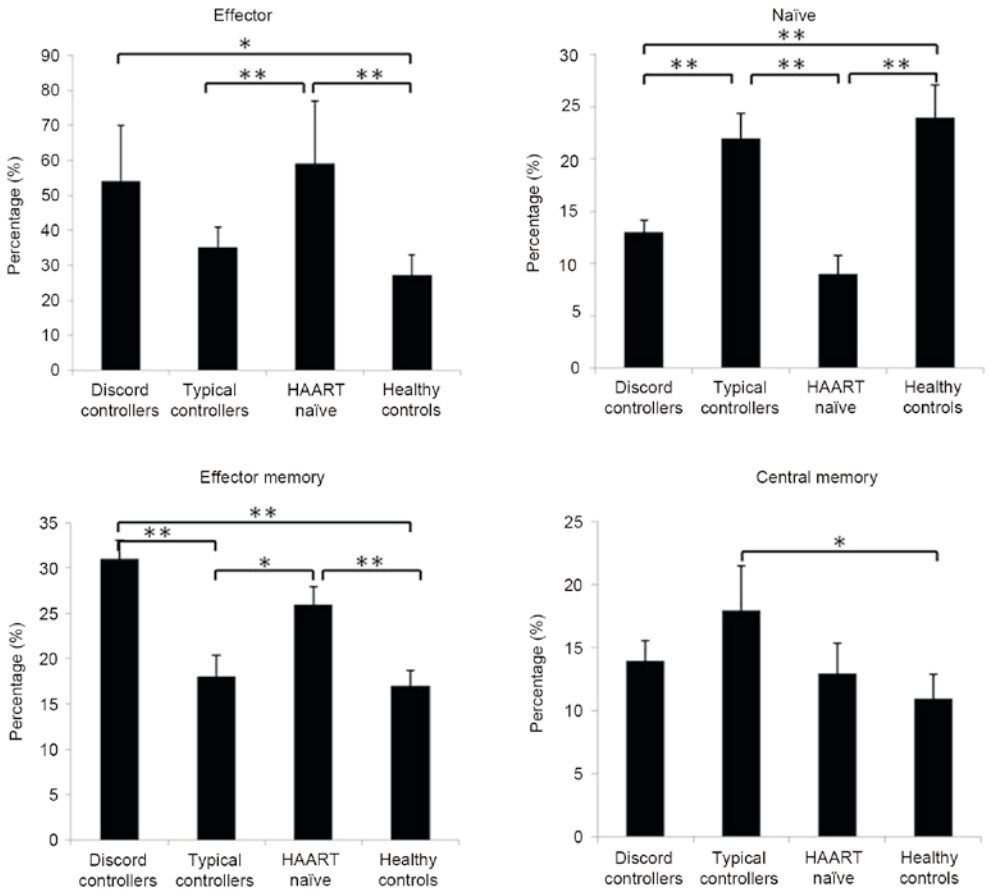

Figure 1. Percentage of CD8+ T cell subsets in patients with human immunodeficiency virus type 1 and healthy controls. The subsets of CD8+ T cells were analyzed using four-color flow cytometry. Based on the expression of CD45RA and CD62L, CD8+ T cells were subdivided into four major subsets: Naïve ($CD45RA^+CD62L^+$), central memory ($CD45RA^-CD62L^+$), effector memory ($CD45RA^-CD62L^-$) and effector ($CD45RA^+CD62L^-$) cells. *P<0.05; **P<0.01. Data are presented as the mean ± standard deviation. HAART, highly active antiretroviral therapy.

a viral RNA load <2,000 copies/ml and <450 CD4+ T cells/mm^3, with the viral load of discord controllers ranging between 100.3 and 1,043.0 copies/ml. In the present study, the viral load of the discord controllers ranged between 1,491 and 23,386 copies/ml. The higher naïve CD8+ T cells and lower CD8+ T cell activation may be associated with lower virus replication, although no direct association between CD8+ T cell subsets and HIV-1 RNA load was found. These results suggested that a more preserved CD8+ T cell compartment is associated with the control of plasma viremia.

The expansion of effector CD8+ T cells is associated with certain microRNAs, which can control gene expression at the post-transcriptional level. miR-155 is an important microRNA, which regulates the immune response and is important in controlling lymphocyte differentiation at multiple levels (25). miR-155 is essential for normal B cell differentiation and antibody production (26), and controls the differentiation of CD4+ T cells into the Th1, Th2, and Th17 subsets of helper T cells (15,27). In addition, miR-155 is essential for efficient antigen presentation by dendritic cells (28). miR-155 is also reported to regulate CD8+ T cell differentiation. Antigen-specific CD8+ effector T cells express high levels of miR-155 (29). Naïve and central memory cells express low levels of miR-155, and effector memory cells express intermediate levels of miR-155 (13). However, the association between miR-155 and CD8+ T cell differentiation in HIV-1-infected individuals has not been reported. In the present study, increased levels of miR-155 were found in CD8+ T cells of HIV-1-infected patients, which was higher in the discord controllers and HAART-naïve patients. The expression of miR-155 in

