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*Original Article*

# MiR-138 regulates dendritic cells mediated Th2-type immune response by regulating the OX40L expression in asthma

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**Abstract:** Objective: The aim of this study was to investigate the mechanisms of miR-138 in regulating Th2 type immune response by targeting OX40 ligand (Ox40L) in vitro. Methods: Serum samples of patients were used to explore the clinical parameter. Wistar rats were used to establish a murine model of asthma. The dual-luciferase report assay was used to detect the regulation of miR-138 on the expression of OX40L. RT-PCR was used to detect miR-138 and OX40L mRNA expression. Mixed lymphocyte reaction (MLR) and Western blot were used to analyze target protein expression. Enzyme linked immune sorbent assay (ELISA) and flow cytometry (FCM) were used to cytokines detection. Results: The level of miR-138 was found to be negatively correlated with the expression of OX40L ($P < 0.05$) and positively correlated with FEV1 ($P < 0.05$). Higher miR-138 and reduced expression of OX40L were observed in dendritic cells (DCs) separated from rat bone marrow. Typically, OX40 and OX40L in asthma group were determined and the results indicated that the two parameters upregulated in compared with healthy control, while the expressions of them were suppressed by over-expression of miR-138. Furthermore, the up-regulation of Th1 cytokines (IL-2 and IFN-γ) and the down-regulation of Th2 cytokines (IL-4 and IL-10) were induced by over-expression miR-138 and meanwhile the decrease of Th1/Th2 was reversed by overexpression of miR-138. Conclusion: In this study, we revealed that miR-138 might regulate Th2-type immune response by down-regulating the OX40L expression in asthma.

**Keywords:** Asthma, miR-138, OX40L, DCs, Th1, Th2, $CD4^+$

## Introduction

Asthma, which can be characterized by airway hyperresponsiveness of allergic diseases and airway inflammation, is one of the most common chronic immunological diseases in human beings [1, 2]. Such cases usually occur in childhood and run throughout the entire life course. Up to a quarter of patients may continue to have relevant symptoms that recur in adulthood [2]. In recent years, research progress in treating asthma is somewhat sluggish and therapeutic schedules have generally suggested empirical approaches in accordance with clinical diagnosis of disease severity rather than the underlying pathogenesis [1]. Studies have shown that the pathogenesis of asthma was connected with certain factors including inflammatory mediator release, inflammatory cell invasion and immune imbalance, among which the imbalance of ratio and function of Th1/Th2 were perceived as the crucial pathogenesis [2, 3]. Under normal circumstances, mutual inhibition between Th1 and Th2 cells is conducive to the balance of immune response in the body [4, 5]. However, Th2 responses and mediated by cytokines including IL-4 will facilitate the occurrence of the aeroallergen and hyperresponsiveness [5]. Th2 cells can increase the secretion of Interleukin-4 (IL-4) and antagonize the emergence of Th1 cells including γ-interferon (IFN-γ) and hence further promote the development of asthma [6].

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) and are involved in the

occurrence and development of a variety of inflammatory diseases, including asthma [7-9]. Recent studies have also revealed that DCs can stimulate the activation of T cells resulting in Th2-type allergic inflammatory reaction [9, 10].

OX40L, also referred to as CD252 or TNFSF4, belongs to superfamily member. It is a type II transmembrane glycoprotein which is mainly expressed on the surface of APC cells, especially DC [11]. OX40 (CD134) is preferentially expressed on the surface of activated regulatory $CD4^+$ T cells. It can specially bind with the OX40L which located on the surface of DCs and initiate a series of reactions which contribute to the maturity of DCs and facilitate the proliferation and survival of $CD4^+$ T cells and the generation of cytokines [12]. Note that OX40L can activate the expression of IL-4 by T cell, inhibit the expression of IFN-γ and participate the immunocyte including mastocyte mediated Th2 immune response [13].

MiRNA is a category of endogenous non-coding RNA with the short sequences of 18-22 bp. It can combine with the 3'-non-coding region of target mRNA (3'-UTR) and inhibit the translation and expression of target gene after transcription. Recent studies have emphasized the miRNAs play a crucial role in controlling allergic airway inflammation and is closely related to the development of asthma, although the clinical data is limited [14]. MiR-138 is more common tumor suppressor miRNA. A recent study has shown that miR-138 can inhibit the proliferation of smooth muscle cells and exert the profound influence on the occurrence of asthma [15]. Besides, miR-138 has been proven to be involved in the regulation of the imbalance of Th1/Th2, indicating the potential mechanisms of in relation to Th2 type immune response [16].

