HIV-1-derived exosomal microRNAs miR88 and miR99 promote the release of cytokines from human alveolar macrophages by binding to TLR8

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Abstract
The human monocytic cell line U937 and human alveolar macrophages were used as in vitro models to explore the role of miR88 and miR99 in the chronic abnormal activation of the body caused by human immunodeficiency virus (HIV). The functions and underlying mechanisms of miR88 and miR99 were studied by real-time quantitative polymerase chain reaction, transwell, and chromatin immunoprecipitation (ChIP) assays. HIV-1-infected cells released miR88 and miR99 into the extracellular space through exosomes, and miR88 and miR99 promoted the release of tumor necrosis factor alpha (TNFα), interleukin (IL)-6, and IL-12 by activating inflammatory factors, such as TLR8, on the surface of macrophages. HIV-derived microRNAs miR88 and miR99 performed these functions by binding to TLR8 and stimulating the release of pro-inflammatory factors from macrophages, such as TNFα, IL-6, and IL-12; these factors may be involved in chronic abnormal immune activation induced by HIV infection.

Keywords
cytokines, macrophage, miR88, miR99, TLR8

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Introduction
Human immunodeficiency virus (HIV) destroys the body’s immune system by infecting CD4+ T cells and leads to the development of acquired immunodeficiency syndrome (AIDS). Based on serological responses and viral nucleic acid sequences, HIV is classified as HIV-1 and HIV-2. Both HIV-1 and HIV-2 can induce AIDS, but HIV-1 is currently the most prevalent HIV type in the world. Since the advent of combined antiretroviral treatment (cART) in 1996, the incidence and mortality of HIV-infected people have been controlled to some extent.1–3 cART can inhibit HIV replication and restores some of the immune functions, but it does not eliminate the virus. Therefore, HIV is still a risk factor for human health.

Exosomes are a class of membrane-bound vesicles secreted by a variety of cells. These vesicles are 30–100 nm in diameter and usually contain proteins and ribonucleic acids (RNAs).4,5 Exosomes have been detected in almost all biological fluids. In some exosomes from virus-infected cells, viral proteins and viral RNA can be present along with
endogenous proteins and RNA. Previous studies have shown that HIV-infected cells can transfer viral proteins and RNA to adjacent uninfected cells via exosomes.\textsuperscript{6,7}

RNA carried in exosomes includes a variety of small RNAs with regulatory effects, such as micro-RNAs (miRNAs). miRNAs are non-coding single-stranded RNA molecules with lengths of 18–24 bp. They function to silence target genes through RNA interference (RNAi) and thus help regulate post-transcriptional expression of genes.\textsuperscript{8–10} In addition, miRNAs perform their regulatory functions by stimulating toll-like receptors (TLRs).\textsuperscript{11,12} Most HIV-derived miRNAs have been reported to perform their regulatory functions through RNAi. However, our previous studies on HIV-infected macrophages revealed that two new HIV-derived exosomal miRNAs, miR88 and miR99, elicit an immune response through TLR8 to induce macrophages that release the cytokine tumor necrosis factor alpha (TNF\textalpha).

Consistent with humans, Simian immunodeficiency virus (SIV) infection also induces chronic abnormal activation of the rhesus monkey immune system, which eventually develops into AIDS.\textsuperscript{13} By contrast, although the virus replicates at high levels after infection in the white-browed macaque, SIV’s natural host, it does not exhibit chronic abnormal immune activation and does not develop into AIDS.\textsuperscript{14} Thus, chronic abnormal immune activation plays an important role in the development of AIDS and is considered the driving force behind HIV’s destruction of the body’s immune system. However, the mechanism by which HIV infection elicits chronic abnormal immune activation remains poorly understood. Studies have shown that the abnormal immune activation is likely associated with viral nucleic acids and proteins; the innate and adaptive immune system responds to viral antigens and translocations of microbial TLR ligands from the intestines to the systemic circulation.\textsuperscript{15–17} The discovery of miR88 and miR99 provided a new entry point to understand the mechanism of chronic abnormal activation of the immune system caused by HIV. However, our understanding of the mechanism of miR88 and miR99 action is still unclear.

