



Effect of DNA Methylation of T Cells on the Development of Experimental Asthma

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ABSTRACT

It has recently been demonstrated that DNA methylation causes differential secretion of T helper 1 (Th1) and Th2 cytokines by effector T cell polarisation. This study investigates changes in the marker genes of T cell subsets after allergen sensitisation and demethylation intervention as well as the T cells' effects on allergic airway inflammation development. The number of T cell subsets and the expression of cytokines were detected by a well-established model of experimental asthma. The number of T cell subsets was detected by flow cytometry, the protein expression was detected by enzyme-linked immunosorbent assay, and the gene expression was detected by real-time polymerase chain reaction. In addition, *in vitro* experiments with Jurkat cells were used to verify the effect of methylase inhibitor 5-Aza-2'-deoxycytidine on the expression of cytokines and genes in the T cells. The expression of Th2 cells, cytokine proteins and genes after allergen sensitisation revealed a significant increase in CD4⁺ T cells. However, analysis of regulatory T cells revealed the opposite results. The decrease of Th2 cells, cytokine proteins, and genes was reversed by a DNA methyltransferase inhibitor *in vitro* and *in vivo*, which also increased the allergen sensitisation status and decreased the inflammatory response. Epigenetics has an impact on disease occurrence. Epigenetic regulation in T cells contributes to the development of experimental asthma, which can be treated pharmacologically.

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Authors' Contribution

LXY and JXF conceived of the study.

JHL and ZR participated in its design and coordination. GL and YQ helped to draft the manuscript. All authors read and approved the final manuscript.

Key words

Asthma, DNA methylation inhibitor, 5-Azacytidine, forkhead box P3, T-regulatory cells, epigenetic regulation

INTRODUCTION

Over 235 million people worldwide are living with asthma, which is one of the leading non-communicable diseases worldwide (Stanescu *et al.*, 2019). Asthma in China is increasing rapidly due to an aging population as well as changes occurring in environment and lifestyle.

Individuals with asthma often have shortness of breath, wheezing caused by airway constriction, mucus

production and long-term remodelling of the lungs (Walsh *et al.*, 2010). Several kinds of inflammatory cells interact in asthmatic lungs, such as mastocytes, basophils, lymphocytes, dendritic cells and eosinophils (Barnes, 2011). It has been known for several decades that asthma is associated with a strong environmental component (Martinez and Vercelli, 2013).

Imbalance in effector T-cell populations plays an important role in the development of chronic inflammatory conditions, including autoimmunity and allergy. Allergic bronchial asthma is a prototypic disease of dominant TH2 activation, which, apart from genetic predisposition, is influenced by environmental stimuli present or absent during fetal life (Martino and Prescott, 2010). Many aspects of airway inflammation are related to the effects of the TH2 cytokines IL-4, IL-5, and IL-13 (Barnes, 2008) which are not adequately counter regulated by TH1 or regulatory T-cell activities. Adequate regulation of the direction of T cell polarization and cytokine gene

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activation or silencing is essential for effective immune function. However, there are few reports on the specific mechanism of regulating the association between T cells and asthma. *In vitro* polarization experiments with naive T cells pointed out that the underlying processes of differentiation into effector T-cell populations are orchestrated by changes in DNA methylation at promoter regions, resulting in differential secretion of TH1/TH2 signature cytokines (Jones and Chen, 2006; Gilbert *et al.*, 2018). DNA methylation is generally thought to play an essential role in T-cell function and failure to maintain DNA methylation patterns in mature T-cells has been implicated in the development of autoimmune disease.

Methods have recently been developed to infer the proportion of immune cell populations in peripheral blood from DNA methylation data (Houseman *et al.*, 2012). Asthma is a consequence of complex gene–environment interactions, and the goal of treatment focus on good asthma control by minimising symptom burden and lowering the risk of exacerbation (Papi *et al.*, 2018). Until recently, genetic studies could not adequately explain the heritability of and susceptibility to the disease, but recent evidence suggests that epigenetic changes may underlie both of these processes (Durham *et al.*, 2010). Therefore, the value of DNA methylation in asthma has been receiving an increasing amount of attention (Baccarelli *et al.*, 2012; Salam *et al.*, 2012; Sofer *et al.*, 2013; Bergougoux *et al.*, 2015; Brook *et al.*, 2015; Devries and Vercelli, 2017; Potaczek *et al.*, 2017; Larouche *et al.*, 2018; Zhang *et al.*, 2018; Zeng, 2013). The activation and skewing of T cells are affected by epigenetic changes (Martino and Prescott, 2010). Effector T cell population imbalances are important in the development of chronic inflammatory conditions, such as allergic bronchial asthma (Lou *et al.*, 2012). Although DNA methylation signatures in CD4⁺T cells differ significantly between atopic and nonatopic patients, the contribution of this level of regulation to the development of the disease phenotype has not been investigated yet (Lou *et al.*, 2012).

