

Mir-20b-Induced Increase in Myeloid-Derived Suppressor Cells in the Lungs of Mice with Chronic Asthma

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Abstract. Bronchial asthma is a common chronic airway inflammatory disease. MicroRNAs (miRNAs) play an important role in the pathogenesis of asthma. We have previously shown that miR-20b can inhibit airway inflammation in asthmatic mice, but the exact mechanism is unknown. In the present study, we show that administration of nasal drops containing miR-20b induced an increase in the percentage of Gr1⁺CD11b⁺ myeloid-derived suppressor cells (MDSCs) in lung tissue from asthmatic mice, and the content of TGF- β in lung tissues also increased after treatment. However, there was no significant change in the number of Gr1⁺CD11b⁺ MDSCs in bone marrow, peripheral blood, or spleens of asthmatic mice receiving the miR-20b nasal drip compared with untreated asthmatic mice. Since MDSCs originate in the bone marrow, these results suggest that miR-20b nasal drops may promote the increase of Gr1⁺CD11b⁺ MDSCs in the lungs of asthmatic mice by the mechanism of inducing expansion.

Key words: miR-20b, asthma, myeloid-derived suppressor cells.

Introduction

Bronchial asthma is one of the most common chronic respiratory diseases. Statistics show that worldwide there are approximately 300 million cases of asthma each year, with 25 million deaths attributed to this disease [1]. There is an upward trend in the incidence and mortality of asthma in all parts of the world, including China [2,3]. Asthma is a heterogeneous disease involving a variety of cells and cellular factors. Patients have reversible and changeable airflow limitations associated with recurrent episodes of wheezing, shortness of breath, cough, and other symptoms. Airway chronic inflammation, high reactivity, and airway remodeling are basic features of asthma [4,5].

A number of studies have shown that microRNAs (miRNAs) play an important role in the pathogenesis of asthma [6]. Feng et al. demonstrated that miR-181a, miR-146a and miR-146b can be used as markers of pro-inflammatory cytokines in asthma

[7]. Simpson et al. found that increased expression of miR-19 is a sign of asthma [8]. Our previous study showed that miR-20b expression was down-regulated in lung tissues from asthmatic mice, and that airway inflammation was relieved using a nasal drip containing miR-20b [9], but the mechanism underlying asthma improvement was not clear. In addition, our previous studies have found that tumor-derived myeloid-derived suppressor cells (MDSCs) inhibited airway inflammation in a TGF- β -dependent manner in asthmatic mice [10]. Fan et al. demonstrated that adoptive transfer of endotoxin-induced MDSCs can effectively suppress symptoms in asthmatic mice, reduce Th2 inflammation and airway remodeling and improve pulmonary function of mice [11]. MDSCs are a heterogeneous mixture of cells derived from the bone marrow and composed of immature myeloid precursor cells [12]. MDSCs inhibit the proliferation and immune function of T, natural killer (NK) and other immune cells mainly by upregulated expression of arginine 1, inducible nitric oxide synthase, reactive oxygen species, and other mechanisms [13]. Since MDSCs play an important role in the pathogenesis of asthma, we examined if miR-20b had an effect on the content of MDSCs in asthmatic mice.

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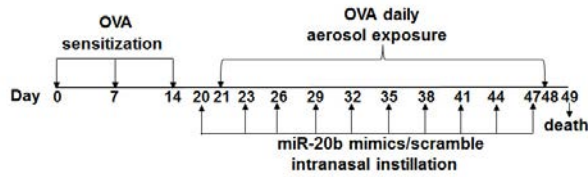


Figure 1. Animal experimental procedures. BALB/c mice were sensitized by intraperitoneal injection of OVA bound to aluminum hydroxide on day 0, 7, and 14 and challenged with aerosolized OVA from day 21 to 48. For miR-20b nasal treatment, 20 μ g miR-20b mimic or scramble in 40 μ l PBS was intranasally delivered from day 20 once every three days for a total of 10 times.