In this study, we continue to deepen our understanding of the functions and mechanisms of miR88 and miR99, aiming to provide a strong theoretical basis for the clinical treatment of AIDS.

**Materials and methods**

**Cell culture, transfection, and treatment**

The human monocytic cell line U937 and the HIV-1-infected U937 cell line were both provided by the AIDS Research and Reference Reagent Program (Bethesda, MD, USA). The cell lines were cultured on RPMI-1640 medium (Hyclone, USA) at 5% CO\textsubscript{2} and 37°C. The RPMI-1640 medium included 10% fetal bovine serum (10099141; Gibco, USA), 10,000 U/mL penicillin, 0.1 mg/mL streptomycin (V900920; Sigma, St. Louis, MO, USA), and 2 mM glutamine. After treatment with 100 nM phorbol myristic acid (PMA) (M1328; Sigma, USA) for 24 h, the cells were differentiated and cultured on a medium containing exosome-depleted fetal bovine serum (A2720801; Gibco, USA).

For the experiment with HIV-1 infection, HIV-1 preparations were obtained from the supernatants of 293T cells 72 h after transfection with HIV-1 full-length plasmid provided by the National Institutes of Health (NIH) (clone: 11739, R5, tropic). The virus suspension containing HIV-1 was added to U937 cell culture medium, and the cells were cultured for 4–5 h.

Human alveolar macrophages (AMs) were provided by healthy volunteers who aged between 18 and 55 years. This study was approved by the Second Hospital of Shanxi Medical University Ethics Committee, and all procedures were performed with written informed consent. AMs were isolated from lavage fluid using the standard techniques of bronchial lavage\textsuperscript{18,19} and were cultured at 37°C and 5% CO\textsubscript{2} on RPMI-1640 medium. AMs in complete medium were treated with miR88 and miR99 (1.0 mg/mL) formulated in LyoVec at the indicated time points (37°C, 5% CO\textsubscript{2}).

**Exosome isolation**

Exosomes were isolated from sera and cell culture conditioned medium using ExoQuick/ExoQuick-TC reagents (System Biosciences, Mountain View, CA, USA) according to the manufacturer’s instructions. First, the flow-through fraction was analyzed for exosomal markers using anti-CD63 primary antibody, anti-rabbit horseradish peroxidase (HRP) conjugate (System Biosciences, USA), and ECL Select Western blotting detection (Amersham, Sweden).
**Immunofluorescence staining**

HIV-1-infected U937 cells were seeded in 12-well plates with round covers and cultured to a logarithmic growth phase. The cells were first differentiated and then rinsed with $1 \times$ phosphate buffered saline (PBS), fixed with 4% paraformaldehyde (158127; Sigma, USA) for 10 min, incubated with 0.5% Triton X-100 in PBS for 20 min at room temperature, blocked with 5% bovine serum albumin (BSA) for 1 h, and then incubated with gag protein antibody (cs-69728; Santa Cruz, USA) overnight at 4°C. The next day, the cells were incubated with a fluorescent secondary antibody (ab6785; Abcam, Cambridge, UK) for 2 h and then with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Finally, they were observed using a fluorescence microscope (Nikon, Japan).

