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# Methyl-cpg-binding Domain Protein 2 Silencing Inhibits Th17 Differentiation of CD4$^+$T cells Induced by Ovalbumin

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## ABSTRACT

**Background:** Little is known about MBD2's epigenetic regulation in the immune pathogenesis of CD4$^+$T cell differentiation.

**Objective:** This study attempted to explore the mechanism of methyl-cpg-binding domain protein 2 (MBD2) in CD4$^+$T cell differentiation stimulated by environmental allergen ovalbumin (OVA).

**Methods:** Mononuclear cells were separated from the spleen tissues of male C57BL/6 mice. The OVA interfered with the differentiation of splenic mononuclear cells and CD4$^+$T cells. The CD4$^+$T cells were obtained by magnetic beads and identified by CD4 labeled antibody. CD4$^+$T cells were transfected with lentivirus to silence MBD2 gene. A methylation quantification kit was used to detect 5-mC levels.

**Results:** The purity of CD4$^+$T cells reached 95.99% after magnetic beads sorting. Treatment with 200 μg/mL OVA stimulated the CD4$^+$T cells differentiation to Th17 cells and promoted the secretion of IL-17. After being induced, the Th17 cell ratio increased. 5-Aza inhibited the Th17 cell differentiation and the IL-17 level in a dose-dependent manner. Under the intervention of the Th17 induction and 5-Aza, MBD2 silencing inhibited the differentiation of Th17 cell, and decreased the IL-17 and 5-mC levels in the cell supernatants. MBD2 silencing reduced the scale of the Th17 cell and IL-17 levels in the OVA-treated CD4$^+$T cells.

**Conclusion:** MBD2 affected IL-17 and 5-mC levels by mediating the Th17 cell differentiation in splenic CD4$^+$T cells that were interfered with 5-Aza. OVA induced Th17 differentiation and increased IL-17 levels, inhibited by MBD2 silencing.

**Keywords:** CD4$^+$T Cell, IL-17, MBD2, Ovalbumin, Th17 Cell

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## INTRODUCTION

Asthma is a clinical syndrome that affects all age groups and is characterized by airway inflammation and hyperresponsiveness [1]. The development of asthma was mainly manifested by systemic inflammation, oxidation, microbial disturbance, cytokine release, free radical damage, and immune response related to the development and the course of asthma [2]. Clinical studies have revealed that the difficulty of asthma treatment is related to the expression of different regulatory molecules of $CD4^+$T cells between atopic and early-onset asthma patients and non-atopic with late-onset asthma patients [3]. Early malnutrition could induce $CD4^+$T cells to show increased activation and proliferation, resulting in susceptibility to experimental asthma [4]. Therefore, novel T cell-based therapies allow us to expand the range of molecular targets to facilitate personalized treatment of asthma disease.

In allergic asthma, exposure to air allergens in sensitized people mobilizes a strong innate and adaptive airway immune response, stimulating eosinophilic airway inflammation, activating and infiltrating allergen-specific $CD4^+$T cells into the airway [5]. $CD4^+$T helper (TH) cells, as well as the regulatory T (Treg) cells, which in reaction to usual allergens play a key role in promoting and suppressing airway inflammation in asthmatic subjects [6]. $CD4^+$T cells were activated to interact with the antigen MHC complex and differentiate into specific subtypes, mainly based on the cytokine microenvironment [7]. All of these required the activation of a set of cytokine signaling, specific transcription factors, and appropriate genes for epigenetic modification on DNA sequences to regulate immune responses [8]. Multidimensional analysis showed that DNA methylation within promoters of a set of core $CD4^+$T cell pathway genes contributes to a low reactive immune response to pneumonia in neonates [9]. DNA methylation may be a mechanistic target for ameliorative therapies for lung infections and immune diseases. Therefore, the regulation of DNA methylation in allergen-specific $CD4^+$T cells may be a worthy target in treating allergic asthma.

MBD2 has a high binding affinity for DNA methylation [10] and is considered a decoder of DNA methylation-encoded information and an executor of the regulatory effects of DNA methylation [11, 12]. MBD2 is associated with immune system function and tumorigenesis, commonly expressed in the lung, liver, and colon, but little is known about its in vivo function [13]. Intratracheal liposomes loaded with MBD2 siRNA protected mice from bleomycin-induced lung injury and fibrosis [14]. Th17 cells and their secretion of IL-17 also play a valuable role in chronic nonspecific airway inflammation and asthma associated with respiratory microbiological infection [15]. However, whether the DNA methylation and MBD2 expression affect the Th17 cell development and function is unknown.

