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MiR-150 impairs inflammatory cytokine production by targeting ARRB-2 after blocking CD28/B7 costimulatory pathway

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Abstract

MiR-150, a major modulator negatively regulating the development and differentiation of various immune cells, is widely involved in orchestrating inflammation. In transplantation immunity, miR-150 can effectively induce immune tolerance, although the underlying mechanisms have not been fully elucidated. In the current study, we found that miR-150 is elevated after blocking CD28/B7 co-stimulatory signaling pathway and impaired IL-2 production by targeting ARRB2. Further investigation suggested that miR-150 not only repressed the level of ARRB2/PDE4 directly but also prevented AKT/ARRB2/PDE4 trimer recruitment into the lipid raft by inhibiting the activities of PI3K and AKT through the cAMP-PKA-Csk signaling pathway. This leads to the interruption of cAMP degradation and subsequently results in inhibition of the NF- κ B pathway and reduced production of both IL-2 and TNF. In conclusion, our study demonstrated that miR-150 can effectively prevent CD28/B7 co-stimulatory signaling transduction, decrease production of inflammatory cytokines, such as IL-2 and TNF, and elicit the induction of immune tolerance. Therefore, miR-150 could become a novel potential therapeutic target in transplantation immunology.

Keywords

MicroRNA; T cell; CD28/B7co-stimulatory signaling pathway; inflammatory cytokine

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Conflict of Interest

We have no conflict of interest in this manuscript.

1. Introduction

MicroRNAs (miRs) are a class of small noncoding RNAs (~22 nucleotides) regulating gene expression primarily at the post-transcriptional level by repressing translation or by promoting degradation of the target messenger RNA (mRNA). The most common mechanism is through the interaction between the 5'-end of the miR (seed region) and the 3' untranslated region (UTR) of their target mRNAs. In addition, some miRNAs can also alter the half-life of their targeted mRNAs by binding to certain domains other than the 3' UTR (1–4). It has been reported that microRNA-150 (miR-150) plays an important role in the pathogenesis of both solid and hematological cancers (5–7). For example, miR-150 promotes the proliferation of gastric cancer (8), inhibits growth and malignant behavior of pancreatic cancer (9), and participates in embryonic development and tumor occurrence by targeting c-Myb (10). MiR-150 may also serve as a potential diagnostic or prognostic marker. Plasma levels of miR-150 have been suggested as a potential diagnostic marker for acute myeloid leukemia (11) and for prognostic evaluation in cancers such as colorectal, esophageal, and non-small cell lung (12–15). In addition, miR-150 has been used as a potential therapeutic target in other diseases. For example, administration of anti-miR-150 carrier inhibited A549 lung cancer (16). MiR-150 is also involved in the regulation of immunity, including immune cell development, proliferation and activation. MiR-150 is also involved in the innate and adaptive immune responses.

MiR-150 is selectively expressed in mature B and T cells, and is an important regulator for differentiation and activation of immune cells (17). Overexpression of miR-150 in mouse model not only blocks B-cell development (18), but also affects B cell differentiation by targeting c-Myb (19). MiR-150 may also play an important role in T cells development through targeting Notch pathway (20). Mi et al demonstrated that miR-150 involves in the cross presentation process of Langerhans cells (21). Zheng et al found that miR-150 deficiency in mice could affect invariant natural killer T (iNKT) cell maturation in both thymus and periphery (22). Moreover, low serum miR-150 levels were observed in patients with multiple sclerosis. Systemic Sclerosis patients with lower serum miR-150 levels tend to have more severe clinical manifestations (15). We have demonstrated that miR-150 could effectively induce immune tolerance in patients with allogeneic hematopoietic stem cell transplantation (allo-HSCT) by regulating CD4+ T cell function. Since miR-150 levels started to decline about four days prior the onset of acute graft-versus-host disease (aGVHD), its levels could be used as a predictor of GVHD. More importantly, the miR-150 levels were negatively correlated with the severity of the immune injury (23). However, the role of miR-150 in the suppression of the immune response and inflammatory cytokines is not well understood.