**Western blot**

The exosomes were extracted using an EXOTC kit (SBI) and lysed with radio-immunoprecipitation assay (RIPA) lysis buffer (V900854; Sigma, USA) to obtain total protein. The protein concentration was determined using a bicinchoninic acid (BCA) protein concentration assay kit (FP0010; Sigma, USA). Equal amounts of protein were electrophoresed on a 10% Bis-Tris gel at 120 V for 60 min. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane at 350 mA for 70 min and blocked with 5% BSA in Tris-buffered saline with Tween 20 (TBST) buffer for 60 min. CD63 primary antibody (ab134045; Abcam, Cambridge, UK) and Tsg101 (ab125011; Abcam, Cambridge, UK) were incubated by gentle shaking at 4°C overnight. The secondary antibody was incubated for 1 h at room temperature. Specific bands were visualized by ECL (Beyotime Biotechnology, Shanghai, China), and gray scale was detected by image laboratory software (Bio-Rad, USA) to quantitatively analyze protein expression.

**Enzyme-linked immunosorbent assay**

TNFα, interleukin (IL)-6, and IL-12 measurements of cell-free macrophage-cultured supernatants were determined by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions, and absorbance was measured at 450 nm using an Emax ELISA plate reader with multi-point data analysis using SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). The detection limit for TNFα was 15.6 pg/mL. Conditioned medium was collected for TNFα, IL-6, and IL-12 analysis by ELISA. All measurements were performed in duplicate, and mean values of four measurements were used for statistical analysis.

For the TNFα analysis, AMs were stimulated with miR88, miR99, a negative control (ssRNA41), a positive control (ssRNA40) (Imgenex), and Lipid A (10 mg/mL) for 24 h (37°C, 5% CO₂), and conditioned medium was collected.

**Real-time quantitative polymerase chain reaction**

miRNAs were extracted using the mirVana miRNA Isolation Kit (Life Technologies, USA). The cDNA was synthesized by reverse transcription of the miRNA using the mirCURY LNA Universal RT microRNA PCR system (Exiqon, Epigenie, USA) and analyzed by quantitative polymerase chain reaction (qPCR). The upstream primer for amplification of vmiR88 was 5′-GAGTGCTTCAAGTACGTGTTG-3′ and the downstream primer for amplification of vmiR99 was 5′-GTAGTGTGCTCCGTCTGTTG-3′. For analysis of cytokine mRNA, adherent cells were treated with Trizol (Applied Biosystems, Foster City, CA, USA) and total RNA was prepared according to the manufacturer’s instructions. The polymerase chain reaction (PCR) primers used were as follows: TNFα forward, 5′-TGCCCTGGCT CAGACATGT-3′; TNFα reverse, 5′-GCTACA TGGAACAGCCTATTGT-3′; IL-6 forward, 5′-AAGCCAGAGCTGTGCAGATGA GTA-3′; IL-6 reverse, 5′-GCTACCTTGGCTCACCTATGGTG-3′; GAPDH forward, 5′-TTTGT CAGACATGT-3′; GAPDH reverse, 5′-GGACGAGCAG TGAGGTCTTT-3′; IL-12 forward, 5′-GTPCCCGTCGCTGTC-3′; IL-12 reverse, 5′-GACGAGCAGCAG TGAGGTCTTT-3′; IL-12 reverse, 5′-TCTCTCTCTTGT TCCCCTCTGA-3′; GAPDH forward, 5′-TTTGT CAGACATGT-3′; GAPDH reverse, 5′-TGGTCCAGGTTTCTACT-3′. GAPDH was used as an internal control, and fold changes were calculated by relative quantification (2−∆∆Ct). Experiments were conducted in three independent trials.
Construction of miRNA expression vector and preparation of lentivirus

The fragments of miR88 (5′-GAGTGCTTCAAGTAGTGTGTG-3′), miR99 (5′-GTAGTGTGTGCCCCTCCTGTTG-3′), and the control miRTAR (5′-CTAACTAGGGAACCCACTGC-3′) were inserted into the pLVX-IRES-mCherry vector in the multiple cloning site to construct pLVX-IRES-mCherry miR88, pLVX-IRES-mCherry miR99, and pLVX-IRES-mCherry miRTAR vectors, respectively. These vectors, together with the control vector, were transfected into 293T cells. After transfection for 12 h, the medium was replaced, and the lentiviruses were collected at 48 and 72 h after transfection.

