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Profiles of MicroRNAs in Interleukin–27-Induced HIV-Resistant T Cells: Identification of a Novel Antiviral MicroRNA

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Objectives: Interleukin-27 (IL-27) is known as an anti-HIV cytokine. We have recently demonstrated that IL-27-pretreatment promotes phytohemagglutinin-stimulated CD4(+) T cells into HIV-1-resistant cells by inhibiting an uncoating step.

Purpose: To further characterize the function of the HIV resistant T cells, we investigated profiles of microRNA in the cells using microRNA sequencing (miRNA-seq) and assessed anti-HIV effect of the microRNAs.

Methods: Phytohemagglutinin-stimulated CD4(+) T cells were treated with or without IL-27 for 3 days. MicroRNA profiles were analyzed using miRNA-seq. To assess anti-HIV effect, T cells or macrophages were transfected with synthesized microRNA mimics and then infected with HIVNL4.3 or HIVAD8. Anti-HIV effect was monitored by a p24 antigen enzyme-linked immunosorbent assay kit. interferon (IFN)-α, IFN-β, or IFN-λ production was quantified using each subtype-specific enzyme-linked immunosorbent assay kit.

Results: A comparative analysis of microRNA profiles indicated that expression of known miRNAs was not significantly changed in IL-27-treated cells compared with untreated T cells; however, a total of 15 novel microRNAs (miRTC1 ~ miRTC15) were identified. Anti-HIV assay using overexpression of each novel microRNA revealed that 10 nM miRTC14 (GenBank accession number: MF281439) remarkably suppressed HIV infection by (99.3 ± 0.27%, n = 9) in macrophages but not in T cells. The inhibition was associated through induction of >1000 pg/mL of IFN-αs and IFN-λ1.

Conclusion: We discovered a total of 15 novel microRNAs in T cells and characterized that miRTC14, one of the novel microRNAs, was a potent IFN-inducing anti-HIV miRNA, implicating that regulation of the expression of miRTC14 may be a potent therapeutic tool for not only HIV but also other virus infection.

Key Words: microRNA, IL-27, interferon, antiviral RNA, CD4(+) T cells, macrophages

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S.G. and X.H. performed assays, analyzed data and wrote the manuscript, Q.C. and D.P. performed assays. J.Q. and B.T.S. analyzed data, J.Y. analyzed data and reviewed the manuscript. W.C. analyzed data and oversaw analyzed data and reviewed the manuscript. T.I. created the project, performed assays, analyzed data, oversaw the project, and wrote the manuscript.

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INTRODUCTION

MicroRNAs (miRNAs) are small (19–22 nucleotides in length) noncoding RNAs that are critical regulators of translation and turnover of messenger RNAs (mRNAs). MiRNAs post-transcriptionally regulate eukaryotic gene expression and play important roles in many biological cellular functions including cell growth, apoptosis, and gene regulation. They are involved in the pathological progress of human diseases such as cancer, vascular disease, immune disease, and infections.[1] These miRNAs generally bind to target areas in the 3′ untranslated region (3′UTR) of mRNAs through the RNA induced silencing complex, which ultimately leads to mRNA degradation or translational repression.[2] More than half of all mRNAs are believed to have 3′UTR containing a complementary sequence of 2–7 nucleotides at the 5′ end of the miRNA (the so-called "seed" sequence).[3] However, it is reported that some miRNAs target the noncanonical regions in mRNAs and regulate cellular functions.[4,5] Because miRNAs were first discovered in 1993, there have been increasing reports of novel miRNAs, and the latest Sanger miRNA database (miRbase.org) has reported nearly 2300 human miRNAs.[6]

Interleukin (IL)-27 is a member of the IL-12 cytokine family (IL-12, IL-23, IL-27, IL-35, and IL-39)[7–10] and forms a heterodimeric cytokine composed of p28 (also known as IL-30) and the Epstein–Barr virus induced gene 3 (EBI3) subunits.[11] It is known that IL-27 is involved in the activation of both innate and adaptive immune responses[9] and is able to suppress different virus species including HIV,[12–15] hepatitis C virus,[16] hepatitis B virus,[17] influenza virus,[18] and cytomegalovirus[19]; therefore, it is considered as a potent antiviral therapeutic reagent. We previously demonstrated that IL-27 is able to induce novel miRNAs in macrophages and dendritic cells (DCs) and identified that 6 novel miRNAs (GenBank accession number: KC832799 ~ KC832805, miRBase name: has-miR-6852, has-miR-7702, has-miR-7703, has-miR-7004, has-miR-7705, and has-miR-7006) in macrophages[20] and 22 novel miRNAs in DCs (GenBank Accession number: KY994043 ~ KY994064),[21] and predicted that some of them target viral genes and potent antiviral RNAs.

Recently, we reported that IL-27-pretreatment of activated primary CD4(+) T cells confers the resistance to HIV infection.[22] To define the mechanism of HIV resistance, the gene expression profile by microarray analysis and proteomic mass analysis using 2-dimensional difference in gel electrophoresis (2D-DIGE) were performed. The microarray analysis displayed no significant differences in the expression of 25,000 messenger RNAs;[23] on contrary, 2D-DIGE analysis demonstrated a significant increase in acetylation of Y box-1 (YB-1), a DNA and RNA binding protein, and we found that the acetylated YB-1 suppressed uncoating of HIV core protein after infection.

In the current study, we analyzed the miRNA profile in the HIV resistant IL-27-treated T cells to further elucidate the function of the cells. We report a total of 15 novel miRNAs and a functional analysis reveals that one of the miRNAs (miRTC14, GenBank Accession number: MF281439) is a powerful anti-HIV miRNA that induces multiple subtypes of interferons.