Here we demonstrated that miR-150 inhibits the CD28/B7 co-stimulatory pathway by targeting ARRB2 and reducing the production of different cytokines including IL-2. In addition, miR-150 downregulates ARRB2/PDE4, subsequently elevates cAMP and activates the cAMP-PKA-Csk inhibitory pathway. MiR-150 is also capable of blocking AKT/ARRB2/PDE4 recruitment into the lipid raft, acting as positive feedback regulation. Our results collectively demonstrated that miR-150 can induce immune tolerance through

regulating the CD4⁺ T cells. Therefore, miR-150 could be a potential therapeutic target in transplantation immunology.

2. Materials and methods

2.1 Plasmid constructions and lentiviruses production

To construct a lentiviral-has-mir-150, miR-150 was cloned into the GV259 vector which expresses EGFP (Shanghai GeneChem Company). The clone was transfected to 293FT cells with pHelper1.0 and pHelper2.0 using Lipofectamine 2000. Supernatant of the culture was harvested, filtered using 0.22 µm filters, and concentrated for 2h at 70,000×g. To construct a lentiviral-CD28/shRNA, the designed DNA fragment was digested with HpaI and XhoI and ligated into the linearized pLB plasmid. PCR was conducted with primers flanking the multiple cloning sites of the pLB plasmid, D28/shRNA1–3 and pLB-CD28/shRNA4 served as template DNA. Cells were co-transfected with plasmids pLB-CD28/shRNA, pCMV-R8.91, and pMD.G using Lipofectamine 2000. Concentration and titration of the lentivirus was performed as described (24).

2.2 Cell culture and infection

Human peripheral blood T cells were purified with anti-human CD4 and anti-biotin beads using auto-MACS (MiltenyiBiotec, Germany). CD4⁺T cells were cultured on plate-bound anti-CD3ε (OKT3, 10 µg/ml) and anti-CD28 (clone CD28.2, 10 µg/ml) (eBioscience) for 48h before infection. The cells were infected with lentiviruses (24). GFP-positive cells were sorted with fluorescence-activated cell sorting (FACS). Cell culture, transfection and stimulation of CD4⁺ T cells (P/I or CD3/CD28 antibody co-ligation) were performed as described (25).

2.3 Real-time PCR

RNAs were isolated from the cells using the AmbionmirVanamiRNA isolation kit (Ambion). Reverse transcription was performed with miR-150-specific primer using the Applied Biosystems TaqMan MicroRNA Assay kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions and U6 was used as endogenous control. Has-miR-150 and U6 TaqMan real-time PCR primers and probes were purchased from Applied Biosystems. For detecting the level of ARRB2 and CD28, total RNA was extracted and cDNA was reversely transcribed. Real-time PCR (Applied Biosystems 7500 Sequence Detection System) was done to measure the mRNA levels of CD28 using beta-actin as an internal control. The relative fold changes of target genes were calculated by the 2^{-Ct} method as described previously.

2.4 Transfection and dual luciferase assay

HEK293T cells were maintained in DMEM (Gibco) in 24-well plates. At about 50% confluence, the cells were transfected with miR-150 and wild-type or mutant 3'UTR of ARRB2. A firefly luciferase reporter construct (300 ng per well) and Renilla luciferase construct (300 ng per well; for normalization) were co-transfected. 24 h after post transfection, cell lysates were made and luciferase activity was measured with the Dual-Glo™ with Luciferase Assay System (E2920, Promega).

2.5 Flow cytometry

For intracellular cytokine staining and FACS analysis, transfected CD4⁺ T cells were stimulated with anti-CD3 (5µg/ml) and anti-CD28 (5µg/ml) Ab for 72h, or a mix of PMA (P-8139, 50 ng/ml) and ionomycin (I-0634, 750 ng/ml) (Sigma-Aldrich, St. Louis) for 4 h at 37°C in a 6-well plate; 10 mg/ml brefeldinA (Sigma) was added. Pelleted cells were fixed, stained and analyzed as previously described (26). For Foxp3 staining, cells were not stimulated; instead, the protocol for the Foxp3 Staining Buffer set was followed. Anti-human IFN-γ-APC, Anti-human IL-4-PE, Anti-human IL-17A-PE, Anti-human Foxp3-PE were purchased from DB. For T cell activity assay, infected CD4⁺T cells stimulated with anti-CD3 and anti-CD28 or PMA and ionomycin were washed twice with staining buffer and anti-human CD4-FITC and anti-human CD69-PE (eBioscience) conjugated mAb was used. Apoptosis was assessed with dual staining, APC-labeled Annexin V and 7-AAD (eBioscience, San Diego, CA), according to the manufacturer's instructions.

