BIOCELL
2023 47(11): 2495-2502

Tech Science Press

MBD2 promotes Th2 differentiation in ovalbumin-induced CD4$^+$ T cells

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Key words: 5-Aza, MBD2, CD4$^+$ T cells, Th2 cells, Ovalbumin

Abstract: Introduction: Allergen-specific CD4$^+$ T cells play a central role in autoimmune disorders, allergies and asthma, with Th2-type immunity being the typical functional response of CD4$^+$ T cells. This study aimed to investigate the role of MBD2 in regulating Th2 cell differentiation. **Methods:** Splenic mononuclear cells were extracted from C57BL/6 mice, and CD4$^+$ T cells were isolated using magnetic beads and confirmed through flow cytometry. Lentivirus was employed to construct MBD2-silenced CD4$^+$ T cells. *In vitro* experiments were performed to treat splenogenic mononuclear cells and CD4$^+$ T cells with Ovalbumin (OVA), and Th2 cell ratios and IL-4 levels were assessed using flow cytometry and ELISA. **Results:** The purity of the isolated CD4$^+$ T cells was 95.73%, confirming successful isolation of primary CD4$^+$ T cells. Compared to the control group, the Th2 cell ratio exhibited an increase in the Th2-induced group. Treatment with 5-Aza (concentrations, 1–100 μM) promoted Th2 cell differentiation and increased IL-4 levels. Notably, when combined with Th2 induction and 10 μM 5-Aza treatment, silencing MBD2 further amplified Th2 cell ratios and elevated IL-4 levels in cell supernatants. Furthermore, OVA (concentration, 200 μg/mL) induced the differentiation of CD4$^+$ T cells into Th2 cells and increased IL-4 secretion. Interestingly, silencing MBD2 significantly increased the Th2 cell ratio and IL-4 levels in OVA-treated CD4$^+$ T cells. **Conclusion:** In summary, OVA promoted CD4$^+$ T cell differentiation into Th2 cells and enhanced IL-4 levels. MBD2 was identified as a mediator of Th2 cell differentiation in splenic-derived CD4$^+$ T cells, influenced by OVA or 5-Aza treatment.

Introduction

Upon activation, naïve CD4$^+$ T cells differentiate into distinct T-helper (Th) subsets, each producing a unique subset of cytokines that play pivotal roles in the immune response against various infections and are also implicated in autoimmune conditions, allergies and asthma (Zhu, 2018). CD4$^+$ T helper cells are essential components of adaptive immune responses to pathogens and are critical for maintaining host immune homeostasis (Yang *et al.*, 2020). Cytokines generated by antigen-presenting cells and other innate immune cells can influence the effector programs of CD4$^+$ T cells, including Th1, Th2 and Th17 cells (Ruterbusch *et al.*, 2020). In asthma, the maturation and function of CD4$^+$ T cells are characterized by Th2/Th17 type immunity (Pacholewska *et al.*, 2017). Therefore, elucidating the mechanism of Th2 cell differentiation in CD4$^+$ T cells could provide significant insight for improving the treatment of autoimmune disorders, allergies and asthma.

Early-life malnutrition triggers hypomethylation of the Th2 cytokine site in CD4$^+$ T cells through the mechanistic target, resulting in cell activation, proliferation, Th2 skew, and increased susceptibility to experimental asthma (Chen *et al.*, 2019). Research has indicated that low concentrations of MBD2 may enhance IL-4 activity (Decramer *et al.*, 2008). Th2 cells are known for producing cytokines such as IL-4, IL-5 and IL-13, among which IL-4's capacity to drive Th2 cell development is pivotal in allergic asthma (Zeng *et al.*, 2018). Notably, high exposure to PM2.5 in the month preceding a visit is independently linked to methylation of the IL-4 CpG24 locus (Prunicki *et al.*, 2021). In addition, it has been shown that reduced DNA methylation in the IL-4 promoter region can intensify Th2-based immune response by naïve HFD CD4$^+$ T cells (Lin *et al.*, 2022). Therefore, the

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Received: 06 June 2023; Accepted: 20 September 2023;
Published: 27 November 2023

Doi: 10.32604/biocell.2023.042617

modulation of Th2 differentiation holds promise for exploring innovative approaches in the treatment of autoimmune disorders, allergies and asthma.

