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MicroRNA-106b regulates pro-allergic properties of dendritic cells and Th2 polarisation by targeting early growth response-2 *in vitro*

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ABSTRACT

Recent evidence has suggested that miRNA is implicated in the immune response of allergic and inflammatory diseases. However, little is known about its role in the mechanism that underlies the establishment of pro-allergic DCs in allergic rhinitis is not fully understood. This study assessed whether and how microRNA (miR)-106b regulates the pro-allergic properties of DCs upon allergen stimulation *in vitro*. Bone marrow-derived dendritic cells (BMDCs) were generated and stimulated with ovalbumin (OVA) to identify the miRNA expression profile. After transfection with miR-106b mimics and inhibitors OVA-activated BMDCs were further evaluated for surface marker expression using flow cytometry, cytokine production using ELISA and subsequent effects on Th2 cell polarisation using flow cytometry. Moreover, the upstream controllers and potential target proteins of miR-106b were examined in a western blot analysis. *Results showed that* MiR-106b expression was significantly inhibited in activated BMDCs upon OVA stimulation ($p < 0.05$). Surface marker expression (e.g., MHC class II, CD80 and CD86) was significantly upregulated after the transfection of an miR-106b inhibitor ($p < 0.05$), and the proportion of GATA-3$^+$ T cells was significantly increased among CD4$^+$ T cells that were cocultured with miR-106b inhibitor-pretreated BMDCs ($p < 0.05$). Conversely, IL-12 production from OVA-activated BMDCs and the proportion of T-bet$^+$ T cells increased significantly in a coculture of CD4$^+$ T cells and miR-106b mimics-transfected BMDCs ($p < 0.05$). The early growth response (Egr)-2 was identified via luciferase reporter assays as a target gene of miR-106b, and significant Egr-2 upregulation was observed in OVA-activated BMDCs following transfection with a miR-106b inhibitor ($p < 0.05$). In *conclusion*, our results suggest that miR-106b negatively regulates the pro-allergic properties of BMDCs and subsequent Th2 polarisation upon OVA stimulation and might represent a promising therapeutic target for allergic inflammation.

1. Introduction

Allergic rhinitis (AR) and asthma are characterised by allergen-initiated, Th2-skewed, eosinophilic airway inflammation [1]. The development of Th2 responses to inhaled allergens represents a malfunction of the adaptive immune system that has been orchestrated by various antigen-presenting cells [2]. Dendritic cells (DCs) are highly specialised antigen-presenting cells that bridge the innate and adaptive immune responses [3]. Upon stimulation from allergens, microbial colonisation and environmental pollution, DCs integrate a variety of stimuli into signals for T cells of the adaptive immune system and subsequently induce Th1, Th2 or other Th cell-based responses [4]. In AR and asthma, immature DCs form a dense network, in which inhaled antigens are continually sampled, and subsequently develop into mature DCs that are thought to play a pivotal role in the initiation and maintenance of Th2 responses [5]. Despite rapid progress in the understanding of the role of DCs in Th2 sensitisation, the key factors required to generate pro-allergic DCs in response to inhaled environmental antigens are not fully understood.

MicroRNAs (miRNAs) are an evolutionarily ancient class of endogenous small noncoding RNAs that posttranscriptionally regulate gene expression by binding to target mRNAs, thereby inhibiting their translation [6]. MiRNA-mediated gene expression regulation is complex, as 60% of all human protein coding genes have been predicted to contain miRNA binding sites in their 3′-untranslated regions (3′-UTR) [7]. Expression profiling has shown that distinct cell types express unique miRNA profiles as well as patterns that change during cellular differentiation and malignant transformation. Recently, miRNAs have been recognised as important in DC differentiation and function [8]. For example, Hashimi ST et al. reported that miR-34 and miR-21 were responsible for the maturation and function of bone marrow-derived dendritic cells (BMDCs) and showed that these miRNAs affect human DC differentiation by targeting the mRNAs encoding the Jagged1 and WNT1 proteins [9,10]. Other studies have identified let-7c, miR-224

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and miR-155 as necessary for normal DC functioning in mice [11–13]. However, how miRNAs might influence the pro-allergic capacities of DCs by tuning mature DC function remains unclear.

In this study, we initially investigated the miRNA expression profile of ovalbumin (OVA)-activated BMDCs using high-throughput sequencing and focused on evaluating the role of miR-106b in the pathophysiology of allergic response. Previously, a microarray study had demonstrated upregulated miR-106b expression in colon cancers with lymph node metastases. Several other studies have shown that miR-106b can regulate cell cycle progression and promote cell migration and metastasis in various types of carcinomas, thus suggesting that miR-106b functions as a pro-tumorigenic molecule [14–16]. However, the roles of miR-106b in regulating the pro-allergic capacity of BMDCs and skewing T cell differentiation have not been well characterised. In this study, we evaluated the importance of miR-106b in OVA-activated BMDC maturation and subsequent Th2 polarisation and investigated the underlying molecular mechanisms *in vitro*.