2.6 Cell proliferation assay

Cell proliferation was assessed by measuring CCK-8(DOJINDO, Japan) dye absorbance, according to the manufacturer's instructions. The infected cells stimulated with anti-CD3 and anti-CD28 or PMA and ionomycin and uninfected CD4⁺ T cells in the presence of PDE4 selective inhibitor rolipram (R-6520, 5–20µM) were adjusted to 1×10⁵/ml and cells were seeded into 96-well cell culture plates. 10µL CCK-8 reagent was added, and the cells were incubated at 37°C with 5% CO₂ for 4h before measuring absorbance under electromagnetic wave at a wavelength of 450 nm.

2.7 Purification of lipid rafts

The CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl were stimulated with anti-CD3 and anti-CD28 before purification of lipid rafts. Isolation of Triton X-100-insoluble lipid rafts was performed as previously described (27, 28). After fractionation, all 12 fractions were tested for LAT (1499-1, Epiomics, Abcam, United Kingdom) content by immunoblotting to verify successful separation. As the majority of LAT segregated into fractions 2 to 5, peak raft fractions were prepared by mixing these fractions. Protein contents of all fractions were measured when PDE4 assays were conducted.

2.8 Lysate preparation and Western blot analysis

Total cell extracts, nuclear and cytosolic extracts were prepared from CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl followed by stimulation with anti-CD3 and anti-CD28 for 30 min. Protein amounts were quantified with the Bradford assay reagent from Bio-Rad (Bio-Rad, USA). Cell extracts were separated by electrophoresis on a denaturing 10–15% polyacrylamide-SDS gel and transferred on to nitrocellulose membranes. Membranes were blocked for 1h with 1×gelatin, and then incubated with specific anti-PDE4D (7953-1, Epiomics, Abcam, UK), anti-Histone H2.X (3522-1, Epiomics, Abcam, UK) anti-AKT, anti-p-AKT, anti-ARRB2, anti-IKK, anti-p-IKK, anti-p-IkB, anti-p-p65 (Cell Signaling Technology, Inc) (1:1000) in 1×gelatin overnight at 4°C. After washing with 1×PBST, membranes were incubated with appropriated horseradish

peroxidase-conjugated secondary antibody (1:5000). Immunoreactive bands were visualized by using ECL detection system, as described by the manufacturer (GE Healthcare, Piscataway, NJ).

2.9 cAMP activity analysis

The infected cells stimulated with anti-CD3 and anti-CD28 or PMA and ionomycin and uninfected CD4⁺ T cells in the presence of rolipram were collected. Parameter™ cAMP Assay (KGE002B, R&D Systems, Inc) was performed in accordance with the manufacturer's instructions. Prior to assay, cells were lysed according to the Cell Lysis Procedure. The cAMP Standard was constructed using the stock solution to produce a dilution series. 50µL of Primary Antibody Solution was added and incubated for 1 hour at room temperature. Each well was washed four times. 50µL of cAMP Conjugate and 100µL of Standard, control, or sample was added to the appropriate wells in 15 minutes of addition of the cAMP Conjugate. 100µL of the appropriate diluents to the NSB and zero standard (B0) was added to wells and incubated for 2 hours and then washed. 200µL of Substrate Solution was added to each well and incubated for 30 minutes at room temperature in the dark. 100µL of Stop Solution was then added to each well. The optical density of each well was determined in 30 minutes using a microplate reader set to 450 nm.

2.10 Protein Kinase Analysis

The CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl followed by stimulation with anti-CD3 and anti-CD28 were collected. PepTag® Assay for Non-Radioactive Detection of cAMP-Dependent Protein Kinase (V5340, Promega) was performed in accordance with the manufacturer's instructions. Cells were washed with PBS and then suspended in 0.5ml of cold PKA extraction buffer and homogenized. A portion of the cAMP-Dependent Protein Kinase, Catalytic Subunit, was diluted to 2µg/ml in PKA dilution buffer. The PepTag® PKA Reaction 5×Buffer, PepTag® A1Peptide, PKA Activator 5×Solution and water were mixed. At time zero, the tube was removed from the ice and incubated in a 30°C water bath for 1 minute. The sample or cAMP-Dependent Protein Kinase, Catalytic Subunit was added and incubated at room temperature for 30 minutes. The reaction was stopped by placing the tube in a boiling water bath or on a 95°C heating block for 10 minutes. The samples were stored at -20°C in the dark until ready to load onto the gel.

