Original Paper

Circulating Exosomal miR-17 Inhibits the Induction of Regulatory T Cells via Suppressing TGFBR II Expression in Rheumatoid Arthritis

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Key Words

Exosome • miR-17 • Treg cells • TGFBR II • Rheumatoid arthritis

Abstract

Background/Aims: A reduced prevalence of circulating regulatory T cells (Tregs) is a hallmark of inflammatory rheumatoid arthritis (RA). However, the underlying mechanisms of alterations of Tregs are unclear. **Methods:** The ratio of Tregs in peripheral blood of healthy controls (HCs) and patients with RA was determined by flow cytometry. MicroRNA (miRNA) expression profiles in exosomes derived from RA patients (RA-exosomes) and in those from HCs (HC-exosomes) were detected by microarray analysis, and miR-17 was measured by quantitative real-time PCR. Transforming growth factor beta receptor II (TGFBR II) expressed by T cells was measured by flow cytometry. The interaction between miR-17 and TGFBR II was evaluated by dual-luciferase reporter assay. **Results:** We found that RA-exosomes can selectively affect Treg differentiation *in vitro*. Several miRNAs are more abundant in the RA-exosomes than in HC-exosomes. Among those upregulated in patients with RA, miR-17 can suppress Treg induction by inhibiting the expression of TGFBR II. **Conclusion:** Our findings imply that altered miRNA expression in RA-exosomes may contribute to the pathogenesis of RA by disrupting the homeostasis of Tregs.

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Introduction

Rheumatoid arthritis (RA) is the most common type of autoimmune disease. It is characterized by synovial inflammation, production of autoantibodies such as anti-citrullinated protein antibody (Ab), and destruction of cartilage and bone. The current understanding of RA pathogenesis is that this equilibrium is disturbed, in part due to qualitative defects of regulatory T cells (Tregs) [1]. Lawson et al. observed a reduced

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prevalence of circulating CD4⁺CD25^{high} Tregs in patients with early active RA[2]. Ehrenstein et al. reported a defect of Tregs in the suppression of pro-inflammatory cytokine production [3], and Nie et al. showed a reduced suppression of proliferating effector cells by Tregs that was TNF-dependent [4]. However, the underlying mechanisms of alterations of Tregs are unclear.

MicroRNAs (miRNAs) are small (~22-nucleotide-long) endogenous noncoding RNAs that function as negative regulators of gene expression through complementary antisense binding to their target mRNAs [5-7]. Alterations of miRNAs in the circulation, inflammatory cell populations, or pathological samples of autoimmune diseases have also been documented [8-11]. miRNA can be present in exosomes, which are extracellular vesicles smaller than 150 nm in diameter. Exosomes can affect the target cells via gene regulation, which is mediated by transfer of miRNAs [12-14]. Critical involvement of exosomes has been demonstrated in various human disorders, including cancer and neurodegenerative diseases [15, 16]. However, exosomal miRNA function in Treg differentiation has not been studied in RA.

In this study, we isolated circulating exosomes from the blood of patients with RA (RA-exosomes) and evaluated the potential pathogenic function of these miRNA-containing exosomes in Treg differentiation. We found that RA-exosomes can selectively affect Tregs *in vitro*. Several miRNAs are more abundant in RA-exosomes than in exosomes from healthy control donors (HC-exosomes). Among those upregulated in patients with RA, miR-17 can suppress Treg induction by inhibiting the expression of transforming growth factor beta receptor II (TGFBR II). Our findings imply that altered miRNA expression in RA-exosomes may contribute to the pathogenesis of RA by disrupting the homeostasis of Tregs.

Materials and Methods

Human subjects

We present demographics of the patients with RA and healthy volunteers who agreed to provide peripheral blood for this study in Table 1. The 2010 ACR/EULAR criteria were used to diagnose RA. None of the patients had received therapy. The Ethics Committee of Lanzhou University Second Hospital approved the study protocol.

