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Original Article

HIV-derived microRNA-99 reduces phagocytic function of macrophages and enhances the release of cytokines via the TLR8 signaling pathway

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Abstract: Aims: The aim of this study was to investigate whether HIV-derived miRNA-99 can regulate phagocytosis of macrophages and induce release of TNF-α, IL-6, or IL-1β. Methods: Phagocytosis of macrophages was measured by confocal microscopy and flow cytometry. The levels of TNF-α, IL-6 and IL-1β released by macrophages were measured by ELISA. Western blotting was employed to examine TLR8 protein expression in macrophages. Results: THP-1 cells that were challenged with PMA (100 ng/ml) for 24 hours became smooth and adherent. In addition, expression of CD11b in macrophages was up to 99%. The phagocytic capabilities of the three groups of macrophages declined, especially in the group of miRNA-99. Levels of TNF-α, IL-6 and IL-1β secreted by macrophages were elevated in Lipid A group and the miRNA-99 group, but not in the miRNA-TAR group. TLR8 protein expression in macrophages transfected with TLR8 plasmids was significantly lower than that in macrophages transfected with control plasmids. In addition, the level of TNF-α in the supernatant from macrophages of the miRNA-99 experimental group was significantly lower than the miRNA-99 control group. No statistical difference was found between the Lipid A experimental group and the Lipid A control group. Conclusions: The present study demonstrates that miRNA-99 decreases the phagocytic capabilities of macrophages, and increases release of TNF-α, IL-6 and IL-1β. In addition, HIV-derived miRNA-99 promotes the release of TNF-α in a TLR8-dependent pathway.

Keywords: microRNA, macrophage, TNF-α, IL-6, IL-1β, phagocytosis, TLR8

Introduction

Acquired immunodeficiency syndrome is human immunodeficiency virus (HIV) infection-induced progressive immune deficiency that is characterized by reduced $CD4^+$ T lymphocytes in human body. Chronic immune activation is the mechanism for persistent progression of HIV infection [1, 2] leading to pathology. Abnormal immune activation is mainly characterized by abnormally elevated levels of immune activation markers such as CD69, CD38 and HLA-DR that are expressed by $CD4^+$ and $CD8^+$ T lymphocytes, as well as elevated inflammatory mediators such as tumor necrosis factor (TNF)-α [3, 4]. MicroRNAs (miRNA or miR) are small non-encoding RNA with 19-24 nucleotides. miRNA molecules silence target genes by RNA interference and regulate cellular gene expression [5]. Virus-encoded miRNA can mutate and modulate the function of host cells. For example, EBV-vmiRNA suppresses host cell coding for CXCL1/ATC gene and induces the occurrence of EBV lymphoma [6]. It has been reported that HIV-1 single strand ribonucleic acid (ssRNA) promotes the release of TNF-α by human macrophages [7], and this effect depends on chromatin recombination in the nucleus which is induced by the binding of ssRNA with TLR8 receptor on cell surface [8]. miRNA-99 is a GU-rich sequence of oligonucleotides derived by HIV, and it has been shown that miRNA-99 promotes the release of TNF-α by alveolar macrophages [9]. Macrophages have various functions, and they regulate immune responses by phagocytosis, antigen presentation, and secretion of cytokines [10]. TNF-α, interleukin (IL)-6, and IL-1β are key cytokines released by macrophages in the inflammatory responses [11, 12]. The influences of miRNA-99 on phagocytosis of macrophages and the release of IL-6 and IL-1 are not clear.

Toll-like receptors (TLRs) are pattern recognition receptors for human immune responses, and they initiate immune responses by the identifying pathogens and inducing the production of pro-inflammatory factors [13, 14]. Surface-type TLRs mainly identifies molecular structures of cell wall of Gram-positive bacteria, such as lipopolysaccharide (LPS), peptidoglycan (PG), derived lipoprotein molecules and lipoteichoicacid (LTA) [15, 16]. For example, TLR5 identifies the components of bacterial flagellin. Regarding intracellular TLRs, TLR9 recognizes Cp G DNA, TLR7 and TLR8 recognize ssRNA, and TLR3 recognizes dsRNA [17, 18]. It has been reported that HCV-encoded miRNA can induce release of the pro-inflammatory factor TNF-α in the microenvironment via TLR8 pathway [19]. In the present study, we investigated changes in macrophage phagocytosis and secretion of inflammatory factors after transfection with miRNA-99 mimics, to understand the effect of HIV-derived miRNA-99 on macrophage function. In addition, the effect of miRNA-99 on the release of TNF-α by macrophages was also studied.

