

MR. WENLONG LIU (Orcid ID : 0000-0003-1296-5144)

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**MicroRNA-181a and microRNA-155 are involved in the regulation of the differentiation and function of regulatory T cells in allergic rhinitis children**

Qingxiang Zeng<sup>1</sup>, PhD, Wenlong Liu<sup>1</sup>, PhD, Renzhong Luo<sup>1</sup>, MD, Gen Lu<sup>2</sup>, MD

1 Department of Otolaryngology, Guangzhou Women and Children's Medical Center, Guangzhou Medical College, Guangzhou, China

2. Department of Respiratory Medicine, Guangzhou Women and Children's Medical Center, Guangzhou Medical College, Guangzhou, China

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Correspondence to:

Wenlong Liu, Department of Otolaryngology, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, No. 9, Jinsui Road, Guangzhou, 510623, China.

Email: lwl20103@163.com

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Gen Lu, Department of Respiratory Medicine, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, No. 9, Jinsui Road, Guangzhou, 510623, China.  
Email: rootlu008@163.com

**MicroRNA-181a and microRNA-155 are involved in the regulation of the differentiation and function of regulatory T cells in allergic rhinitis children**

QX Zeng, WL Liu, RZ Luo, MD, G Lu

**Background:** Regulatory T cells (Tregs) play central roles in limiting airway allergic inflammation and preventing inappropriate Th2 responses to environmental allergens. This study aims to evaluate the role of miR-181a and miR-155 in the regulation of the differentiation and function of Tregs through both in vivo and in vitro studies.

**Methods:** The CD4<sup>+</sup> T cell and Tregs were purified from peripheral blood mononuclear cells (PBMCs) in allergic rhinitis (AR) children, respectively. The miR-155/181a mimics and inhibitors were transfected into CD4<sup>+</sup> T cells and Tregs. The differentiation and function of Tregs were evaluated by flow cytometry and enzyme-linked immunosorbent assay. AR mice models were established and miR-155/181a mimics or inhibitors were injected through tail vein. The Tregs cell percentage and function from mice were compared among different groups.

**Results:** The miR-181a up-regulated the release of interleukin (IL)-10 and TGF- $\beta$ , whereas the miR-155 promoted Tregs differentiation in CD4<sup>+</sup> T cells. Similarly, we also found that miR-155 promote Tregs proliferation directly reacted through suppressor of cytokine signaling 1 (SOCS1) and sirtuin1 (SIRT1) signaling pathway, whereas miR-181a up-regulated mRNA expression of IL-10 and TGF- $\beta$  through phosphatidylinositol 3-OH kinase (PI3K)/Akt pathway. We also found that miR-155/181a affect Tregs percentage and function in mice model.

**Conclusion:** Our findings suggest that miR-181a and miR-155 were closely correlated with the proliferation and function of Tregs in AR, providing new potential treatment target.

**Key words:** allergic rhinitis, children, regulatory T cells, miR-181a, miR-155

## Introduction

Allergic rhinitis (AR) has been attributed to Th1/Th2 imbalance and inappropriate Th2 responses to environmental allergens in the past. However, this paradigm is expanded by identification of novel T helper families, such as regulatory T cell (Treg), Th17 and Th9 cells [1]. Of these cells, Tregs may play central roles in the regulation of Th balance by inhibiting the differentiation of T helper 2 (Th2) and limit airway allergic inflammation [2,3]. Tregs suppresses excessive immune response and keeps immune homeostasis via directly contacting immune cells or producing of immunosuppressive cytokines including IL-10, IL-35, and TGF- $\beta$  [4].

MicroRNAs (miRNAs), 21 to 23-nt RNA molecules, are first found in mammals and have been implicated various biological activities [5]. Recent studies provided evidence that miRNAs play critical roles in the immune function of Tregs through the regulation of two key miRNA-processing enzymes, Dicer or Drosha [6,7]. Our unpublished study has shown that children with AR had significantly lower levels of miR-155 and miR-181a than healthy controls and the miR-155 and miR-181a level were correlated with the number and function of Treg. However, the direct regulation of miR-155 and miR-181a on Tregs in AR were not established well.

MiR-155 is believed to be involved in the pathogenesis of AR since its expression is up-regulated in multiple immune cell lineages, including T lymphocytes, B lymphocytes, mast cells, fibroblasts, macrophages and dendritic cells [8-10]. On the other hand, the inhibition of miR-181a negatively affected both positive and negative selection of double positive cells. Its expression is high in immature T cells and low in differentiated Th1 and Th2 cells [11]. However, the role of miR-181a in Tregs is not clear.