Ovalbumin (OVA) was used to construct a cellular or animal sensitization model of asthma [16]. Experimental asthmatic models of BALB/ C mice stimulated by the OVA showed inflammatory cell infiltration and excessive mucus secretion increased $CD4^+$IL-$17A^+$cell count, and serum IL-13, IL-5, and IL-17A levels resulting in an imbalance of $CD4^+$T cell subtypes in mice [17]. Expression of the MBD2 increased in the $CD4^+$T cells, separated from the peripheral blood of asthmatic patients [18]. The Th17 cells are critical in the pathogenesis of neutrophil-dominated asthma [19]. MBD2 may downregulate SOCS3 expression to promote the differentiation of the Th17 cells in severe asthma [20]. However, the role of the MBD2 in OVA-induced allergic asthma remains unknown. In this study, we isolated mononuclear cells from the spleen tissues of male C57BL/6 mice, and the splenogenic $CD4^+$T cells were separated by magnetic beads. The primary $CD4^+$T cells were used

to probe the mechanism of MBD2 expression and T cell subtype differentiation under the OVA, in order to gain rationale for the treatment of allergic asthma.

## MATERIALS AND METHODS

*Isolation of Splenic Mononuclear Cells from Mice*

Male C57BL/6 mice were intraperitoneally injected with chloral hydrate (10%, 3 mL/kg) and soaked in 70% alcohol for disinfection for 2~3 min. Mice spleens were aseptically separated at a super clean table and then transferred to PBS containing 2 × cyanin-streptomycin to wash away the blood stains. The 400 mesh cell filter was placed on a 50 mL centrifuge tube and moistened with fetal bovine serum (FBS)-1640 medium. The spleen tissue was cut up on a strainer. The piston of the sterile syringe gently grinds the spleen on a strainer to cause scattered mononuclear cells to enter the culture medium. The suspension of mononuclear cells was diverted to a 15 mL tube, followed by 400g centrifugation for 5 min to obtain cell precipitation. 5 mL of erythrocyte lysate was added, gently blown, mixed, lysed for 5 min, and centrifuged at 400g for 5 min. Then, 5 mL PBS was added to re-suspend the cells, the supernatant was centrifuged again and the cells were re-suspended. 10 μL of suspended cells were used for cell counting. This experiment was approved by the Ethics Committee of the Affiliated Hospital of Guilin Medical University (NO.201703190).

*OVA Co-culture Stimulates Mononuclear Cell Differentiation*

First, the optimal concentration of the OVA (ZY9000, Shanghai Zeye Biotechnology Co., Ltd) was screened from the wild-type splenogenic mononuclear cells. The specific groups are as follows: The control group (splenogenic mononuclear cell culture without intervention), the 50 μg/mL OVA group (add 50 μg/mL OVA to the control group), 100 μg/mL OVA group (add 100 μg/mL OVA to the control group) and 200 μg/mL OVA group (add 200 μg/mL OVA to the control group). After the intervention of different concentrations of the OVA, the $CD4^+$T cells were isolated and purified by immunomagnetic bead sorting.

*Sorting and Identification of $CD4^+$T Cells*

$CD4^+$T cells were separated by a mouse $CD4^+$T cell magnetic bead separation kit (130-104-454, Miltenyi). The mononuclear cell suspension was cleaned with PBS for centrifugation. Buffer (40 μL) and biotin-labeled cocktail antibody (10 μL) were added to every $10^7$ cells and mixed well for incubation. Then, buffer (30 μL) and biotin magnetic beads (20 μL) were added to $10^7$ cells, mixed, and incubated at 4°C for 10 min. During the incubation, the LS separation column (130-042-401, Miltenyi) was moistened with 3 mL Buffer. After the incubation, the cell suspension was added to the LS separation column to collect the effluent. The supernatant was discarded after the centrifugation of the effluent, and the precipitation was suspended on a 2 mL 10%FBS-1640 complete medium. The magnetic beads were separated and the initial $CD4^+$T cells (Th0 cells) were obtained. The unsorted cells were used as the control. After sorting, the purity was detected using a CD4-labeled antibody (17-0042-82, ebioscience).

*$CD4^+$T Cell Differentiation and 5-Aza Intervention*

The $CD4^+$T cells were divided into the control group, the Th17-induced group, and the 5-Aza group. The control group was only intervened with CD3 (2 μg/mL, 100340, Biolegend)+CD28 (2 μg/mL, 102116, Biolegend) for 4 days. The Th17-induced group was cultured with CD3 (2 μg/mL)+CD28 (2 μg/mL)+IFN-γ (10 μg/mL, 505701, Biolegend)+IL-4 (10 μg/mL, 504102, Biolegend)+TGF-β1 (5 ng/mL, 763102, Biolegend)+IL-6 (20 ng/mL, 216-16-2, peprotech)+IL-23 (50 ng/mL, 589002,

Biolegend) for 4 d. The 5-Aza group was stimulated with 1, 10, and 100 μM 5-Aza (ab142744, Abcam) for half an hour. before the addition of cytokines required for the Th17 differentiation.