In allergic asthma, specific the airway and participate in both innate and adaptive immune responses within the airway (Ling and Luster, 2016). Early-life exposure to exogenous substances that bind to aryl hydrocarbon receptors (AhR) has been shown to induce DNA methylation bias and functional alterations in $CD4^+$ T cells (Burke *et al.*, 2021). At the same time, Th2 cells play a role in promoting IgE-mediated sensitization, airway hyperresponsiveness, and eosinophilia (Muehling *et al.*, 2017). However, the precise role of the DNA methylation inhibitor, 5-Aza-2′-deoxycytidine (5-Aza), in the differentiation of Th2 immune cells into $CD4^+$ T cells in respiratory diseases remains unclear.

Methyl-CpG-binding domain protein 2 (MBD2) interprets the information encoded within DNA methylomes by binding to methylated CpG DNA, thereby exerting regulatory control over target gene expression at the transcriptional level (Yue *et al.*, 2022). Notably, in patients with severe asthma, MBD2 exhibits a negative correlation with Th2 cells (Chen *et al.*, 2021). Furthermore, in the absence of MBD2, dendritic cells display a significantly impaired capacity to initiate a Th2 immune response against parasitic worms or allergens (Cook *et al.*, 2015). Thus, we hypothesized that MBD2 might play a role in mediating the differentiation of Th2 cells in asthma, although the specific mechanisms remain unclear. Therefore, in this study, we aimed to investigate the influence of MBD2 on the differentiation of Th2 cells in $CD4^+$ T cells induced by 5-Aza (Brand *et al.*, 2012) or OVA (Lertnimitphun *et al.*, 2021) to increase our understanding on asthma and propose novel insights that could be used to improve treatment outcomes.

Materials and Methods

Cell origin and grouping

Spleen-derived mononuclear cells and $CD4^+$ T cells were extracted from male C57BL/6 mice, as described in our previous research (Jiang *et al.*, 2023), and splenogenic mononuclear cells were categorized into the following groups: Control, 50, 100, and 200 μg/mL OVA (323–339, Invivogen, France).

$CD4^+$ T cells, with or without 200 μg/mL OVA treatment, were divided into wild-type, sh-NC, and sh-MBD2 groups. The construction of MBD2-silenced $CD4^+$ T cells was carried out in accordance with our previous study (Jiang *et al.*, 2023).

Based on these designated groups, Th2-induced differentiation and 5-Aza treatment were performed, and the groups were divided into control, Th2-induced, 5-Aza, and Th2+5-Aza groups. The cells were incubated with 2 μg/mL anti-CD3 (100340, Biolegend, San Diego, California, USA) + 2 μg/mL anti-CD28 (102116, Biolegend, San Diego, California, USA) in control. Th2-induction was incubated with 2 μg/mL anti-CD3 + 2 μg/mL anti-CD28 + 10 ng/mL anti-IL-12 (505307, Biolegend, San Diego, California, USA) + 10 μg/mL IL-4 (214-14-500, peprotech, Cranberry, New Jersey, USA) and treated for 4 days. Moreover, 5-Aza (1, 10 and 100 μM, ab142744, Abcam, Cambridge, UK) were performed half 1 h before the Th2 induction.

Flow cytometry

Cells from each group were suspended and stimulated with a stimulation cocktail, following which the cells were washed and centrifuged with 0.5% BSA-PBS (1 mL). Then, 500 μL of cell fixation buffer was added to fix the cells, and the cell precipitate was resuscitated with 1 × membrane-breaking buffer (1 mL), followed by resuspension. Each cell group was treated and thoroughly mixed with CD4-APC antibody (0.5 μL, 17-0042-82, ebioscience, San Diego, California, USA), CD4-APC antibody (0.5 μL, 17-0042-82, ebioscience, San Diego, California, USA) or IL-4-FITC antibody (2 μL, 53-7041-82, ebioscience, San Diego, California, USA). After incubation, staining tubes and single staining tubes were prepared, and the cells were washed and resuspended in 0.5% BSA-PBS for flow cytometry (A00-1-1102, Beckman, Pasadena, California, USA) detection.

IL-4 level

For IL-4 level analysis, cell culture supernatant samples were centrifuged at 1,000 g at 2°C–8°C for 15 min, and the resulting supernatant was used for detection. The level of IL-4 was analyzed using an IL-4 assay kit (KE10010, Proteintech, Chicago, USA) and a multifunctional enzyme label analyzer (MB-530, HEALES, Shenzhen, China).