Materials and Methods

Reagents and mice. Chicken ovalbumin (OVA) and aluminum hydroxide (Inject Alum) are Sigma and PIERCE products, respectively. TGF- β ELISA kit was purchased from Wuhan Huamei Company. All fluorescent antibodies are from eBioscience. MiR-20b mimics and scramble were designed and synthesized by the Shanghai Jima company. The sequences for miR-20b mimics and miR-20b scramble are as follows: miR-20b mimics sense, CAA AGU GCU CAU AGU GCA GGU AG, antisense, ACC UGC ACU AUG AGC ACU UUG UU, miR-20b scramble sense: UUC UCC GAA CGU GUC ACG UTT, antisense: ACG UGA CAC GUU CGG AGA ATT. The YLS-8A multi-purpose nebulizer is the product of Jinan Yi Yan Technology Development Co., Ltd. The ELISA microplate reader was purchased from BioTek. BALB/c mice were provided by the experimental animal center of Bengbu Medical College, and all of the animal experiments were approved by the Ethics Committee of Bengbu Medical College.

Establishment and operation of animal models. BALB/c mice were randomly divided into four groups: Normal control group (Control), asthma group (Asthma), miR-20b mimics treatment asthma group (miR-20b mimics) and miR-20b scramble treatment asthma group (miR-20b scramble). Asthmatic model mice were generated by intraperitoneal injections of 200 μ l induced sensitivity solution (containing OVA 50 μ g and aluminum hydroxide 2 mg) on days 0, 7 and 14. Beginning on day 21, the mice were placed in an atomizing inhalation chamber for daily, 30-min inhalation of atomized 5% OVA solution for a total of four weeks. Mice in the control group were treated with PBS instead of OVA. Mice in the miR-20b mimics group received a nasal drip beginning on day 20, consisting of 20 μ g miR-20b mimics in 40 μ l PBS once every three days for a total of 10 applications. In the miR-20b scramble group, scrambled miR-20b sequence replaced miR-20b mimics (Figure 1).

Preparation of lung tissue single cell suspensions. After each group of mice was atomized for the final time, lung tissues were removed 24 h later, homogenized, and resuspended in 3 ml of RPMI-1640 complete medium. We then added collagenase (0.27 mg/ml), hyaluronidase (0.42 mg/ml), and DNA enzymes I (4.2 μ g/ml), and the mixture was incubated at 37°C for 40 min, with gentle mixing every 8–10 min. The digested lung tissue was further homogenized and subjected to membrane filtration, followed by sedimentation for 20 min on ice and centrifugation at 600 rpm for 2 min to remove most of the sediment. The supernatant was carefully removed and centrifuged for 10 min at 2000 rpm. The precipitate was then treated with RBC lysis buffer for 10 min to remove red blood cells. Cell suspensions were washed twice with PBS and reserved for later use.

Preparation of spleen cell suspensions. Mice spleens were removed 24 h after the final atomization of each group. The spleens were cut into pieces, resuspended, and filtered through 400 mesh membranes. Filtrates were treated with RBC lysis buffer for 10 min to remove red cells. The cell suspensions were washed twice with PBS and reserved for later use.

Peripheral blood preparation. Mouse peripheral blood was collected into anticoagulant, treated with RBC lysis buffer to remove red cells, and the final cell suspension was washed twice with PBS and stored.

Flow cytometry. Single cell suspensions were washed twice with staining buffer, then adjusted to a concentration of 3×10^5 cells/100 μ l with staining buffer. The cell suspension was stained with FITC-Gr1, PE-cy5-CD11b (concentration according to the manufacturer's instructions). Stained cells were detected using a FACSCalibur flow cytometer and the data was analysed using 5.7.2 Flowjo software.

ELISA. The supernatants from mice lung tissue homogenates were collected and the content of TGF- β was detected by ELISA. For detailed approaches, see kit manual.