*MBD2-silenced CD4$^+$T Cell Construction*

The cells were set into 3 groups. One group was the wild group (Th0 cells that were directly sorted were inoculated without lentivirus and polycoagulant as the blank control), and the other two groups were transfected with empty lentivirus (shRNA-empty) and the lentivirus (shRNA-MBD2) that was the optimal silencing target of MBD2. The detailed steps were as follows: Th0 cells were laid on a 12-well plate at the rate of $2\times10^6$. The amount of virus required for transfection was calculated. The lentiviruses to be transfected (empty lentiviruses and the lentiviruses with the best silent target for MBD2) were respectively taken out and defrozen on ice for use. The optimal silencing target of MBD2 was selected by quantitative reverse transcription PCR (RT-qPCR). The primer sequences of the three targets are shown in Table 1. Lentivirus and polycoagulant (infection-enhancing) were then mixed into two groups of 12-well plates inoculated with initial CD4+T cells to be transfected into wells. Three groups of cells were cultured at 37°C, 5% $CO_2$, and saturated humidity for 12 hrs. The cell supernatant was discarded and the cell precipitation was left for future use. In addition, the transfected cell precipitates could be cultured in a complete medium for 72 hrs and the transfection effect could be observed under a microscope.

*RT-qPCR*

The total RNA was separated by a Trizol reagent (Thermo, USA). The mRNA (CW2569, CWBio, China) kit was applied to synthesize cDNA. The mRNA levels of genes (Table 1) were tested by SYBR (Beijing Kangwei Century, CW2601, China) and $2^{-\Delta\Delta CT}$ methods.

*MBD2-silenced CD4$^+$T Cell Differentiation and 5-Aza Intervention*

After the MBD2 gene was silenced by transfection of CD4$^+$T cells, the cells were set into three groups: the wild group (Th0 cells after direct separation), the shRNA-empty group (Th0 cells transfected with lentivirus), and the shRNA-MBD2 group (Th0 cells transfected with lentivirus which is the best target for MBD2 silencing). The above three groups of cell precipitate were suspended on 10% FBS-1640 complete medium and added into 12-well plates. The groups were divided into the control group, the Th17-induced group, the 5-Aza, and the Th17+5-Aza group, respectively. The method for inducing differentiation of MBD2-silenced CD4$^+$T cells was the same as above. The 5-Aza was added for contrast intervention, noting that all the 5-Aza interventions were performed in half an hour before the addition of cytokines required for the Th17 differentiation.

**Table 1. The primer sequences**

| Gene | Primer sequence (5′→3′) |
|---|---|
| MBD2 target 1 | F: CGGATGAATGAACAACCACGT |
| | R: ACGTGGTTGTTCATTCATCCG |
| MBD2 target 2 | F: GTTTGGCTTAACACATCTCAA |
| | R: TTGAGATGTGTTAAGCCAAAC |
| MBD2 target 3 | F: GAAGAGCGAGTCCAACAAGTA |
| | R: TACTTGTTGGACTCGCTCTTC |
| MBD2 | F: CCGGCAAGATGATGCCTAGT |
| | R: GCAGAGAGGTGCACACAAAC |
| β-actin | F: ACATCCGTAAAGACCTCTATGCC |
| | R: TACTCCTGCTTGCTGATCCAC |

MBD2: methyl-cpg-binding domain protein 2

*Flow Cytometry*

After the above group intervention, the cells were collected by centrifugation and suspended with 10% FBS 1640 (500 µL), and added to a cell stimulation cocktail (1 µL, plus protein transport inhibitors) for 6 hrs at 37°C. Then the cells were washed with 1 mL 0.5% BSA-PBS and centrifuged at 350 g for 5 min. 500 µL Intracellular buffer was used for resuspension, fixation, and centrifugation. 1 mL 1×Permeabilization Buffer was added to resuspend the precipitation, 350 g centrifugation for 5 min. Re-suspension of cell precipitates using 100 µL 1×Permeabilization Buffer and addition of 0.5 µL CD4-APC antibody (17-0042-82, Ebioscience), and 0.7 µL IL-17A-PE antibody (12-7177-81, ebioscience) for incubation, and set a dye-free tube and a single dye tube. The cells were suspended with 150 µL 0.5% BSA-PBS and tested by the flow cytometry (A00-1-1102, Beckman).

*ELISA*

The supernatant of cell culture in each treatment group was centrifuged for 15 min at 2-8°C 1000 g, and the supernatant could be immediately detected. IL-17 (KE10020, Proteintech) kits, as well as ELISA reader (MB-530, huisong) were used to analyze the IL-17 levels in the cell supernatant.