MATERIALS AND METHODS

Ethics Statement

All experimental procedures in these studies were approved by the National Cancer Institute at Frederick and National Institute of Allergy and Infectious Diseases (NIAID) Institutional Review Board and performed in accordance with the relevant guidelines and regulations. Participants were informed written consent before blood being drawn.

Preparation of Cells and Viruses

CD4(+) T cells and CD14(+) monocytes were isolated from peripheral blood mononuclear cells of healthy donors.[12,15,24] HIV-resisting IL-27-treated T cells (27-Tc) and control T cells (Ctrl-Tc) were prepared as previously described.[22] In brief, T cells were stimulated with phytohemagglutinin (MiliporeSigma, St. Louis, MO) and then cultured for an additional three-days with or without 100 ng/mL of IL-27 (R&D Systems, Minneapolis, MN) in completed RPMI-1640 (Thermo Fisher Scientific, Waltham, MA) medium (RP-10) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Quality Biological, Gaithersburg, MD), 10 μg/mL of gentamicin (Thermo Fisher Scientific), and 20 units/mL of IL-2 (MiliporeSigma).[22] Monocyte-derived macrophages (MDMs) were differentiated from CD14(+) monocytes using 25 ng/mL of macrophage colony–stimulating factor (M-CSF) (R&D Systems) in macrophage serum-free media (Thermo Fisher Scientific) as previously described.[18] IL-27-differentiated MDMs (I-Mac) were induced by culturing monocytes with 25 ng/mL M-CSF and 100 ng/mL IL-27 (R&D Systems) for 7 days.[18] Both MDMs were maintained in completed DMEM (D-10) containing 10% FBS, 25 mM HEPES, and 10 μg/mL of gentamicin. HEK293T cells were obtained from ATCC (Manassas, VA) and maintained in D-10. HIVAD8 and HIVNL4.3 virus stocks were prepared using a plasmid encoding the full length of HIVAD8[25] and HIVNL4.3 genes,[26] and the 50% tissue culture infectious doses ($TCID_{50s}$) of each stock were determined as previously described.[12,27] HIVLuc-V[28,29] was produced by cotransfecting HEK293T cells with pNL4-3ΔEnv-Luc[18,28,29] and pLTR-VSVG using a TransIT-293 transfection kit (Mirus, Madison, WI) as previously described.[18]

Preparation of MiRNA Library and Sequencing of MiRNAs

Total RNA from Ctrl-Tc and 27-Tc was extracted using QIAzol Lysis Reagent (Qiagen, Germantown, MD). A total of 6 miRNA libraries were prepared using the NEBNext Multiplex Small RNA Library Prep for the Illumina protocol (New England BioLabs, Ipswich, MA) and sequenced on the Hiseq2500 using v4 chemistry for single end sequencing (San Diego, CA) at the sequencing facility, Frederick National Laboratory for Cancer Research. A quality check of the miRNA-seq data indicated that 93% or more of the bases for all samples had a Phred quality score of Q30 or greater, and all samples yielded between 53 and 62 million reads. All small RNA sequences have been deposited into the NCBI SRA database under BioProject ID PRJNA602725.

Differential Expression Analysis

Differential miRNA expression analysis was performed as previously described.[21] Raw reads were cleaned by Cutadapt v1.10,[30] then mapped to the human reference genome hg38 using the Burrows–Wheeler aligner, BWA (v0.7.10-r789) with one mismatch.[31] Known miRNA read counts were determined by the bedtools v2.26[32] with miRBase (v21).[33] The read count matrix was analyzed using the edgeR package 3.16.5[34] in R version 3.2.3. The significant miRNAs were selected based on fold changes >1.5 or ≤ 1.5, false discovery rates <0.05, and at least one sample count (maximum read counts) >50.

Discovery of Candidates of Novel miRNAs

Discovery of novel miRNAs was performed as previously described.[21] In brief, novel miRNA candidates were generated by miRDeep2 algorithm.[35] Other RNAs, such as snoRNAs, snRNAs, tRNAs, rRNAs, Y-RNAs, etc., were eliminated using RFam (v12.1) (https://rfam.xfam.org/) and

GENCODE v24 (https://www.gencodegenes.org/human/release_24.html) annotation. Top candidates of interests were selected based on their read counts or *P* values. These sequences have been deposited into the GenBank database under accession number: MF281428 ~ MF281440 (Table 1).

Quantitative Real Time-Polymerase Chain Reaction

To quantify the expression of miRNAs and interferons (IFNs), real-time quantitative real time-polymerase chain reaction (qRT-PCR) was performed using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) with the a CFX96 real-time system (Bio-Rad, Hercules, CA) as previously described[20,36] All probes used in this study are listed in Table 3, Supplemental Digital Content, http://links.lww.com/QAI/B571.

miRNA Transfection

MiRNA mimics were synthesized by Thermo Fisher Scientific. Transfection of miRNAs into T cells and MDMs was conducted using 4D-Nucleofector (Lonza, Walkersville, MD) with the EO-115 program and RNAiMAX (Thermo Fisher Scientific),[37] respectively, following the protocols from the vendors. Cell viability was determined by trypan blue exclusion assay as previously described.[12] MiRNA mimic negative controls were obtained from Thermo Fisher Scientific (control 1: Cat # 4464058, control 2: Cat # AM17110) and GE-Healthcare (Chicago, IL) (control 3: Cat # CN0010000-01-05). An siRNA negative control was purchased from Thermo Fisher Scientific.