Isolation of exosomes

Exosomes were purified from the plasma according to the manufacturer's instructions (Cell Guidance Systems, Cambridge, UK). Briefly, plasma samples were subjected to sequential centrifugation at 1200 ×g for 15 min and at 2000 ×g for 20 min, and supernatant was collected after the final centrifugation at 10,000 ×g for 45 min. The resulting supernatant was mixed with a one-fifth volume of polyethylene glycol buffer (30% PEG 6000, 50 mmol/L HEPES, 1 mol/L NaCl). Exosomes were pelleted by centrifugation at 10,000 ×g for 30 min and resuspended in phosphate-buffered saline (PBS). The size distribution and purity of the collected exosomes was determined using a NanoSight LM10 nanoparticle analysis system (NanoSight Ltd, Malvern, UK). More than 95% of the extracellular vesicles' size was smaller 200 nm, and the purity of the collected exosomes was higher than 95%.

CD4⁺T cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy control (HC) donor blood samples by Ficoll centrifugation (Axis-Shield Diagnostics, Dundee, UK). CD4⁺T cells (greater than 95% CD4⁺T cells) were purified from PBMCs using a MACS CD4⁺T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

Table 1. Clinicopathological characteristics of patients (n = 25)

Variables	No. and (%)
No. of patients	25
Age (median; range), years	60.2; 29–78
Sex (male/female)	5/20 (20.0/80.0)
RF (positive/negative)	22/3 (88.0/12.0)
CRP (mean;range), (mg/dL)	2.8; 0.3–10.4
Krenn's score	
4	2 (8.0)
5	2 (8.0)
6	4 (16.0)
7	8 (32.0)
8	5 (20.0)
9	4 (16.0)

RNA isolation and quantitative real-time PCR

Total RNA from exosomes was isolated using TRIzol reagent (Takara, Dalian, China). Before performing quantitative RT-PCR assays (qPCR), RNA was reverse transcribed into miRNA cDNA and total cDNA using a One-Step PrimeScript miRNA cDNA Synthesis Kit (Takara) and PrimeScript RT reagent Kit (Takara), respectively. The mRNA and miRNA expression levels were detected by qPCR with an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara) according to the manufacturer's instructions, and were normalized versus GAPDH mRNA and small nuclear RNA U6, respectively. The Ct value of miR-17, miR-19b, miR-121, and TGFBR II was quantified using the 2- $\Delta\Delta C_t$ method.

Cell culture

To determine the roles of exosomes and miRNAs, CD4⁺ T cells were cultured with exosomes before or after being transfected with miRNA mimics, inhibitor, and small interfering RNAs (siRNAs) as indicated. RPMI-1640 (Life Technologies, Carlsbad, CA) was used for the culture. The exosomes were derived from the same amount of the plasma and the CD4⁺ T cells were prepared from HCs. Culture cells were stimulated with 1 μ g/mL anti-CD3 monoclonal Ab (mAb) (OKT3) and 1 μ g/mL anti-CD28 mAb (Beckman Coulter, Brea, CA) for 1 h at 37°C before cells were added. In the differentiation assay, CD4⁺ T cells were polarized towards Tregs with 10 ng/mL TGF- β and 100 U/mL IL2 (PeproTech, Rocky Hill, NJ). To stain intracellular cytokines, cultured cells were stimulated with 30 ng/mL phorbol-myristate-acetate (Sigma-Aldrich, St. Louis, MO), 200 ng/mL ionomycin (Sigma-Aldrich), and 1 μ M monensin (Sigma-Aldrich) for 4 h before staining.

Flow cytometry

The Abs used for flow cytometry were FITC-conjugated mouse anti-human CD4, PE-conjugated mouse anti-human Foxp3, APC-conjugated mouse anti-human TGFBR II, and PECY-Cy5.5-conjugated mouse anti-human CD25 from BD Biosciences (San Jose, CA). The cultured cells were collected, washed twice, and resuspended in 100 μ L of PBS containing 0.1% bovine serum albumin (BSA). These cells were stained and labeled with either specific Abs or the appropriate isotype controls. The cells were incubated on ice for 30 min, washed with PBS containing 0.1% NaN₃ and 0.5% BSA, and then fixed in 1% paraformaldehyde solution. Analyses were performed using FACScan and CellQuest software (BD Biosciences).