*DNA methylation levels*

The Methylated DNA Quantification Kit (ab117128, Colorimetric) was used to quantify overall DNA methylation by specifically measuring 5-methylcytosine (5-mC) levels in a microplate-based form. The specific steps were as follows: extracting DNA from each group of cells and binding the DNA into the detection well. Then, the test well was washed, the captured antibody, the test antibody, and the enhancer solution were added respectively. Finally, the developer solution was added, and measured the absorbance OD value. The content of the 5-mC was calculated according to the formula in the kit instructions.

*Data Analysis*

The statistical analysis of data was done by the Graphpad Prism8.0 statistical software in this study. The test was done for the normality and homogeneity of variance, and the test for normal distribution and homogeneity of variance. The comparison between the two groups was conducted by unpaired T-test. One-way ANOVA was used for the comparison of the three groups and the ones above. Tukey's was used for the post-facto test. $P<0.05$ represented a significant difference.

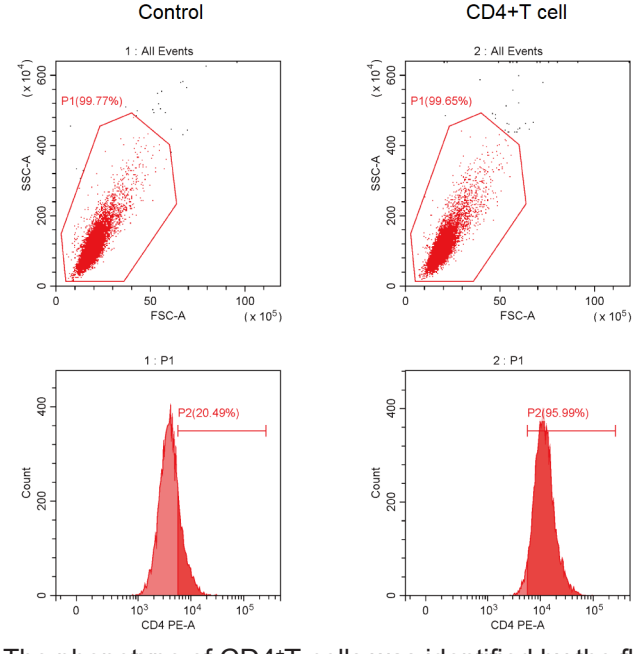


**Figure 1.** The phenotype of CD4⁺T-cells was identified by the flow cytometry.

## RESULTS

*Purity Identification and Detection of CD4⁺T cells*

We first isolated mouse splenic mononuclear cells and obtained the CD4⁺T cells by magnetic bead sorting. The purity of the CD4⁺T cells reached 95.99% after magnetic beads sorting, proving that the cell purity was good and could be used for subsequent experiments (Figure 1). The results showed that we successfully obtained high-purity primary splenic CD4⁺T cells from mice.

*5-Aza Interfered with the Th17 Cell Differentiation*

We further induced the Th17 cell differentiation from mouse spleen-derived CD4⁺T cells and intervened with DNA methylation inhibitors (5-Aza). Compared with the normal group, the proportion of the Th17 cell increased in the Th17-induced group. Moreover, the 5-Aza treatment inhibited the Th17 cell differentiation (Figures 2A, B). We also found the dose-dependent inhibition of the IL-17 levels by the 5-Aza (Figure 2C). The results showed that the 5-Aza interfered with the Th17 differentiation of spleen-derived CD4⁺T cells. And we selected 10 μM 5-Aza for subsequent experiments to explore the mechanism of methylation in CD4⁺T cell differentiation.

*Screening of MBD2 Optimal Silencing Target and Observation of CD4⁺T Cell*

We further screened the optimal silencing target of the MBD2 gene. Compared with the wild group, the expression of the MBD2 gene in the shRNA-empty group had no significant change, and the silencing effect of the target 3 gene was the best (Figure 3A). MBD2-silenced CD4⁺T cells were constructed, and the microscopic observation showed that the