Statistical analysis

Data analysis was performed using GraphPad Prism 8.0 software (San Diego, California, USA). Measurement data are presented as mean ± standard deviation. Normality and homogeneity of variance tests were conducted to ensure data met the assumptions of the analysis. For comparisons between two groups, an unpaired *t*-test was used, while one-way analysis of variance (ANOVA) or repeated measures ANOVA was used comparing multiple groups. Tukey's *post-hoc* test was performed when appropriate. $p < 0.05$ was used to determine statistical significance.

Results

Purity identification of mouse $CD4^+$ T cells after positive sorting

Flow cytometry analysis confirmed the purity of $CD4^+$ T cells to be 95.73% (Fig. 1), demonstrating the successful isolation of primary $CD4^+$ T cells derived from mouse spleen, thereby allowing us to proceed with subsequent experiments.

5-Aza induced the differentiation of Th2 cell and IL-4 secretion

Following Th2-induction, we observed an increase in the Th2 cell ratio (Figs. 2A and 2B). Moreover, 5-Aza treatment demonstrated a dose-dependent promotion of Th2 cell differentiation (Figs. 2A and 2B) and enhanced IL-4 secretion (Fig. 2C). Based on these findings, we selected 10 μM 5-Aza for subsequent experiments, demonstrating that 5-Aza can induce Th2 differentiation from $CD4^+$ T cells.

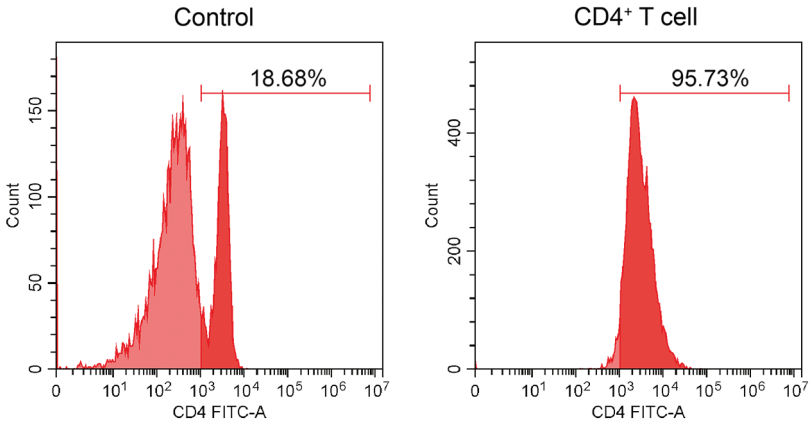

FIGURE 1. Flow cytometry for identifying the purity of $CD4^+$ T cells.