In the present study, we aimed to explore the regulatory effects of miR-155/181a on the differentiation and function of Tregs through both *in vivo* and *in vitro* studies.

## **Methods**

### *Patients*

Twenty patients with AR (7-12 years old, Male:Female, 11:9) were recruited from January 2017 to June 2017. The diagnosis of AR was made according to the Allergic Rhinitis and its Impact on Asthma (ARIA) guideline (2010) after confirmation of disease history,

nasal symptoms, clinical examination, skin prick test and/or specific IgE measurement [12].

Patients with following conditions were excluded: history of other allergic diseases, other upper and lower respiratory diseases, use of systemic or topical corticosteroids within the past 4 weeks. Our study were approved by local committee and the informed consent was obtained.

#### *Tregs differentiation and culture*

CD4<sup>+</sup> T cells were purified from peripheral blood mononuclear cells (PBMCs) using Whole Blood CD4 MicroBeads (MACS Miltenyi Biotec, Bergisch Gladbach, Germany) as described in the manufacturer's instructions (purity >95%). For Tregs differentiation, CD4<sup>+</sup> T cells ( $7 \times 10^5/\text{mL}$ ) were incubated with anti-CD3/CD28 Dynabeads (Invitrogen, Carlsbad, Germany) for 36 hour and rhIL-2 and TGF- $\beta$  for the following four days in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated fetal calf serum (Biochrom, Berlin, Germany), penicillin (100 IU/mL), streptomycin (100  $\mu\text{g/mL}$ ), sodium pyruvate, and L-glutamine (Gibco, Life Technologies, Darmstadt, Germany) at 37 °C with 5 % CO<sub>2</sub> in air.

#### *Tregs purification and culture*

Tregs were purified from peripheral blood mononuclear cells (PBMCs) using the Regulatory T Cell Isolation Kit (Miltenyi Biotec, Germany) according to the instructions. Then the anti-CD3/CD28-coated beads (Invitrogen, Carlsbad, Germany) were used for stimulation of Tregs by co-culture a 1:1 cell-to-bead ratio for 36 hours. The rhIL-2 and

TGF- $\beta$  was added for four additional days' culture. The Tregs were cultured as described above.

#### *MiRNA mimic or inhibitor transfection and signaling pathway inhibitors*

In different groups, the transfection was performed using 1  $\mu$  M miRNA mimics, inhibitors, scrambled control, miRNA mimics control in serum-free ACCELL medium (Thermo Scientific, Lafayette, Colorado, USA) for 1 h at 37°C. Signaling pathway inhibitors (1  $\mu$  M) such as LY294002 (phosphatidylinositol 3-OH kinase (PI3K)/Akt inhibitor), suppressor of cytokine signaling 1 (SOCS1), sirtuin-1 (SIRT1), si-SOCS1 and si-SIRT1 were added in different groups. A scrambled oligonucleotide (GenePharm, Shanghai, China) was used as a control. The knock down of SOCS1 and SIRT1 were confirmed by PCR.

#### *Animal models*

The animal experiments were approved by local ethics committee boards. Four-week-old C57BL/6 mice were purchased from Shanghai Experimental Animal Center (Shanghai, China) and fed with a standard chow diet. The study included 56 mice, which were grouped into the control (12 mice), AR (12 mice), and intervention groups (32 mice).

A subcutaneous injection of ovalbumin (100  $\mu$ g OVA) mixed with 1.6 mg Al(OH)<sub>3</sub> in 0.9% NaCl on day 0 was given for sensitization. One week later (day 7), a second subcutaneous injection of 100  $\mu$ g OVA was given and Al(OH)<sub>3</sub> was given in the control mice. On day 14 to 18, the mice were challenged nasally with 10  $\mu$ g of OVA in 40  $\mu$ L of PBS (20  $\mu$ L per nostril) or saline for controls. One day after the final challenge, the mice

were anaesthetized and exsanguinated. Peripheral blood was collected from the abdominal vena cava.

For miRNA test *in vivo*, 100 nmol miRNA mimics, inhibitors or miRNA control or LY294002, SIRT1, SOCS1, si-SOCS1 and si-SIRT1 (Thermo Scientific, Lafayette, Colorado, USA) were given to mice intranasally 2 h before each OVA challenge.