Dysregulation of epigenetic events leads to many diseases, and inhibiting these epigenetic changes is clinically important. There are major classes of epigenetic drugs already in use, such as 5-Azacytidine (5-Aza-C; Vidaza) and decitabine (Dacogen) (DNA methyltransferase inhibitors) (Seelan *et al.*, 2018; White *et al.*, 2006). However, the value of the DNA methylation signature in CD4⁺ T cells for the development of the disease phenotype remains unclear. Analysis of CD4⁺ T cells revealed a significant increase in DNA methylation at the IFN- γ promoter after allergen sensitization/challenge, furthermore, the increase in DNA methylation at the IFN- γ promoter could be reversed with a DNA methyltransferase (DNMT) inhibitor *in vitro* and *in vivo* with beneficial

effects on sensitization status and allergic phenotype.

T lymphocytes are the coordinators of adaptive immunity. Naive T cells can differentiate into Th1, Th2, or Th17 and Treg cells, but how they differentiate depends on environmental co-stimulatory signals. Some studies have found that the primary human CD4⁺ T cells are not easily passaged and cannot be used for subsequent studies. In addition, Jurkat T lymphocytes are reported to have similar functions to primary human CD4⁺T cells. In this study, Jurkat T cells are selected as the model of T lymphocytes. In view of the different polarization directions, we stimulated the Th2-type differentiation of Jurkat T cells to form a T-cell model of Th2-type inflammatory response, intervened the polarized Jurkat T cells with methylase inhibitors, and observed the morphology of Jurkat cells, as well as Th2/Treg expression changes. This study intends to investigate whether the modifications in epigenetic signatures during the process of allergic sensitisation are sufficient to drive Th2 cell polarisation and subsequent phenotype development.

MATERIALS AND METHODS

Experimental animals

Adult male sprague dawley (SD) rats (180–220 g) were obtained from the Animal Experiment Center of Xinjiang Medical University in Xinjiang, China. The SD rats were bred at this facility under specific pathogen-free (SCXK2011-0004) conditions for animal experiments. The animals were randomly divided into four groups, with 10 animals in each group, as follows the normal group, modelgroup, 5-Aza-0.5 group and 5-Aza-5 group.

Sensitisation and aerosol challenge

Methods reported in the literature were used to make the disease models (Mincham *et al.*, 2019). The animals were sensitised to ovalbumin (OVA) and given two intraperitoneal injections consisting of 10% OVA (grade VI; Sigma-Aldrich, Germany) emulsified in 10% Al(OH)₃ (Qilu Animal Health Products Co., Ltd, China) on days, 7 and 14. The control group given two intraperitoneal injections consisting of phosphate-buffered saline (PBS) emulsified in 10% Al(OH)₃. From day 15 to day 29, the animals were exposed to aerosolised OVA (3% wt/vol diluted in PBS) for 30 min. A schematic illustration of the experimental schedule for sensitisation, aerosol exposure and drug administration are shown in Figure 1.

5-Aza-2'-deoxycytidine treatment

The animals intraperitoneally received 5-Aza-2'-deoxycytidine (5-Aza; Sigma-Aldrich, Germany) 0.2 mg/kg body weight dissolved in 100 mL of PBS. The animals in the control group received PBS. This treatment occurred

every day after the OVA challenge for a total treatment duration of seven days.

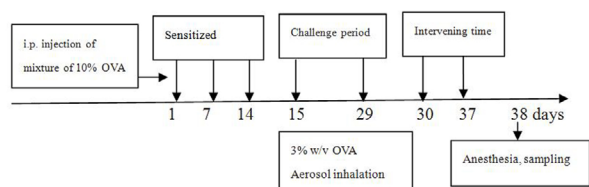


Fig. 1. A schematic illustration of the experimental schedule for sensitization and aerosol challenge and drug administration.

Isolation of peripheral blood lymphocytes and differential CD4⁺ T cell counts

Seven days after the 5-Aza treatment was completed, the peripheral blood of the rats was disaggregated. The erythrocytes were haemolyzed and washed, and peripheral blood mononuclear cells (2×10^6 cells) were resuspended and incubated. Next, the CD4⁺ T cells were stained with different antibodies (BD Biosciences, San Jose, CA). After the staining, each tube added 500 μ l of PBS lotion. Flow cytometry (FACS Calibur; Becton-Dickenson, San Jose, CA, USA) was used to detect the different cells. The T helper 1 (Th1), Th2 and Th17 and regulatory T (Treg) cells (Elabscience Biotechnology Co. Ltd, China) were used for the flow cytometry.