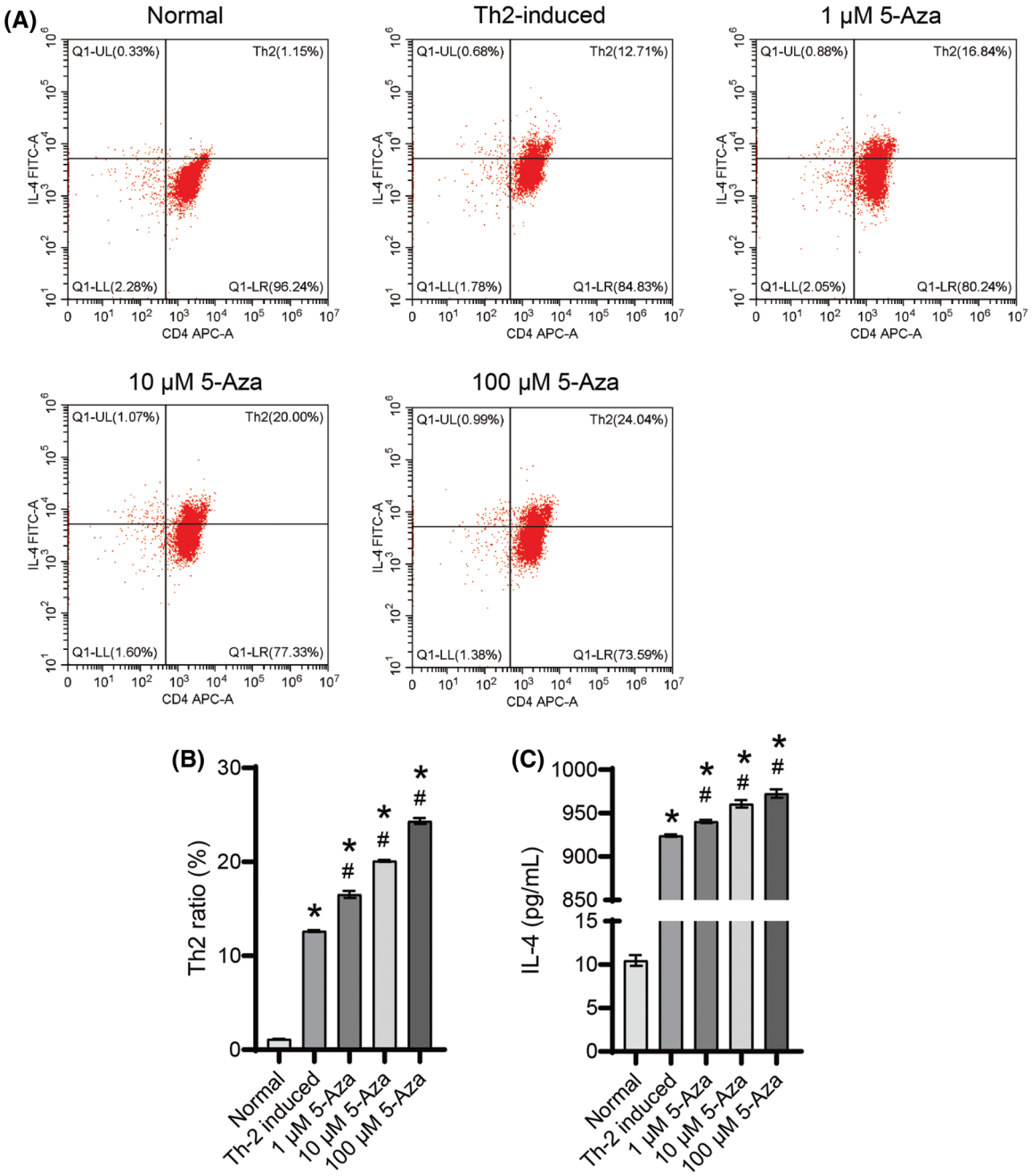

FIGURE 2. 5-Aza induced Th2 differentiation from $CD4^+$ T cells. (A) and (B) Th2 cell ratio. (C) IL-4 level. $^*p < 0.05$ *vs.* normal; $^{\#}p < 0.05$ *vs.* Th2-induced. n = 3.