#### *Determination of the proliferation of Tregs*

After culture and stimulation, Tregs were labeled with [<sup>3</sup>H]thymidine (0.5 mCi/well, Amersham-Pharmacia Biotech, Cologno Monzese, Italy) for 12 hours of culture and the radioactivity was detected by a  $\beta$ -cell-plate scintillation counter (Wallac, Gaithersburg, MD).

#### *T cell suppression assays*

CD25 negative CD4 T cells were also purified from PBMCs and labeled with 10 mM carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies). Tregs treated by different mimics or inhibitors were cultured with CFSE-labeled CD25 negative CD4 T cells with a 1:1 ratio for 4 days. Flow cytometry was performed to confirm the proliferation of CD25 negative CD4 T cells.

#### *Quantitative Real-time PCR (qRT-PCR)*

For mRNA expression, total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, California). For miRNA, total RNA was transcribed into cDNA reversely by

Taqman microRNA reverse transcription kit and Taqman microRNA assays (Invitrogen) according to the manufacturer's instructions. The data were calculated using the  $2^{-\Delta \Delta C_t}$  method and normalized to RNA U6 controls.

### *Statistical Analysis*

The data are presented as mean  $\pm$  SD. The differences between groups were determined by the Kruskal-Wallis H test or the nonparametric Mann-Whitney U test. A  $P < 0.05$  was set as statistically significant.

## **Results**

### *MiR-155 promotes the differentiation of Tregs through SOCS1 and SIRT1 pathway*

CD4<sup>+</sup> T cells were transfected with miR-155 mimics or inhibitors to investigate the function of miR-155 in Tregs differentiation. We found that the percentage of Tregs in CD4<sup>+</sup> T cells were significantly higher in CD4<sup>+</sup> T cells when transfected with miR-155 mimics than in those transfected with miR-control and miR-155 inhibitor (Figure 1A). Consistently, the mRNA level of forkhead/winged helix transcription factor (Foxp3) were increased in miR-155 mimics group compared with miR-control and miR-155 inhibitor group (Figure 1B). We also found that SOCS1 and si-SIRT1 suppress miR-155 mediated Tregs differentiation, suggesting that SOCS1 and SIRT1 was involved in miR-155 mediated Tregs differentiation (Figure 1A, B). However, the mRNA expression of IL-10 and TGF- $\beta$  in cell culture supernatant were not different between groups (Figure 1C, D).

### *MiR-181a regulates the function of Tregs and the expression of IL-10 and TGF- $\beta$ by Tregs*

Our results showed that both the mRNA of IL-10 and TGF- $\beta$  from supernatant were significantly higher in CD4<sup>+</sup> T cells transfected with miR-181a mimics than in those transfected with miR-control and miR-181a inhibitor (Figure 2). We also found that LY294002 (phosphatidylinositol 3-OH kinase (PI3K)/Akt inhibitor) and SIRT1 inhibitor suppress miR-181 mediated enhanced Tregs function (Figure 2). However, the Tregs differentiation were not affected by miR-181a (Data not shown).

### *MiR-155/181a regulated Tregs proliferation and function in Tregs*

To explore the direct effect of miR-155/181a in Tregs function, we purified Tregs and transfected with miR-155/181a. Similarly, our results showed that the miR-155 promoted proliferation of Tregs through SOCS1 and SIRT1 pathway and miR-181a up-regulated mRNA expression of IL-10 and TGF- $\beta$  through PI3K/Akt and SIRT1 pathway. We also found that Tregs transfected with miR-181a had enhanced suppression function in T cell suppression assays, which was inhibited by PI3K/Akt inhibitor (Figure 3). We found no effect of miR-181a mimic on proliferation of Treg cells and miR-155 on the expression of IL-10 and TGF- $\beta$ . Our results also showed that the miR-155 mimic had no effect on suppression by Tregs.

### *A proliferative imbalance of Tregs and miR-155/181a expression in AR mice*

As shown in Figure 4, the number of Tregs and Foxp3 level from peripheral blood of AR mice decreased significantly compared with control mice. The serum levels of TGF- $\beta$

and IL-10 in AR mice were significantly lower than did from control mice. RT-PCR also showed significantly decreased expression of miR-155 and miR-181a in peripheral blood mononuclear cells (PBMCs) of AR mice.