Statistical analysis. Data were expressed as means $\pm$ standard deviation and analyzed using SPSS 16.0 statistical software. Groups were compared using analysis of variance and $P < 0.05$ was considered statistically significant.

Results

The content of Gr1⁺CD11b⁺ MDSCs was increased in the lung tissue of asthmatic mice with miR-20b intranasal instillation. Lung tissues

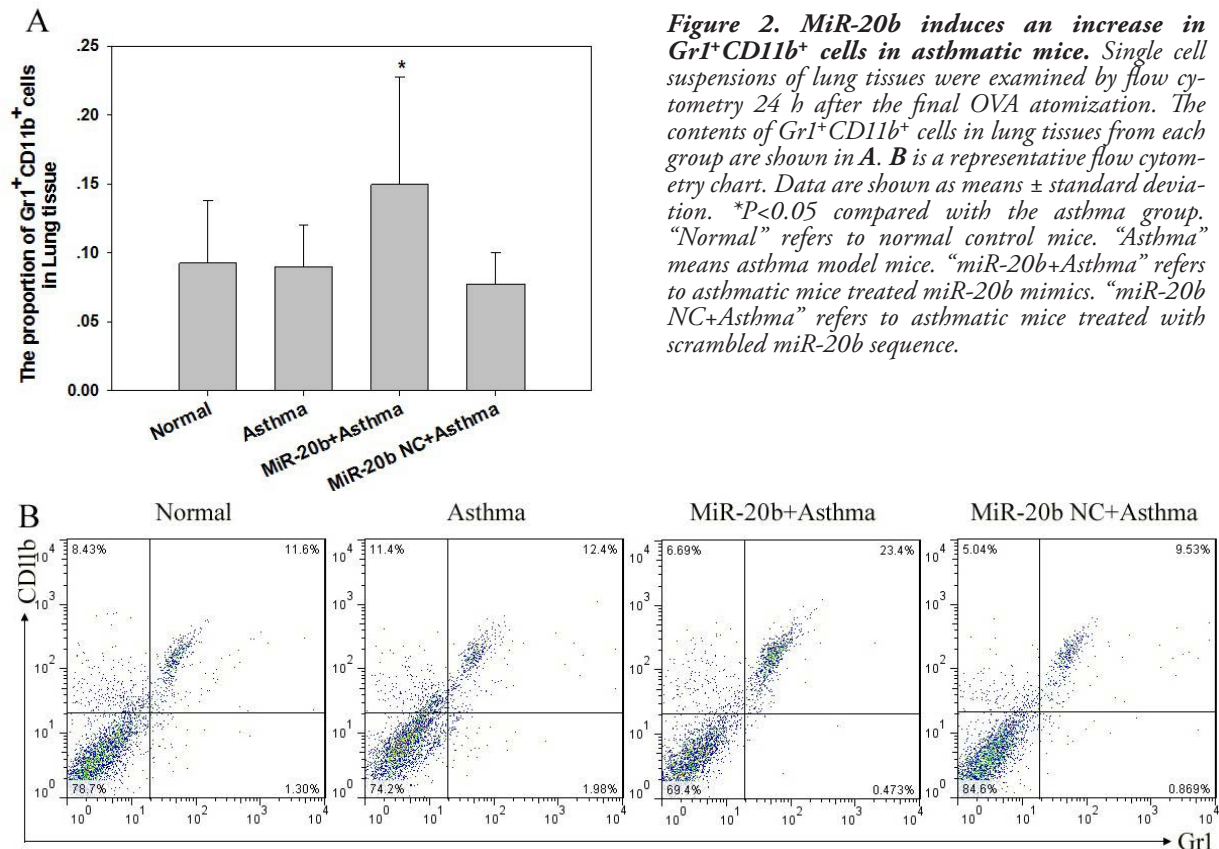


Figure 2. MiR-20b induces an increase in Gr1⁺CD11b⁺ cells in asthmatic mice. Single cell suspensions of lung tissues were examined by flow cytometry 24 h after the final OVA atomization. The contents of Gr1⁺CD11b⁺ cells in lung tissues from each group are shown in A. B is a representative flow cytometry chart. Data are shown as means $\pm$ standard deviation. * $P < 0.05$ compared with the asthma group. "Normal" refers to normal control mice. "Asthma" means asthma model mice. "miR-20b+Asthma" refers to asthmatic mice treated miR-20b mimics. "miR-20b NC+Asthma" refers to asthmatic mice treated with scrambled miR-20b sequence.

were removed from the each group of mice 24 h after the final OVA atomization. Single cell suspensions were prepared and the content of Gr1⁺CD11b⁺ MDSCs was detected by flow cytometry. The results are shown in **Figure 2**. Compared with the asthma group, the proportion of Gr1⁺CD11b⁺ MDSCs was significantly increased ($P < 0.05$) in the miR-20b nasal drip asthma group. It was noteworthy that the proportion of Gr1⁺CD11b⁺ cells in the asthma group was not different from the control group.