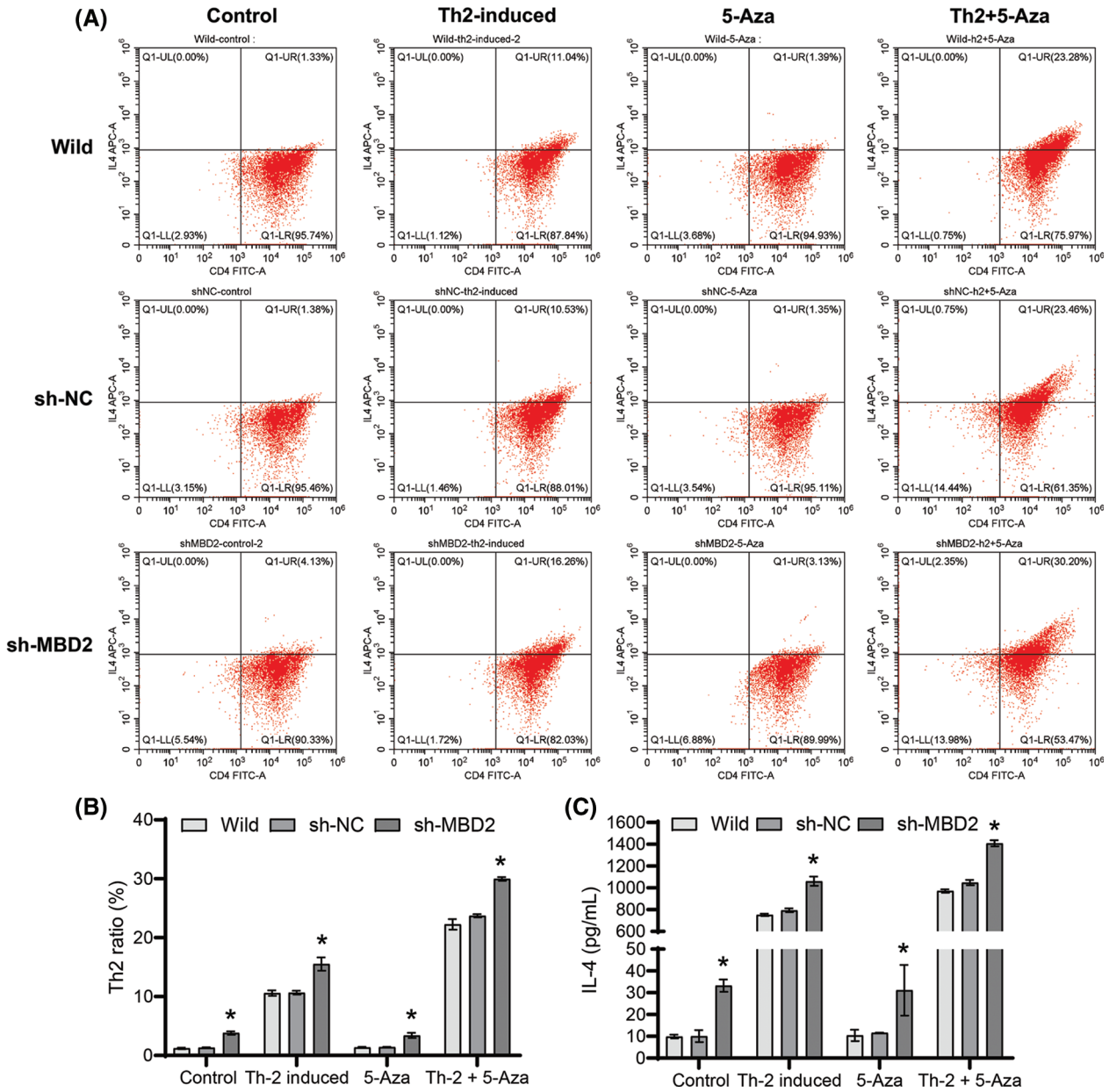

FIGURE 3. MBD2-mediated Th2 cell differentiation. (A) and (B) Th2 cells proportion. (C) IL-4 level. *$p < 0.05$ *vs.* sh-NC in Control, Th2-induced, 5-Aza, or Th2+5-Aza groups. n = 3.

MBD2 affected the differentiation of Th2 cells

Silencing MBD2 promoted Th2 differentiation from $CD4^+$ T cells under the influence of both 10 μM 5-Aza and Th2 induction (Figs. 3A and 3B) and also increased IL-4 levels in the cell supernatant when subjected to 10 μM 5-Aza and Th2 induction (Fig. 3C). These findings indicate that silencing MBD2 could induce Th2 cell differentiation.

OVA promoted Th2 cell differentiation and the secretion of IL-4

The proportion of Th2 cells significantly increased with increasing OVA doses (Figs. 4A and 4B). Furthermore, our analysis of secreted factors from splenogenic mononuclear cells exposed to OVA treatment revealed a substantial increase in IL-4 levels within the OVA treatment group compared to the control group (Fig. 4C). Importantly, the highest observed Th2 cell ratio and IL-4 levels were in the 200 μg/mL OVA group compared to the other groups (Fig. 4C). These findings demonstrate that OVA treatment promotes Th2 cell differentiation and increases IL-4 secretion.

Silencing of MBD2 promoted the role of OVA on Th2 cell differentiation

No significant change was observed in the ratio of Th2 cells and IL-4 levels between the wild-type and sh-NC groups (Figs. 5A–5C), but a significant increase was observed in both the ratio of Th2 cells and the IL-4 levels in the sh-MBD2 group (Figs. 5A–5C), suggesting that silencing MBD2 promotes Th2 cell differentiation and enhances IL-4 levels induced by OVA.

Discussion

The addition of various cytokine combinations to induce initial $CD4^+$ T cell activation *in vitro* is a valuable strategy for assessing the development of specific $CD4^+$ T helper