Costimulatory molecules, such as OX40/OX40L, play a crucial role in regulating the differentiation and balance of Th1/Th2 cells and the overexpression of OX40L from DCs appears particularly important [17].

MicroRNA (miRNA) has been the research hotspot in recent years. It is of vital importance at the level of transcriptional and regulation and participate in physiological process, including the growth and development, tumor formation and immunoregulation. However, whether there exists regulatory mechanism of miRNA expressed by OX40L in the DCs has not been addressed in literature. Therefore, the exploration and verification of relevant miRNA may provide valuable information for the changeover of asthma at the molecular level.

## Materials and methods

### *Asthma serum samples detection*

Serum samples were obtained from 15 patients with acute episode of asthma and 15 patients with stable asthma. 15 healthy persons were served as the control group. Clinical parameters (lung function and FeNO) were recorded. The expression of miR-138 in serum samples was measured using the RT-PCR kits (Invitrogen) and the OX40L expression was detected using ELISA kits (Sigma-Aldrich, USA).

### *Vectors, transfection and luciferase reporter assay*

The pMIR-3'UTR-WT was generated by cloning the PCR product into a pMIR-REPORT luciferase miRNA expression reporter vector (Ambio, Austin, Texas, USA). The sequence from the 3'-UTR (3'-untranslated regions) of OX40L was amplified by PCR using genomic cDNA of $CD4^+$ T cells. The pMIR-3'UTR-MUT was generated based on pMIR-3'UTR-WT by Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA); the vector was mutated in the putative miRNA-138 binding site. Each vector (pMIR-3'UTR-WT and pMIR-3'UTR-MUT) was transfected along with miRNA-138 mimics and miRNA-138 mimics control into 293T cells (American Type Culture Collection, Manassas, VA, USA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Cells were cultured in DMEM with 10% FBS at 37°C under 5% $CO_2$ for 48 h. Macdonald et al Luciferase activity was measured using the Dual-Luciferase Reporter 1000 Assay Kit (Promega Corporation) according to the manufacturer's protocol.

### *DC generation*

Based on the methods described by Macdonald et al [18] and with some modifications, bone marrow cells from femurs and tibiae of wistar rats which used were ages 8 weeks to 10

weeks were collected, then after low centrifugation at 1500 rpm for 3-5 min, discarding the supernatant, RBC Lysis Buffer was added at a ratio of 1:10 at room temperature for 5 min. After low centrifugation, the cell precipitate was washed with PBS twice and the cell suspension were collected and used in the experiments.

*Separation and purification of DCs*

DCs were isolated by automated magnetic cell sorting kit (Miltenyi-Biotec, Germany) according to the manufacturer's protocol. Briefly, the DC suspension isolated was treated at 4°C with anti-DC (OX62) microbeads for 15 min and followed by washing and magnetic separation.

The DCs were resuspended in 200 μl of PBS after centrifugation and 10 μl of various antibodies (CD80-FITC, CD86-FITC, MHC-II-APE) were added and an isotype control group was established. After incubation at 4°C for 30 min in the dark, the cells were washed once with PBS and analyzed using a flow cytometer.

*Transfection*

DCs were seeded into 6-well plate and cultured in RPMI 1640 (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 20 ng/ml rGM-CSF, 10 ng/ml rrIL-4 and 1% streptomycin at 37°C in a 5% $CO_2$ atmosphere, medium was replenished every 3 days; at day 4 the supernatant was removed and replaced with fresh medium at $2 \times 10^6$ cells per well. Using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), miR-138 mimic and miR-138 mimic, control were transfected into DC according to the manufacturer's recommendations. After 12 h of transfection, the cells were reseeded in 6-well plates with complete culture medium.