HIV-1 Replication and Infection Assays

T cells or MDMs were infected with HIVLuc-V at 100 ng/mL p24, HIVNL4.3 at 1000 $TCID_{50}/10^6$ cells, or HIVAD8 at 5000 $TCID_{50}/10^6$ cells.[12] HIVLuc–V-infected cells were cultured for 48 hours, and then viral infection was quantified using the Bright-Glo Luciferase kit (Promega, Madison, WI).[18] HIV replication in T cells and MDMs was monitored on 7 and 14 days after infection, respectively, using a p24 antigen enzyme-linked immunosorbent assay (ELISA) kit (Perkin Elmer, Boston, MA).[14]

Enzyme-Linked Immunosorbent Assay

Concentrations of interferon (IFN)-α, β, and λ in culture supernatants from transfected cells were measured using the VeriKine-HS Human IFN-α All Subtype ELISA Kit (PBL Assay Science, Piscataway, NJ), the VeriKine-HS Human IFN-β ELISA Kit (PBL Assay Science), and the human IL-29 ELISA kit (Invitrogen, Carlsbad, CA), respectively, following the manufacturer's instructions.

Neutralization of IFN Using B18R

MDMs were transfected with 10 nM miRNAs as described above and cultured for a total of 72 hours in the absence or presence of 1 μg/mL B18R (R&D Systems). For a positive control of the neutralization effect for B18R, untransfected MDMs were cultured with IFN-α (100 units/mL) (R&D System).

Statistical Analysis

Results were representative of at least 3 independent experiments. The values are expressed as means ± SD or ± SE. The statistical significance was determined by the Student *t* test. $P < 0.05$ was considered to indicate a statistically significant difference between the experimental groups.

TABLE 1. Sequence and Genomic Location of Novel miRNAs*

miRNA Name	GenBank Accession Number	Mature miR Sequence 5′–3′†	Precursor Genomic Location
miRTC1	MF281428	ccccacugcuaaauuugacug	chr2:88229575..88229659:−
miRTC2	MF281429	aucugugggauuaugacug	chr8:55909398..55909449:−
miRTC3	MF281430	aacauagcgagaccccgucucua	chr19:11536523..11536595:−
miRTC4	MF281431	uccccaguacccccacca	chr1:156887614..156887687:+
miRTC5	MF281432	gcauuggugguucaguggu	chr8:105496291..105496332:+
miRTC6	MF281433	aaggagcucacagucuauug	chr8:130477073..130477146:+
miRTC7	MF281434	aucccagacgagcccccc	chr20:56273039..56273100:−
miRTC8	MF281435	gcaggacgguggccaug	chr16:72055503..72055548:−
miRTC9	MF281436	cuccuggcuggcucgcca	chr18:2292241..2292322:−
miRTC10	MF281437	uacucucucggacaagcuguaggu	chr7:139044100..139044157:−
miRTC11	MF281438	aucugugggauuaugac	chr8:46040269..46040318:−
miRTC12	MF281439	gucuacggccauaccacc	chr9:63815546..63815633:+
miRTC13	MF281440	caaaaacugugauuacuuuug	chr18:64238466..64238547:−
miRTC14	MF281428	aacgcugcgaccuagauguauucu	chr18:74148958..74149017:+
miRTC15	MF281429	ucccuguucuccuucccugucc	chrX:53411910..53411974:−

*The novel miRNAs were identified using miRDeep2 and RFam (v12.1), as described in the Materials and Methods.
†The sequence data were submitted to the National Center for Biotechnology Information Read Archive under accession No. PRJNA602725.

RESULTS

Identification of 15 Novel miRNAs in CD4+ T Cells

To compare the miRNA profiles of untreated CD4(+) T cells (Ctrl-Tc) and IL-27-treated CD4(+) T cells (27-Tc), RNAs were extracted from cells from 3 independent donors, and miRNA-seq was conducted. The significantly expressed miRNAs were determined using 3 criteria: (1) the read counts of the miRNA of interest in 27-TC were > 1.5-fold greater or less than in Ctrl-Tc, (2) the false discovery rate was less than 0.05, and (3) at least the maximum count of 1 sample count was more than 50. We found a total of 1110 known miRNAs in the 6 samples. The average number of sequence reads for known miRNAs in Ctrl-Tc was 56,445 (range: 0–3, 311 to 324), compared with 53,394 (range: 0–3, 248 to 442) in 27-Tc. Principal component analysis demonstrated that proportion of variants in the small RNA expression in 27-Tc was less than the donor-dependent variants (see Fig. 1, Supplemental Digital Content, http://links.lww.com/QAI/B571), suggesting that IL-27 may not differentially regulate expression of the miRNAs in T cells.

To further characterize the miRNA profile in these cell types, we sought to identify novel miRNAs and then discovered a total of 15 novel miRNAs (miRTC1 ~ miRTC15) (Table 1). The average number of reads of the novel miRNAs for Ctrl-Tc and 27-Tc was 115 reads (range: 0–1 to 419) and 114 (range: 2–1 to 287), respectively (see Fig. 2, Supplemental Digital Content, http://links.lww.com/QAI/B571). The novel miRNAs were traced back to their possible genomic locations to define whether secondary stem-loop structures could form. These 15 miRNAs could all form the appropriate stem-loop structures as shown by the predicted secondary structures (see Fig. 3, Supplemental Digital Content, http://links.lww.com/QAI/B571). To validate the expression of each of miRNA, we performed a semiquantitative real time PCR using specific probes for each miRNA. We used a total of 12 probes for the 15 novel miRNAs (the probes for miRTC7, 9 and 11 were not able to be synthesized, because miRTC7 and 9 contain a high GC content and miRTC11 possesses a high similarity [89.5%] to miRTC2). Because of the high similarity between miRTC2 and 11, the probe for miRTC2 detects both miRNAs. The qPCR assay confirmed the expression of all tested miRNAs in Ctrl-Tc and 27-Tc from the 3 donors; however, IL-27-treatment did not significantly alter the expression levels of these miRNAs (Fig. 1A). Using the same probes, we also assessed the expression of the novel miRNAs in MDMs and IL–27-induced MDMs (I-Mac)[18] (Fig. 1B). The expression of each miRNA was consistently detected in each MDM sets from all 3 donors without any significant changes in I-Mac. Of note, the expression of miRTC14 in T cells and MDMs was relatively lower than other miRNAs.