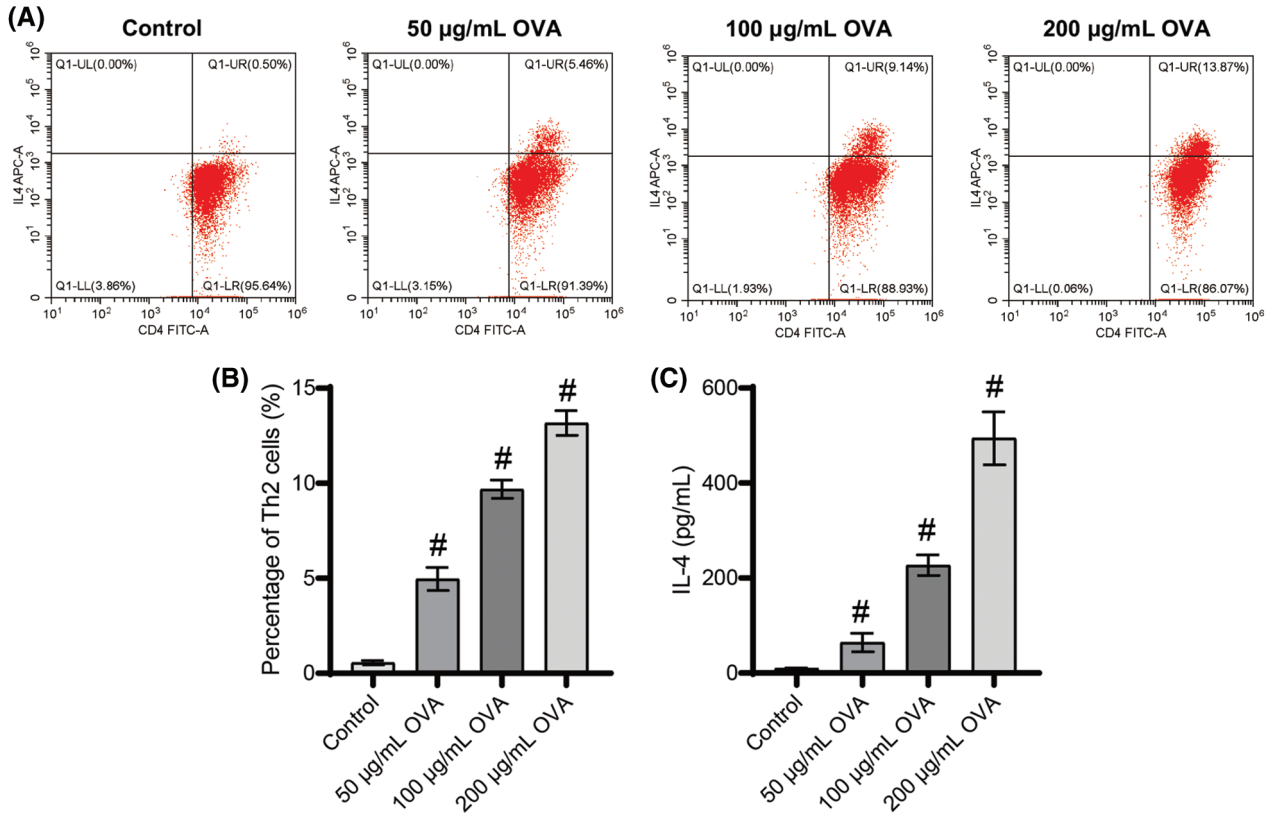

FIGURE 4. Different doses of OVA induced Th2 cell differentiation. (A) and (B) Ratio of Th2 cells. (C) IL-4 level. $^{\#}p < 0.05$ *vs*. Control. n = 3.

subpopulations (Flaherty and Reynolds, 2015). Immature $CD4^+$ T lymphocytes differentiate into highly allergic T helper 2 cells, inducing the expression of interleukin IL-4, inhibiting the expression of interferon IFN-γ, and accompanied by uniform DNA methylation of these gene promoters (Tang *et al.*, 2012). 5-Aza serves as a DNA methyltransferase inhibitor, enhancing the function and stability of regulatory T cells (Varanasi *et al.*, 2017). Although treatment with varying concentrations of 5-Aza (1–100 μM) has been shown to promote Th2 cell differentiation and elevate IL-4 levels and DNA methylation is known to play a role in Th2 cell differentiation, the precise mechanisms in asthma warrant further investigation.

$CD4^+$ T-helper (Th) cells are pivotal for maintaining immune homeostasis and host defense, and their dysregulation contributes to the development of autoimmune diseases critical for the development of various autoimmune diseases (Pawlak *et al.*, 2020). This intricate process involves the activation of signal transducers, transcriptional activators, and master regulatory transcription factors that guide the function of each distinct helper T lymphocyte subtype (Chalmin *et al.*, 2018). Notably, 5-Aza-2'-deoxycytidine-mediated demethylation was shown to enhance the differentiation of naïve T cells into regulatory T cells (Tregs) in chronic HBV infection (Fang *et al.*, 2021). Our findings indicate that silencing MBD2 facilitates Th2 cell differentiation in $CD4^+$ T cells and enhances IL-4 levels in cell supernatants, especially when combined with 10 μM 5-Aza and Th2 induction.