Oligonucleotide transfection

Transfected miR-17, miR-19b, miR-121 mimic, miR-17 inhibitor, negative control (NC) duplex, and siRNA against TGFBR II (siTGFBR II), were all synthesized by GenePharma (Shanghai, China). Oligonucleotide transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The transfection rate was approximately 90%.

Statistical analysis

Results are expressed as the mean $\pm$ standard error of the mean (SEM) and statistically evaluated by use of the two-tailed unpaired *t* test. A *P* value less than 0.05 was considered statistically significant. Statistical analyses were performed by GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA).

Results

Treg frequency is decreased in RA patients and inhibited by RA-exosomes

Given the critical contribution of cellular immunity in RA patients, we analyzed the ratio of Tregs in peripheral blood of HCs and patients with RA by flow cytometry. As shown in Fig. 1A and B, Treg frequency was significantly decreased in RA patients compared with HCs.

To investigate the function of the circulating exosomes in RA patients, we purified exosomes from the plasma of HCs and RA patients. The average size of the vesicles purified from the plasma samples was 89.5 nm (*n* = 10). The size of most of the purified vesicles was consistent with that of exosomes, which are smaller than 150 nm (Fig. 1C). Furthermore, they expressed the conventional exosome markers CD9 and CD63 (Fig. 1D).

Fig. 1. Treg frequency is decreased in RA patients and inhibited by RA-exosomes. **A** and **B**, Dot plot and histogram of flow cytometry data. **A**, Representative Treg frequency of HC and RA patients. **B**, The data indicate the mean $\pm$ SEM of 13 subjects. **C**, Representative size distribution of purified exosomes. Using a NanoSight LM10 nanoparticle analysis system, the size was analyzed three times for each sample. **D**, Western blot analysis for CD9 and CD63. The PBMC and exosome samples (EV1 and EV2) were collected from RA patients. Each lane was loaded with 5 μ g of protein for blotting. **E** and **F**, Frequencies of Tregs among CD4⁺ T cells after culture with exosomes derived from HC and RA patients. **F**, One representative experiment is shown. **E**, Data indicate the mean $\pm$ SEM of three independent experiments.

Fig. 2. Exosomal miR-17 is increased and inversely correlates with Treg frequency in RA patients. A, Heat map of miRNA expression profile in the exosomes from HC and RA patients. RNA was extracted from exosomes, which were isolated from the plasma of one HC and one RA patient. B–D, Quantification of the candidate miRNAs by RT-qPCR. The total amount of RNAs in the exosomes from the same amount of plasma was also examined. E–G, Correlation between the frequency of Tregs and exosomal miRNAs in patients with RA. The data indicate the mean±SEM of 15 subjects.

Given that Treg frequency was decreased in RA patients, we focused on the effect of exosomes on Tregs. We cultured peripheral blood CD4⁺ T cells from HCs with anti-CD3 mAb and anti-CD28 mAb in the presence of RA-exosomes or HC-exosomes. Interestingly, RA-exosomes resulted in a decreased ratio of Tregs compared with HC-exosomes (Fig. 1E and F).

Exosomal miR-17 is increased and inversely correlates with Treg frequency in RA patients

To determine how RA-exosomes affect Tregs, we further analyzed miRNA expression profiles in RA-exosomes as compared with those in HC-exosomes. Microarray analysis results showed remarkable differences in miRNA profiles between RA-exosomes and HC-exosomes (Fig. 2A). Next, we selected three miRNAs, namely, miR-17, miR-19b, and miR-121, for further analysis, the expression of which showed significant differences between RA-exosomes and HC-exosomes (Fig. 2B–D).