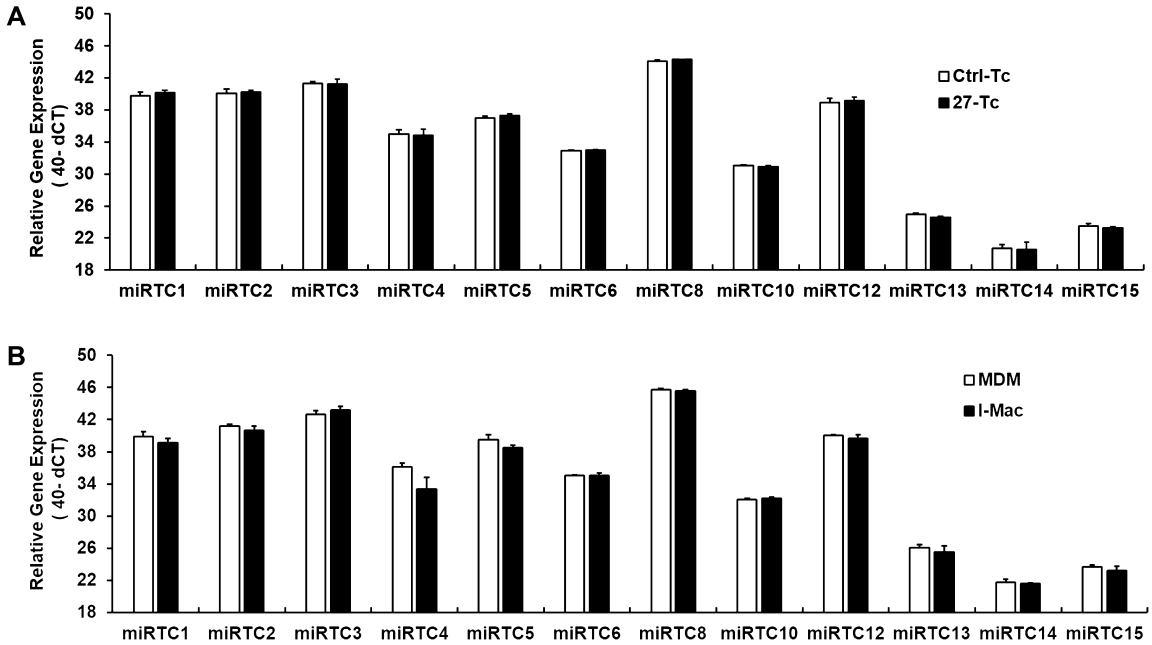


FIGURE 1. Comparison of baseline novel miRNA expression in T cells and MDMs. CD4(+) T cells and CD14(+) monocytes were isolated from peripheral blood mononuclear cells of 3 independent healthy donors. A, T cells were stimulated with phytohemagglutinin for 3 days and then cultured for additional 3 days without (Ctrl-Tc) or with 100 ng/mLIL-27 (27-Tc) in the presence of 20 units/mL of IL-2. B, Monocytes were differentiated into macrophages in the presence of 25 ng/mL M-CSF alone (MDMs) or 25 ng/mL M-CSF with 100 ng/mL IL-27 (I-Mac) for 7 days. Total RNA from Ctrl-Tc, 27-Tc, MDMs, and I-Mac were extracted, and the expression of each novel miRNA was quantified by real time qRT-PCR. Gene-specific probes were custom-made by Thermo Fisher Scientific. As an internal control, the small nuclear protein RNU44 probe was used. Base line of the expression of each miRNA was calculated by comparing the Ct values of each miRNA with the Ct value of RNU44 and then subtracting this from 40 (40-delta Ct).[74] Results represent mean ± SE (n = 3) of 3 independent assays.

Novel miRNAs Inhibit HIV in MDMs

To assess the anti-HIV effect by overexpression of each novel miRNA in T cells, miRTC1 ~ miRTC14 mimic (excluding miRTC7, 9, 11, 15: miRTC15 mimic was not be successfully synthesized) were chemically synthesized. T cells were transfected with each miRNA mimic using the 4D-Nucleofector, and then infected with either HIVNL4.3 or HIVLuc-V. Although overexpressed each miRNA was confirmed by a qRT-PCR (see Fig. 4A, Supplemental Digital Content, http://links.lww.com/QAI/B571), and cell viability using trypan blue exclusion assay was not significant changed (viability was 80–90%); none of the overexpressed miRTCs had a significant impact on either HIVNL4.3 replication or HIVLuc-V infection (see Fig. 5, Supplemental Digital Content, http://links.lww.com/QAI/B571); thus, we concluded that the novel miRNAs had no anti-HIV property in T cells. To further characterize a potential role of each miRNA, MDMs were transfected with each miRNA mimic using RNAiMAX and then infected with either HIVAD8 or HIVLuc-V. The overexpression of each miRTC in MDMs was confirmed by qRT-PCR (see Fig. 4B, Supplemental Digital Content, http://links.lww.com/QAI/B571). Of interest, miRTC5, miRTC10, and miRTC14 significantly suppressed HIV HIVAD8 replication, and miRTC3, miRTC10, and miRTC14 remarkably abolished HIVLuc-V infection (Fig. 2A). Trypan exclusion assay demonstrated none of miR mimics had impact on cytotoxicity: cell viabilities of miRCtrl-transfected, miRTC10-transfected, and miRTC14-transfected MDMs were 87 ± 5.0%, 90 ± 5.4%, and 83 ± 4.0%, respectively ($P > 0.05$). Taken together, we found that miRTC10 and 14 possess anti-HIV property in MDMs but not in T cells. MiRTC14 mimic was the strongest inhibitor of HIVAD8 and HIVLuc-V infection. Thus, we mainly focused on the miRTC14-mediated antiviral effect in subsequent experiments.