Small interfering RNA–mediated knockdown for TLR8

RNAi-mediated knockdown of TLR8 was performed using synthetic duplex RNA oligonucleotides (small interfering RNA (siRNA); Thermo Scientific). Target sequences for TLR8 were GAA CGGAAAUCCCGGUAUA, CAGAAUAGCAGGCGUAACA, GUGCAGCAAUCGUCGACUA, and CUUCCAAACUUAUCGACUA. The non-targeting irrelevant siRNA#1 from Thermo Scientific was used as a control. Macrophages were electroporated with 100 nM siRNA using Amaxa Nucleofector system following the manufacturer’s protocol (Amaxa).

Transwell experiment

Transwell plates (25 mm diameter, 0.4 μm pore size; Corning, New York, USA) were used to conduct experiments in three groups. The upper layer was inoculated with 2 × 10⁴ HEK293 cells transfected with miR88, miR99, and control miRTAR lentivirus, and the lower layer was inoculated with 4 × 10⁴ AMs. After co-culture for 3 days, the lower AM culture medium was collected, and the contents of TNFα (ab181421; Abcam, Cambridge, UK), IL-6 (ab46027; Abcam, Cambridge, UK), and IL-12 (ab62822; Abcam, Cambridge, UK) were detected using an ELISA kit.

Chromatin immunoprecipitation assay

The HEK293 stable cell line expressing full-length human TLR8 was obtained from Imgenex. U937 exosomes, U1 exosomes, miR88, miR99, and control were transfected to the AMs by cationic lipofection. After transfection for 24 h, the cells were fixed with formaldehyde, disrupted by sonication, and precipitated with the addition of the TLR8 antibody and Protein A (A1489; ABelonal, Wuhan, China). The non-specific interactions were eluted to obtain a complex decrosslinking, and the nucleic acid fragment was purified for qPCR analysis.

Data analyses

Comparisons between groups were assessed by two-way analysis of variance using Prism 6.0 software (GraphPad software, San Diego, CA, USA) or one-way analysis of variance. Post hoc analysis was performed using InStat 3.0 statistical software (GraphPad software, San Diego, CA, USA) and Dunnett’s multiple comparison test. The results are presented as mean ± SEM. A P-value < 0.05 was considered statistically significant. All experiments were performed in triplicate.

Result

HIV-1-infected U937 cells released exosomal miRNAs, miR88 and miR99

After the U937 cells were infected with HIV-1 virus for 24 h, immunofluorescence staining was performed on the core protein p24 encoded by the highly conserved HIV-1 gag gene to determine whether U937 cells were infected with HIV-1. Fluorescence microscopy results showed clear green fluorescence in U937 cells, demonstrating that HIV-1 virus had effectively infected U937 cells (U1) (Figure 1(a)).

Exosomes in the U937 and U1 cell culture medium were extracted and subjected to Western blot analysis using cell lysate as a control. The results showed the exosomal markers CD63 and Tsg101 were successfully expressed in the extract (Figure 1(b)).

Extracellular exosomes of U937 and U1 cells were extracted and real-time quantitative polymerase chain reaction (RT-qPCR) was used to detect miR88 and miR99 levels. Compared to U937 exosomes, the U1 exosomes contained a large amount of miR88 and miR99 (Figure 1(c)).

miR88 and miR99 promoted the release of cytokines TNFα, IL-6, and IL-12 from AMs

The expression vectors for miR88, miR99, and the control miRTAR were constructed and were called...
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pLVX-IRES-mCherry miR88, pLVX-IRES-mCherry miR99, and pLVX-IRES-mCherry miRTAR, respectively. The lentiviruses obtained from these expression vectors were used to infect HEK293 cells. Fluorescence microscopy showed clear red fluorescence in HEK293, indicating that the lentivirus effectively infected the cells (Figure 2(a)). The miRNAs of the lentivirus-infected HEK293 cells were extracted and subjected to reverse transcription. RT-qPCR was used to determine the expression levels of miR88 and miR99. The results showed that the levels of miR88 and miR99 in HEK293 cells infected with lentivirus were significantly higher than those in the control, indicating that miR88 and miR99 were efficiently expressed in HEK293 cells (Figure 2(b)).