Research has demonstrated that low-dose 5-Aza (1–10 μM) promotes the generation and differentiation of regulatory T (Treg) cells and enhances their immune-suppressive function (Han *et al.*, 2021). Similarly, human $CD4^+$ $CD25^-$ T cells treated with low-dose 5-Aza-2'-deoxycytidine (5 μM) exhibit reduced proliferation without inhibitory effects (Kehrmann *et al.*, 2014). These findings align with our use of a 10 μM 5-Aza dosage in this present study. Further, animal experiments also support the benefits of low-dose 5-Aza in immune regulation. For instance, short-term pretreatment with 5-Aza (0.15 mg/kg) significantly inhibits disease activity in experimental autoimmune encephalomyelitis mice and improves inflammatory responses in the central nervous system (Chan *et al.*, 2014). Low-dose (0.15 mg/kg) 5-Aza significantly inhibits FoxP3 methylation, rebalances the Treg/Th17 ratio, and mitigates liver damage and inflammation in mice with primary biliary cholangitis (Jiang *et al.*, 2021). Taken together, our study highlights that low-dose 5-Aza (10 μM) effectively promotes Th2 cell differentiation and modulates the immune system.

Identifying biomarkers for allergic asthma holds great potential for disease monitoring, early diagnosis, and treatment (Breiteneder *et al.*, 2020). $CD4^+$ T cells expressing chemoreceptor homologous molecules, such as CRTH2, play a pivotal role in determining Th2 cell subsets (Palikhe *et al.*, 2016). These cells are identified as allergen-specific central memory Th2 cells, and an increased presence of circulating $CD4^+$ $CRTH2^+$ T cells has been observed to be a distinct feature of severe asthma (Palikhe *et al.*, 2016; Shrestha Palikhe *et al.*, 2021). OVA-related sensitization and Th2 immune response are crucial in mediating asthma (Lin *et al.*, 2022). Our study demonstrates that a high dose of