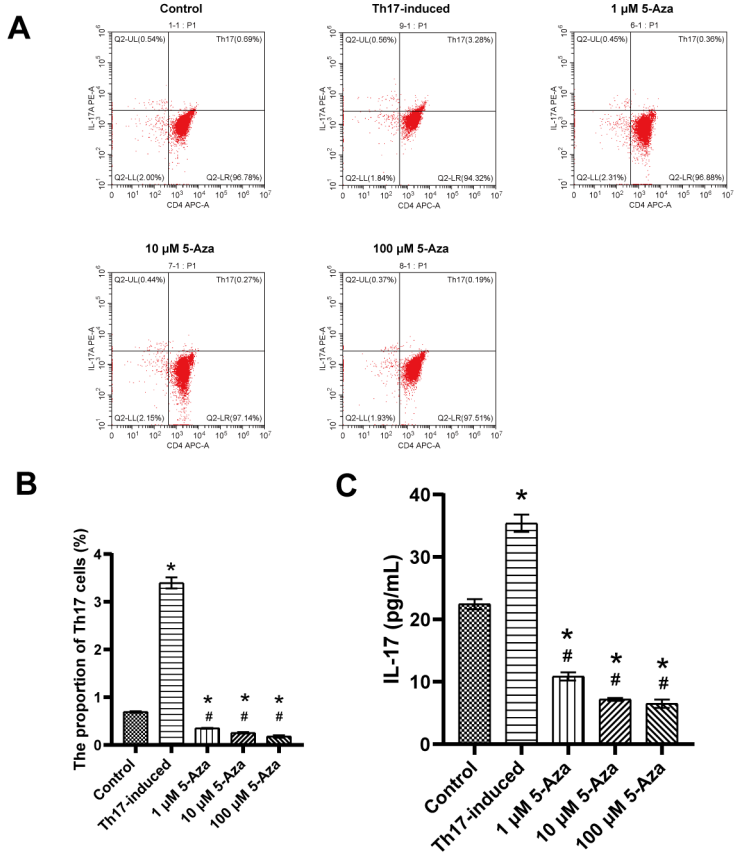


**Figure 2.** 5-Aza affected the differentiation of splenic CD4⁺T cells. (A, B) The Th17 cell ratio was analyzed by the flow cytometry. (C) The IL-17 levels were analyzed by ELISA. *Compared with the normal group, P<0.05; #Compared with the Th17-induced group, P<0.05.

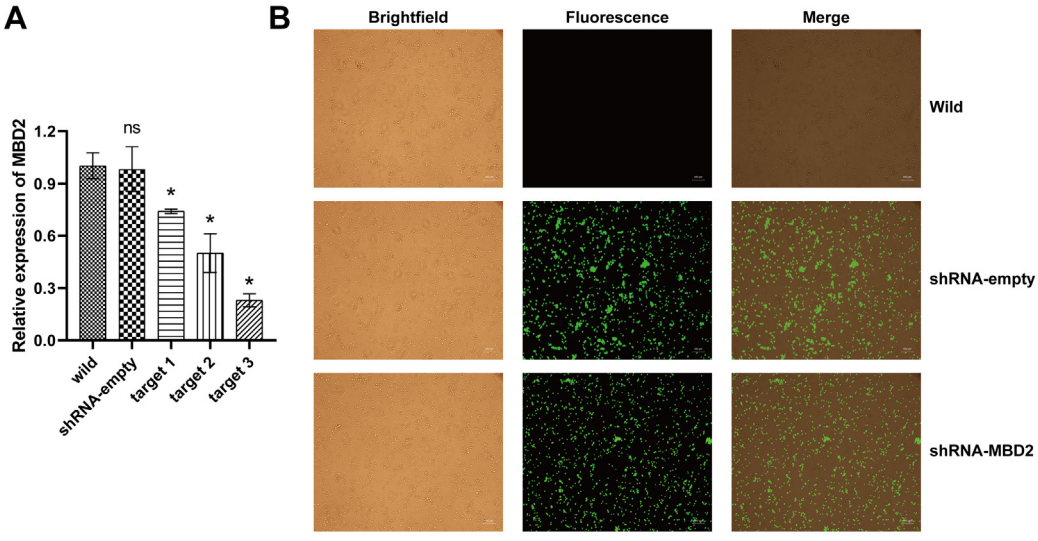


**Figure 3.** MBD2 optimal silencing target screening and cell construction. (A) Screening of the most suitable silencing target for PCR detection of MBD2. (B) Effect of $CD4^+$T cells transfected with MBD2 by lentivirus. *Compared with the wild or the shRNA-empty group, P<0.05. MBD2: methyl-cpg-binding domain protein 2

construction of MBD2-silenced lentivirus transfected $CD4^+$T cells was successful (Figure 3B). The results showed that we successfully constructed the sh-MBD2 $CD4^+$T cells.

### *MBD2 Affected the Differentiation of Th17 by DNA Methylation*

We constructed the $CD4^+$T cells that silenced MBD2 and showed that MBD2 silencing inhibited the Th17 differentiation

