



MiR-202-5p/MATN2 are associated with regulatory T-cells differentiation and function in allergic rhinitis

Li Wang¹ · Xin Yang¹ · Wencai Li² · Xicheng Song¹ · Shasha Kang¹

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Abstract

Regulatory T cells (Tregs) play a crucial role in allergic rhinitis (AR). However, the mechanism of how Tregs are regulated in AR is poorly understood. Here, we aimed to explore the role of Tregs in AR and how Tregs were regulated by miR-202-5p, which was demonstrated to be important in AR. Peripheral blood mononuclear cells (PBMC) were isolated from collected blood samples. Tregs were purified using Regulatory T Cell Isolation Kit, and differentiated from isolated CD4 T cells using recombinant human interleukin-2 (rhIL-2) and transforming growth factor beta (TGF- β). mRNA expression levels of miR-202-5p, matrilin-2 (MATN2), TGF- β 1 and interleukin-10 (IL-10) were detected by real-time PCR. The concentrations of IL-4, interleukin-17 (IL-17), IL-10, interferon gamma (IFN- γ) and TGF- β 1 were detected by enzyme-linked immunosorbent assay (ELISA). MATN2 protein level was detected by Western blot. MiR-202-5p expression dramatically elevated in PBMCs, CD4⁺ T cells and Tregs of AR patients. In vitro, miR-202-5p promoted Tregs differentiation via targeting MATN2. MiR-202-5p/MATN2 axis mediated Tregs proliferation and functions. MiR-202-5p/MATN2 are associated with regulatory T-cells differentiation and function in allergic rhinitis.

Keywords MiR-202-5p · MATN2 · Tregs · AR · Differentiation

Introduction

Allergic rhinitis (AR) is one of the most common inflammatory reactions, which is characterized by hypersecretion of mucus, enhanced serum immunoglobulin E (IgE) level and increased infiltration of eosinophil [1, 2]. Helper T-cell 1/2 (Th1/Th2) imbalance and abnormal Th2 response contribute to AR development [3]. Clinically, AR is diagnosed by the symptoms of rhinorrhea, sneezing, itching and nasal

obstruction [1]. Recently, AR incidence has increased [4]. More importantly, it serves as the risk factor for bronchial asthma development, which seriously influences the life quality of patients [5]. Therefore, an improved understanding of how AR develops is needed for effective AR treatment.

Regulatory T cells (Tregs) are involved in immune system balance and play a crucial role in proliferation and activation of CD4/CD8 T cells, immune tolerance, and immune response. Tumor growth factor β (TGF- β) and interleukin 10 (IL-10) secreted by Tregs regulate Th1/Th2-mediated inflammation [6, 7], Th2 differentiation and therefore suppresses airway allergic inflammation [8, 9]. Accumulating evidence demonstrated the effect of Tregs in AR [10]. In children with AR, the frequency of Tregs is much lower than healthy control. Moreover, Tregs frequency is negatively associated with total IgE concentration [11], indicating the role of Tregs in AR development. However, how Tregs are regulated in AR remains unclear.

Our previous study showed that in AR development, miR-202-5p promoted type 2 macrophages (M2) polarization by targeting matrilin-2 (MATN2) [12], indicating the regulating effect of miR-202-5p in AR development. Therefore, in the present study, we aimed to investigate the effect of

Li Wang, Xin Yang, Wencai Li contributed equally to this work.

- ✉ Li Wang
wangli7986@163.com
- ✉ Xicheng Song
song_xicheng@163.com
- ✉ Shasha Kang
kangshashayt@163.com

¹ Department of Otorhinolaryngology Head and Neck Surgery, Yuhuangding Hospital, Yantai 264000, Shandong, China

² Department of Cardiovascular Medicine, Yuhuangding Hospital, Yantai 264000, Shandong, China

miR-202-5p/MATN2 in Tregs differentiation and function during AR development, trying to provide the basis for potential clinical applications of miR-202-5p/MATN2 in AR treatment.