#### *Tregs proliferation and function by miR-155/181a regulation in AR mice*

To prove the direct effect of miR-155/181a in regulation of Treg, the mice received systemic administration of miR-155/181a mimics or inhibitors *in vivo*. We found a significant reduction in the frequency of Tregs and Foxp3 level in PBMCs from miR-155 inhibitor group compared with miR-155 mimics group (Figure 5A, B). Our results showed that both the mRNA level of IL-10 and TGF- $\beta$  from PBMCs were significantly higher in AR mice treated with miR-181a mimics than in those with control and miR-181a inhibitor group (Figure 5C, D).

#### **Discussion**

In this study, we first demonstrated that miR-155 promotes the differentiation of Treg, whereas miR-181a regulates the expression of IL-10 and TGF- $\beta$  in AR, providing new mechanism in the pathogenesis of AR.

Tregs functions in maintenance of self-tolerance and preventing the development of various inflammatory diseases by directly contacting effective immune cells and secreting anti-inflammatory cytokines [4]. Our previous unpublished studies had demonstrated that AR children had decreased Tregs number and function [13].

MiRNAs are about 23 nt small non-coding RNAs that regulate mRNA expression at the posttranscriptional level by directing mRNA degradation or transcriptional repression [14]. In the present study, we found that miR-155 promote Tregs differentiation and this process mediated through SOCS1 and SIRT1 pathway. On the other hand, the function of Tregs was not affected when anti-miR-155 was introduced, which was manifested as unchanged IL-10 and TGF- $\beta$  level and T cell suppression function. Consistently, previous studies also suggested that miR-155 was indispensable for Tregs differentiation but did not affect the function of Tregs in regulating conventional T cells [15,16]. More specifically, the number of Tregs in the thymus and periphery from miR-155-deficient mice were significantly lower, but the expression of CD25, CTLA-4 or glucocorticoid induced TNF receptor-related protein were normal [15], suggesting that the function of Tregs was not affected.

Targeting SIRT1 is known to regulate the Tregs differentiation and function by up-regulating Foxp3 expression and by Foxp3 lysine  $\epsilon$ -aminodeacetylation leading to ubiquitination and proteasomal degradation [17,18]. SOCS1 regulates the activation, development and differentiation of T cells by negatively affecting the Janus kinase (JAK)/STAT signaling pathway [19,20]. Therefore, we also blocked SIRT1 by specific inhibitor and added SOCS1, then miR-155 mediated Tregs differentiation was inhibited significantly, confirming that miR-155 regulate Tregs differentiation through SIRT1 and SOCS1 pathway. Consistently, Lu's study also showed that proliferation of Tregs cells in the thymus was observed after specific deletion of SOCS1. However, the SIRT1 in our study presented opposite role in the regulation of Tregs compared with Beier's studies. These discrepancy may be explained by different disease backgrounds and models used in different

studies. Interestingly, a recent study by Marcel also reported that loss of Sirt1 compromised Treg cell survival, resulting in antigen-induced T cell proliferation and inflammation in two mouse models [21].

In the present study, we also found that miR-181a can enhance Tregs function, demonstrated as increased IL-10 and TGF- $\beta$  level and T cell suppression assays. MiR-181a is highly expressed in thymocytes, and its abnormal expression impairs both positive and negative thymic selection [22,23]. Inconsistently, Serr's results showed that blocking miRNA181a promoted Tregs proliferation in murine and humanized models *in vivo* [24]. Zhou's report also suggested that transfection with miR-181a precursor reduced the Tregs numbers in chronic HCV infection [25]. These inconsistent results suggested that miR-181a may exert pro-inflammatory or anti-inflammatory effect in different backgrounds. We also found that miR-181a regulated the function of Tregs through PI3K/Akt pathway, which was consistent with previous report [26].

We used both CD4<sup>+</sup> T cells and Tregs cell as experimental model in this study. Our pre-test showed that miR mimic had similar effect on both cells from AR and control children, so we only provided data from AR donors. First, we found that miR-181a and miR-155 can promote Tregs differentiation and enhanced function in CD4<sup>+</sup> T cells. To avoid the effect of other cell in CD4<sup>+</sup> T cells, we also purified Tregs and treated Tregs with miR-181a/miR-155. Our results showed that miR-181a and miR-155 had the similar effect in both CD4<sup>+</sup> T cells and Tregs cells, suggesting that miR-181a and miR-155 acted on Tregs independent of other cells.