*RNA extraction and quantitative real time PCR (q RT-PCR)*

Total RNA samples were obtained using TRIzol (Invitrogen, USA) isolation method and cDNA was synthesized using one step PrimeScript miRNA cDNA Synthesis Kit (Takara Biotechnology, Dalian, China). The qRT-PCR analysis for miRNAs was confirmed using TaqMan miRNA assays (Applied Biosystems, USA). Amplification reaction was performed using Step OnePlus™ Real-Time PCR System (Applied Biosystems). U6 snRNA and GAPDH were respectively used as endogenous control for miR-138 mRNA expression and OX40L mRNA expression. Moreover, the relative expression level of miR-138 and OX40L were computed using the $2^{-\Delta\Delta Ct}$ analysis method.

*Western blotting*

Total proteins were extracted from $CD4^+$ T cells using RIPA lysis buffer (Beyotime, China) and quantified with the BAC assay (Pierce, IL) after transfecting for 48 h. Equal amounts of protein were subjected to SDS-PAGE under reducing conditions and transferred onto polyvinylidene difluoride membrane (PVDF) (Millipore Corp, MA). After blocking in Tris Buffered Saline with Tween containing 5% non-fat dry milk for 2 h, the target proteins were cultured overnight at 4°C with anti-human antibodies of OX40L or GAPDH, furthermore, it was incubated for 1 h with the corresponding goat anti-rabbit secondary antibodies at room temperature. A fluorescent Western blotting detection system was used.

*Establishment of murine model of asthma and Model evaluation*

20 healthy Wistar rats with 8-10 weeks old were divided into two equal groups. They were denoted as control group and experimental group, respectively. The murine model of asthma was established using the methods of ovalbumin (OVA) sensitization and stimulation. The sensitization liquid (10 mg OVA) were injected into the rats of experimental group in the first and seventh day. From the 15th day on, the rats were inflicted by inhalation of 50 mL of 2% OVA/PBS. The inhalation frequency was one time (30 min) every day and the duration was one week. The control groups were injected and atomization inhaled with normal saline (NS) instead of sensitization liquid.

The evaluation was performed based on the behavior analysis of rats. The occurrence of the asthma symptom of rats such as wheeze, cough and dysphoria during atomization process was considered to hit the target.

*Isolation and purification of $CD4^+$ T lymphocytes*

$CD4^+$ T cells were isolated from venous peripheral blood samples from rats with asthma and healthy rats. The peripheral blood samples of

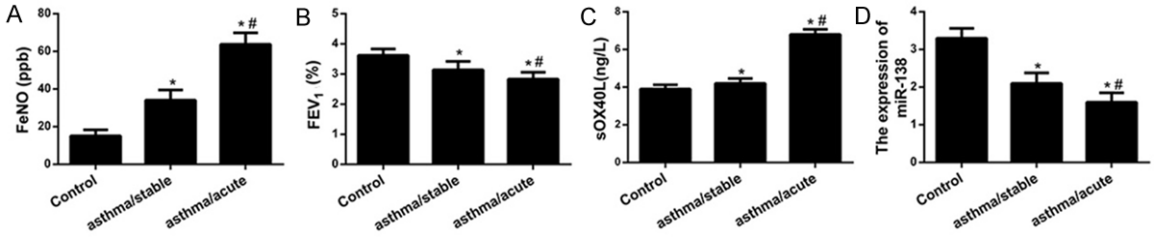


**Figure 1.** Clinical parameters (FeNO, FEV1) and the expressions of OX40L and miR-138 of patients with bronchial asthma. Control: healthy person; asthma/stable: the patient with stable bronchial asthma; asthma/acute: the patient with acute attack of bronchial asthma; *Patient with stable asthma differ significantly ($P < 0.05$) with control; #Patient with acute attack of bronchial asthma differ significantly ($P < 0.05$) with patient with stable bronchial asthma.

**Table 1.** Correlation analysis of miR-138 with OX40L, FEV1, FEV1% pred and FeNO.

| Correlations | MiR-138 | |
|---|---|---|
| | r | *p* |
| OX40L | -0.462 | 0.013 |
| FeNO | -0.042 | 0.723 |
| FEV1 | +0.127 | 0.045 |
| FEV1% pred | +0.205 | 0.038 |

rat were collected and the monocyte was extracted using the Percoll separating medium. The purification of $CD4^+$ T lymphocytes was conducted by automated magnetic cell sorting kit (Miltenyi-Biotec, Germany) according to the manufacturer's protocol.