Methods

Patients

In this study, we recruited a total of 30 AR patients with 70.0% of male (the mean age is 33.6 years with range 21–52), and 10 healthy volunteers with 70.0% of male (the mean age is 32.3 years with range 22–56) in Yuhuangding Hospital from January 2017 to June 2018. The patients with chronic rhinosinusitis, nasal polyps, and infectious rhinitis were excluded. Total IgE (IU/ml) of AR patients was 521.9 ± 158.4 and specific IgE (IU/ml) was 95.7 ± 10.9 . This study was approved by Yuhuangding Hospital (#YHDH1305LC148). Informed consent was obtained from all the patients and healthy volunteers. The diagnosis and management of AR were carried out by our physicians following the routine procedures. Patients that had any of the following conditions were excluded: cancer, severe organ diseases, autoimmune diseases, under treatment with immune suppressors or immunotherapy for any reason.

MiR-202-5p mimics, miR-202-5p inhibitors and MATN2 transfection

The sequence of miR-202-5p mimics is 3'-GUUUCUUAUACGUAUCCUU-5', and miR-202-5p inhibitor is 3'-AAGGAUACGUAUAUGAAGAAAC-5'. In different groups, the transfection was performed using 1 μ M miRNA mimics, inhibitors, human recombinant MATN2 (HZbscience), vehicle control, miRNA mimics control in serum-free ACCELL medium (Thermo Scientific, Lafayette, Colorado, USA) for 1 h at 37 °C.

Tregs purification and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the whole blood of healthy volunteers and AR patients. Then, we used the Tregs cell Isolation Kit (Miltenyi Biotec, Germany) to purify Tregs following the manufacturer's protocol. Then, we used anti-CD3/CD28 beads (Invitrogen, Waltham, MA, USA) to stimulate Tregs at the ratio of 1:1 cell-to-bead coculture for 36 h, followed by rhIL-2 and TGF- β administration for another 4 days of culture in RPMI 1640 medium. The medium was supplied with 10% fetal calf serum, sodium pyruvate, L-glutamine, 100 IU/mL penicillin and 100 μ g/mL streptomycin at 37 °C with 5% CO₂.

Tregs differentiation

The whole blood CD4 microbeads (Miltenyi, Germany) were used to isolate CD4+ T cells following the manufacturer's protocol. After isolation, CD4+ T purity was > 95%. For Tregs differentiation, the CD4+ T cells at the concentration of 7×10^5 cells/mL were cocultured with anti-CD3/CD28 beads (Invitrogen, USA) for 36 h. Then, rhIL-2 and TGF- β administration was performed for another 4 days in RPMI 1640 medium. The medium was supplied with 10% fetal calf serum, sodium pyruvate, L-glutamine, 100 IU/mL penicillin and 100 μ g/mL streptomycin at 37 °C with 5% CO₂.

RNA isolation and qRT-PCR

The RNAs were isolated from PBMCs, CD4+ T cell and Tregs using TRIzol reagent. Then, we performed reverse transcription for cDNA synthesis using ReverTra Ace- α kit following the manufacturer's protocol. SYBR-Green mix was used to amplify gene expression. $2^{-\Delta\Delta C_t}$ method was used to detect relative expression levels. U6 and GAPDH were the controls of microRNAs and MATN2, respectively. The sequences of the primers for MATN2 were sense 5'-AGACACTGGATTGCTTGAC-3' and antisense 5'-ACACCGTGGAACACTACA-3'. The sequences of the primers for GAPDH were sense 5'-CCCCTTCATTGACCTCAACT-3' and antisense 5'-ATGACCTTGCCCACAGCCTT-3'. miRNAs relative expression was analyzed by quantifying the miRNAs stem-loop. The sequences of the primers for U6 were sense 5'-GCTTCGGCAGCACATATACTAAAAT-3', antisense 5'-CGCTTCACGAATTTGCGTGTTCAT-3' and reverse transcription primer was 5'-CGCTTCACGAATTTGCGTGTTCAT-3'. The sequences of the primers for miR202-5p were sense 5'-TCGGCAGGTTTCCTATGCATAT-3', antisense 5'-CTCAACTGGTGTCTGGA-3' and reverse transcription primer was 5'-CTCAACTGGTGTCTGGAGTCGGCAATTCAGTTGAGC CAAAGAAC-3'.