We investigated whether a relationship exists between the frequency of the Tregs and these three miRNAs (miR-17, miR-19b, and miR-121) in RA patients. A negative correlation between miR-17 and Treg frequency was observed, but no correlation between the Tregs and either of the remaining two miRNAs was observed (Fig. 2E–G).

Fig. 3. RA-exosomes inhibit the differentiation of Tregs via miR-17. A, CD4⁺ T cells were cultured in media supplemented with PBS, HC-exosomes, or RA-exosomes, with or without miR-17 inhibitor. The data indicate the mean±SEM of three independent experiments. B, CD4⁺ T cells were transfected with miR-17, miR-19b, or miR-121 mimic. The data are representative of three independent experiments.

RA-exosomes inhibit the differentiation of Tregs by miR-17

To investigate the effect of miR-17, miR-19b, and miR-121 on Treg differentiation, CD4⁺ T cells from HCs were transfected with miR-17, miR-19b, or miR-121 mimic, cultured with anti-CD3 and anti-CD28 mAbs for 72 h, and then the frequencies of Treg populations were evaluated by flow cytometry. The frequency of Tregs was significantly decreased after transfection with miR-17 mimic, while the frequency of Tregs was not affected after transfection with miR-19b or miR-121 mimic (Fig. 3B). Importantly, miR-17 inhibitor significantly upregulated the frequency of Tregs in the RA-exosome group, and the rate of increase in Treg frequency after treatment with the miR-17 inhibitor was greater in the presence of RA-exosomes than HC-exosomes (Fig. 3A).

miR-17 inhibits Treg differentiation through targeting TGFBR II

To determine the mechanism of miR-17-mediated inhibition of Treg induction, we have attempted to identify target genes of miR-17. Using an online database for target prediction, Target Scan Human, we were able to identify candidate genes targeted by miR-17. Given that TGF- β signaling pathways promote induction of Tregs, we evaluated the effect of miR-17 on target gene TGFBR II expressed by T cells. CD4⁺ T cells were transfected with miR-17 and cultured in the presence of anti-CD3 and anti-CD28 mAbs for 72 h. Notably, the expression of TGFBR II on the cultured CD4⁺ T cells was significantly decreased (Fig. 4A).

To investigate whether miR-17 regulates Treg differentiation via the TGFBR II 3' untranslated region (3'-UTR), we performed a luciferase reporter assay. HEK 293 T cells were transiently transfected with these constructs and miR-17 mimic or NC. miR-17 mimic significantly suppressed the luciferase activity of reporter genes containing the wild-type 3'-UTR of TGFBR II (Fig. 4B). In contrast, the inhibition was fully rescued when all target sites were mutated (Fig. 4B).

Fig. 4. miR-17 inhibits Treg differentiation through targeting TGFBR II. **A**, CD4⁺ T cells were transfected with miR-17 and then cultured under stimulation with anti-CD3 and anti-CD28 mAbs for 72 h. Representative histograms of the expression levels of TGFBR II are shown. **B**, Luciferase activity of the wild-type TGFBR II 3'-UTR reporter vectors was decreased by overexpression of miR-17 in HEK 293 cells, while it was not significantly decreased when all binding sites were mutated compared with the negative control. **C**, Western blot analysis of CD4⁺ T cells transfected with either siRNA against TGFBR II (siTGFBR II-1 and -2) or NC-siRNA (siRNA). The data shown are representative of three independent experiments. **D**, CD4⁺ T cells were transfected with two siRNAs targeting TGFBR II and cultured under stimulation with anti-CD3 and anti-CD28 mAbs for 72 h. The data are representative of three independent experiments. **E**, The frequency of Tregs and the expression of TGFBR II on CD4⁺ T cells were compared between the groups with lower and higher amounts of miR-17 in exosomes in the blood. **F**, Correlation analysis between the amount of miR-17 in CD4⁺ T cells and the expression of TGFBR II on CD4⁺ T cells in RA patients. MFI, mean fluorescence intensity.