*Mixed lymphocyte reaction (MLR) in vitro*

According to method of [19], the $CD4^+$ T cells were seeded at a density of $2 \times 10^5$ cells per well into 96-well culture plates and incubated at 37°C in a humidified 5% $CO_2$ for 48 h. Subsequently, mixed lymphocyte reaction (MLR) was performed by co culture of $CD4^+$ T cells with normal DCs or DCs of over-expression miR-138 in vitro. The expression of T cells was tested by 3H-TdR.

*Enzyme-linked immunosorbent assay (ELISA)*

According to method described by [20] with slight modifications, the supernatant of MLR was measured with IL-2, IFN-γ, IL-4 and IL-10 ELISA kits (Sigma-Aldrich, USA). The concentration of cytokines was calculated according to the corresponding OD value.

*Flow cytometry*

Two different samples of MLR incubated with anti-CD4-FITC and anti-IL-4-PE or anti-CD4-FITC and anti-IL-4-PE respectively for 30 min. The percentage of Th1 or Th2 were analyzed by flow cytometry using a FACScan (BD Biosciences) based on the method of [21]. The expression of OX40 and OX40L were determined with the same method.

*Statistical analysis*

All date were analyzed with SPSS13.0, Pearson's correlation analysis was used to explore the relationship between the parameters of samples. Student's t-test was used to analyze differences between two groups. One-way ANOVA analysis was used to determine the multi-sample analysis. All statistical tests were two-sided, and *P* value less than 0.05 was considered significant.

## Results

*Negative correlation between miR-138 and OX40L*

Detection of patient serum demonstrated that OX40L in patients with asthma was high expression and miR-138 was low expression. Besides, according to the Pearson's correlation analysis, a significant negative correlation was found between the expression level of miR-138 and the protein expression of OX40L ($P < 0.05$); there was no correlation between miR-138 and FeNO ($P > 0.05$) and miR-138 was positively correlated ($P < 0.05$) with FEV1 (**Figure 1**; **Table 1**).

*Prediction and identification of OX40L targeted miR-138*

MiR-138 was predicted to target human OX40L using bioinformatics databases. To verify the direct relationship between miR-138 and hu-