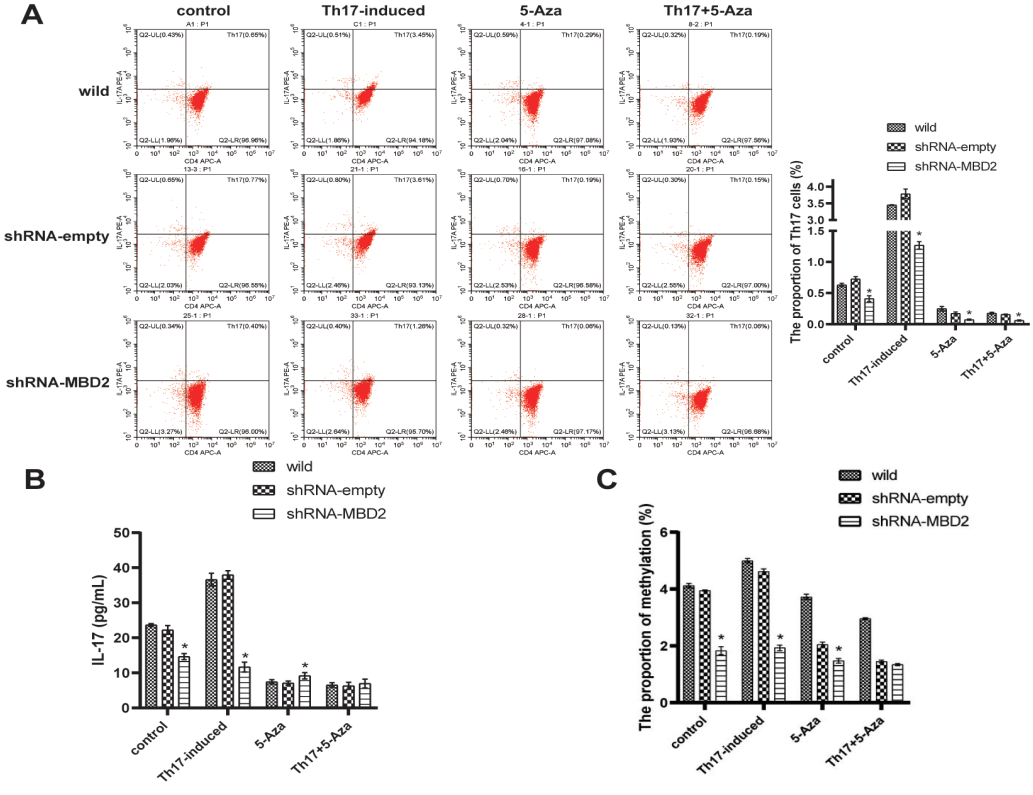


**Figure 4.** MBD2-mediated Th17 cell differentiation by DNA methylation. (A) Th17 cell proportion was detected by the flow cytometry. (B) The levels of IL-17 in the cell supernatant were analyzed by ELISA. (C) The 5-mC level was detected in the cell supernatant. *Compared with the shRNA-empty group, P<0.05. MBD2: methyl-cpg-binding domain protein 2

of CD4+T cells and reduced the IL-17 level in the cell supernatant under Th17 induction and 5-Aza intervention (Figures 4A, B). The DNA methylation showed that MBD2 decreased the level of 5-mC after the Th17 induction and 5-Aza intervention (Figure 4C). MBD2 affected the DNA methylation on the Th17 cell differentiation.

*OVA Promoted IL-17/Th17 Cell Differentiation*

Then we treated splenogenicmouse mononuclear cells of mice with 50~200 μg/mL OVA, respectively. The flow cytometry and magnetic beads sorting analysis showed that the proportion of Th17 cells proportion in the CD4+T cells increased with the dose of the OVA, among which the proportion of theTh17 cells increased significantly in a dose-dependent manner (Figures 5A, B). IL-17 level also increased in the OVA treatment group (Figure 5C). OVA stimulated the differentiation of the Th17 cells.

*MBD2 Silencing Affected the Regulatory Effect of OVA on Th17 Cell Differentiation.*

To further analyze the mechanism of the OVA-induced the CD4+T cell differentiation, we transfected CD4+T cells with MBD2 optimal silencing target lentivirus and stimulated them with 200 μg/mL OVA to observe the CD4+T cells differentiation. The Th17 cell ratio in the shRNA-MBD2 group significantly reduced in contrast with the shRNA-empty group (Figures 6A, B). In contrast with the wild group, the IL-17 level in the shRNA-empty group did not change significantly (Figure 6C). The levels of IL-17 decreased in the shRNA-MBD2 group in contrast with the shRNA-empty group (Figure 6C). MBD2 silencing inhibits the Th17 cell differentiation induced by the OVA.