Western blot

Primary extracts from CD4+ T cells or Tregs were separated with 10% SDS-polyacrylamide gels. Then, the proteins were transferred to ECLTM membranes. The membranes were blocked using 5% milk, followed by incubation with primary anti-MATN2 (Abcam, USA) or anti- β -actin (Abcam, USA) antibodies at the concentration of 1:1000 dilution. Horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, USA) were added to develop signals using the ECL system (GE, UK).

ELISA

The concentrations of IL-4, IL-17, IFN- γ , IL-10, and TGF- β were detected by the commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol.

Flow cytometry

Flow cytometry was used to detect the percentage of Tregs in CD4+ T cells. A total of 1×10^6 cells were first stained with surface markers. Then, intracellular staining was performed on the cells using the Regulatory T Cell Staining Kit (eBioscience, San Diego, CA, USA) following the manufacturer's instructions. Flow cytometry was performed using the BD Fortessa (BD Bioscience, Franklin Lakes, NJ).

T-cell suppression assays

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

Statistical analysis

All data presented in this study as a mean $\pm$ standard deviation (SD). Data were analyzed using the Student's *t* test or one-way ANOVA analysis. Experiments were repeated in triplicate. $p < 0.05$ was considered to be statistically significant.

Results

MiR-202-5p expression increased in AR patients

To explore the effect of miR-202-5p in AR, we first detected the mRNA expression level of miR-202-5p in isolated PBMCs, CD4+ T cells and Tregs from the peripheral blood of 30 AR patients (AR) and ten healthy volunteers (control), respectively. As shown in Fig. 1a, miR-202-5p expression level was much higher in AR patients than healthy control. Moreover, in isolated CD4 T cells from both groups, the miR-202-5p level significantly increased in AR group when compared with control group (Fig. 1b). Similarly, the miR-202-5p level in Tregs from peripheral blood of the AR group dramatically increased (Fig. 1c). These results demonstrated the association of miR-202-5p and AR and the potential role of miR-202-5p in Tregs.

MiR-202-5p inhibited Tregs differentiation in vitro

We next further detected the role of miR-202-5p in Tregs differentiation. Tregs proportion significantly decreased after miR-202-5p treatment, while dramatically increased after adding miR-202-5p inhibitor (Fig. 2a) compared with the negative control (miR-NC), suggesting that miR-202-5p inhibited Tregs differentiation.

The effect of miR-202-5p on Tregs-associated cytokines was further investigated. The concentrations of IL-4 and IL-17A were significantly increased after miR-202-5p treatment and decreased after miR-202-5p inhibitor administration compared with miR-NC (Fig. 2c, e). In contrast, some other related cytokines such as IFN- γ , IL-10, and TGF- β 1 obviously decreased upon the miR-202-5p treatment, while it increased after miR-202-5p inhibitor administration (Fig. 2b, d, f). The results from cytokines suggested that miR-202-5p impacted the immune imbalance.