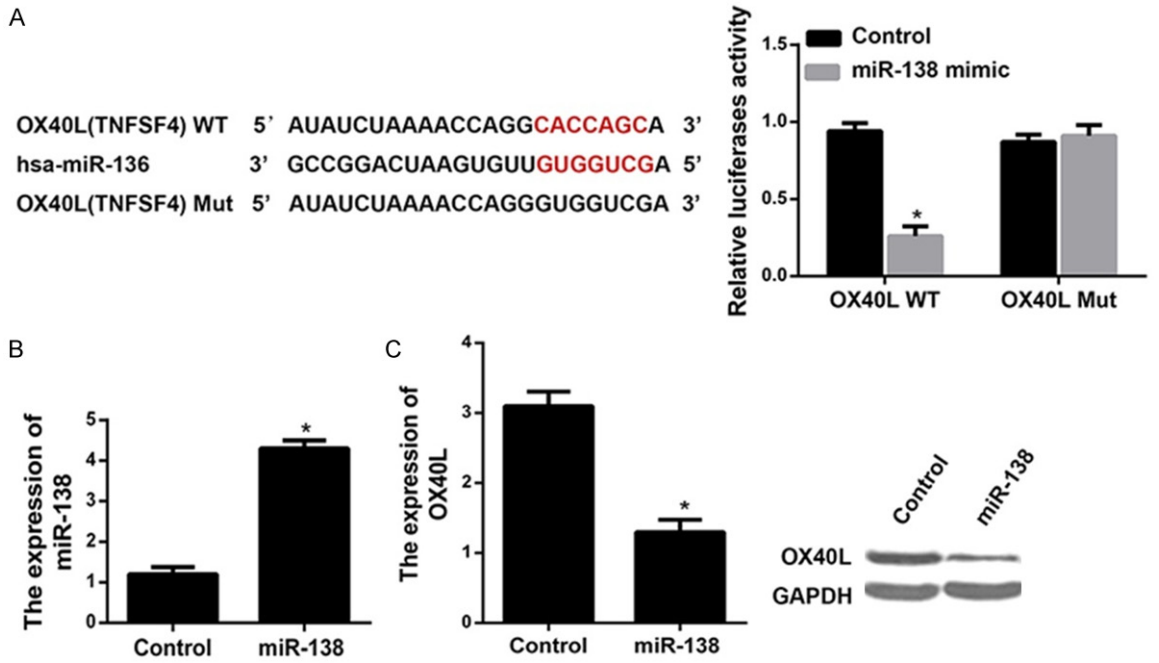


**Figure 2.** MiR-138 regulates the expression of OX40L in DCs. A: The wild miR-138 binding sequence and the mutant miR-138 binding sequence in the 3'-UTR of the OX40L and Luciferase activity assay of the target regulation of miR-138 to OX40L 3'-UTR; B: Transfection of miR-138 mimic in separated and purified rat bone marrow-derived cells, detection of the expression of miR138 via RT-PCR; C: The protein expressions levels of OX40L using Western blot (miR-138 target regulates OX40L; the overexpress of miR-138 suppressed the express of OX40L mRNA and protein). $^{*}P < 0.05$ versus control group.

man OX40L, the dual-luciferase reporter was used. The results showed that the luciferase activity in the OX40L-wt with mimics group was significantly reduced and the mutation of the sites in the 3'-UTRs abrogated the repressions (**Figure 2A**), indicating that miR-138 directly targeted OX40L. Moreover, it can be found that when miR-138 was overexpressed the levels of expression of OX40L mRNA and OX40L protein were obviously reduced (**Figure 2B**, **2C**), indicating that overexpression of miR-138 inhibited the expression of OX40L.

*Overexpression of miR-138 inhibits OX40L expression and regulates the balance of Th1/Th2*

To investigate the role of miR-138 in the interaction of OX40/OX40L and the pathogenesis of asthma, a murine model of asthma was established. The results shown that the proliferation of T cells increased in the asthmatic rats (**Figure 3A**). Besides, the OX40 and OX40L upregulated in asthma group compared with healthy control, while the expression of them was suppressed by over-expression of miR-138 (**Figure 3B**, **3C**). In addition, the expression of Th1 cytokines (IL-2 and IFN-γ) was reduced in asthmatic rats compared with healthy control (**Figure 4A**), while the expression of Th2 cytokines (IL-4 and IL-10) was increased in asthmatic rats compared with healthy control (**Figure 4B**). But the expression of Th2 was reversed by over-expression miR-138 (**Figure 4C**, **4D**). In conclusion, it was obtained that the up-regulation of Th1 cytokines (IL-2 and IFN-γ) and the down-regulation of Th2 cytokines (IL-4 and IL-10) were induced by over-expression miR-138 and meanwhile the decrease of Th1/Th2 was reversed by over-expression.

## Discussion

Asthma is a chronic inflammatory disease of airway induced by interactions of inflammatory cells and mediators. It is generally considered to be caused by overproduction of Th2 cytokines including IL-4 and IL-5 resulting from allergen-specific T cells [5, 22]. Based on the results of Western Blot and MLR, we have found that the abnormal high expression of OX40L in the serum of patients with bronchial