Finally, we established OVA-induced mouse model to prove the *in vitro* results.

Consistently, we found that Tregs treated with miR-155/181a presented with increased number and enhanced function, suggesting that the miR-155/181a may be served as important direct target of Treg.

## **Conclusions**

In summary, our findings suggest that miR-181a and miR-155 were deeply involved in the proliferation and function of Tregs through SOCS1, SIRT1 and PI3K/Akt pathway in AR, which may be potential treatment target in future.

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Not applicable.

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## **Conflicts of interest**

The authors declare that they have no competing interests.

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### Figure legend

Figure 1 MiR-155 regulates Tregs differentiation and function in CD4<sup>+</sup> T cells. (A, B). Proliferation of Tregs and Foxp3 expression by Tregs after treated by scrambled control (Scr), miR-control, miR-155 mimics, miR-155 inhibitor, SOCS1, SIRT1, si-SOCS1 and si-SIRT1. (C, D). The mRNA expression of IL-10 and TGF- $\beta$  by Tregs after treated by miR-155 mimics, miR-155 inhibitor, SOCS1, SIRT1, si-SOCS1 and si-SIRT1. \*  $P < 0.05$ , compared with controls, \* \*  $P < 0.05$ , compared with miR-155 mimics.

Figure 2 MiR-181a regulates Tregs differentiation and function in CD4<sup>+</sup> T cells. (A, B). The mRNA expression of IL-10 and TGF- $\beta$  by Tregs after treated by scrambled control (Scr), miR-control, miR-181a mimics, miR-181a inhibitor, LY, SIRT1, and si-SIRT1. (C, D). Proliferation of Tregs and Foxp3 expression by Tregs after treated by scrambled control (Scr), miR-control, miR-181a mimics, miR-181a inhibitor, LY, SIRT1, and si-SIRT1. \*  $P < 0.05$ , compared with controls, \* \*  $P < 0.05$ , compared with miR-181a mimics.

Figure 3 MiR-155 or miR-181a regulated Tregs proliferation and function in Tregs. (A). Proliferation of Tregs after treated by scrambled control (Scr), miR-control, miR-181a mimics, miR-181a inhibitor, miR-155 mimics, miR-155 inhibitor, SOCS1, SIRT1, si-SOCS1 and si-SIRT1. (B, C). The mRNA expression of IL-10 and TGF-  $\beta$  by Tregs after treated by scrambled control (Scr), miR-control, miR-181a mimics, miR-181a inhibitor, miR-155 mimics, miR-155 inhibitor, LY, SIRT1, and si-SIRT1. (D). The suppression function of Tregs after treated by scrambled control (Scr), miR-control, miR-181a mimics, miR-181a inhibitor, miR-155 mimics, miR-155 inhibitor, LY, SIRT1, and si-SIRT1. The suppression percentages of CD25 negative CD4 T cells proliferation by Tregs in each culture system were determined as  $100 - (\text{the percent divide in coculture} / \text{the percent divide in CD25 negative CD4 T cells alone}) \times 100 \%$ . \*  $P < 0.05$ , compared with controls, In Figure A and B, \* \*  $P < 0.05$ , compared with miR-155 mimics, In Figure C and D, \* \*  $P < 0.05$ , compared with miR-181a mimics.

Figure 4 Decreased proliferation and function of Tregs and miR-155/181a expression in peripheral blood from AR mice. (A,B) The number of Tregs and Foxp3 level in peripheral blood from from AR mice decreased significantly compared with control mice. (C,D) The serum levels of TGF-  $\beta$  and IL-10 in AR mice were significantly lower than did from control mice. (E,F) Decreased expression of miR-155 and miR-181a in peripheral blood mononuclear cells (PBMCs) of AR mice compared with controls.

Figure 5 Tregs proliferation and function by miR-155/181a regulation in AR mice. (A,B)

Proliferation of Tregs and Foxp3 expression by Tregs after treated by miR-control, miR-155 mimics, miR-155 inhibitor, SOCS1, SIRT1, si-SOCS1 and si-SIRT1 in mice. (C,D) The mRNA expression of IL-10 and TGF- $\beta$  by Tregs after treated by miR-control, miR-181a mimics, miR-181a inhibitor, LY, SIRT1, and si-SIRT1 in mice.