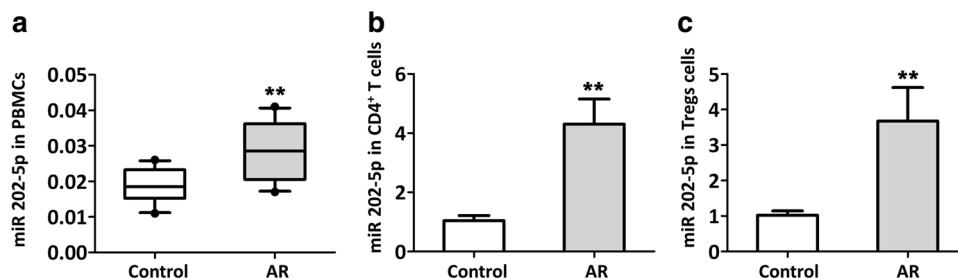
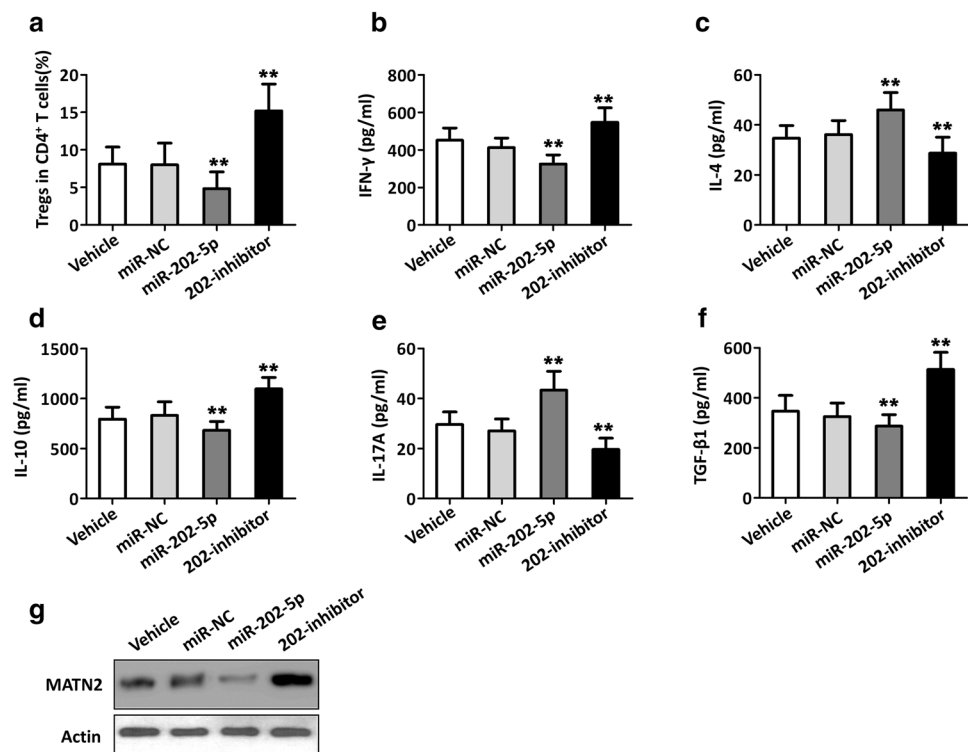


Fig. 1 Highly expressed miR-202-5p in peripheral blood mononuclear cells (PBMCs), CD4+ T cells and Tregs cells from AR patients. **a** MiR-202-5p expression in PBMCs from 30 AR patients and 10 healthy controls were measured by qRT-PCR. Whisker plots (min. to the max.). **b**, **c** qRT-PCR analysis of the relative expression of miR-

202-5p in CD4+ T cells and Tregs cells from AR patients or healthy controls. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; ** $p < 0.01$, compared to the PHA-stimulated PBMC vehicle group healthy controls (ctrl group)

Fig. 2 MiR-202-5p impacted the immune imbalance and promoted the differentiation of Treg cells in vitro. **a** The addition of miR-202-5p upregulated the proportion of Treg cells in CD4⁺ subsets compared to the PHA-stimulated PBMC group (vehicle group), while the inhibitor of miR-202-5p was in contrast. **b–f** The addition of miR-202-5p led to the upregulation of IL-4 and IL-17 levels and the downregulation of IFN- γ , IL-10, and TGF- β 1 levels, while the inhibitor of miR-202-5p was in contrast. **g** The expression of MATN2 in the sorted CD4⁺ T cells transfected with miR-202-5p mimics or miR-202-5p inhibitors was detected by Western blot. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; ** $p < 0.01$, compared to the PHA-stimulated PBMC vehicle group (vehicle group)