Materials and methods

Cells

Human monocytic leukemia THP-1 cells were obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in RPIM-1640 medium (Boster, Wuhan, China) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Waltham, MA, USA) under 37°C and 5% CO_2. The cells were passaged every two days, and those in logarithmic growth were seeded onto 6-well plates at a density of 5×10^5/ml. In each well, propylene glycol monomethyl ether acetate (PMA; 100 ng/ml) was added. The cells were cultured for 48 hours before observation using inverted microscopy.

Macrophages were divided into 8 groups: control group, Lipid A group, miRNA-TAR group, miRNA-99 group, TLR8 plasmid + microRNA99 group, blank plasmid + miRNA-99 group, TLR8 plasmid + Lipid A group, and blank plasmid + Lipid A group. Each group was tested in 6 replicate wells.

Transfection with miRNA-99 or miR-TAR

To transfect the cells with miRNA-99 or miR-TAR, Eppendorf tubes with lyophilized powder of miRNA-99 or miRNA-TAR (Haoran Biological Technology, Shanghai, China) were centrifuged for 5 minutes. Water treated with diethy pyrocarbonate was then added before mixing. Dissolved miRNA-99 or miRNA-TAR (1-3 μg) was mixed with 100 μl Lyovec (InivivoGen, San Diego, CA, USA) to obtain Lyovec-RNA complex, which was transfected into THP-1 cells. The sequence of miRNA-99 was GUAGUGUGUGCCCGUCUGUUG, while that of miRNA-TAR was CUAACUAGGGAACCCACUGC.

Silencing of TLR8 gene

To silence TLR8 gene, small-interfering RNA (siRNA) of TLR8 gene plasmid and blank plasmid were designed and produced by Genechem, Shanghai, China. The sequences of the siRNA were: 5'-GATCCCcgATTCCATTAAGCAATACTACTCGAGTAGTATTGCTTAATGGAATCGTTTTTGGAT-3' and 5'-AGCTATCCAAAAAcgATTCCATTAAGCAATACTACTCGAGTAGTATTGCTTAATGGAATCGGG-3'. Based on a previous study [7], we altered the sequences of TLR8 to silence different segments of TLR8, and observed the silencing effects.

The plasmids were extracted using Endo-free Plasmid Mini Kit II (Qiagen, Hilden, Germany), and plasmid concentration was determined by a reader (450 μg/ml; RT-6000 model, Ratyo, Shenzhen, China). SG Cell Line 4D-Ncleofector X nuclear transfection system (Lonza, Basel, Switzerland) was used to transfect the plasmids. Expression of green fluorescence protein inside the cells was observed using fluorescence confocal microscopy (Olympus, Tokyo, Japan).

Western blotting

Cells in each group were trypsinized and collected. Precooled Radio-Immunoprecipitation Assay (RIPA) lysis buffer (600 μl; 50 mM Tris-base, 1 mM EDTA, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% TritonX-100, 1% sodium deoxycholate; Beyotime Institute of Biotechnology, Shanghai, China) was then added to the samples. After lysis for 30 minutes on ice, the mixture was centrifuged at 12,000 rpm and 4°C for 10 minutes. The supernatant was used to determine protein concentration by bicinchoninic acid (BCA) protein concentration determination kit (RTP7102, Real-Times Biotechnology Co., Ltd., Beijing, China). Protein samples (6 μg) were then mixed with 5 × sodi-