Lung histology

The left lungs of the rats were fixed with 4% paraformaldehyde through the trachea, removed and subsequently stored in 4% paraformaldehyde. Then, they were embedded in paraffin, and 3-mm sections were stained with haematoxylin/eosin. The right lungs of the rats were cryopreserved at -80°C for real-time polymerase chain reaction (RT-PCR).

Measurement of cytokine concentrations

The cytokine concentrations of IL-17, IFN- γ , IL-4 and Foxp3 in the plasma were measured by an ELISA kit (Elabscience Biotechnology Co. Ltd, China) according to the manufacturer's instructions.

Quantitative PCR

After the total RNA of the lungs was extracted (TRIzol, Invitrogen, US), it was reverse transcribed (Omniscript RT Kit; Qiagen, Hilden, Germany) using oligo (dT) primers. The QuantiTectSYBR Green PCR Kits (Qiagen) were used for the reverse transcription PCR (RT-PCR) of IL-17, IFN- γ , IL-4, Foxp3 and β -Actin (Actb; housekeeping control). The RT-PCR data were analysed according to the previously described $2^{-\Delta\Delta C_t}$ method and

normalised to Actb gene transcription levels.

RT-PCR primer sequence

Actb: (F) 5'-GGAGATTACTGCCCTGGCTCCTA-3'
(R) 5'-GACTCATCGTACTCCTGCTTGCTG-3'
IFN- γ : (F) 5'-TGTCATCGAATCGCACCTGAT-3'
(R) 5'-GCTTTGTGCTGGATCTGTGG-3'
IL-4: (F) 5'-TCCACGGATGTAACGACAGC-3'
(R) 5'-TGGTGTTCTTGTGTCGTA-3'
IL-17: (F) 5'-TCCAGCAAGAGATCCTGGTC-3'
(R) 5'-TGGCGGACAATAGAGGAAAC-3'
Foxp3: (F) 5'-ACTGCCAAGCAGATCACCTC-3'
(R) 5'-CTGCATAGCTCCAGCTTCT-3'

Cell preparation

For the *in vitro* experiments, human leukemic Jurkat (clone E6-1) T cells were provided by the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. The details of the Jurkat cell culture are shown on the official website (<https://www.cellbank.org.cn/search-detail.php?id=197>). Cells in the exponential growth phase were treated by 5-Aza at concentrations of 0, 2, 5 and 10.0 $\mu\text{mol/l}$ for 24, 48 and 72 h. Untreated cells were used as the control. The Jurkat cells were cultured in an RPMI-1640 medium with 10% foetal bovine serum (37°C , 5% CO_2).

RNA extraction and RT-PCR for Jurkat cells

The extraction of total RNA was conducted according to the manufacturer's instructions. Here, DNase I (Thermo Fisher Scientific Inc.) was used to remove genomic DNA contamination from the total RNA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal standard. The fold differences were determined when the IL-4, IL-13, STAT6 and Foxp3 expressions were compared with untreated cells.

RT-PCR primer sequence,

GAPDH: (F) 5'-GACCTG ACCTGCCG CTA-3'
(R) 5'-AGGAG GGGTGTGCTGT-3'
IL-4: (F) 5'-AGCAG TCC ACAGGC ACAAG-3'
(R) 5'-CTG GGTGGCTTCCTTCACAG-3'
IL-13: (F) 5'-CATGGCGCTTTTGTGACCA-3'
(R) 5'-AGCTGTGAGGTTGATGCTCC-3'
STAT6: (F) 5'-ATTCACCACGCGGGATTCT-3'
(R) 5'-ACTCAGGGACCACTTGCTTG-3'
Foxp3: (F) 5'-ACTGCCAAGCAGATCACCTC-3'
(R) 5'-CTGCATAGCTCCAGCTTCT-3'

Measurement of proliferation for Jurkat cells

The test groups were divided into five subgroups according to different concentrations: 1 $\mu\text{mol/l}$, 2 $\mu\text{mol/l}$, 5 $\mu\text{mol/l}$ and 10 $\mu\text{mol/l}$. The cell morphology was observed by microscopy. The cells were counted after being cultured for 24, 48 and 72 h, and a growth curve was drawn.

Table I. Rat weights and lung weights.

	Control (n=9)	Sham model (n=10)	5-Aza		F	P
			0.5mg (n=10)	5mg (n=10)		
Rat weight (g)	408.33±31.06	405.20±29.96	397.30±31.36	402.30±31.17	0.769	p=0.577
Lung weight (g)	2.41±0.23	2.73±0.21	2.66±0.31	2.34±0.27	3.239	0.013

The influence of different concentrations of 5-Aza on the T cell proliferation was investigated using Cell Counting Kit-8 (BestBio, China).

Measurement of cytokine concentrations for Jurkat cells

The protein expression level of IL-4, IL-13, STAT6 and Foxp3 were detected by an ELISA kit at concentrations of 0 µmol/l, 5 µmol/l and 10.0 µmol/l.