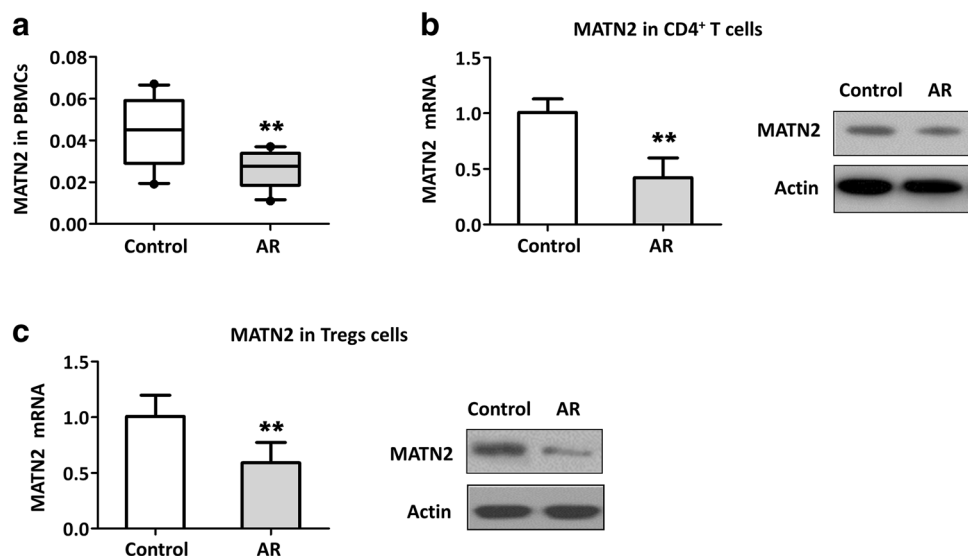


The target of miR-202-5p was further investigated. As shown in Fig. 2g, MATN2 protein level in miR-202-5p mimic transfected CD4⁺ T cells dramatically decreased, while it increased in miR-202-5p inhibitor transfected CD4⁺ T cells, suggesting that MATN2 is the functional target of miR-202-5p.

MATN2 expression decreased in AR patients

Given the crucial role of miR-202-5p in AR and MATN2 being the functional target of miR-202-5p, we next detected the expression levels of MATN2 in AR patients. As shown in Fig. 3a, MATN2 mRNA expression level significantly decreased in PBMC of AR patients when compared with control healthy volunteers. In CD4⁺ T cells from AR patients, the mRNA and protein levels of

Fig. 3 Lowly expressed MATN2 in peripheral blood mononuclear cells (PBMCs), CD4⁺ T cells and Tregs cells from AR patients. **a** MATN2 expression in PBMCs from 30 AR patients and 10 healthy controls were measured by qRT-PCR. Whisker plots (min. to max.). **b, c** qRT-PCR and Western blot analysis of the relative expression of MATN2 in CD4⁺ T cells and Tregs cells from AR patients or healthy controls. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; ** $p < 0.01$, compared to the PHA-stimulated PBMC vehicle group (vehicle group)



MATN2 were much lower than that in control groups (Fig. 3b). Moreover, MATN2 mRNA and protein levels in Tregs from AR patients were much lower than that in healthy control (Fig. 3c). All the results demonstrated that MATN2 expression levels decreased in AR patients.

MATN2 promoted Tregs differentiation in vitro

To further explore the effect of MATN2 in AR and Tregs differentiation, we first added MATN2 in CD4⁺ T cells in vitro. As shown in Fig. 4a, MATN2 dramatically enhanced Tregs percentage in CD4⁺ T cells which is opposite to the effect of miR-202-5p. However, additional miR-202-5p administration (MATN2 + 202) based on MATN2 significantly suppressed Tregs differentiation in vitro. MATN2 dramatically inhibited the concentrations of IL-4 and IL-17A, while it enhanced the concentrations of IFN- γ , IL-10, and TGF- β 1, which is opposite to the role of miR-202-5p. In addition, additional miR-202-5p administration (MATN2 + 202) significantly attenuated the miR-202-5p effect in Tregs-associated cytokines production (Fig. 4b–f). These data demonstrated that MATN2 impacted the immune imbalance and promoted the differentiation of Treg cells in vitro.