MiR-20b increased the content of TGF- β in lung tissue of asthmatic mice. We have previously shown that MDSCs inhibit airway inflammation in asthmatic mice in a TGF- β -dependent manner. In the present experiment, lung tissues from each group of mice were taken 24 h after the last OVA atomization on the 49th day of the experimental process. The contents of TGF- β in lung tissue homogenates were detected by ELISA and are shown in **Figure 3**. As expected, TGF- β levels were significantly higher in asthmatic mice treated with miR-20b mimics compared with the untreated asthma group ($P < 0.01$). No change in the content of

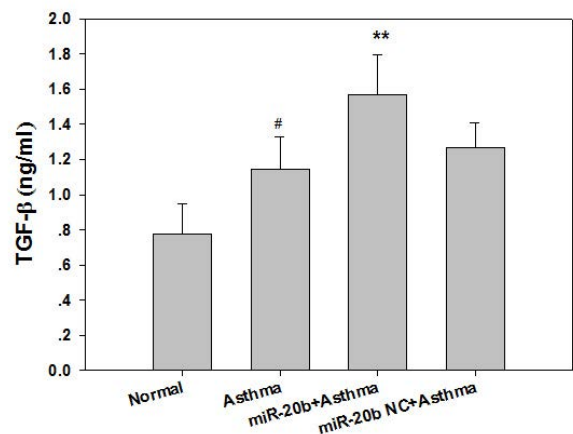


Figure 3. MiR-20b increases the content of TGF- β in lung tissue of asthmatic mice. Lung tissue from each group of mice was removed on the 49th day of the experimental process, and TGF- β content in the supernatant of lung tissue homogenates was assayed by ELISA. Data are expressed as means $\pm$ standard deviation. ** $P < 0.01$ compared with the asthma group. # $P < 0.01$ compared with the normal group. Figure labels are defined in Figure 2.

TGF- β was detected in the lungs of asthmatic mice treated with the scrambled miR-20b control sequence.