The transwell co-culture experiment was next carried out with HEK293 cells infected with miR88 and miR99 lentivirus inoculated in the upper chamber, and normal AMs cultured in the lower layer. Three days later, the lower AM culture medium was collected, and the expression of cytokines TNFα, IL-6, and IL-12 in normal AMs of the lower chamber was measured by ELISA. The results showed the expression of those cytokines had significantly increased (Figure 2(c)).

Exomes from HEK293 cells infected with miR88 and miR99 lentivirus were extracted. A total of 120 μU of these purified exosomes were used to challenge AMs. As a control, AMs were challenged with the same amount of exosomes from pLVX-IRES-mCherry miR88, pLVX-IRES-mCherry miR99, pLVX-IRES-mCherry miRTAR, and a negative control. AM culture medium was collected after treatment, and the expression of cytokines TNFα, IL-6, and IL-12 was measured by ELISA. We observed that pLVX-IRES-mCherry miR88 and pLVX-IRES-mCherry miR99 resulted in a significant increase in TNFα, IL-6, and IL-12 release (Figure 2(d)).

Interactions between miR88/miR99 and macrophage TLR8

AMs were stimulated with the medium control, U937 exosomes, U1 exosomes, and positive control (miR88 and miR99). After 1 day of transfection, the culture supernatant and cells were separately collected and lysed. The TLR8 antibody was added to the cell lysate for a chromatin immunoprecipitation (ChIP) assay, and the levels of miR88 and miR99 in the immunoprecipitate were determined by real-time polymerase chain reaction (RT-qPCR). The test results showed that the miR88 and miR99 levels in the U1 exosomes were significantly higher than U937 exosomes and the control group (Figure 3(a)).

Next, we treated human AMs with those exosomes and positive control (miR99 and miR88). AM culture medium was collected, and the expression of cytokines TNFα, IL-6, and IL-12 was measured by ELISA. Treatment with U1 exosomes
Figure 2. miR88 and miR99 promote cytokine released from alveolar macrophages. (a) Fluorescence microscopy showing pLVX-IRES-mCherry miR88, pLVX-IRES-mCherry miR99, and the control pLVX-IRES-mCherry miRTAR, expressed in HEK293 cells. (b) RT-qPCR detection showing miR88 and miR99 lentiviruses expression in HEK293 cells. (c) ELISA results showing the expression of TNF\(\alpha\), IL-6, and IL-12 in alveolar macrophages increased significantly after co-culture with a transwell of HEK293 cells infected with miR88 and miR99 lentiviruses. (d) ELISA results showing that the expression of cytokines TNF\(\alpha\), IL-6, and IL-12 significantly increased after co-culture with 120 \(\mu\)L of purified exosomes.

\(***\) \(P < 0.001\).
Figure 3. Interaction between miR88 and miR99 with TLR8 of alveolar macrophages. (a) ChIP and RT-qPCR results showing that the levels of miR88 and miR99 in anti-TLR8 immunoprecipitates in the U1 exosomes were significantly higher than U937 exosomes and the control group. (b) ELISA results showing treatment of AM with U1 exosomes, resulting significant increase in the expression of cytokines TNFα, IL-6, and IL-12.

*P < 0.05; **P < 0.01; ***P < 0.001.
and positive control resulted in a significant increase in TNFα, IL-6, and IL-12 release. (Figure 3(b)).