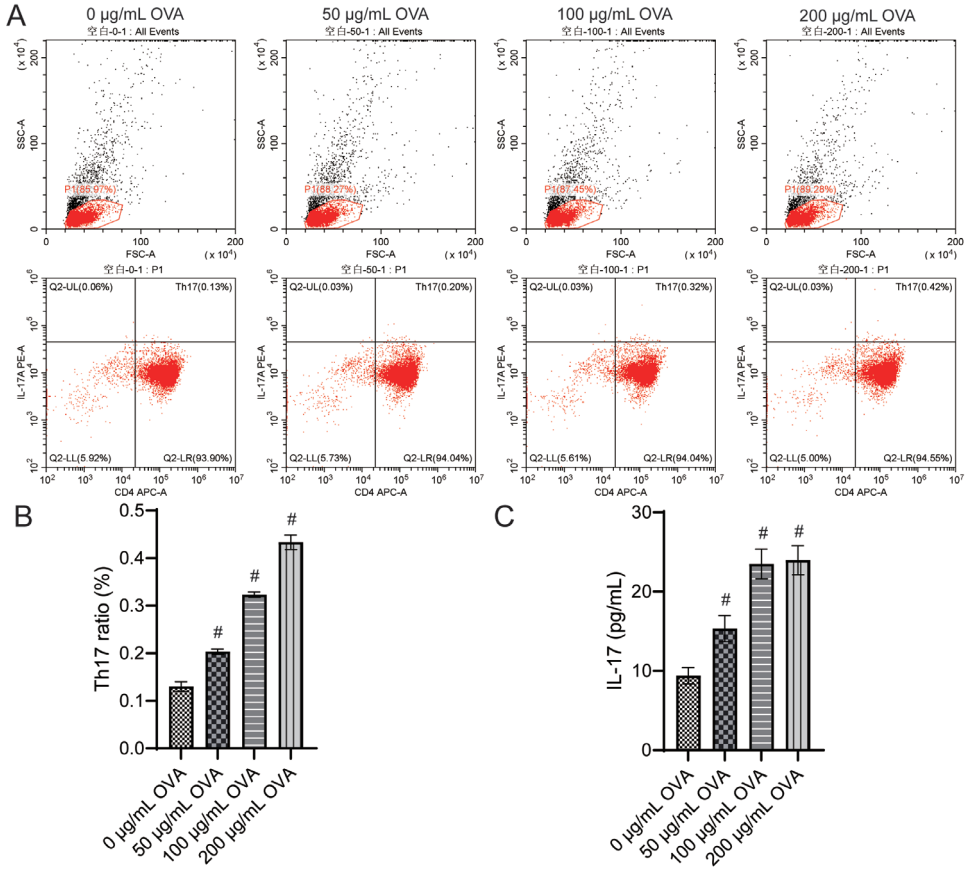


**Figure 5.** The OVA-promoted Th17 cell differentiation. (A, B) The flow cytometry analysis of the proportion of Th17 cells under the different concentrations of the OVA. (C) ELISA was used to analyze the IL-17 level. # indicated P<0.05 vs the 0 μg/mL OVA group. OVA: Ovalbumin

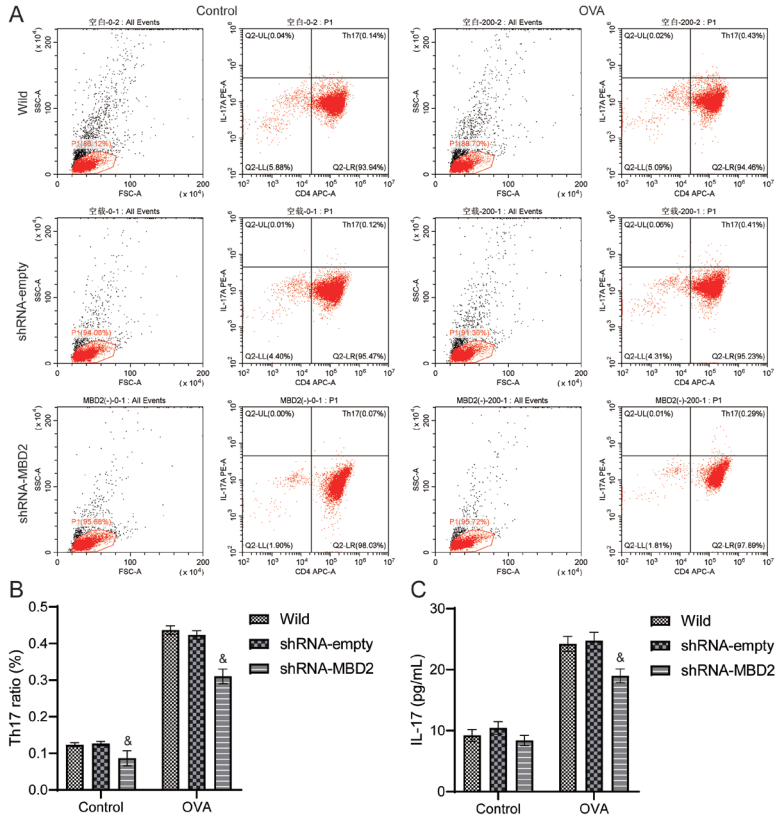


**Figure 6.** MBD2 silencing affected the regulatory effect of the OVA on Th17 cell differentiation. (A, B) The flow cytometry analysis of the Th17 cell proportion. (C) The level of IL-17 was analyzed by ELISA. & indicated P<0.05 vs the shRNA-empty group. OVA: Ovalbumin; MBD2: methyl-cpg-binding domain protein 2