To evaluate the effect of TGFBR II on Treg differentiation, peripheral blood CD4⁺T cells were transfected with various siRNAs and cultured in the presence of anti-CD3 and anti-CD28 mAbs for 72 h before flow cytometry analysis. We confirmed that TGFBR II was knocked down by the three different siRNAs (Fig. 4C), which resulted in a similar reduction in the frequency of Tregs (Fig. 4D).

To obtain supportive evidence for the alteration of the miR-17/TGFBR II axis in patients with RA *in vivo*, we isolated PBMCs from HCs and RA patients. Strikingly, the results showed that the expression of TGFBR II on CD4⁺ T cells was significantly lower in the miR-17-high group than in the miR-17-low group (Fig. 4E). Moreover, a significant negative correlation between them was also detected (Fig. 4F).

Discussion

In the present study, we describe a novel effect of exosomes on Tregs in RA patients: RA-exosomes can suppress Treg induction by the transfer of miR-17, and then miR-17 inhibits the differentiation of Tregs by targeting the expression of TGFBR II.

Tregs have been identified as sentinels of the immune response, keeping aberrant immune reactions in check and preventing autoimmunity. We and other groups have shown that a reduction of Treg frequency is an immunological hallmark of RA [3, 4, 17]. Because exosomes were shown to contribute to intercellular communication by transporting signals into the target cells either close to or distant from the cells of exosome origin [12, 16], we investigated the effect of RA-exosomes on Treg differentiation and found that RA-exosomes could inhibit Treg differentiation *in vitro*. Because miRNA has been shown to play an important role in the differentiation and function of immune cells [18-23], we next analyzed miRNA expression profiles in RA-exosomes as compared with those in HC-exosomes by microarray analysis. The results showed remarkable differences in miRNA profiles between RA-exosomes and HC-exosomes. Moreover, a negative correlation between miR-17 and Treg frequency was observed. The frequency of Tregs was significantly decreased after transfection with miR-17, and miR-17 inhibitor significantly upregulated the frequency of Tregs in the RA-exosome group. Together, these results suggest that RA-exosomes prevent the differentiation of Tregs through transporting miR-17 into T cells.

miRNAs are short noncoding RNAs with a length of 18–22 nucleotides that cause mRNA cleavage and subsequent degradation by binding to the complementary 3'-UTR of the mRNA. miR-17 has been shown to control iNKT cell oncogenes via modulation of TGF- β signaling [24], and TGF- β signaling plays an important role in Treg differentiation [25]. Therefore, we evaluated the effect of miR-17 on TGFBR II expressed by T cells and found that the expression of TGFBR II on the cultured CD4⁺ T cells was significantly decreased after transfecting with miR-17, and siTGFBR II resulted in a similar reduction in the frequency of Tregs, suggesting that miR-17 regulated the Treg differentiation by inhibiting TGFBR II expression. Moreover, luciferase reporter assay results showed that the miR-17 mimic significantly suppressed the luciferase activity of reporter genes that contain the wild-type 3'-UTR of TGFBR II and the inhibition was fully rescued when all target sites were mutated, which suggests that TGFBR II is the target gene of miR-17 and miR-17 inhibits the Treg differentiation by targeting TGFBR II expression.

In this study, we found that RA-exosomes can selectively affect Tregs *in vitro*. Several miRNAs are more abundant in RA-exosomes than in HC-exosomes. Among those upregulated in patients with RA, miR-17 can suppress Treg induction by inhibiting the expression of TGFBR II. Our findings imply that altered miRNA expression in RA-exosomes may contribute to the pathogenesis of RA by disrupting the homeostasis of Tregs.

Conclusion

Our findings imply that altered miRNA expression in RA-exosomes may contribute to the pathogenesis of RA by disrupting the homeostasis of Tregs.

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Disclosure Statement

The authors declare no conflicts of interest.

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