To further characterize the miRTC14 effect, we performed a dose response assay using different concentrations of either miRTC14 or a control nontargeting miRNA (miRCtrl) with HIVLuc-V infection in MDMs. As shown in Figure 2B, miRTC14, but not miRCtrl inhibited viral infection in a dose dependent manner, and in the presence of 10 nM, miRTC14 inhibited HIVLuc-V infection by (99.3 ± 0.27%, n = 9) ($P < 0.001$) and qRT-PCR confirmed that the miRTC14 and the miRCtrl expressed in the cells in a dose-dependent manner (Fig. 2C).These results further indicated that miRTC14 is a powerful anti-HIV miRNA.

MiRTC14 is an Interferon-Inducing RNA

It has been empirically shown that the transfection efficiency of nucleic acids into primary cells is significantly lower than that into cell lines[38,39] ; therefore, we first speculated that miRTC14 could stimulate cells as an

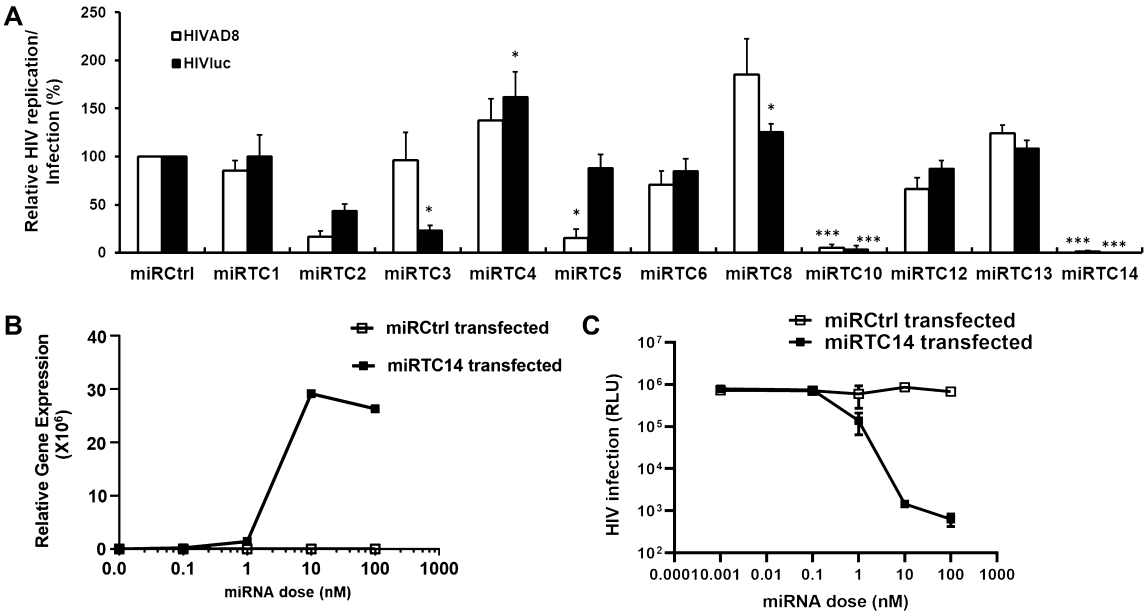


FIGURE 2. Characterization of the anti-HIV effect of each novel miRNA in MDMs. A, MDMs were transfected with 10 nM miRNA using RNAimax lipofectamine for 72 hours and then infected with HIVAD8 (opened bars) or HIVLuc-V (closed bars) as described in the Materials and Methods. HIVAD8 and HIVLuc-V infected cells were culture for 14- and 2-days, respectively. HIV replication was monitored by a p24 antigen capture kit (PerkinElmer) and Luciferase was detected by the Bright-Glo (Promega). Each experiment was performed triplicate using cells from 3 independent donors. Results represent relative infection compared to HIV infection value in miRCtrl-transfected MDMs. Data indicate mean ± SE (n = 3). *$P < 0.05$, ***$P < 0.001$. B, MDMs were transfected with 0, 0.1, 1.0, 10.0, and 100.0 nM of miRCtrl or miRTC14 mimic for 72 hours, and total RNA was extracted, and change of miRTC14 expression was quantified using qRT-PCR, results indicate relative gene expression increase dose dependently in miRTC14 transfected cells. C, MDMs were transfected with 0, 0.1, 1.0, 10.0, and 100.0 nM miRCtrl or miRTC14 mimic for 72 hours and then infected with HIVLuc-V. HIV infection was monitored at 48 hours after infection by the luciferase activity using the Bright Glo. Results represent mean ± SD. ***$P < 0.001$.

extracellular RNA ligand to induce the observed anti-HIV effect. To address this hypothesis, MDMs were treated with 10 nM miRTC14 alone without transfection lipid, transfection lipid alone, miRTC14 mimic alone, or a mixture of miRTC14 and the lipid. Only the mixture of miRTC14 with lipid induced a high levels of anti-HIV effect (99.3 ± 0.27%, n = 9) (Fig. 3A), indicating that only the transfected miRTC14 prompted the anti-HIV effect. To elucidate whether miRTC14 directly interacts HIVAD8 transcripts as the noncanonical mechanism,[4,5] a target prediction analysis was performed using 4 prediction tools (miRanda,[40] RNA22,[41] IntaRNA,[42] and RNAHybrid[43]) with minimum free energy ≤50 kcal/mol cutoff. Intriguingly, the analysis revealed that miRTC14 potentially targets HIVAD8 *env, gag, pol*, *and vif* genes (see Fig. 6, Supplemental Digital Content, http://links.lww.com/QAI/B571). Given the presumed rarity of transfection into MDMs, despite the result from the prediction analysis, we hypothesized that the miRTC14-transfected cells may produce an antiviral soluble factor(s) that inhibits HIV *in tra*ns.