2.11 Csk, Lck and PI3K activity assays

The infected cells stimulated with anti-CD3 and anti-CD28 and uninfected CD4⁺ T cells in the presence of PI3K inhibitor LY294002 (L9908, 5–20µM) (Sigma-Aldrich, St. Louis) were collected. Csk (GMS50199.1), Lck (GMS50215.1) and PI3K activity assay kits (GMS50058.1) (GENMED SCIENTIFICS INC. U.S.A) were performed in accordance with the manufacturer's instruction. Prior to assay, cells were treated according to the Cell Prepare Procedure. Added Reagent C, D, E, F to 96-wells plate successively and then shaken gently and incubated for 2 h at 30°C. Reagent G and sample were added to the wells and shaken gently. The optical density of each well was determined at 0 and 5 minute using a microplate reader set to 340 nm. The total activity of sample is OD340 (0 minute) – OD340

(5 minute). Activity was calculated: $[(\text{sample} - \text{background}) \times \text{dilution times X}] / [0.005(\text{mL}) \times 6.22 \times 5(\text{minute})] = \text{U/mL} \div (\text{sample concentration}) \text{ mg/mL} = \text{U/mg}$.

2.12 ELISA

For the detection of IL-2 and IFN- γ production, the supernatants of infected cell cultures stimulated with anti-CD3 and anti-CD28 for 24h and uninfected CD4⁺ T cells in the presence of Akt inhibitor (sc-203811, 5–20 μ M) (Santa Cruz) were collected, and the manufacturer's instructions were followed. The wells were coated with antigen dilution and incubated for 2 h. The solution was removed and the plate washed three times with PBS. The remaining protein-binding sites were blocked and plates incubated for at least 2 h at room temperature and then washed. Each dilution was added to an antigen-coated well in duplicate and incubated for 2 h at room temperature and then washed. Secondary antispecies antibodies were then added and the plate was incubated for 2 h and then washed. Substrate solution was added and absorbance measured at 405 nm. An end-point measurement was performed after 1 h. The titer was defined as the dilution of serum giving an optical density (OD) of 0.2 above the background of the ELISA after a 1h reaction.

2.13 Statistical considerations

All values are expressed as the mean $\pm$ SD. Statistical comparisons between groups were performed using one-way ANOVA followed by Student t test, $p < 0.05$ is considered statistically significant.

3. Results

3.1 MiR-150 targets and downregulates ARRB2

MiR-150 is involved in the development and differentiation of immune cells, and exerts a negative regulating role to the immune response. We have reported that post allogeneic hematopoietic stem cell transplantation (HSCT), miR-150 levels in CD4⁺ T cells decreased significantly in the patients 4 days before the onset of aGVHD (23). Bioinformatics analyses identified 44 potential miR-150 targets in all three microRNA data bases: miRbase, miRanda and Targetscan (Supplemental Figure 1A). Among them, *ARRB2* appears to be a strong candidate for aGVHD development. As an downstream molecule in CD28 costimulatory signaling pathway, ARRB2 plays an important role in T cell activation through interacting with PDE4 (26). In addition, miR-150 is highly complementary with the 3'-UTR of the *ARRB2*. We decided to determine whether ARRB2 is regulated by miR-150. We first measured the ARRB2 levels in human CD4⁺ T lymphocytes overexpressing miR-150. As shown in Figure 1A and compared to either mock transfection or the cells transfected with empty vector, MiR-150 increased by approximately 2.54 times in cells transfected with miR-150 expression vector. The mRNA level of ARRB2 is not affected (Figure 1B), whereas its protein levels was significant decreased when miR-150 is overexpressed (Figure 1C). To further confirm that downregulated ARRB2 is indeed targeted by miR-150, we inserted the wild type or the mutant 3'-UTR of the ARRB2 (Figure 1D) into the luciferase reporter plasmid. The miR-150 overexpression vector was co-transfected with either reporter plasmid, and luciferase activity was measured. As shown in Figure 1E, overexpression of miR-150 significantly repressed fluorescence activity controlled by the

wild type 3'-UTR of ARRB2. But the activity was not affected by the mutant 3'-UTR of ARRB2 although the transfection efficiency was about the same in different transfections (Supplemental Figure 1B). These data collectively demonstrated that ARRB2 is downregulated by miR-150 in human CD4⁺ T lymphocytes.