Statistical analysis

The SPSS 20.0 software (IBM Corporation, Endicott, NY, USA) was used in this study. The data were expressed as the mean ± standard deviation. Comparisons were made by a one-way ANOVA or unpaired Student's t-test. A P-value <0.05 was considered statistically significant.

RESULTS

Effect of modeling and drug treatment on body weight and rat lung

The weights of the rats in the different groups were determined before sacrifice. The between-group comparisons were comparable ($P > 0.05$) (Table I). However, there were significant differences between the weights of the lungs in the different groups ($P < 0.05$) (Table I). According to the anatomical map (Fig. 2), it can be seen that the normal group's lung tissue appeared clean and elastic. The lung tissue of the model group and other groups that were sensitised and challenged with OVA aerosols was covered with congestion points and looked eroded.

5-Aza could alleviate the infiltration of inflammatory cells in lung tissue

The lung tissues of rats in each group were stained with HE and analyzed by microscope to observe the pathological changes of lung tissue. In the normal control group (Fig. 3A), the bronchial and lung tissues were normal. The intact alveolar septum of all levels of bronchial epithelium was normal. The tissue cells are arranged neatly. There was less infiltration of inflammatory cells. The bronchial mucosal epithelial lumen was narrowed in the model group. The basal layer was thickened and the wall smooth muscle was hypertrophic. Airway edema in submucosal tissue. A large number of eosinophils, lymphocytes and

monocytes infiltrated (Fig. 3B, E). After 5-Aza treatment, airway submucosal edema was improved, and there was still a small amount of inflammatory cell infiltration, but less than that in the model group (Fig. 3C, D).

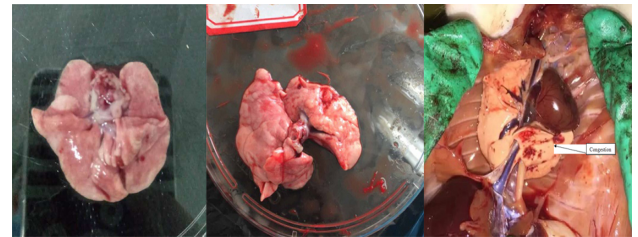


Fig. 2. Pulmonary congestion was observed in lung tissue from the constructed asthmatic model rats. Normal lung tissue appears clean and elastic, but the lung tissue of the model group and other groups that were with poor elasticity. (A) Normal lung tissue; (B) Model lung tissue; (C) In vivo lung (5-Aza groups).

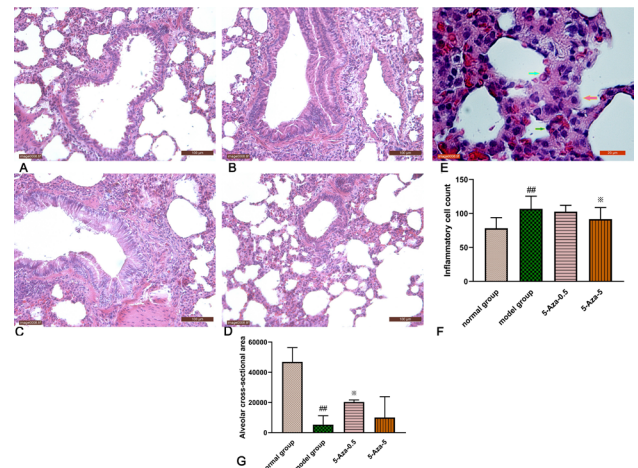


Fig. 3. Lung inflammation in lungs in different groups ($\times 10$). Compared with the model group, the 5-aza group had less lumen stenosis, mucosal epithelial development and bronchial secretion storage. (A), Normal group; (B), Model group; (C), 5-Aza-0.5 group; (D), 5-Aza-5 group. (E), Graph of inflammatory cell infiltration in HPF of model group. Lymphocytes (green arrows), eosinophils (blue arrows), and monocytes (pink arrows). (F), Statistics of the number of inflammatory cells in HPF. (G), Semiquantitative analysis of alveolar cross-sectional area.

We quantified the level of inflammation by counting inflammatory cells in HPFs in each group (Fig. 3F). The results were consistent with the results of HE. The number of inflammatory cells in the model group was significantly higher than that in the normal group ($P < 0.01$). The semi-quantitative analysis of the alveolar cross-sectional area was performed based on the optical density values (IOD) collected by the software Image pro plus, and the results are shown in Figure 3G. The cross-sectional area of alveoli in the model group was smaller than that in the normal group ($P < 0.01$). The alveolar cross-sectional area of the 5-Aza-0.5 group was larger than that of the model group ($P < 0.05$).