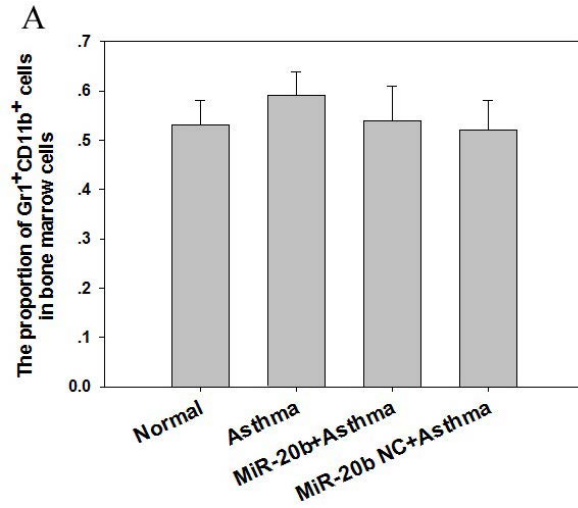


Figure 4. MiR-20b has no effect on the content of Gr1⁺CD11b⁺ MDSCs in bone marrow of asthmatic mice. Bone marrow cells from each group of mice were taken on the 49th day of the experimental process, and the proportion of Gr1⁺CD11b⁺ cells was assayed by flow cytometry. The contents of Gr1⁺CD11b⁺ cells in the bone marrow from each group are shown in **A**. **B** is a representative flow cytometry chart. Data are expressed as means $\pm$ standard deviation. Figure labels are defined in Figure 2.

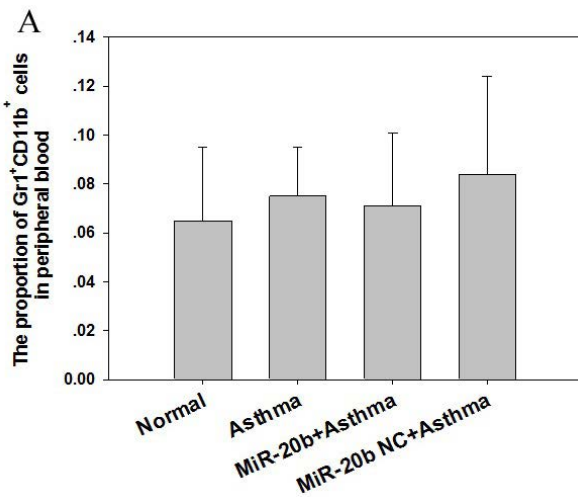
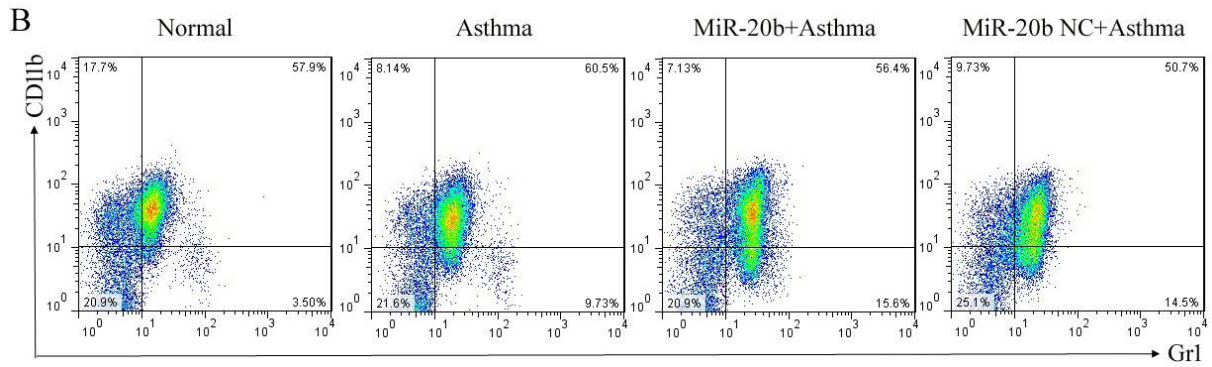
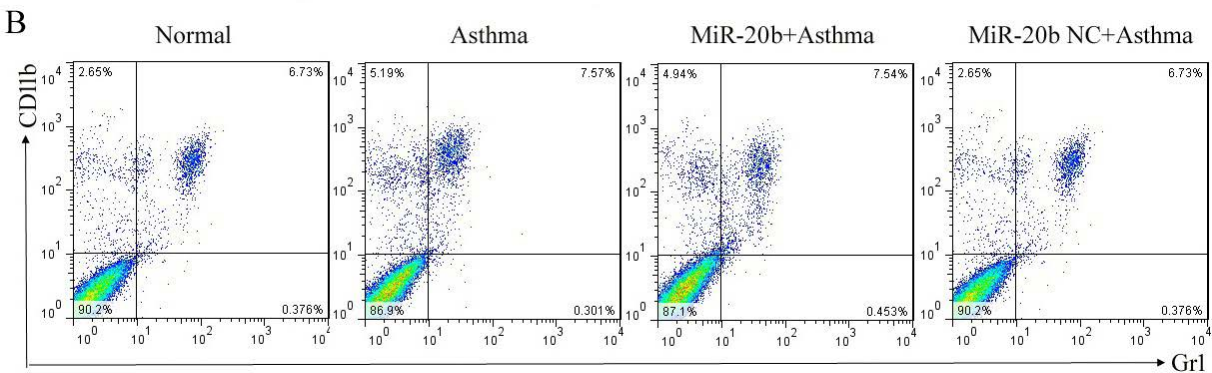