3.2 MiR-150 participates in the modulation of CD28/B7 co-stimulatory pathway

Our previous study showed that blocking CD28/B7 signaling pathway reduced aGVHD occurrence through immune differentiation and cytokine production (24). To investigate whether miR-150 regulates T cell function through the CD28/B7 costimulatory pathway, we constructed lentiviral vector carrying CD28-shRNA (Figure 2A) to block costimulatory pathway and evaluated the expression level of miR-150. Figure 2B shows that compared with the control group miR-150 expression levels were significantly elevated. Conversely, overexpression of LV-150 reduced the proportion of Th1 and Th17 cells and increased Th2 cells, after anti-CD3 and CD28 mAb stimulation (Figure 2C). This indicates that miR-150 acts as a key negative regulator of Th1 responses. We also demonstrated that compared with the LV-Ctrl group, LV-150 did not affect T cell subsets upon PMA + ionomycin stimulation (Supplemental Figure 2). To the cells in the LV-150 group, anti-CD3 + CD28 mAb stimulation reduced proliferation and increased apoptosis (Figure 2D and 2E). In contrast, cell proliferation and apoptosis were not affected with PMA + ionomycin stimulation (Figure 2D and 2E). These results demonstrated that blockade of the CD28/B7 costimulatory pathway could influence the expression of miR-150, and miR-150 regulates T cell function through the CD28/B7 costimulatory pathway.

3.3 MiR-150 increases the level of cAMP and impairs CD4⁺ T cells activation by targeting ARRB2

In T cells, ARRB2 and cAMP-specific phosphodiesterase PDE4 form dimer in lipid rafts, resulting in hydrolysis of cAMP to ensure complete activation of T cells (26,28). We assessed the protein levels of ARRB2 and PDE4 in the lipid rafts and found that overexpression of miR-150 resulted in decreased levels of both ARRB2 and PDE4 proteins in lipid rafts (Figure 3A) although total protein levels of PDE4 in CD4⁺ T cells were not significantly changed (Fig 3B). These data suggest that miR-150 reduced recruitment of PDE4 into the lipid rafts. We also found that overexpression of miR-150 significantly increased cAMP levels, comparable to the results obtained from using the PDE4 inhibitor rolipram (Figure 3C). Moreover, after restoration of ARRB2 expression in CD4⁺ T cells, cAMP levels becomes normal (Figure 3D). Finally we showed that co-transfection of LV-ARRB2 with LV-mir-150 restored T cell differentiation (Fig 2C), proliferation (Fig 3E) and viability (Fig 3F) compared to the LV-150 and PDE4 inhibitor rolipram groups. These results altogether suggest that miR-150 affects the function of T cells by regulating the cAMP levels via ARRB2.

3.4 MiR-150 blocks the activation of Lck by positively regulating the cAMP-PKA-Csk pathway

Cyclic AMP is a key molecule in the cAMP-PKA-Csk axis and plays an important role in the inhibition of T cell activation through phosphorylation of Lck (29–32). To demonstrate that miR-150 plays an important role through this axis, the activity of PKA, Csk

phosphorylation, and the activity of Lck in CD4⁺ cells were estimated when miR-150 is overexpressed. Our data show that overexpression of miR-150 increased the activities of PKA (Figure 4A) and Csk (Figure 4B), but decreased the activity of Lck (Figure 4C). Combination of these effects impaired T cell activation. In addition, CD69 expression (Figure 4D) was significantly decreased. These results indicate that miR-150 negatively affects T cell function by inhibiting Lck activity through the cAMP-PKA-Csk axis.