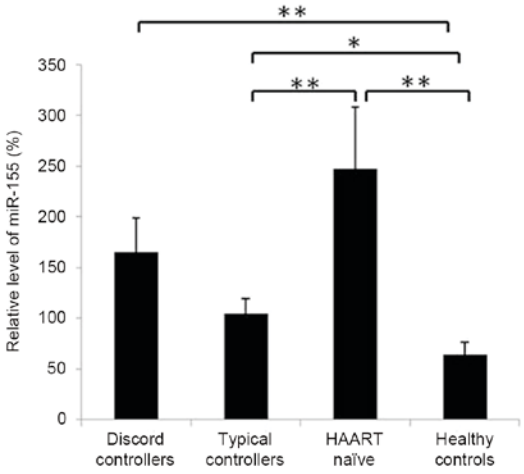

Figure 2. Relative levels of miR-155 in CD8+ T cells of patients with human immunodeficiency virustype1 and healthy controls. CD8+ T lymphocytes were purified from peripheral blood mononuclear cells using MACS human CD8 microbeads. Total RNA was isolated and miR-155 was detected using reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean ± standard deviation. *P<0.05; **P<0.01. miR, microRNA; HAART, highly active antiretroviral therapy.

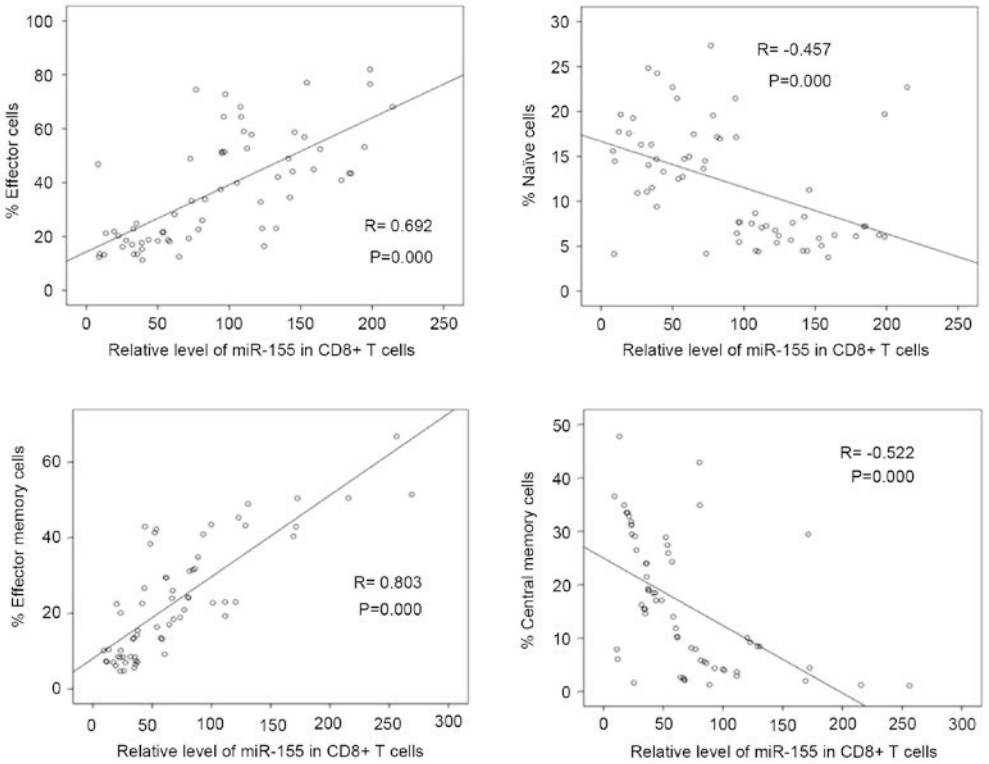

Figure 3. Correlation between levels of miR-155 and percentages of $CD8^+$ T cell subsets inpatients with human immunodeficiency virus1 infection. Spearman's correlation test was used. miR, microRNA.

$CD8^+$ T cells was positively correlated with the percentages of effector and effector memory $CD8^+$ T cells, and negatively correlated with the percentages of naïve and central memory $CD8^+$ T cells. These results were consistent with those of previous studies on other pathogen infections. Lind *et al* (30) demonstrated that miR-155 was essential for optimal $CD8^+$ T cell responses against influenza virus and *Listeria* infection, and was crucial for the generation of $CD8^+$ T cell memory against pathogens. Tsai *et al* (14) showed that miR-155 was an important regulator in effector and memory virus-specific $CD8^+$ T cell responses in murid herpes virus 68-infected mice. Compared with the wild-type mice, chimeric mice lacking miR-155 in $CD8^+$ T cells showed a weaker effector response and a skewing toward memory precursor cells with significantly higher viral titers (14). However, the present study found no correlation between the levels of miR-155 levels and virus replication.

The expression of miR-155 has been reported to be associated with HIV infection. miR-155 can affect disease progression by regulating the transformation of naïve Tregs and naïve CD4 subsets into activate subsets (31,32). In addition, miR-155 exerts an anti-HIV-1 effect by targeting several HIV-1 dependency factors involved in post-entry and pre-integration events, for example, DC-SIGN and TRIM32, leading to severely reduced HIV-1 infection (33). The present study suggested that miR-155 may be an important regulator of the CTL response in patients with HIV. However, how the expression of miR-155 is altered by HIV-1 infection remains to be elucidated, as does the causal association between the expression of miR-155 and virus replication. The mechanisms underlying altered levels of miR-155 in the $CD8^+$ T compartment of HIV-1-infected patients requires further investigation. A limitation of the present study is that miR-155 was detected in total $CD8^+$ T cells, but not in subsets. Further investigations on the expression of miR-155 in subsets of $CD8^+$ T cells in patients with HIV-1 may provide additional information onmiR-155 and the differentiation of $CD8^+$ T cells.

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