5-Aza decreased the cell counts of Th2 and Th17 cells and increased the cell counts of Treg cells

As shown in Figure 4A-D, there was no significant difference in the number of Th1 cells in peripheral blood of each group ($P > 0.05$). The cell numbers of Th2, Th17 and Treg cells were significantly different among the groups ($P < 0.05$). The percentage of Th2, Th17 cells in the model group was significantly higher than that in the normal group ($P < 0.05$). The percentage of Th2, Th17 cells in the 5-Aza-0.5 treatment group was lower than that in the model group ($P < 0.05$). The percentage of Treg cells in the model group was lower than that in the normal group ($P < 0.05$). The percentage of Treg cells in the 5-Aza-5 group was lower than that in the blank control group ($P < 0.05$). The distribution of the flow cytometry is documented in Supplementary Figure 1.

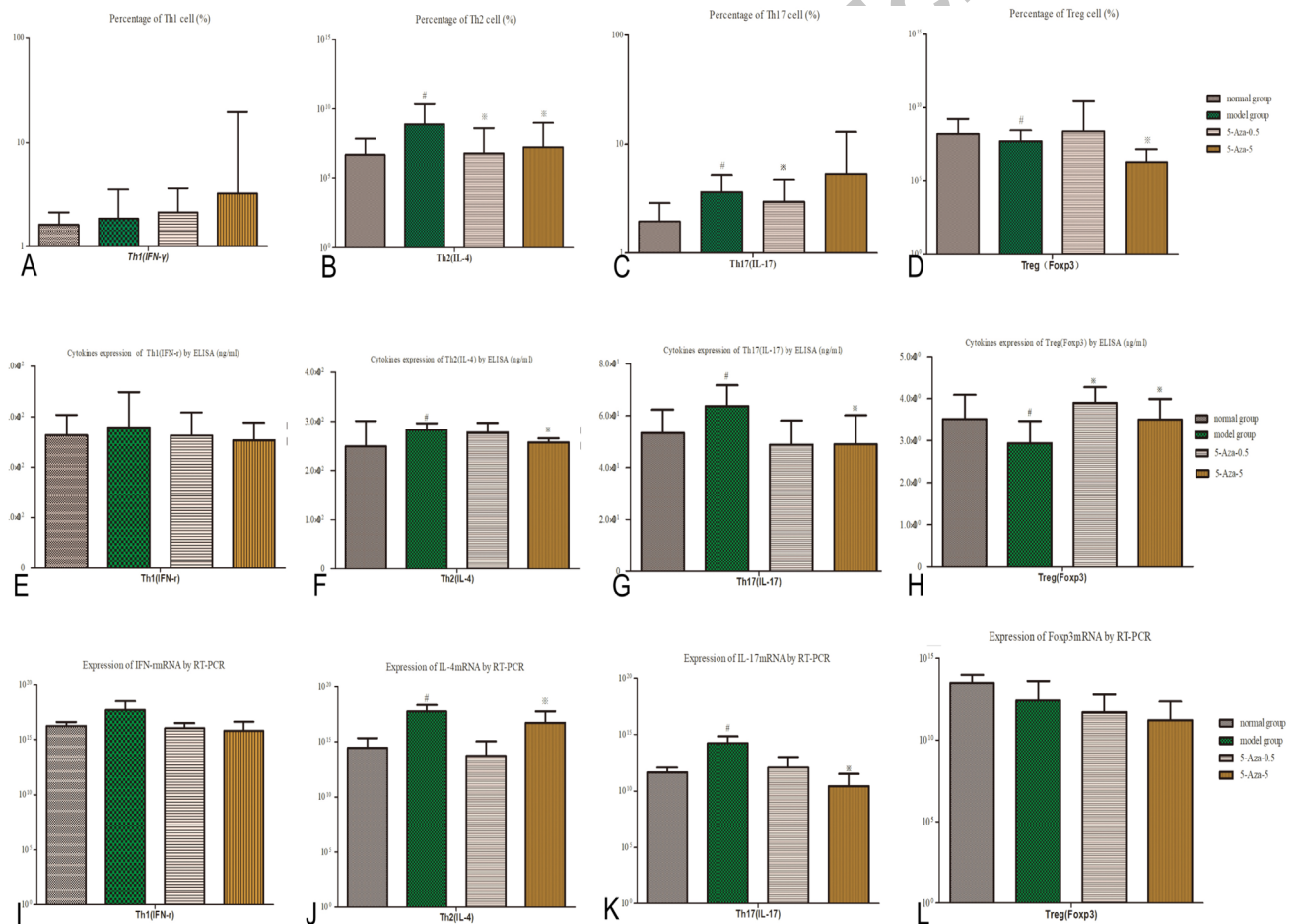


Fig. 4. The results of cell counts, cytokine expressions and RT-PCR used for Th1, Th2, Th17, Treg. (A-D) The results of cell counts: The DNMT inhibitor 5-Aza decreased cell counts of Th2, Th17 cells and increased cell counts of Treg cells; (E-H) The results of cytokine expressions: The DNMT inhibitor 5-Aza decreased cytokine expressions of Th2, Th17 cells and increased cytokine expressions of Treg cells; (I-L) The results of RT-PCR: The DNMT inhibitor 5-Aza inhibits transcription levels. The * in the figure indicates that there were statistical differences compared with the blank control group.