## DISCUSSION

Asthma was a heterogeneous disease [21]. Abnormal activation of T cells was the driving link of the pathophysiological changes of asthma [21]. Asthma included heterogeneous clinical subtypes driven by different pathophysiological mechanisms [22]. The characteristic of helper CD4$^+$T cells could be used to characterize the regulation of inflammatory environment in patients with acute exacerbation of asthma and stability [22]. Early life diagnosis, sensitization, intratasthmatic types, disease surveillance, and treatment progression were key motivations for exploring biomarkers for allergic asthma, and multiple biomarkers were provided for potential intratasthmatic types associated with T cell phenotypes such as Th1, Th2, Th9, Th17, and Tregs and their major cytokines [23]. Our study found that the OVA-stimulated allergic asthma was accompanied by an increase in the Th17 cells and IL-17 levels, suggesting that changes in the Th17 cell phenotypes are associated with the development of allergic asthma.

Cytokine promoted the establishment of effector T-helper lineages during the activation of naive CD4$^+$T-cells *in vitro* and can be used as a first step in evaluating the development of promoting or inhibiting certain CD4$^+$T-helper subsets [24]. The DNA methyltransferase inhibitor 5-Aza increases the stability and feature of regulatory T cells and may be an effective means of controlling virus-induced inflammatory lesions [25]. The 5-Aza treatment prevented experimental autoimmune encephalomyelitis development and inhibits central nervous system inflammation by increasing Treg cell count and inhibiting peripheral effector cells [26]. In our study, the 5-Aza treatment inhibited the Th17 (IL-17) induced differentiation of CD4$^+$T cells.

DNA methylation was gained upon the act of the Th17 cells differentiation in $CD4^+$T cells, but the mechanism is still blank.

Exposure to PM2.5 enhanced the expression of glutamate-oxaloacetate transaminase 1 (Got1) through the accumulationofAhRsand2-hydroxyglutaric acid, thereby inhibiting the activity of 10-11 translocated methylcytosine-dioxygenase 2, resulting in hypermethylation at the P3 site of the forkhead box and impaired differentiation of Treg cells, exacerbating asthma [27]. MBD2 regulated a range of genes expression related to optimal DC function [28]. In naive cells, the DNA was mainly methylated [29]. Our study showed that the MBD2 silencing inhibited the Th17 differentiation of $CD4^+$T cells and reduced IL-17 and 5-mC levels in the cell supernatants under Th17 induction and the 5-Aza intervention. The loss of the MBD2 leads to a deficiency in reading information encoded by the DNA methylation conversion, which disrupts t-BET/Hlx homeostasis and inhibits the Th17 differentiation [30]. Therefore, our study demonstrated that the MBD2 had a hand in the Th17 cells differentiation of $CD4^+$T cells by regulating epigenetic modification of the DNA methylation, which might provide us with a new method for the treatment of autoimmune diseases or asthma.

Clinical studies have shown that asthma was related to a higher frequency of double-positive Th2/Th17 cells in bronchoalveolar lavage fluid, and the disease features greater airway obstruction and high reactivity [31, 32]. Elevated levels of IL-17A in bronchoalveolar lavage fluid, and flow cytometry showed an increased percentage of the Th17 cells [33]. In our study, the OVA treatment stimulated $CD4^+$T cells differentiation in the Th17 cells and promoted the secretion of IL-17. In the OVA-induced asthmatic mice when exposed to LPS, neutrophil-dominated airway inflammation, increased IL-17A levels in the Th17 cells in airway hyperresponsiveness, bronchoalveolar lavage fluid, spleen, and hilar lymph nodes were observed [34]. The above studies demonstrated that the OVA-stimulated allergic asthma was accompanied by an increase in the Th17 cells and IL-17 levels, consistent with our results. Epigenetic studies have demonstrated that the MBD2 mediated immune cell responses during inflammation [35]. MBD2 played a key role in promoting treg-specific demethylation (TSDR) demethylation, Foxp3 expression, and Treg inhibitory function [36]. Our study also found that MBD2 silencing during the OVA-induced $CD4^+$T cells *in vitro* significantly downregulated the ratio of Th17 cell and IL-17 levels. These findings suggested that MBD2 might play an action in the differentiation of $CD4^+$T cell subtypes in asthma. This study may provide us with a new method for the stereotyping and treatment of asthma.

## ACKNOWLEDGMENTS

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## ETHICS

This work was approved by the ethics committee of the Affiliated Hospital of Guilin Medical University (NO.201703190). The maintenance and care of experimental animals comply with National Institute of Health guidelines for the humane use of laboratory animals.

**Conflict of Interest:** None declared.

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