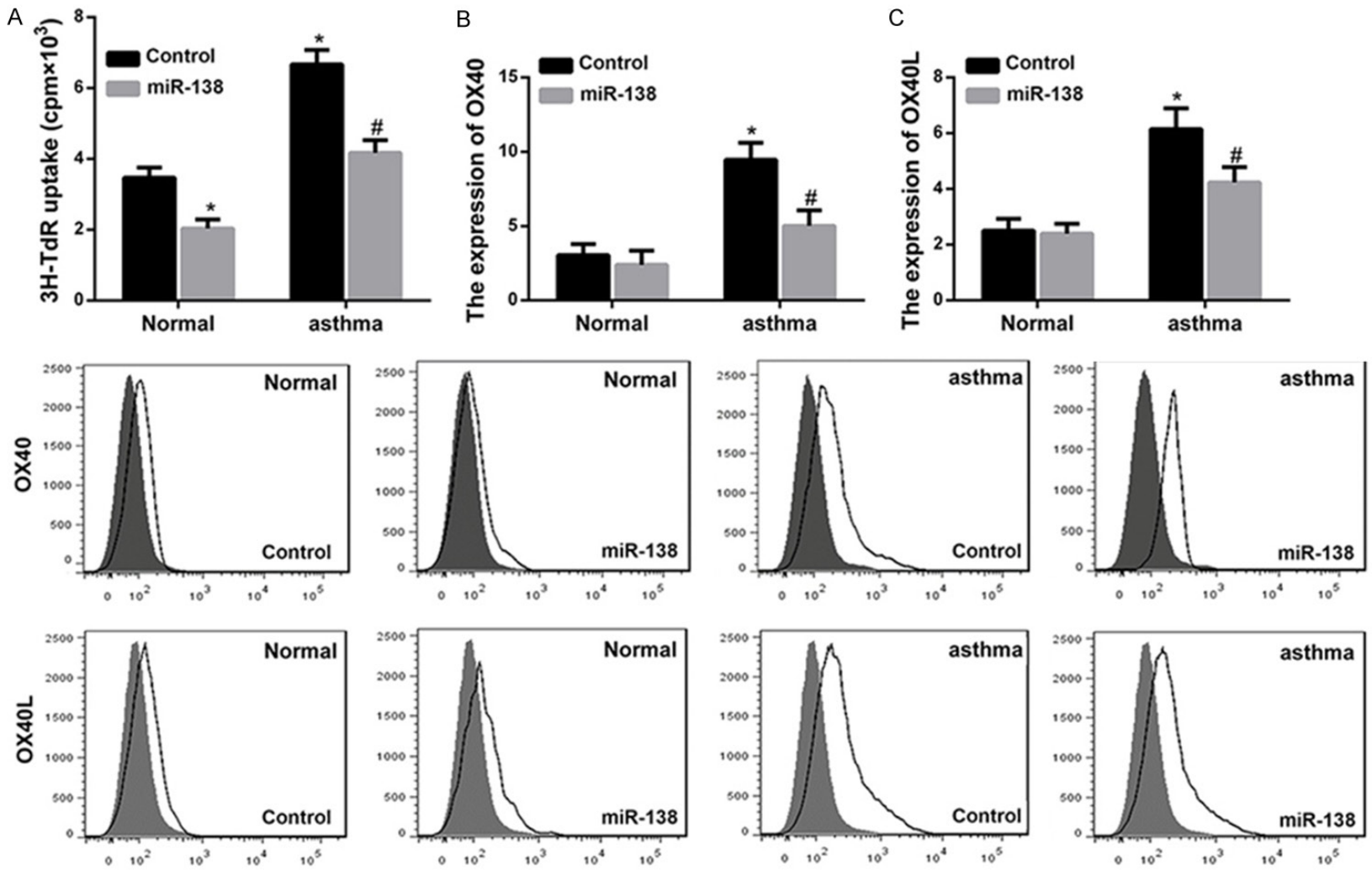


**Figure 3.** Overexpression of miR-138 inhibits OX40L expression and proliferation of $CD4^+$ T cells. The establishment of the murine model with bronchial asthma. The venous peripheral blood monouclear cells of healthy rats and rats with asthma were separated. $CD4^+$ T cells were sorted by magnetic beads and mixed with healthy DCs and DCs overexpressed by miR-138 and cultivated. A: The proliferation of $CD4^+$ T cells using 3H-TdR. *The proliferation of $CD4^+$ T cells in asthma group differ significantly ($P < 0.05$) with control. #Overexpression of miR-138 inhibits $CD4^+$ T cells proliferation; B: Determination of expression of OX40 using flow cytometer. *The expression of OX40 in asthma group differ significantly ($P < 0.05$) with control. #Overexpression of miR-138 inhibits OX40 expression; C: Determination of expression of OX40L using flow cytometer. *The expression of OX40L in asthma group differ significantly ($P < 0.05$) with control. #Overexpression of miR-138 inhibits OX40L expression.