5-Aza inhibits the expression of cytokine genes and reduces the release of inflammatory factors

According to the manufacturer's instructions of Elisa kit, the content of inflammatory factors produced by Th2 and other cells in the lung tissues of each group was detected. The results are shown in Figure 4E-H. Compared with the normal group, the contents of IFN- γ , IL-4, IL-17 and Foxp3 were increased. After 5-Aza treatment, their content was significantly down-regulated ($P < 0.05$). In order to verify the hypothesis that the changes in the content of inflammatory factors are due to the differential expression of the corresponding genes. The mRNA levels of their corresponding genes were detected by qRT-PCR. The results were consistent with the content detection trend (Fig. 4, Supplementary Fig. 1).

5-Aza inhibited Jurkat T cell proliferation

The Jurkat cell morphology was observed by microscopy. As shown in Figure 5A, the Jurkat cells showed round contours, transparent and uniform cytoplasm and good refraction and looked like bunches of grapes in suspension. After 24h of 5-Aza treatment, Jurkat cells exhibited shrunken bodies, attenuated refraction and increased intracellular granules. With the passage of time, Jurkat T cell proliferation was inhibited to a greater extent (Fig. 5).

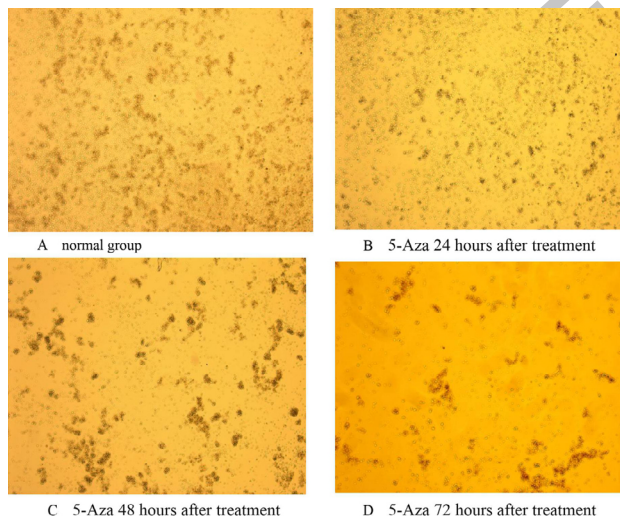


Fig. 5. Jurkat T cell morphology at different time periods (10 $\mu\text{mol/L}$ concentration). The inhibition of 5-Aza on the proliferation of Jurkat cells had a dose-and time-dependent manner. (A) Normal group; (B). 5-Aza 24 h after treatment; (C). 5-Aza 48 h after treatment; (D). 5-Aza 72 h after treatment.

With the increase of 5-Aza concentration from 0 $\mu\text{mol/L}$ to 10 $\mu\text{mol/L}$, the cell number significantly decreased, and cell lysis increased. At the same

concentration, the inhibition rate of the 1–10 $\mu\text{mol/L}$ dose groups increased with the extension of the intervention. When the intervention concentration reached 5 $\mu\text{mol/L}$, the inhibition rates at 24, 48 and 72 h were 51.8%, 58.9% and 50.1%, respectively ($P < 0.05$). When the intervention concentration was 10 $\mu\text{mol/L}$, the inhibition rates at 24, 48 and 72 h were 42.7%, 47.5% and 54.7%, respectively ($P < 0.05$) (Fig. 6). This showed that the inhibition of 5-Aza on the proliferation of the Jurkat cells had a dose and time-dependent manner.

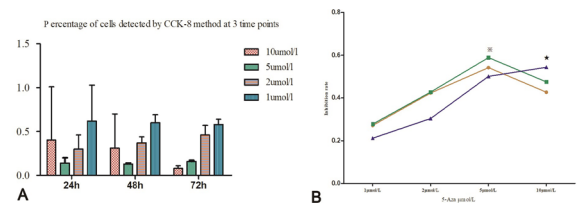


Fig. 6. The inhibition rate of Jurkat T cell proliferation. (A), Cell viability at three time points detected by CCK-8. (B), Different concentrations of 5-Aza inhibited Jurkat T lymphocytes and the inhibition rate increased as 5-Aza concentration increased.