3.5 MiR-150 inhibits PI3K activity and blocks AKT/ARRB2/PDE4 recruitment into lipid rafts

Lck activation promotes the phosphorylation of the CD28 intracellular SH2 domain, which combines with and then phosphorylates the P85 subunit of PI3K to produce PIP3 (33). PIP3, in turn, phosphorylates AKT and recruits PDE4/ARRB2 to form trimers in the lipid rafts (34). To verify the involvement of miR-150 in this process, we evaluated PI3K activity and the phosphorylation level of AKT in CD4⁺ T cells when miR-150 is overexpressed. The cells in LV-150 group show decreased PI3K activity (Figure 5A) and decreased levels of p-AKT without affecting the total AKT level (Fig 5B), which is similar to that of PI3K inhibition. Also similar to that of PI3K inhibition, in the miR-150 group the levels of both AKT and trimers in the lipid rafts were significantly reduced (Figure 5C). These results suggest that miR-150 not only affect the function of PDE4 via ARRB2 but also block the interaction between PI3K and CD28 via activation of the cAMP-PKA-Csk signaling pathway, which further inhibits AKT/ARRB2/PDE4 recruitment into the raft and thus playing an important role in this positive feedback regulation.

3.6 MiR-150 impairs production of inflammatory cytokines by inhibiting the Akt/IKK/NF- κ B pathway

Activation of AKT also leads to IKB kinase (IKB kinase, IKK) phosphorylation, activation of NF- κ B signaling pathway and cytokine production in lymphocytes (35). Our results demonstrate that miR-150 can inhibit AKT activity. To elucidate whether miR-150 also regulates the NF- κ B signaling pathway by altering AKT phosphorylation, we overexpressed miR-150 and detected IKB kinase activity and P65 levels in CD4⁺ T cells. Treatment with AKT inhibitor serves as a positive control. The cells in the LV-150 group showed decreased levels of p-IKK β and p-I κ B α without changing the level of IKK β in cytoplasm (Figure 6A). MiR-150 also resulted in increased levels of p65 in the cytoplasm and decreased the levels in the nucleus (Figure 6B). Further analysis of NF- κ B downstream Th1 cytokines showed decreased levels of IL-2 and TNF- α after miR-150 overexpression. This is similar to that seen in the positive control with AKT inhibitor (Figure 6C). These results suggest that miR-150 affects production of CD4⁺ T cell-associated cytokines through regulation of the NF-KB signaling pathway.

4. Discussion

MicroRNAs are widely involved in the regulation of the innate and adaptive immune systems, playing important roles in maintaining immune homeostasis. MiR-150 is a critical negative regulator of various immune cell functions via apoptosis, survival, and proliferation. Acute GVHD involves a series of immune injuries targeting the host organs mediated by donor T lymphocytes after allo-HSCT. Prognostic biomarkers and therapeutic

strategies for aGVHD are important priorities for clinical development. Our previous study found that patients undergoing acute transplant injury after allo-HSCT showed decreased miR-150 levels in CD4⁺ T cells at least four days prior to the onset of aGVHD, and the patients without injury showed significantly increased miR-150 expression levels. These results imply not only that miR-150 can be used to predict the occurrence of aGVHD, it may also be involved in the induction of immune tolerance in allo-HSCT (23).

miR-150 plays multiple roles by targeting different pathways in different immune cells, such as preventing early B cell development and maturation by targeting c-Myb, hampering iNKT maturation and decreasing cytokines production, and regulating bone marrow-derived mononuclear cell mobilization and migration (36) by targeting CXCR4. We found that in CD4⁺ T cells, miR-150 targeted ARRB2, which works as an important downstream intracellular molecule in the CD28/B7 costimulatory signaling pathway and plays key roles in the full activation of T cells and in the presence and development of aGVHD mediated by donor T lymphocytes (26, 37). In the current study, we demonstrated that miR-150 regulates T cell function through CD28/B7 co-stimulation pathways.

ARRB2 is a negative regulator of G protein-coupled receptors and serves as a scaffold. Through recruiting of a variety of functional proteins, ARRB2 participates in different signal transduction pathways and regulates the transcription of specific genes (38). After CD28/B7 co-stimulation, ARRB2 dimerize with PDE4, a cAMP-specific phosphodiesterase involved in the hydrolysis of cAMP (26, 39, 40), an important intracellular signaling molecule that inhibits T cell function and mitosis (41, 42). We demonstrated that miR-150 can reduce the ARRB2/PDE4 dimer recruited into the lipid raft and increase cAMP levels. Whereas LV-miR-150 and LV-ARRB2 co-transfection effectively reduced the levels of cAMP, and restored T cell function. These findings indicate that miR-150 regulates cAMP levels by targeting ARRB2 and thus induces immune tolerance.