To further confirm that there was an interaction between miR88/miR99 and TLR, we further transfected the GFP-LTR8-transfected HEK293 cells with the liposome control group (LyoVec), unrelated miR (LyoVec-VmiRTAR), and experimental group miR88 and miR99 (Figure 4(a)). After 1 day of transfection, the culture supernatant and cells were collected separately and lysed. The TLR8 antibody was added to the cell lysate for ChIP experiments, and the expression levels of miR88 and miR99 in the immunoprecipitate were determined by RT-PCR. The results of the assay showed that the expression levels of miR88 and miR99 in the experimental group were significantly higher than those in the liposome control (LyoVec), irrespective of the miR control (LyoVec-VmiRTAR) (Figure 4(b)).

The expression level of TNFα in the cell culture supernatant was measured by ELISA. The results showed that the expression levels of TNFα in the experimental groups of miR88 and miR99 were significantly higher than those in the liposome control (LyoVec) and were irrespective of the miR control (LyoVec-VmiRTAR) (Figure 4(c)).

miR88 and miR99 binding to TLR8 to promote cytokines release

The respective antisense RNA antagonists of miR88 and miR99, antagonir88 (5′-CACAC ACUACUUGAAGCACUC-3′) and antagonir99 (5′-CAACAGACG GGCACACACUAC-3′), were added to AMs. After the addition of the antagonist for 1 h, miR88 and miR99 were correspondingly added. At the same time, a normal AM control and a control without an antagonist (AM) were added to miR88 and miR99, respectively. The cell culture supernatant was collected, and the changes in expression of cytokines TNFα, IL-6, and IL-12 were determined by ELISA. The results showed that the expression levels of cytokines TNFα, IL-6, and IL-12 were significantly decreased after the addition of the corresponding antagonist (Figure 5(a)).

In order to further determine the interaction between miR88/miR99 and TLR8, RNAi-mediated knockdown of TLR8 was performed. TLR8 knockdown was assessed by Western blot probed with anti-TLR8 (Figure 5(b)). We then analyzed the expression levels of TNFα in AMs treated with miR88, miR99, US (unstimulated), a negative control (ssRNA41), and a positive control (lipid A and ssRNA40). The results showed that the expression levels of TNFα were significantly decreased after TLR8 knockdown with both miR88 and miR99 and implied that vmiR88 and vmiR99 promoted TNFα release by binding to TLR8 (Figure 5(b)).

We determined the time course for mRNA and protein expression of cytokines TNFα, IL-6, and IL-12. miR99-stimulated TNFα release was very rapid, and maximal TNFα release was observed by 6 h, which was much faster than the 24 h for induction of TNFα mRNA expression. The expression levels of IL-6 and IL-12 were very similar to TNFα, but were found to be TNFα-dependent (Figure 5(c)).

Discussion

miRNAs play important roles in HIV-1 infection and the pathogenesis of AIDS. The HIV-1 regulates miRNA expression in host cells, thereby enabling its latency and replication.20,21 It not only interacts with the miRNA of the host cell but also encodes some miRNAs of its own. These HIV-1-derived exosomal miRNAs are involved in the transcription and post-transcriptional regulation of the HIV-1 genome through RNAi, or they participate in the regulation of the cell cycle or cell function of the host.22–24 Our previous study found two novel HIV-1-derived exosomal miRNAs, miR88 and miR99 (denoted as vmiR88 and vmiR99). They promoted the release of cytokine TNFα from host cells by stimulating TLR8, which may be related to the chronic immune activation associated with HIV-1 infection.