5-Aza changed cytokine transcription levels of Jurkat T cells

The actual expression of IL-4, STAT6 and Foxp3 mRNA in the model group was higher than that in the normal group, but the IL-13 mRNA expression showed no significant difference between the two groups ($P > 0.05$). This suggested that the Jurkat cells polarised toward Th2 after stimulation. The actual expression of STAT6 mRNA after high-dose drug intervention was different from that in the model group ($P < 0.05$), and high-dose intervention might decreased the expression of STAT6 mRNA. The expression of Foxp3 mRNA in the high-dose group was higher than that in the PBS group ($P < 0.05$). Thus, methylase inhibitors could upregulate the expression of Foxp3 mRNA (Fig. 7).

DISCUSSION

Allergen-specific Th cells produce cytokines that induce many of the hallmark features of asthma. Cytokine producing many Th subsets, and these subsets all play a role in the development of asthma (Yu *et al.*, 2012). For example, IL-22 acts in either pro-inflammatory or anti-inflammatory ways depending on the specific cytokine microenvironment in individual tissues. As a result, a thorough understanding of the many pathways involved in asthma is required for effective therapeutic development. The pathways that regulate the differentiation in allergic asthma are beginning to be better understood.

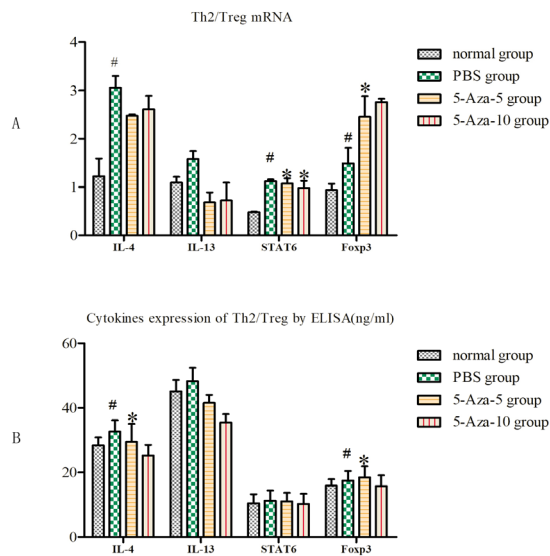


Fig. 7. The results of Th2/Treg gene and transcription factor mRNA expression (A) and cytokines protein expression (B): The 5-Aza treatment could down-regulate the mRNA expression and cytokines protein expression of Th2/Treg gene and transcription. Compared to the normal group, # $P < 0.05$. Compared to PBS group, * $P < 0.05$.

The results of this experiment showed that the proportions of Th2 and Th17 cells in the model group were higher than those in the control group. This indicates that the level of asthma model cell levels mainly increased in the Th2 and Th17 cells. The expression of Th1 cells in the model group was also higher than that in the control group, but there was no statistical difference, indicating that the Th2-based inflammatory response was accompanied by an increase in Th1 cells, which was consistent with the results of some scholars (Verma *et al.*, 2013).

Reduced whole-blood DNA methylation at 14 CpG sites which acquired after birth are strongly associated with childhood asthma, which indicates the activation of eosinophils and cytotoxic T cells in childhood asthma (Xu *et al.*, 2018). While DNA methylation signatures are associated with asthma, their significance is not clear (Collison *et al.*, 2013). Direct methylation of DNA is associated with genetic repression. Such methylation is lost at genetic enhancers in the Th2 locus during Th2 differentiation (Devries and Vercelli, 2016), and it has been implicated in control of the Foxp3 locus (Lee *et al.*, 2002). In addition, DNMT3A negatively regulates IL-13 in Th2 cells and allergic airway inflammation (Floess *et al.*, 2007).

The DNA methylation promoted the stable differentiation of lung CD4⁺ T cells to the Th2 phenotype.

These two genes (*Foxp3* and *DNMT3A*) are

critical modulators of Th1/Th2 balance and play vital roles in asthma pathogenesis. But the development and differentiation of T helper cells are influenced by *DNMT1* gene. DNMT1 is thought to be the major enzyme responsible for post replicative maintenance of DNA methylation, and maintained the methylation status during replication and also critical for the development, differentiation and regulation of Th1 and Th2 cells.