MiR-202-5p/MATN2 regulated Tregs proliferation and function

To further investigate the effect of miR-202-5p/MATN2 in Tregs, we isolated Tregs from human PBMCs and treated them with PHA (vehicle), miR-NC, miR-202-5p, miR-202-5p inhibitor, MATN2, and MATN2 + miR-202-5p. As shown in Fig. 5a, miR-202-5p inhibitor, and MATN2 significantly enhanced Tregs proliferation. However, additional miR-202-5p administration (MATN2 + 202) obviously inhibited proliferating Tregs. The mRNA expression levels of IL-10 and TGF- β 1 were inhibited by miR-202-5p, while they were enhanced by miR-202-5p inhibitor and MATN2. Additional miR-202-5p treatment based on MATN2 (MATN2 + 202) inhibited IL-10 and TGF- β 1 mRNA levels (Fig. 5b, c). Similarly, MATN2 + 202 attenuated the promoting role of miR-202-5p inhibitor or MATN2 on the suppression function of Tregs (Fig. 5d). These data demonstrated that MiR-202-5p/MATN2 seems to be involved in the regulation of Tregs proliferation and function.

Discussion

The present study indicated that the highly expressed miR-202-5p in PBMCs, CD4⁺ T cells and Tregs from AR patients regulates the differentiation and function of Tregs

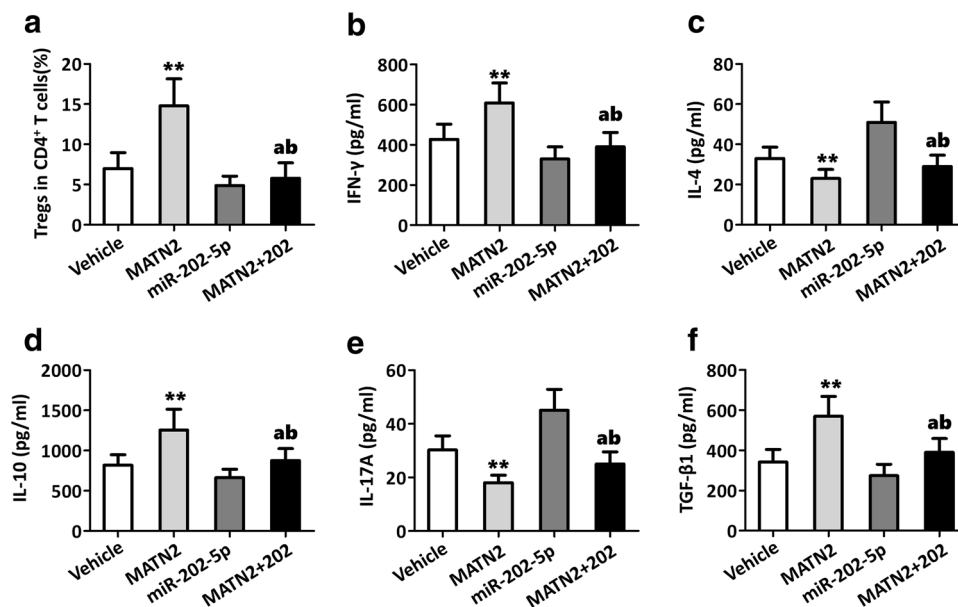


Fig. 4 MATN2 impacted the immune imbalance and promoted the differentiation of Treg cells in vitro. **a** The addition of MATN2 upregulated the proportion of Treg cells in CD4⁺ subsets, while the addition of miR-202-5p weakened the effect of MATN2. **b–f** The addition of MATN2 led to the downregulation of IL-4 and IL-17 levels and the upregulation of IFN- γ , IL-10, and TGF- β 1 levels, while

the addition of miR-202-5p weakened the effect of MATN2. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; ** $p < 0.01$, compared to the PHA-stimulated PBMC vehicle group; a, $p < 0.01$ compared to the MATN2 group; b, $p < 0.05$ compared to the miR-202-5p group