2. Materials and methods

2.1. Culture and stimulation of murine BMDCs

Male BALB/c mice, aged 6–8 weeks, were obtained from the Guangdong Medical Experimental Animal Centre (Guangzhou, China) and housed under pathogen-free conditions. Mice protocols were approved by the Animal Care and Use Committee of Eye and ENT Hospital of Fudan University Murine BMDCs were generated according to our previous description [17]. Briefly, bone marrow progenitors were cultured in the presence of GM-CSF (10 ng/mL) and IL-4 (10 ng/mL; both were purchased from PeproTech, Rocky Hill, NJ, USA). After a total of 6 days in culture, non-adherent cells and loosely adherent proliferating BMDC aggregates were harvested as immature BMDCs for future use. Mature BMDCs were obtained after a 24-h stimulation with 100 g/mL of OVA (grade V; Sigma-Aldrich, St Louis, MO, USA). All experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. RNA preparation and Solexa high-throughput sequencing

OVA-activated and control BMDCs (three replicates per subtype) were subjected to Solexa sequencing. Total RNA was extracted from the BMDCs using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA was purified directly for the Solexa sequencing analysis using an Illumina Genome Analyzer (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, a pair of Solexa adaptors was ligated to the 5′ and 3′-ends of the total RNA, and the small RNA molecules were amplified for 17 cycles via reverse transcription PCR with a PCR kit (Invitrogen). The approximately 30-bp fragments, which comprised small RNA plus adaptors, were isolated from an agarose gel. The purified RNA was directly used for cluster generation and sequencing analysis on an Illumina Genome Analyzer (Illumina) according to the manufacturer's instructions. Next, the differences in the miRNA quantities between the two groups were determined by comparing the log2 ratios of the two subgroups. This work was performed at the Beijing Genomics Institute (BGI, Shenzhen, China).

To compare the expression levels of known and novel miRNAs between the two groups, a log2-ratio figure plot and scatter plot were used to show the differential expression levels (Fig. S1). The normalisation procedures followed the BGI standard protocol: (1) miRNA expression in the two groups was normalised to obtain expression values in reads per million (RPM) as follows: Normalised expression = actual miRNA count / total count of clean reads × 1,000,000 and (2) the fold-change and p value were calculated from the normalised expression. Next, the log2 ratio plot and scatter plot were generated. The fold change was calculated as the log2 (activated/control), and the P-value formula was as follows:

$$P(y/x) = \left(\frac{N2}{N1}\right)^{Y} \frac{(x+y)!}{x!y!\left(1+\frac{N2}{N1}\right)^{(X+Y+1)}}.$$

In this formula, the total control BMDC clean tag number is defined as N1, and the total activated BMDC clean tag number as N2; miRNA A holds x tags in the control and y tags in the activated libraries. The false discovery rate (FDR) was also estimated in order to determine the P-value threshold. An FDR <0.01 with an absolute log2 value (activated/control) ≥1.0 was used as the criterion for determining the significance of a difference in the miRNA expression. At a normalised expression zero, FDR was changed to 0.01; additionally, some miRNAs with ratios <1 in both samples were not subjected to the differential analysis.

2.3. MiRNA quantification via quantitative reverse transcription PCR (qRT-PCR) analysis

The total RNA was subjected to reverse transcription using a reverse transcription kit (Tiangen Biotech, Beijing, China) with specific stem-loop primers to generate cDNA. MiRNA expression was quantified using a SYBR QPCR kit (Toyobo, Osaka, Japan) and an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The relative miRNA levels were normalised to the U6 small nuclear RNA levels.

2.4. Transfection of miR-106b mimics or inhibitors or control dsRNA

For the BMDC transfection experiments, miR-106b mimics or inhibitors or the dsRNA controls (Ribo Biotechnology, Guangzhou, China) were diluted with OptiMEM I medium (Invitrogen). Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer's instructions to transfect murine BMDCs (5×10^5 cells/mL) with miR-106b mimics or inhibitors or dsRNA controls (50 nM). After 24 h, the cells were resuspended in RPMI 1640 with 10% FBS and subjected to further experimentation.

2.5. Mixed lymphocyte reactions

$CD4^+$ T cells were purified from BALB/c mice as previously described [19]. Briefly, splenic tissues from naive mice were passed through a stainless steel sieve to harvest the cells. $CD4^+$ T cells were purified (>95%, confirmed by flow cytometry) by negative selection using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. $CD4^+$ T cells were co-cultured in a 10:1 ratio with BMDCs that had been pretreated with OVA (100 μg/mL, 24 h) in 96-well U-bottom plates. After 3 days, the cells were analysed via flow cytometry.

2.6. Flow cytometric analysis of DC marker expression and CD4 + T cell subsets

BMDCs (5×10^5 cells/mL) were washed and incubated with FITC-conjugated anti-MHC class II, anti-CD80 and anti-CD86 antibodies (eBioscience, San Diego, CA, USA) for 20 min at 4 °C in the dark. The samples were washed and analysed within 24 h on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Appropriately conjugated isotype-matched control antibodies were used as negative controls. To analyse $CD4^+$ T cell polarisation, co-cultured $CD4^+$ T cells were surface stained with APC-conjugated anti-mouse CD4. The cells were then fixed and permeabilised with a fixation/permeabilisation buffer (eBioscience) prior to intracellular staining with PE-conjugated anti-T-bet and