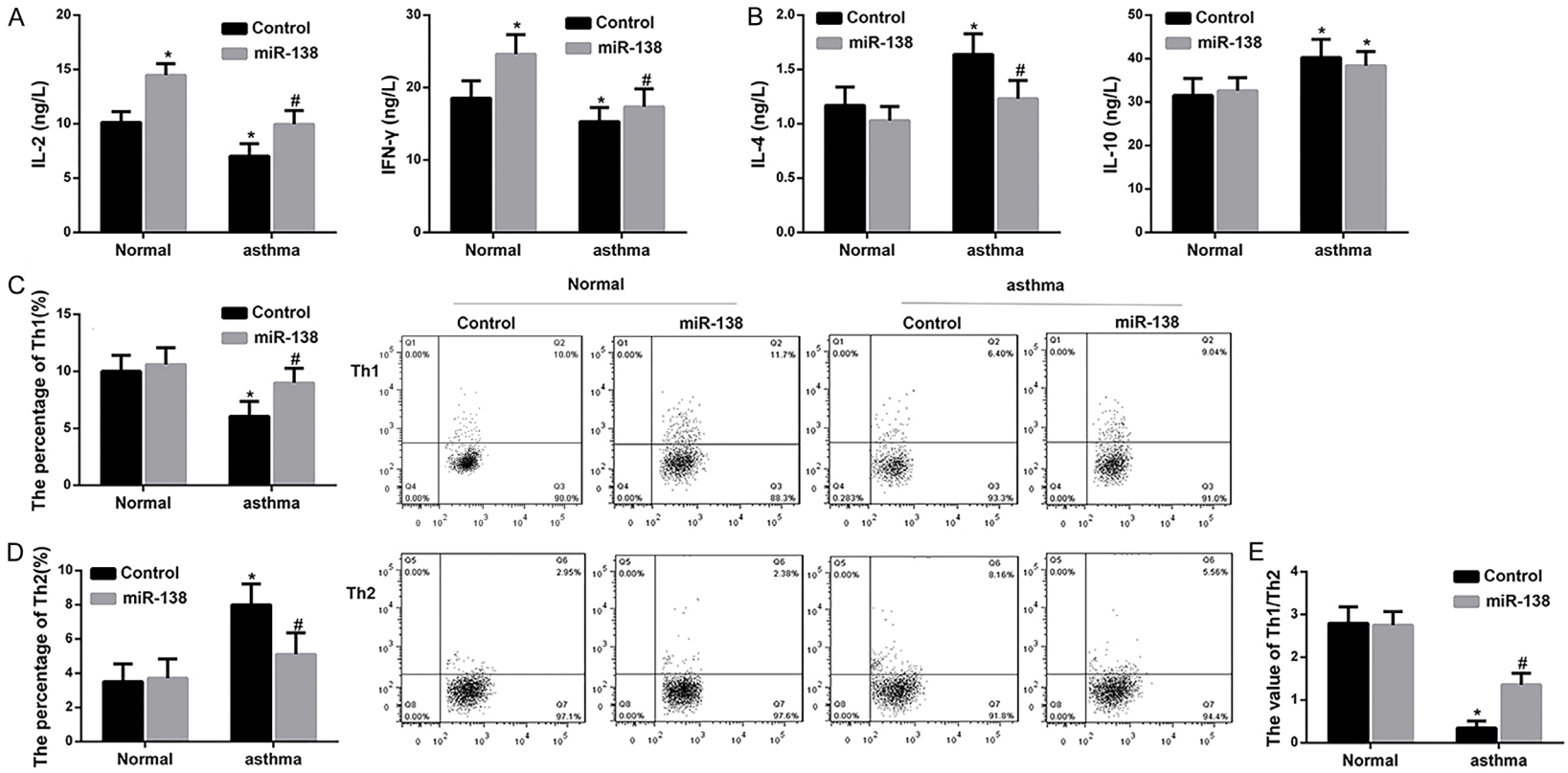


**Figure 4.** Overexpression of miR-138 regulates the cytokines related to Th1 and Th2 in CD4$^+$ T cells by MLR (CD4$^+$ T cells were sorted by magnetic beads and mixed with healthy DCs and DCs overexpressed by miR-138 and cultivated in vitro). A: The measurement of Th1 cytokines (IL-2 and IFN-γ) with ELISA; B: The expression of Th2 cytokines (IL-4 and IL-10); C: Determination of expression of Th1 cell account using flow cytometer; D: Determination of expression of Th2 cell account using flow cytometer; E: The ratio of cell account of Th1 and Th2. $^*P < 0.05$ versus mimic control. $^\#P < 0.05$ versus mimic control of the same group.