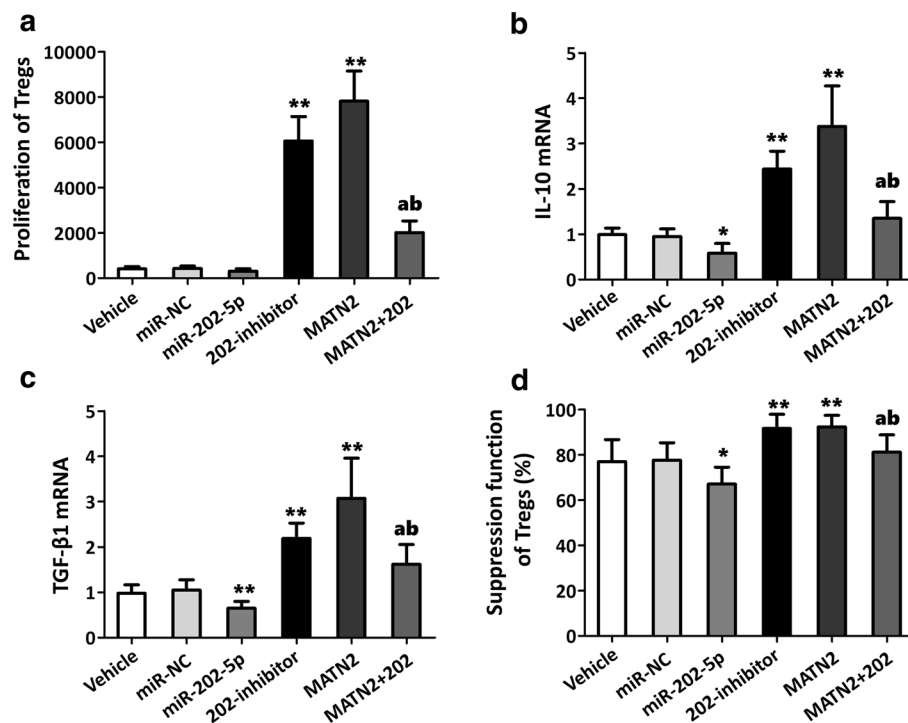


Fig. 5 miR-202-5p/MATN2 regulated Tregs proliferation and function in Tregs. **a** The proliferation of Tregs after treated by PHA (vehicle), miR-NC, miR-202-5p, miR-202-5p inhibitor, MATN2, and MATN2+miR-202-5p. **b–c** The mRNA expression of IL-10 and TGF-β1 by Tregs after treated by PHA (vehicle), miR-NC, miR-202-5p, miR-202-5p inhibitor, MATN2, and MATN2+miR-202-5p. **d** The suppression function of Tregs after treated by PHA (vehicle), miR-NC, miR-202-5p, miR-202-5p inhibitor, MATN2,

and MATN2+miR-202-5p. The suppression percentages of CD4+CD25⁺ T cells proliferation by Tregs in each culture system were determined as $100 - (\text{the percent divide in coculture} / \text{the percent divide in CD4+CD25}^+ \text{ T cells alone}) \times 100\%$. The data represent the mean $\pm$ SD. Experiments were repeated in triplicate; * $p < 0.05$ and ** $p < 0.01$, compared to the PHA-stimulated PBMC vehicle group (vehicle group), a, $p < 0.01$ compared to the MATN2 group; b, $p < 0.05$ compared to the miR-202-5p group

via targeting MATN2, which is consistent with our previous study. Moreover, the concentrations of Tregs-related cytokines altered after miR-202-5p or miR-202-5p inhibitor treatment.