In this study, we found that higher levels of miR88 and miR99 expression were present in the exosomes extracted from the culture supernatant of HIV-1-infected U937 cells that were differentiated by PMA treatment. Co-cultures of HEK293 cells infected with miR88 and miR99 lentivirus with human AMs in a transwell showed that miR88 and miR99 promoted the release of cytokines TNFα, IL-6, and IL-12. This result indicated HIV-1-derived exosomal miR88 and miR99 were released into the extracellular space of the host through exosomes and had the potential to continue to act on adjacent uninfected HIV cells to activate the immune response.
Figure 4. Interaction between miR88 and miR99 and TLR8 transfected with HEK293 cells. (a) Fluorescence microscopy showing that the GFP-LTR8 plasmid was expressed in HEK293 cells. (b) RT-qPCR results showing that the levels of miR88 and miR99 in the immunoprecipitates were significantly higher than those in the liposome control (LyoVec), but not in the miRTAR. (c) ELISA results showing that the expression of TNFα in the experimental group transfected with miR88 and miR99 was significantly higher than that in the liposome control (LyoVec) and unrelated miR control (miRTAR).

*P < 0.05; ***P < 0.001
Figure 5. miR88 and miR99 binding to TLR8 to promote cytokines release. (a) ELISA assay showing that antagomir88 and antagomir99, the antagonists corresponding to miR88 and miR99, significantly downregulated the levels of TNFα, IL-6, and IL-12 cytokines in macrophages overexpressed by vmiRNA88 and vmiRNA99. (b) TLR8 knockdown determined by Western blot probed with anti-TLR8. The results show that the expression levels of TNFα significantly decreased after TLR8 knockdown with both miR88 and miR99. (c) Time course for mRNA and protein expression of cytokines TNFα, IL-6, and IL-12. Alveolar macrophages treated with miR99-stimulated (1.0 mg/mL) at the indicated time points (h). Conditioned medium analyzed by ELISA. Total RNA was isolated from cell extracts, and expression of TNF (normalized by GAPD) was analyzed by RT-qPCR. Those cytokines’ release was much faster than mRNA expression.

**P < 0.01; ***P < 0.001.
Previous studies have shown that miR88 and miR99 achieve the above functions by stimulating TLR8, which belongs to a family of highly conserved pathogen pattern recognition receptors. Activation of this family of receptors is closely related to innate and adaptive immunity. The cytokines TNFα, IL-6, and IL-12, among others, are the main effectors of this receptor family. Studies have shown that the natural ligand for TLR8 is a single-stranded RNA rich in GU sequences.25 Previous studies also showed that miR88 and miR99 contained 71% and 76% GU, respectively. Therefore, we speculated that the stimulation of TLR8 by miR88 and miR99 was likely to be achieved by directly binding to TLR8 receptor. The results of the ChIP experiment provided additional evidence that there was indeed a direct interaction between miR88, miR99, and TLR8. The use of miR88- and miR99-corresponding antagonists effectively blocked the miRNA from promoting the release of cytokines TNFα, IL-6, and IL-12 from human AMs. The expression level of TNFα was significantly decreased after TLR8 knockdown and implied that miR88 and miR99 promoted TNFα release by binding to TLR8.

In general, miRNA function is characterized as RNAi through targeted gene silencing of mRNAs at the translational level,26 but in recently reported studies,27,28 miRNA supported other important biological functions, such as serving as direct agonists for cell signaling and as ligands of TLR8. We determined the time course for mRNA and protein expression of cytokines TNFα, IL-6, and IL-12. Consistent with the literature reports, miR99-mediated TNFα, IL-6, and IL-12 release was much faster than miR99-targeted gene transcription and translation of those cytokines. HIV-derived miR88 and miR99 can be released at low copy numbers; the observed biological effects most likely represent TLR8 signaling activation that can provide amplification in a signal cascade and stimulate physiologically relevant responses resulting in cytokine release. Taken together, our results confirm that miR88 and miR99 have the function of promoting macrophage release of cytokines and activating the immune response.

In summary, our study found that HIV-1-derived miRNAs miR88 and miR99 were released into the extracellular space by exosomes and interacted with TLR8 on the surface of macrophages, which resulted in activating immune responses and promoting the release of cytokines TNFα, IL-6, and IL-12. These findings have important implications for understanding the pathogenesis of HIV-1 and AIDS and provide a theoretical basis for the clinical treatment of AIDS.

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