Figure 5. MiR-20b nasal drops had no effect on the content of Gr1⁺CD11b⁺ MDSCs in peripheral blood of asthmatic mice. On the 49th day of the experimental procedure, peripheral blood was drawn from each group of mice and the proportion of Gr1⁺CD11b⁺ cells was detected by flow cytometry. **A** shows the statistical proportion of Gr1⁺CD11b⁺ cells in peripheral blood from each group of mice. **B** is a representative flow cytometry chart. Data are expressed as means $\pm$ standard deviation. Figure labels are defined in Figure 2.



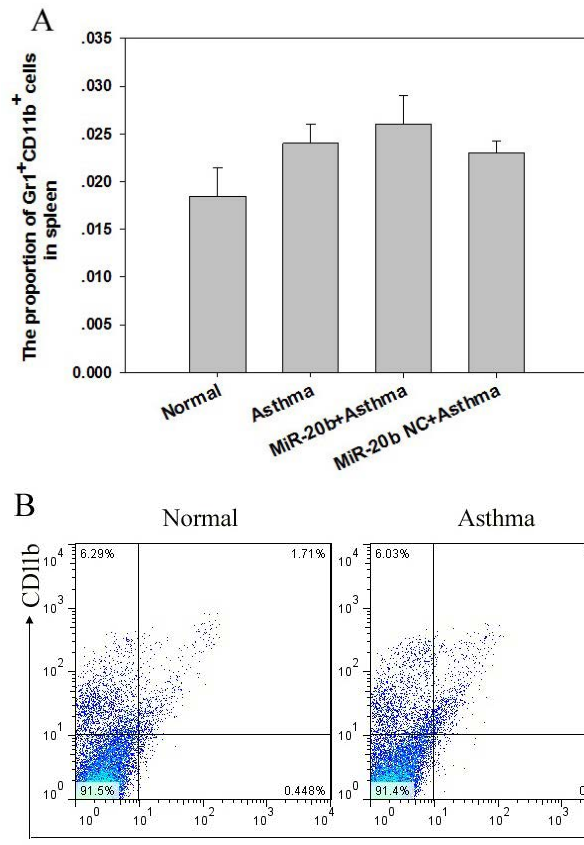


Figure 6. MiR-20b nasal drops had no effect on the content of Gr1⁺CD11b⁺ MDSCs in spleens of asthmatic mice. Spleens were removed from each group of mice 24 h after the final OVA atomization on the 49th day of the experimental procedure and the proportion of Gr1⁺CD11b⁺ cells was analyzed by flow cytometry. **A** shows the statistical proportion of Gr1⁺CD11b⁺ cells in spleen cells from each group of mice. **B** is a representative flow cytometry chart. Figure labels are defined in Figure 2.