Interferons (IFNs) are a well-investigated anti-HIV proteins[44] and are produced from MDMs on stimulation; thus, we presumed that the transfected miRTC14 might induce IFNs and inhibit HIV. To dissect the IFN induction by miRTC14 transfection, we used a soluble vaccinia virus-encoded type I-IFN receptor (B18R), previously shown to have a potent Type-I IFN neutralizing effect.[45,46] In the absence or presence of 1 μg/mL of B18R, miRTC14 transfection was performed, and then the anti-HIV effect was assessed using MDMs. As a positive control for the neutralizing effect, cells were treated with 10 units/mL of IFN-α. In the presence of B18R, the miRTC14-mediated anti-HIV activity was suppressed (Fig. 3B), suggesting that miRTC14 induces Type-I IFN.

Based on receptor usages, human IFNs have been classified into 3 major types (type-I, type-II, and type-III IFNs). Type-I IFN consists of 5 subtypes, α, β, ε, κ, and ω and human Type-III IFN comprises 4 λ subtypes. Because type-I and type-III IFNs are secreted from macrophages,[14,37,47] we quantified the concentrations of not only type-I but also type-III IFNs in the culture supernatants of miRTC1 ~ miRTC14 or miRCtrl-transfected cells (see Fig. 10, Supplemental Digital Content, http://links.lww.com/QAI/B571). Preliminary screening experiments showed that

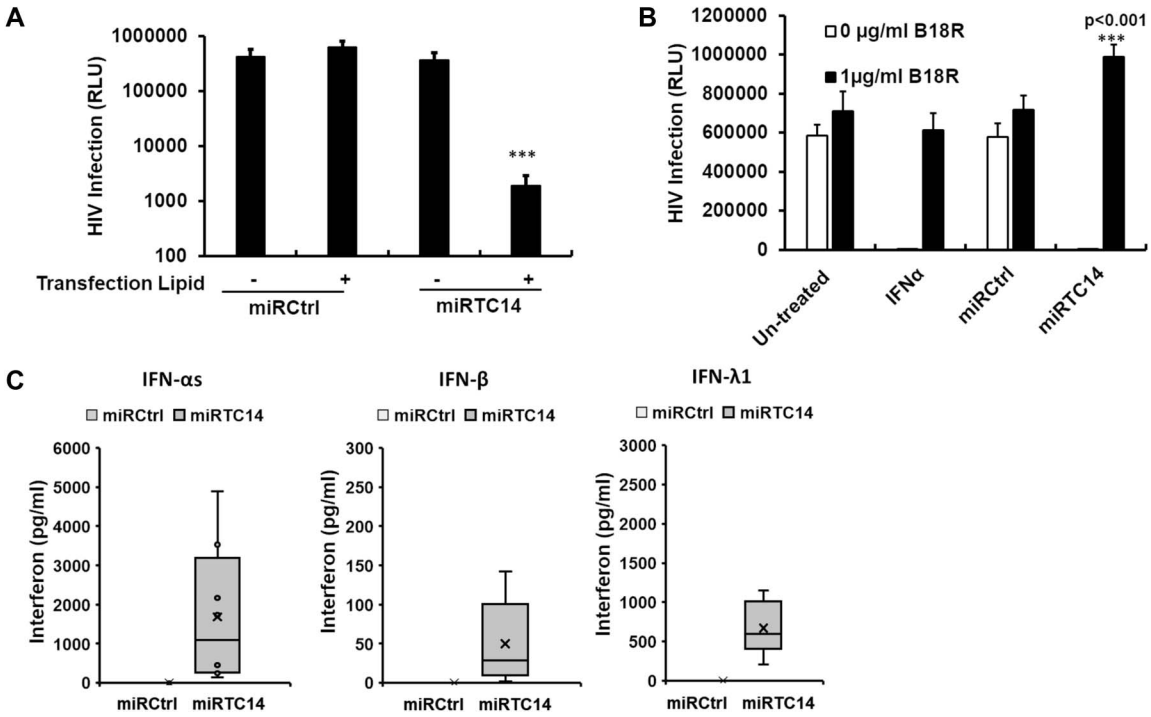


FIGURE 3. miRTC14 significantly induces type-I and type-III IFNs. A, Impact of RNA transfection lipid on the anti-HIV effect. MDMs were treated with a transfection lipid reagent, lipofectamine RNAiMAX, miRNA (10 nM miRCtrl or miRTC14), or miRNA with the lipid for 72 hours. The cells were then infected with HIVLuc-V. HIV-infection was monitored by the luciferase activity. The data are a representative result from 2 independent experiments, the results show mean ± SD ***$P < 0.001$. B, MDMs were transfected with 10 nM miRCtrl or miRTC14 and cultured in the absence or presence of 1 μg/mL of B18R for a total 72 hours. The transfected cells were infected with HIVLuc-V and then incubated for 48 hours. HIV infection was monitored by the luciferase activity. As a control, untreated cells or IFN-α (R&D systems)-treated cells were also cultured with or without the B18R. The data are a representative result from 2 independent experiments, data shown are mean ± SD (n = 3). ***$P < 0.001$. C, Quantitation of concentrations of IFNs in transfection supernatants. MDMs were transfected with 10 nM of miRCtrl or miRTC14 for 72 hours, and, then, cell-free supernatants were collected. Concentrations of IFN-α, β, and α were determined through ELISA kits. Detection limits of IFN-αs (all subtypes), β, and λs (all subtypes) were 1.25, 1.2, and 15.6 pg/mL, respectively. The box plots show data the results from 8 donors for IFN-αs and IFN-λs and 5 donors for IFN-β.