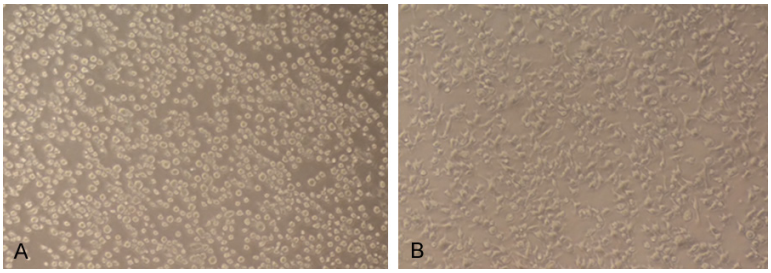

Figure 1. Morphology of THP-1 cells after stimulation with PMA. (A) Shapes of THP-1cells (A) before and (B) after treatment with PMA. Magnification, 100 ×.

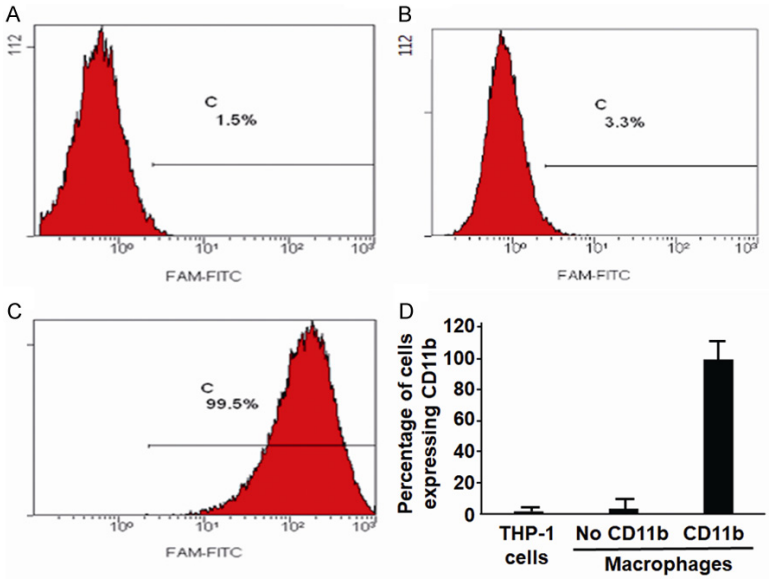

Figure 2. Expression of CD11b in macrophages determined by flow cytometry. A. THP-1 cells (without CD11b). B. Macrophages without CD11b after treatment with PMA for 24 hours. C. Macrophages with CD11b after treatment with PMA for 24 hours. D. The percentage of cells expressing CD11b.

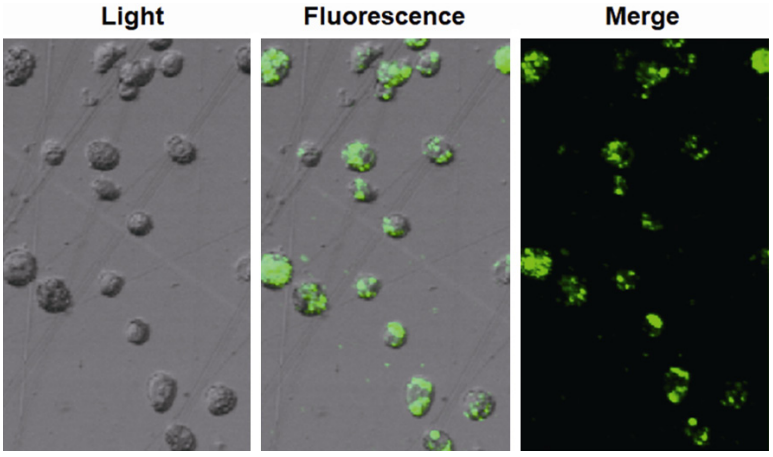

Figure 3. Macrophages that phagocytose E. coli labeled with green fluorescence protein. Fluorescence confocal microscopy was used to observe macrophages with green fluorescence. The three pictures were obtained from the same field. Magnification, 400 ×.