asthma and the expression of OX40L significantly increased in $CD4^+$ T cells from asthma patients, indicating that OX40L plays a crucial role in the pathogenesis of asthma. Previous studies have reported that the noncoding RNAs can suppress the expression of protein-coding by binding to the target sequence at the 3'-UTR of the target gene [23]. To be specific, miR-138 can promote proliferation and migration of smooth muscle cells in db/db mice by down-regulation of SIRT1 and suppress invasion and metastasis of ovarian cancer cell by targeting SRY-related high mobility group box 4 (SOX4) and hypoxia-inducible factor-1α (HIF-1α) [24, 25]. miR-138-5p could contribute to gefitinib resistance in non-small cell lung cancer (NSCLC) cells through negatively regulating G protein-coupled receptor 124 (GPR124) [26]. In brief, several biological processes in diverse diseases are closely related to the down-regulation of miR-138. Therefore, in the study, miR-138 was chosen to be the targeted miRNA for OX40L. To detect the relationship between miR-138 and OX40L, the expression of miR-138 and OX40L were determined initially and the results revealed that miR-138 decreased significantly in asthma patients and exhibited a significantly negative correlation with OX40L. Moreover, detection of reciprocal relationship between miR-138 levels and OX40L expression demonstrated that the 3'-UTR OX40L was indeed a target for miR-138. Simultaneously, as results shown that over-expressed miR-138 inhibit the expression of OX40L from DCs. These results were consistent with former study.

Recent studies have found that several miRNAs are potential therapeutic targets for asthma. For instance, antagonism of microRNA-126 could inhibit the effector function of Th2 cells and the progression of allergic airways disease [27]. It was confirmed that MiRNA-138 could regulate the balance of Th1/Th2 via targeting RUNX3; and microRNA could upregulate T cells in asthma airway and herein stimulate the production of TH2 cytokine [16, 28]. As was reported previously, $CD4^+$ T cells could differentiate into Th1, Th2 and other type of T cells depending on cytokines, the nature of the encountered and environmental signals stimulus itself [29]. Another study showed that the balance of Th1 and Th2 is relevant to the development of asthma [30]. In addition, OX40L plays a critical role in the differentiation of T cells and has an influence on the balance of Th1/Th2 [31]. Therefore, to investigate the role that miR-138 plays in the differentiation of T cell via targeting OX40L, the murine model of asthma was established. We found that T cells were prominently increased in the asthma group and over-expressed miR-138 inhibited the proliferation of T cell. Besides, the OX40L as well as OX40 were significantly reduced due to the hyper-expression of miR-138 in the asthma group. These suggesting that miRNA-138 could regulate the T cells differentiation via targeting OX40L. It has been known that OX40 is mainly expressed by Th2 cells; while OX40L is predominantly expressed by antigen-presenting cells, especially dendritic cells, which are resulted from overproduction of Th2 cytokines (IL-4, IL-10) by allergen-specific T cells [21]. The expression of OX40L was suppressed as a result of the overexpression of miR-138. In addition, the balance of Th1/Th2 was broken and shifted to overexpress of Th2-related cytokines. Remarkably, miR-138 has been proposed to target the 3'-UTRs of the OX40L and control OX40 receptor and cytokine signaling via a negative feedback loop, thus reducing the production of inflammatory cytokines (IL-4, IL-10). Th2 cells are crucial for causing disease by producing key cytokines, including IL-4 and IL-10 [32]. DCs are essential for priming and Th2 differentiation of naive $CD4^+$ T cells towards aeroallergens, and in recent years it has been well established that these cells play a pivotal role in the initiation phase of allergic asthma [33]. To verify whether the miR-138 regulates the levels of T cell and cytokines through direct targeting of 3'-UTR of OX40L, we detected the interaction between miR-138 overexpression and cytokines from asthma patients in our study. The results showed that the overexpression of miR-138 increased Th1-related cytokines (IL-2, IFN-γ), induced the Th2-related cytokines (IL-4, IL-10) and also decreased the Th1/Th2. Taken all together, these results indicated that the overexpression of miR-138 induce the deviation of Th1/Th2 by resulting in OX40L expression, which may suppress the development of asthma.

In summary, our study demonstrates that miR-138 plays an important role in asthma. Up-regulation miR-138 can suppress the proliferation of the $CD4^+$ T cell and lead to a balance of Th1/Th2 via targeting 3'-UTRs of OX40L, which is related to the pathogenesis of asthma.

Moreover, this study provides valuable information which may help to elucidate the pathogenesis of asthma and implicates miR-138 as a potential therapeutic target for asthma.

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## Disclosure of conflict of interest

None.

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