miRTC14 was the highest inducer of type I and type III IFNs. In the supernatants of miRCtrl #1-transfected cells, only IFN-α was detected at a low level (1.67 ± 0.67 pg/mL, n = 8), whereas all other miRTC mimics (except miRTC10 mimic), 2 different negative control miRNAs (miRCtrl #2 and #3) and a different form of control small RNA (siRNACtrl) produced IFNs below a detectable level. In the miRTC14-transfected supernatants, although a donor dependency was observed in the induction of IFNs, both type-I and type-III IFNs were detected: IFN-α (1680 ± 625 pg/mL, n = 8, $P < 0.01$), IFN-β (49.9 ± 25.0 pg/mL, n = 5, $P < 0.05$), and IFN-λs (668 ± 124 pg/mL, n = 7, $P < 0.001$) Figure 3C. In the human genome, IFN-α and IFN-λ composed of 13 and 4 subtypes, respectively. To define which subtypes of IFN genes were induced by miRTC14, qRT-PCR was performed. The result demonstrated that miRTC14 significantly enhanced *IFNA1, IFNA2, IFNA8, IFNA13*, *IFNA14, IFNB*, and *IFNL1* expression more than 100-fold (Fig. 4A, B).

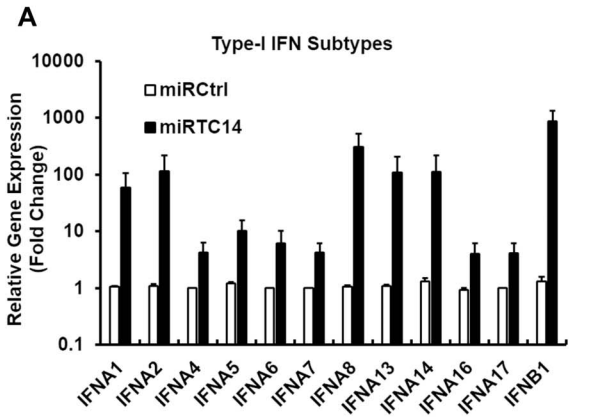
To understand the mechanism of the IFN-induction, potential targets of miRTC14 were predicted using 4 miRNA prediction tools: miRanda,[40] miRDB,[48,49] TargetScan,[50,51] and MR-microT.[52] A total of 1 gene was identified as common predicted genes by all 4 tools, and 15 genes were predicted by 3 of the 4 programs (see Fig. 7, Tables 1 and 2, Supplemental Digital Content, http://links.lww.com/QAI/B571). We checked the LUC7 such as 3 pre-mRNA splicing factor (LUC7L3) which is predicted by 4 tools by real-time PCR assay that demonstrated the LUC7L3 was upregulated (see Fig. 8, Supplemental Digital Content, http://links.lww.com/QAI/B571). LUC7L3 is a nuclear protein which is involved in pre-mRNA splicing.[53,54]

After cross-referencing with the genes associated with regulation of *IFN* activation, 2 genes, histone deacetylase 4 (HDAC4)[55,56] and phosphatidylserine synthase 1 (PTDSS1)[57] were predicted as potential targets for miRT14. Because both gene products are known to negatively regulate IFN gene activation, we expected that miRTC14 could downregulate the gene expression and subsequently induce the IFN genes activation. A real-time PCR assay, however, demonstrated that the expression of both genes was not changed by the miRTC14 in MDMs (see Fig. 9, Supplemental Digital Content, http://links.lww.com/QAI/B571), implicating that miRTC14 induces IFNs through some uncharacterized mechanisms.

DISCUSSION

We have previously reported that IL-27 pulse-treatment of T cells produced HIV-resistant cells without significant changes in the expression of mRNAs compared with untreated cells.[22] In the current study, to further characterize IL-27 effect in the T cells, miRNAs profile was analyzed. Although a profile of known miRNAs did not display significant differences between 27-Tc and Ctrl-Tc, we discovered a total of 15 novel miRNAs in both cell types. The expression was not significantly changed by IL-27 treatment. Overexpression of miRTC14 using mimic miRTC14 into MDMs, but not Ctrl-T cells, resulted in a robust anti-HIV effect through the induction of high amounts of multiple subtypes of IFNs. Thus, the miRTC14 is a novel IFN-inducing anti-viral miRNA, and this anti-HIV effect is caused by an extensively investigated IFN-mediated mechanism(s).

A validation assay by qRT-PCR demonstrated that IL-27 had no impact on the expression of any of novel miRNAs, and miRTC14 expression in T cells and MDM was relatively lower than other miRNAs in both cell types. MiRTC14 (GenBank accession number: MF281439) is encoded in the translocase of the inner mitochondrial membrane 21 (TIMM21) gene on chromosome 8 as an exonic miRNA. Although the function and regulation of TIMM21 has not been well investigated yet, a study of the mechanism of TIMM21 gene regulation may provide a new insight into miRNA-mediated antiviral effect; therefore, it would be interesting to monitor miRTC14 expression and TIMM21 expression in HIV-infected patients or other diseases.