um dodecyl sulfate loading buffer before denaturation in boiling water bath for 10 minutes. Afterwards, the samples were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (90 V, 3 h). The resolved proteins were transferred to polyvinylidene difluoride membranes on ice (160 mA, 40 minutes) and blocked with 50 g/L skim milk at room temperature for 2 hours. Then, the membranes were incubated with rabbit anti-human TLR8 polyclonal primary antibody (1:1,000; Elabscience, Wuhan, China) and rabbit anti-human β-actin primary antibody (1:4,000; Bioworld Technology, St. Louis Park, MN, USA) at 4°C overnight. After extensive washing with phosphate-buffered saline with Tween 20 for 5 times of 5 minutes, the membranes were incubated with goat anti-rabbit IgG secondary antibody (1:2,000; Elabscience, Wuhan, China) for 1 hour at room temperature before washing with phosphate-buffered saline with Tween 20 for 5 times of 5 minutes. Then, the membrane was developed with enhanced chemiluminescence detection kit (Sigma-Aldrich, St. Louis, MO, USA) for imaging. Image lab v3.0 software (Bio-Rad, Hercules, CA, USA) was used to acquire and analyze imaging signals. The relative content of TLR8 protein was expressed as TLR8/β-actin ratio.

Determination of phagocytosis

Under a confocal microscope, green fluorescence was observed for FITC-Escherichia coli (E. coli). After removing supernatants, macroph-

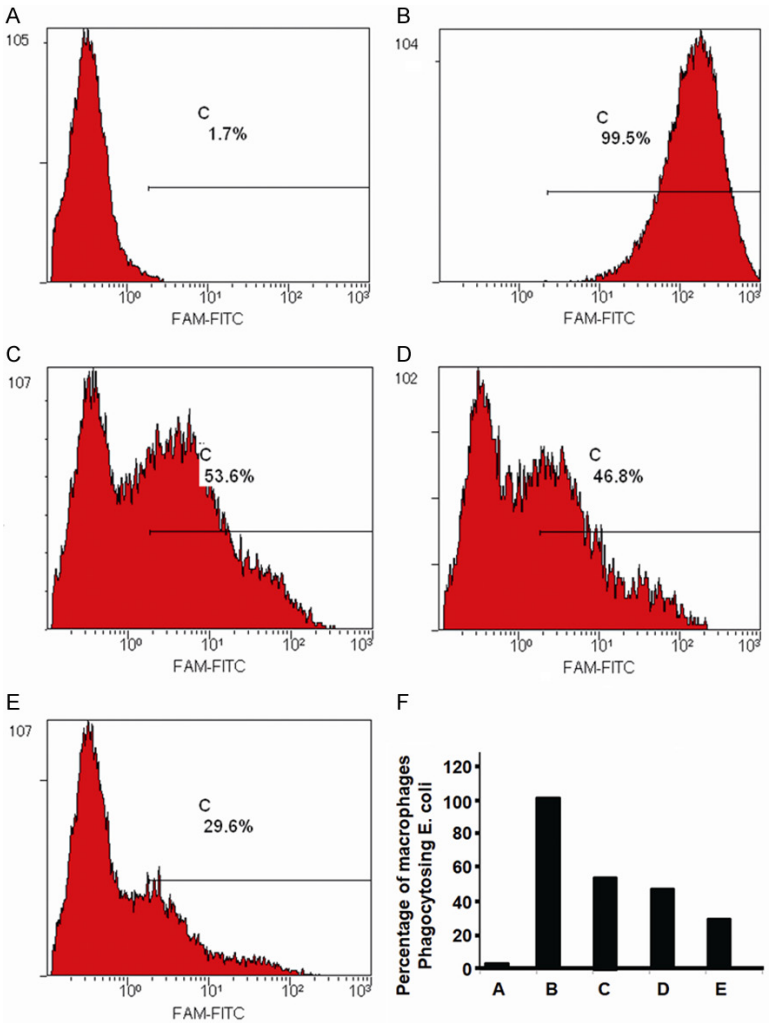

Figure 4. Phagocytosis of E. coli by macrophages. A-E. Flow cytometric analysis of the percentages of macrophages with fluorescence in the following groups: A. Macrophages without FITC-E. coli; B. Macrophages with FITC-E. coli; C. Macrophages with FITC-E. coli + Lipid A; D. Macrophages with FITC-E. coli + miR-TAR; E. macrophages with FITC-E. coli + miRNA-99. F. Histograms of the percentages of macrophages that phagocytosed FITC-labeled E. coli.