MiR-20b had no effect on the content of Gr1⁺CD11b⁺ MDSCs in the bone marrow of asthmatic mice. Since MDSCs are derived from the bone marrow, we examined the content of Gr1⁺CD11b⁺ MDSCs in the bone marrow of each group of mice on the 49th day of the experimental procedure. There was no significant change in the number of Gr1⁺CD11b⁺ MDSCs in the bone marrow of miR-20b mimics-treated asthmatic mice compared with the untreated asthma group (**Figure 4**). This showed that miR-20b nasal drops had no significant effect on bone marrow mobilization of Gr1⁺CD11b⁺ MDSCs in asthmatic mice.

MiR-20b nasal drops had no effect on the content of Gr1⁺CD11b⁺ MDSCs in the peripheral blood and spleens of asthmatic mice. Because bone marrow-derived MDSCs can be transported in the peripheral blood and stored in the spleen, we analyzed the content of MDSCs in the peripheral blood and spleen by flow cytometry. The results are presented in **Figures 5 & 6**. Compared with the asthma group, the content of Gr1⁺CD11b⁺ MDSCs in the peripheral blood and spleen of the miR-20b mimics-treated asthmatic group showed no significant change.

Discussion

In this study, we demonstrated that the proportion of Gr1⁺CD11b⁺ MDSCs in the lung tissue of asthmatic mice increased after treatment with miR-20b nasal drops, but the application of these drops had no significant effect on the content of MDSCs in the bone marrow, peripheral blood, or spleen. MDSCs are a heterogeneous group of cells composed of macrophages, granulocytes and dendritic cells in the early stages of differentiation [14]. Under physiological conditions, the bone marrow and spleen contain a certain proportion of MDSCs. However, the content of MDSCs increases rapidly under pathological conditions such as cancer, infection, and stress [15]. In mice, the cells expressing myeloid markers Gr1 and CD11b are identified as MDSCs. According to their morphological and phenotypic characteristics, MDSCs are divided into granulocytic and monocytic MDSCs [16]. MDSCs inhibit the proliferation and function of immune cells such as T and NK cells through induction of arginase 1, inducible nitric oxide synthase and reactive oxygen species and thus play an important role in immune regulation [17]. miRNAs are endogenous small RNAs

19–24 nucleotides in length which regulate target gene expression through the degradation of target gene mRNA or translational inhibition of target gene expression [18,19]. Several miRNAs have been shown to influence the generation and function of MDSCs. Zhang et al. reported that miR-20a and miR-17-5p can reduce the inhibitory potential of MDSCs by regulating STAT3 [20]. In contrast, miR-21 and miR-181b increased the production of MDSCs and promoted their immunosuppressive function in advanced sepsis patients [21]. Mei et al. showed that miR-200c can promote the inhibitory potential of MDSCs by regulating the expression of PTEN and FOG2 [22]. Huang et al. demonstrated that miR-34a may inhibit MDSC apoptosis by targeting the p2rx7, Tia1, and plekhf1 genes and thus contribute to the amplification of MDSCs [23]. Our study showed that miR-20b induced an increase in the number of MDSCs in the lung tissue of asthmatic mice, but had no effect on the content of MDSCs in the bone marrow, peripheral blood, or spleen. MiR-20b belongs to the miR-106a-363 gene family, which can form a larger miR-17 family with the miR-17-92 and miR-106b-25 gene clusters [24]. Recent studies have indicated that miR-20b is associated with the occurrence and development of many tumors and is closely related to cell proliferation, differentiation, and apoptosis [25]. MDSCs originate in the bone marrow, and while we showed that miR-20b induced MDSCs expansion in mouse lung tissue, the administration of miR-20b via nasal drops may not be an effective method for mobilizing MDSCs in bone marrow. In summary, the number of Gr1⁺CD11b⁺ MDSCs in the lung tissue of asthmatic mice increased after administration of miR-20b nasal drops, but there was no significant change in the number of MDSCs in the bone marrow, peripheral blood, or spleens of miR-20b-treated mice. MiR-20b may thus promote an increase of MDSCs in the lungs of asthmatic mice by the mechanism of inducing expansion.

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