cAMP inhibits the activity of Lck through activation of its downstream PKA-Csk. In addition, Lck is an important regulator of T cell activation, not only activating downstream ZAP70 to regulate cytokine transcription in TCR downstream (43, 44) but also functioning as an important activator to phosphorylate the CD28 intracellular YNNM motif. Lck activates PI3K through phosphorylation of the YNNM motif, which promotes the transformation of PIP and PIP2 into PIP3 (33, 45–47). In turn, PIP3 phosphorylates AKT and mediates AKT/ARRB2/PDE4 trimer recruitment to the rafts (48). We found that miR-150 inhibited the activation of Lck by increasing the cAMP levels, thereby sequentially inhibiting the activities of PI3K and AKT, blocking the recruitment of trimers into the raft, and thereby blocking T cell activation. Therefore, miR-150 not only reduces ARRB2/PDE4 expression to influence PDE4 function but also blocked the interaction between PI3K and CD28 via activation of the cAMP-PKA-Csk signaling pathway. In addition, miR-150 also inhibits the incorporation of AKT/ARRB2/PDE4 into the lipid rafts, and thereby induces immune tolerance through a positive feedback regulation circuitry.

MiR-150 is capable of inhibiting AKT activity by regulating cAMP levels, and in T lymphocytes AKT can phosphorylate I κ B kinase (IKK) (32). Thereby, miR-150 indirectly promotes I κ B phosphorylation and dissociation from NF- κ B, which then translocates into

the nucleus. We found that miR-150 inhibits AKT phosphorylation and the NF- κ B signaling pathway. Cytokine imbalance is an important factor mediating immune injury and transplantation rejection. Our previous studies confirmed that Th1 differentiation is responsible for immune rejection and that Th1 cytokine expression was positively correlated with aGVHD (23). Here we demonstrated that miR-150 affects downstream cytokines by regulating the NF- κ B signaling pathway and negatively regulate T cell function.

In conclusion, we demonstrated that miR-150 can block CD28/B7 costimulatory signaling, effectively decreasing the production of cytokines by targeting ARRB2, which collectively leads to immunologic suppression. MiR-150 can also reduce the amount of ARRB2/PDE4 recruited into lipid rafts by targeting ARRB2, and inhibit AKT/ARRB2/PDE4 recruitment into the rafts via positive feedback of the cAMP-PKA-Csk signaling pathway. Therefore, miR-150 affects the production levels of Th1 cytokines by positively regulating the expression of cAMP.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

miRs	microRNAs
mRNA	messenger RNA
allo-HSCT	allogeneic hematopoietic stem cell transplantation
aGVHD	acute graft-versus-host disease

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FIGURE 1. MiR-150 targets ARRB2

(A) CD4⁺ T cells were transiently transfected with no vector (Mock), with empty lentivirus vector (LV-Ctrl) or miR-150-expressing vector (LV-miR-150). The miR-150 levels in the transfected cells were estimated by real-time PCR. Result showed stable increased expression of up to 2.55 times after LV-miR-150 transfection. (B) Relative expression level of ARRB2 mRNA in CD4⁺ T cells mock transfected or transfected with LV-miR-150 or LV-Ctrl. (C) Immunoblot analysis and quantification of ARRB2 protein in CD4⁺ T cells mock transfected or transfected with LV-miR-150 or LV-Ctrl. (D) Interference site between miR-150 and ARRB2 mRNA 3'-UTR and constructed mutations in the ARRB2 3'-UTR. (E) Luciferase activity of reporter carrying the mutated (Mut) or wild-type (WT) ARRB2 3'-UTR cotransfected into HEK293T cells with miR-150. ** $p < 0.01$. Error bar represents the SD from three independent experiments.