age pellets were washed with sterile phosphate-buffered saline twice to remove residual miRNA. Then, antibiotics-free and serum-free RPMI-1640 medium was added. Afterwards, 50 μl E. coli solution was added onto cells at a ratio of 100:1. After culturing the cells for 2 hours in the dark, cells were observed under a confocal microscope for green fluorescence. After washing with phosphate-buffered saline twice, the macrophages that were attached to the bottom were scraped off and prepared into single-cell suspension using RPMI-1640 medium. Flow cytometry was then performed to determine phagocytic rate (FC500; Beckman-Coulter, Brea, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

Reagents were added onto differentiated macrophages in 24-well plates before incubation for 24 hours. Then, supernatants were collected and subjected to ELISA for measuring the concentrations of TNF-α, IL-6 and IL-1β using relevant ELISA kits (Boster, Wuhan, China) according to the manufacturer's manuals.

Statistical analysis

The results were analyzed using SPSS 17.0 statistical software (IBM, Armonk, NY, USA). The data are expressed as mean ± standard deviation. Single-factor analysis of variance was used to compare differences among groups. $P < 0.05$ indicated statistically significant differences.

Results

PMA stimulation alters the morphology of THP-1 cells

To observe the effect of PMA stimulation on the shapes of THP-1 cells, inverted microscopy was performed. The cells showed separated floating status, round, or nearly round shapes, and grapevine clustering (**Figure 1A**). After treatment with PMA, the cells adhered to the bottom, protruded pseudopods and exhibited spindle or polygon shapes (**Figure 1B**). The result suggests that PMA stimulation alters the morphology of THP-1 cells.

Most THP-1 cells have differentiated to macrophages for further experiments

To determine the expression of CD11b on the surface of macrophages, flow cytometry was carried out. The positive rate of CD11b on THP-1 cell surface was over 99% (**Figure 2**). The result indicates that most THP-1 cells

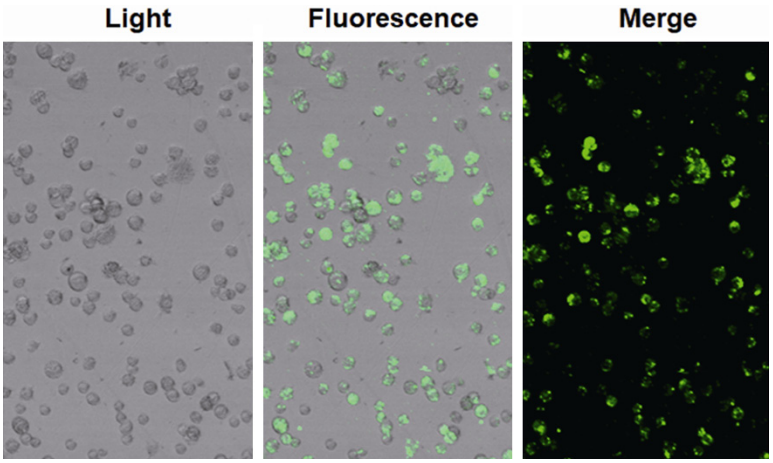

Figure 5. Macrophages that were transfected with control and TLR8 plasmids. Fluorescence confocal microscopy was used to observe macrophages with green fluorescence. The three pictures were obtained from the same field. Magnification, 400 ×.