MicroRNAs are small non-coding RNAs with a length of about 23 nt [13]. The main role of microRNAs is to regulate mRNA expression levels of target genes through directing the degradation or modulating transcriptional repression of target mRNA [14]. Therefore, microRNAs play crucial roles in various biological processes [15]. In the occurrence and formation of osteosarcoma, miR-202-5p was indicated to be suppressed in osteosarcoma tissue and osteosarcoma cell lines. MiR-202-5p overexpression inhibited cell migration and cell invasion of osteosarcoma. Mechanically, miR-202-5p directly targeted rho-associated coiled-coil-containing protein kinase 1 expression and suppressed its expression levels [16], indicating the potential role of miR-202-5p in osteosarcoma treatment. In colorectal carcinoma (CRC) tissues, miR-202-5p expression levels dramatically decreased. The overexpression of miR-202-5p suppressed the growth and metastasis of CRC cells. SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin

subfamily c member 1 (SMARCC1) serves as the functional target of miR-202-5p in CRC, which is evidenced by luciferase reporter assay. Moreover, SMARCC1 overexpression could rescue the inhibiting effect of miR-202-5p in colorectal carcinoma progression [17]. There are more studies about the effect of miR-202-5p in cancer development [18]. However, the role of miR-202-5p in AR is poorly explored. Our previous study demonstrated that miR-202-5p expression level in AR mucosal tissues is much higher than in healthy controls. MiR-202-5p promoted M2 polarization in AR through targeting MATN [12]. In this present study, we further detected the role of miR-202-5p on Tregs in AR. Consistently, miR-202-5p expression in AR significantly increased when compared with healthy control. MiR-202-5p suppressed Tregs percentage in CD4⁺ T cells and inhibited the concentrations of Tregs-associated cytokines such as IFN-γ, IL-10, and TGF-β1, while it enhanced IL-4 and IL-17 levels. As expected, miR-202-5p inhibitor showed the opposite effect of the cytokine levels.

The biological function of MATN2 was found to be crucial for promoting Schwann cell migration, neurite outgrowth, skin wound healing and skeletal muscle regeneration

[19]. In an autoimmune encephalomyelitis mouse model, MATN2 deficiency led to reduced axon damage and disease severity. Moreover, the MATN2-induced pro-inflammatory response was eliminated in Myd88 knock-out mice, indicating the important role of MATN2 in neuroaxonal injury response [20]. However, no study reported the effect of MATN2 in AR before our group. The previous study from our group [12] and this study demonstrated that MATN2 expression levels decreased in PBMCs, CD4+ T cells and Tregs of AR patients. MATN2 was the functional target of miR-202-5p, which inhibited MATN2 expression in sorted CD4+ T cells. MATN2 administration dramatically enhanced Tregs percentage in CD4+ T cells and the concentrations of Tregs-associated cytokines. Moreover, MATN2 attenuated the inhibiting role of miR-202-5p in Tregs proliferation and function.

It is important to note the limitations of this study. First, all data were from an in vitro study. The mouse model of AR should be used to further mimic the AR progress and detect the biological role of miR-202-5p/MATN2 in AR development. Second, PBMCs, CD4+ T cells and Tregs all were isolated from the whole blood of AR patients and healthy controls, while non-mucosal tissues were collected to verify the conclusions of this study.

In summary, we explored the biological role of miR-202-5p/MATN2 in Tregs differentiation and function in AR. We provided evidence that miR-202-5p expression dramatically elevated in PBMCs, CD4+ T cells and Tregs of AR patients. In vitro, miR-202-5p promoted Tregs differentiation via targeting MATN2. MiR-202-5p/MATN2 axis mediated Tregs proliferation and function.

Thus, MiR-202-5p/MATN2 are associated with regulatory T-cells differentiation and function in allergic rhinitis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals The procedures were approved by the human ethics committee of Yuhuangding Hospital (#YHDH1305LC148).

Informed consent Informed consent has been derived from each participant.

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