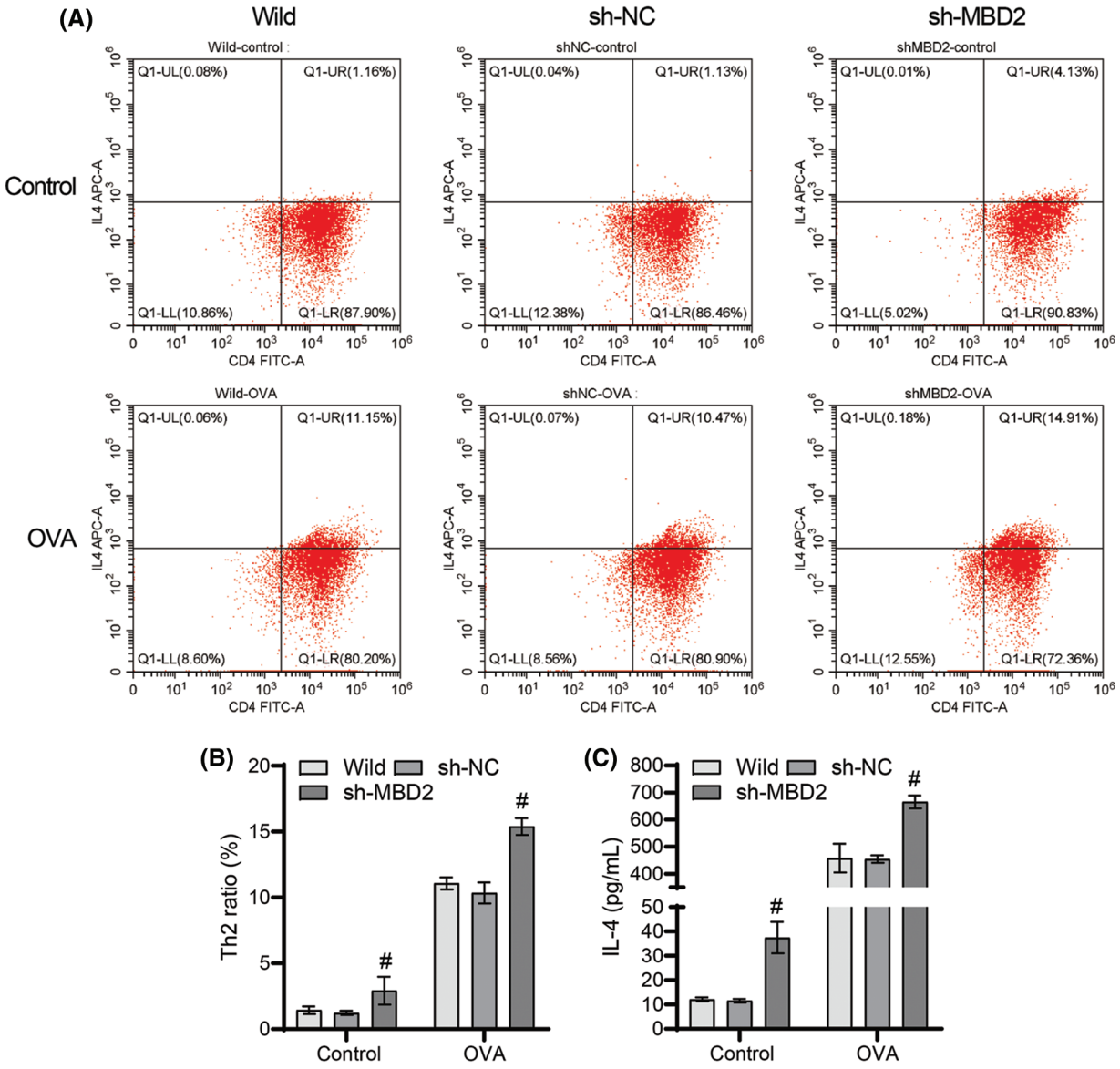

FIGURE 5. Silencing of MBD2 promotes the action of OVA on Th2 cell differentiation. (A) and (B) Th2 cell proportion. (C) IL-4 levels. $^{\#}p < 0.05$ *vs.* sh-NC. n = 3.

OVA induces an increase in Th2 cells and the secretion of the cytokine IL-4, highlighting the significance of OVA in the immune response associated with allergic asthma.

MBD2 expression was found to be elevated in peripheral blood $CD4^+$ T cells from individuals with asthma (Jia *et al.*, 2017). Asthma is characterized by abnormal accumulation, differentiation, or function of memory $CD4^+$ T cells that produce Th2 cells (Seumois *et al.*, 2014). Notably, macrophage-specific knockout of MBD2 has been shown to protect mice from OVA-induced allergic airway inflammation and inhibit M2 procedures (Wu *et al.*, 2022). In our study, MBD2 silencing was found to significantly increase the ratio of Th2 cells and the levels of IL-4 in OVA-induced $CD4^+$ T cells, providing evidence that MBD2 promotes Th2 cell differentiation from $CD4^+$ T cells induced by OVA.

It is important to acknowledge that our research only confirmed the expression of MBD2 in OVA-induced $CD4^+$ T cells and its role in promoting Th2 cell differentiation in cell experiments. However, due to limitations in experimental funding and technology at our institution, we were unable to generate or acquire a sufficient number of MBD2 knockout mice for *in vivo* experiments, underscoring the need for further investigation in the future.

In conclusion, this study shows that OVA can induce Th2 cell differentiation and increase the IL-4 levels in $CD4^+$ T cells and suggests that MBD2 plays a key role in modulating IL-4 levels by mediating the differentiation of Th2 cells in splenic-derived $CD4^+$ T cells in response to OVA or 5-Aza treatment.

Acknowledgement: The authors would like to thank the Affiliated Hospital of Guilin Medical University for their technical assistance.

Funding Statement: This work was supported by grants from the National Natural Science Foundation of China (Nos. 81760009 and 81560007).

Author Contributions: The authors confirm contribution to the paper as follows: Q. Pan: Writing-original draft; Conceptualization. Y. Jiang: Writing-original draft; Methodology. L. Li, X. Du, Q. Han, F. Ling, R. Li, S. Chu, L. Mai, and J. Huang: Data curation; Formal analysis; Visualization. L. Ma: Design; Writing-review & editing; Project administration. All authors reviewed the results and approved the final version of the manuscript.

Availability of Data and Materials: All data generated or analyzed during this study are included in this article.

Ethics Approval: This experiment was approved by the ethics committee of the Affiliated Hospital of Guilin Medical University (No. 201703190). All methods were carried out in accordance with relevant guidelines and regulations.

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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