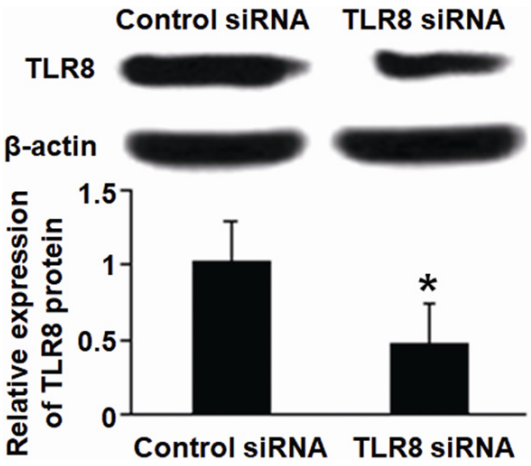

Figure 6. Expression of TLR8 protein in macrophages transfected with control siRNA and TLR8 siRNA. Western blotting was used to determine the expression of protein. *, P < 0.05 compared with control siRNA group.

have differentiated to macrophages for further experiments.

Macrophages have phagocytosed E. coli that are labeled by green fluorescence

To visualize E. coli and calculate phagocytic rate, fluorescence confocal microscopy and flow cytometry were employed. The data show that the cytoplasm of macrophages has green fluorescence (**Figure 3**). Flow cytometry showed that macrophages had the highest percentage of cells that phagocytosed E. coli (**Figure 4A-E**). The result suggests that macrophages have phagocytosed E. coli that is labeled by green fluorescence.

TLR8 and control plasmids are successfully transfected into macrophages

To observe TLR8 plasmids in macrophages, fluorescence confocal microscopy was carried out. The data showed that macrophages had green fluorescence (**Figure 5**). The result suggests that TLR8 and control plasmids are successfully transfected into macrophages.

The siRNA of TLR8 silences the expression of TLR8 protein in macrophages

To determine the expression of TLR8 protein, Western blotting was employed. The data showed that the level of TLR8 protein in macrophages transfected with TLR8 siRNA plasmids were significantly lower than that in control group ($P < 0.05$) (**Figure 6**). The result indicates that the siRNA of TLR8 silences the expression of TLR8 protein in macrophages.

miRNA-99 stimulates release of TNF-α, IL-6 and IL-1β by macrophages, and promotes the release of TNF-α in a TLR8-dependent pathway

To measure the contents of TNF-α, IL-6 and IL-1β proteins in the supernatants of macrophage culture medium, ELISA was performed. The data show that levels of TNF-α, IL-6 and IL-1β in supernatants of Lipid A group and miRNA-99 group were significantly higher than the control group ($P < 0.05$ for all), but those in miR-TAR group were not significantly different from the control group ($P > 0.05$ for all). Moreover, the levels of TNF-α, IL-6 and IL-1β in supernatants of miRNA-99 group were significantly higher than those of the Lipid A group ($P < 0.05$ for all) (**Tables 1-3**). After silencing TLR8 using its siRNA, the content of TNF-α in miRNA-99 experimental group was significantly lower than that in miRNA-99 control group ($P < 0.05$). In addition, the content of TNF-α in Lipid A

Table 1. Content of TNF-α in the supernatants of macrophage culture medium (mean ± standard deviation)

Groups	Number of wells	TNF-α (pg/mL)
Control	6	48.19 ± 5.46
Lipid A	6	250.01 ± 7.79*
miR-TAR	6	52.55 ± 7.29#,§
miRNA-99	6	876.29 ± 6.08*,#

Note: *, P < 0.05 compared with control group; #, P < 0.05 compared with Lipid A group; §, P < 0.05 compared with miRNA-TAR group.

Table 2. Content of IL-6 in the supernatants of macrophage culture medium (mean ± standard deviation)

Groups	Number of wells	IL-6 (pg/mL)
Control	6	11.40 ± 2.28
Lipid A	6	61.99 ± 2.94*
miR-TAR	6	12.67 ± 5.39#,§
miRNA-99	6	133.23 ± 3.28*,#

Note: *, P < 0.05 compared with control group; #, P < 0.05 compared with Lipid A group; §, P < 0.05 compared with miRNA-TAR group.