FIGURE 2. MiR-150 participates in the modulation of CD28/B7 co-stimulative pathway (A) CD28 mRNA and protein expression in CD4⁺ T cells were significantly decreased after over-expression of CD28/shRNA1–4 compared to the normal control group ($P < 0.01$). (B) Quantitative PCR analysis of miR-150 in CD4⁺ T cells after transfection of LV-CD28/shRNA1–4. (C) Intracellular staining of IFN- γ , IL-4 and IL-17A in CD4⁺ T cells mock infected or infected with LV-Ctrl, LV-miR-150, and LV-miR-150+ARRB2. Cells were examined by intracellular staining after stimulation by anti-CD3 and CD28 mAb. Data are representative of three independent experiments. (D, E) CD4⁺ T cells mock transfected or

transfected with LV-miR-150 or LV-Ctrl were respectively stimulated with anti-CD3 mAb plus anti-CD28 mAb, and Ionomycin plus PMA. Proliferation of CD4⁺ T cells was measured by CCK-8, and apoptosis of CD4⁺ T cells were measured by Annexin V and 7-AAD. * $p < 0.05$, ** $p < 0.01$. Error bar represents the SD from three independent experiments.

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FIGURE 3. MiR-150 increases the level of cAMP and thus impairs CD4⁺ T cells activation by targeting ARRB2

(A) Immunoblot analysis and quantification of ARRB2 and PDE4 in lipid rafts from CD4⁺ T cells mock transfected or transfected with LV-miR-150 or LV-Ctrl post-stimulation by anti-CD3 and CD28 mAb. (B) Immunoblot analysis and quantification of PDE4 in the total lysates of CD4⁺ T cells mock transfected or transfected with LV-miR-150 or LV-Ctrl after stimulation by anti-CD3 and CD28 mAb. (C) The cAMP concentrations in CD4⁺ T cells mock transfected or transfected with LV-miR-150 or LV-Ctrl and administrated of different concentrations of PDE4 inhibitor Rolipram. (D) The cAMP concentrations in CD4⁺ T cells mock transfected or transfected with LV-Ctrl, LV-miR-150 and LV-miR-150+ARRB2 and administrated of Rolipram. (E–F) Analysis of proliferation and apoptosis of CD4⁺ T cells mock transfected or transfected with LV-Ctrl, LV-miR-150 and LV-miR-150+ARRB2 and administrated of Rolipram. * $p < 0.05$, ** $p < 0.01$. Error bar represents the SD from three independent experiments.

FIGURE 4. MiR-150 blocks the activation of Lck by positively regulating the cAMP-PKA-Csk pathway

(A–C) The activity of PKA, Csk and Lck in CD4⁺ T cells mock transfected or transfected with LV-miR-150 or LV-Ctrl after stimulation by anti-CD3 and CD28 mAb. (D) FCM analysis of CD69 positive cells in CD4⁺ T cells mock transfected or transfected with LV-miR-150 or LV-Ctrl after stimulation by anti-CD3 and CD28 mAb. * $p < 0.05$, ** $p < 0.01$. Error bar represents the SD from three independent experiments.

FIGURE 5. MiR-150 inhibits the activity of PI3K and blocks AKT/ARRB2/PDE4 recruitment into lipid rafts

(A) PI3K activity in CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl and administrated of different concentrations of PI3K inhibitor LY-294002. (B) Immunoblot analysis and quantification of Akt and p-Akt in the total lysates of CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl and administrated of different concentrations of PI3K inhibitor LY-294002. (C) Immunoblot analysis and quantification of Akt in lipid rafts of CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl and administrated of different concentrations of PI3K inhibitor LY-294002. * $p < 0.05$, ** $p < 0.01$. Error bar represents the SD from three independent experiments.

FIGURE 6. MiR-150 impairs inflammatory cytokines production by inhibiting the Akt-IKK-NF-κB pathway

(A) Immunoblot analysis and quantification of IKKβ, p-IKKβ and p-IκB in the cytoplasm of CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl and administrated of different concentrations of Akt inhibitor. (B) Immunoblot analysis and quantification of p65 protein in the cytosolic and nuclear of CD4⁺ T cells mock transfected or transfected with LV-miR-150, LV-Ctrl and administrated of different concentrations of Akt inhibitor. β-actin was used as the cytosolic endogenous control and histoneH2AX as the nuclear endogenous control. (C) levels of IL-2 and TNF-α in CD4⁺ T cells mock transfected or

transfected with LV-miR-150, LV-Ctrl and administrated of different concentrations of Akt inhibitor. * $p < 0.05$, ** $p < 0.01$. Error bar represents the SD from three independent experiments.

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