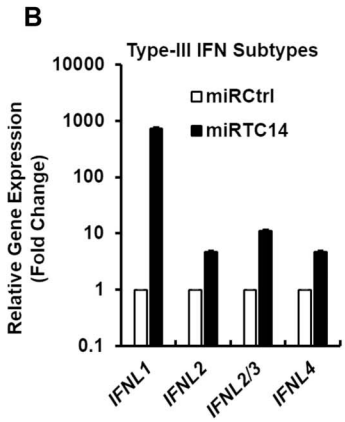


Table: 1

IFN subtypes	p values
IFNA1	0.001
IFNA2	0.007
IFNA4	0.029
IFNA5	0.138
IFNA6	0.033
IFNA7	< 0.0001
IFNA8	0.001
IFNA13	< 0.0001
IFNA14	0.019
IFNA16	< 0.0001
IFNA17	< 0.0001
IFNB1	0.064
IFNL1	0.002
IFNL2	0.224
IFNL2/3	0.188
IFNL4	0.213

Figure 4. miRTC14 induces the activation of multiple subtypes of IFN genes. MDMs were transfected with 10 nM miRCtrl or miRTC14 and cultured for 48 hours. Total RNAs were extracted from the cells, and real time qRT-PCR was performed using a gene-specific probe for each subtype of IFNs. Gene expression is presented as relative expression units compared with miRCtrl-transfection after normalization to GAPDH.[36] Results represent ± SE from 3 independent experiments. The inserted table shows the corresponding *P* values of all the type of interferon gene expressions.

RNA transfection induces IFNs from MDMs or other cell types as an innate-immune response. This induction is mediated through cytosolic RNA sensors, for example, retinoic acid-inducible gene I (RIG-I) and melanoma differentiation–associated gene 5 (MDA5).[58,59] RIG-I and MDA5 recognize short and long RNAs, respectively.[60,61] Both of these RNA sensors are activated under certain conditions that introduce double-stranded RNA (dsRNA) molecules with a loop structure with a 5′-triphosphate or diphosphate group.[62,63] It has been reported that the minimum required length of dsRNA for RIG-I is 10–14 bp dsRNA with a stem loop containing a 5′-triphosphate.[64,65] In the current study, we used a synthesized miRTC14 mimic consisting of a 24-bp dsRNA with neither a loop structure nor the 5′ triphosphate. Although miRCtrl contains the same structure, it had no effect on the induction of IFNs; therefore, it is plausible that the mechanism underscoring the induction of IFNs via miRTC14 may be a sequence-dependent and a RIG-I independent mechanism. The overexpression of miRTC14 in $CD4^+$ T cells had no direct impact on HIV replication; however, because IFNs inhibit HIV replication in T cells,[14] the replication would be inhibited by a coculture with miRTC14-transfeced MDMs. To further characterize function of miRTCs, coculture of HIV-infected T cells[66,67] with each miRTC-transfected MDMs may provide a new set of evidence of miRTC function. HIV replication may be inhibited by an IFN-independent manner, such as endosomal transmission[68] or uncharacterized factors from the miRTC-transfected MDMs. This study may provide a new insight of miRNA function.

A target prediction analysis resulted in that miRTC14 potentially targets HIV genes (see Fig. 6, Supplemental Digital Content, http://links.lww.com/QAI/B571), suggesting that miRTC14 may inhibit HIV through not only the IFN-induction and but also the canonical miRNA mechanism. MiRTC3 partially suppressed HIVLuc-V infection (single round infection) by 77 ± 5.2% ($P < 0.05$) within 48 hours, however, it did not inhibit HIVAD8 replication (multiple round infection) within14 days after infection. It was assumed that the remaining HIV infection in the presence of miRTC3 propagated during the long 14-day incubation, consequently resulting in the detection of HIV replication. Intriguingly, miRTC5 inhibited HIVAD8 by 82 ± 9.0% ($P < 0.05$) but not HIVLuc-V, presuming that miRTC5 may interrupt a step of multiple round infection, for example, assembly or budding step,[69,70] or cell-to-cell transfusion step. It is reported that miR-146a interacts Gag and facilitates Gag degradation, subsequently it inhibits HIV replication.[71] Because a target prediction analysis showed that miRTC5 targets *gag* gene with a lower probability, the anti-HIV effect of miRTC5 may involve an unconventional mechanism as does miR-146a. Further investigation may reveal the mechanism behind the anti-HIV effect by miRTC5.

MiRNAs in viral infection can play dual roles either by helping the body to protect against it or by helping the virus for infection.[72] In our study, we demonstrated that IL-27 did not enhance the expression of endogenous miRTC14 in T cells, but when the miRNA was overexpressed in MDMs, it can initiate a series of IFN induced signaling which inhibit HIV. The regulation of the expression of miRTC14 in macrophage may be a potent therapeutic target.

In summary, during a course of analysis of miRNA profiles in 27-Tc and Ctrl-Tc, we discovered a total of 15 miRNAs and verified that miRTC14 is an IFN-inducing miRNA. It has been reported that microRNA-30e* is an IFN-inducing miRNA by the NFk-dependent pathway,[73] miRTC14 may prompt the IFN production through a similar pathway. Further studies are needed to delineate the underlying mechanism of this miRNA-mediated IFN induction, and such studies could also shed light on the regulation of IFN gene activation and off-target effects of miRNA transfection.

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ERRATUM

Bacterial Vaginosis and Risk of HIV Infection in the Context of *CD101* Gene Variation: Erratum

In the December 15, 2020 issue of *JAIDS*, the license for the article by Wanga et al (Bacterial Vaginosis and Risk of HIV Infection in the Context of *CD101* Gene Variation. *JAIDS* 2020;85(5):584-587) has been changed in compliance with funding requirements. The article is published under the creative commons license CC BY.
This has been corrected online.