Table 3. Content of IL-1β in the supernatants of macrophage culture medium (mean ± standard deviation)

Groups	Number of wells	IL-1β (pg/mL)
Control	6	8.10 ± 1.79
Lipid A	6	36.97 ± 2.42*
miR-TAR	6	8.83 ± 1.8#,§
miRNA-99	6	65.95 ± 3.12*,#

Note: *, P < 0.05 compared with control group; #, P < 0.05 compared with Lipid A group; §, P < 0.05 compared with miRNA-TAR group.

Table 4. Content of TNF-α in the supernatants of macrophage culture medium after silencing TLR8 gene (mean ± standard deviation)

Groups	Number of wells	TNF-α (pg/mL)
miRNA-99 experimental	6	425.03 ± 1.10*
miRNA-99 control	6	820.24 ± 3.52
Lipid A experimental	6	212.32 ± 6.14
Lipid A control	6	271.53 ± 2.04

Note: *, P < 0.05 compared with miRNA-99 control group.

experimental group was not significantly different from that in Lipid A control group (P > 0.05) (**Table 4**) The results suggest that miRNA-99 stimulates release of TNF-α, IL-6 and IL-1β by macrophages, and promotes the release of TNF-α in a TLR8-dependent pathway.

Discussion

After HIV-1 infection in humans, even if effective antiretroviral therapy is performed, AIDS pathology is still progressing [20, 21]. Chronic immune activation of macrophages caused by HIV-1 is an important mechanism by which HIV induces immune damage in addition to $CD4^+$ T lymphocyte depletion. miRNA can regulate cell transcription by complementation with mRNA as RNA interference. HIV-derived miRNA-99 does not rely on RNA interference way, but regulates cellular functions by binding to TLR8.

Macrophages are the first line of defense against natural immunity, and bactericidal activity is their most basic function in immune defense reaction against infection. The present study shows that the phagocytic ability of macrophages in the Lipid A group is lower than that in the control group, while the phagocytic rate in miRNA-99 group is even lower than that in the Lipid A group. A decrease in macrophage phagocytic activity may prevent pathogens to be eliminated, and this also reduces antigen presentation capability of macrophages, thereby reducing specific lymphocyte immune responses. This explains why the incidence of opportunistic infections (especially pulmonary infections) is increased in HIV-infected patients.

TNF-α is a small-molecule protein secreted by macrophages. It is one of the earliest and most important inflammatory factors in the body against infections. Low levels of TNF-α protect the body against bacterial infections or damages caused by tumor cells and tissue injuries. However, excessive production of TNF-α leads to overexpression of inflammatory factors such as HIL-IB, IL-6, IL-8 and IL-1β, which recruit and activate other immune cells, amplify inflammatory responses, and cause immune activation states. Our study shows that TNF-α, IL-6 and IL-1β levels in miRNA-99 group are significantly higher than those in the Lipid A group, suggesting that miRNA is more powerful than Lipid A in promoting inflammation and damaging cells, tissues and organs.

TLRs are important members of the family of pathogen-associated molecular patterns (PA-

MPs). After binding with PAMPs, TLRs activates IRF3, IRF7, AP-1 and NF-κB to induce the production of antiviral proteins, IFNs and a large number of inflammatory factors [22, 23]. TLR7 and TLR8 can recognize ssRNA. In the present study, we have silenced TLR8 protein expression by RNA interference. Furthermore, treatment with miRNA-99 significantly reduces the content of TNF-α in culture supernatant of macrophages, suggesting that miRNA-99 stimulates the release of TNF-α by macrophages via TLR8 pathway.

In conclusion, the present study demonstrates that miRNA-99 stimulates macrophages to release pro-inflammatory cytokines by activating TLR8 pathway. Additionally, miRNA-99 reduces macrophage phagocytosis of pathogens, resulting in decreased antigen presentation ability of macrophages. These data suggest that miRNA-99 may be an important mechanism for chronic immune activation in AIDS. The present study helps to elucidate the pathogenesis of chronic immune activation induced by HIV infection, and provides new experimental evidence for the development of drugs targeting miRNA-99.

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Disclosure of conflict of interest

None.

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