Verma *et al.* (2013) and Wu *et al.* (2013) found that DNMT1 expression level was low in all the tissues (lung, trachea and BALF cells) of asthmatic in comparison to normal mice. They analyzed Th1/Th2 profile in the PBMC of asthmatic and normal mice by flow cytometry. Based on cytokine profile presence of IFN- γ represents Th1 cells and IL-4 and IL-5 represents Th2 cells. In asthmatic mice there was an increased percentage of Th2 cells producing IL-4 and IL-5 compared to normal. In contrast, Th1 cells producing IFN- γ decreases in asthmatic mice compared to normal mice. This is consistent with the results of our study, suggesting that the asthma model is dominated by increased Th2 cytokines. Meanwhile they found that the expression level of DNMT1, STAT3, IFN- γ , and IL-6 were lower and Socs3, IL-4 and IL-5 were higher in all the tissues (Lung, trachea) and BALF cells of OVA/OVA mice in comparison to normal tissues showed the least. It is suggested that the expression of Th2 cells in asthma increases at the same time, and Th1 cells also increase, which is also highly consistent with our conclusion. This indicates that the pathogenesis of asthma is related to the dynamic balance of Th1/Th2. They further performed bisulphite sequencing to reconfirm the methylation status of the DNMT1 promoter and observed >85 % CpG methylation in the DNMT1 promoter of lung, trachea and BALF cells. Where as in normal mice methylation was not observed in lung. This result further validates our findings in clinical asthma patients, that is, the onset of asthma may be related to gene methylation at specific sites. This discovery Inspired us that the pathogenesis of asthma may be related to gene methylation at specific loci. Our study demonstrated our findings by intervening demethylation with methylase inhibitors. This study assumes greater significance in designing and developing to therapeutic means.

The results of Wu *et al.* (2013) and Sun *et al.* (2020) suggest that studies have shown that 5-Aza treatment can change the number of Treg cells in T cell subsets and effectively inhibit the symptoms of asthma. Here, it could also be seen that methylase inhibitors can affect the differentiation and expression of T cells. The results of this study showed that after the rats were sensitised and challenged, the proportions of Th1, Th2 and T17 cells were all higher than those of the normal control group, and the proportion of Treg cells was lower than that of the normal group. However, after intervention with

5-Aza, the proportion of Th1 cells increased and did not decrease, indicating that the expression of Th1 cells might not be affected by methylase inhibitors. In other words, the demethylation failed to reduce the expression of Th1 cells, which was inconsistent with our clinical practice. The degree of methylation of IFN- γ in clinical asthma patients was no different from that in the normal group, and the degree of methylation of IL-4 was higher than that in the control group. Therefore, after the intervention of demethylation, the expression of Th2 cells showed a downward trend. However, the dose of methylase inhibitor intervention appeared to be inconsistent with theory. Although high-dose 5-Aza could reduce the expression of Th2 cells, the effect was not as good as that of low-dose 5-Aza. At the same time, in the results of this experiment, after the intervention of 5-Aza in the Th17 cells, the high-dose intervention caused the proportion of Th17 cells to rise and not drop, which might have been related to the dose and pharmacokinetics.

Asthma prevalence is influenced by environmental factors. New research shows that PM2.5 impaired could aggravate asthma in an AhR-dependent manner (Wang *et al.*, 2018). Studies show that decreased IL-2 production and increased IL-4 cytokine promote methylation (Brand *et al.*, 2012). In the results of this experiment, the Jurkat cells could form Th2 polarisation after stimulation, which indicated that stimulation factors play an important role in Th cell differentiation and further indicated the influence of the environment on the disease. After methylase inhibitor intervention in the Jurkat cells, the transcription and translation levels of the IL-4/IL-13/STAT6 gene were changed, the expression of Foxp3 mRNA was upregulated, and the expression of Foxp3 protein was downregulated. This suggested that the intervention of methylation could be used to form a new dynamic balance model of Th2 hyperinflammation.

In a preliminary study from Urumqi, Xinjiang (mentioned previously), it was found that the IL-41SR gene in asthma was higher than that in the control group, which suggested that hypermethylation might be one of the key factors in the pathogenesis of asthma. Additionally, the CD4⁺ T cells also displayed increased DNA methylation of the IFN- γ promoter, protein, gene, and cell count expression of IL-4 and IL-17 increase after allergen sensitisation and challenge; however, Treg cell decreases were observed. The increase in a Th2-type inflammatory reaction may be reversed with DNMT inhibitors, which could be confirmed using experiments.

Previous studies have found that the effect of 5-Aza can differ for the same gene under different environmental settings (Seelan *et al.*, 2018). Our study reported that epigenetic regulation in T cells contributes to the development of experimental asthma and can be targeted

pharmacologically.

There were some shortcomings in this experiment. One was the need to detect more susceptible factors and transcription factor levels so that the reasons for the three levels of inconsistent expression could be identified. The effect of methylation on asthma must also be studied. Subsequent studies will further explore the effect of methylation of some target genes on asthma through methylation sequencing.

CONCLUSION

Epigenetics has an impact on disease occurrence. Epigenetic regulation in T cells contributes to the development of experimental asthma, which can be treated pharmacologically.

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Ethics approval and consent to participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the ethics committee of Xinjiang Medical University, Ethics number: SCXK2011-0004.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

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Supplementary material

There is supplementary material associated with this article. Access the material online at: <https://dx.doi.org/10.17582/journal.pjz/20220609090644>

Statement of conflict of interest

The authors have declared no conflict of interest.

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Supplementary Material

Effect of DNA Methylation of T Cells on the Development of Experimental Asthma

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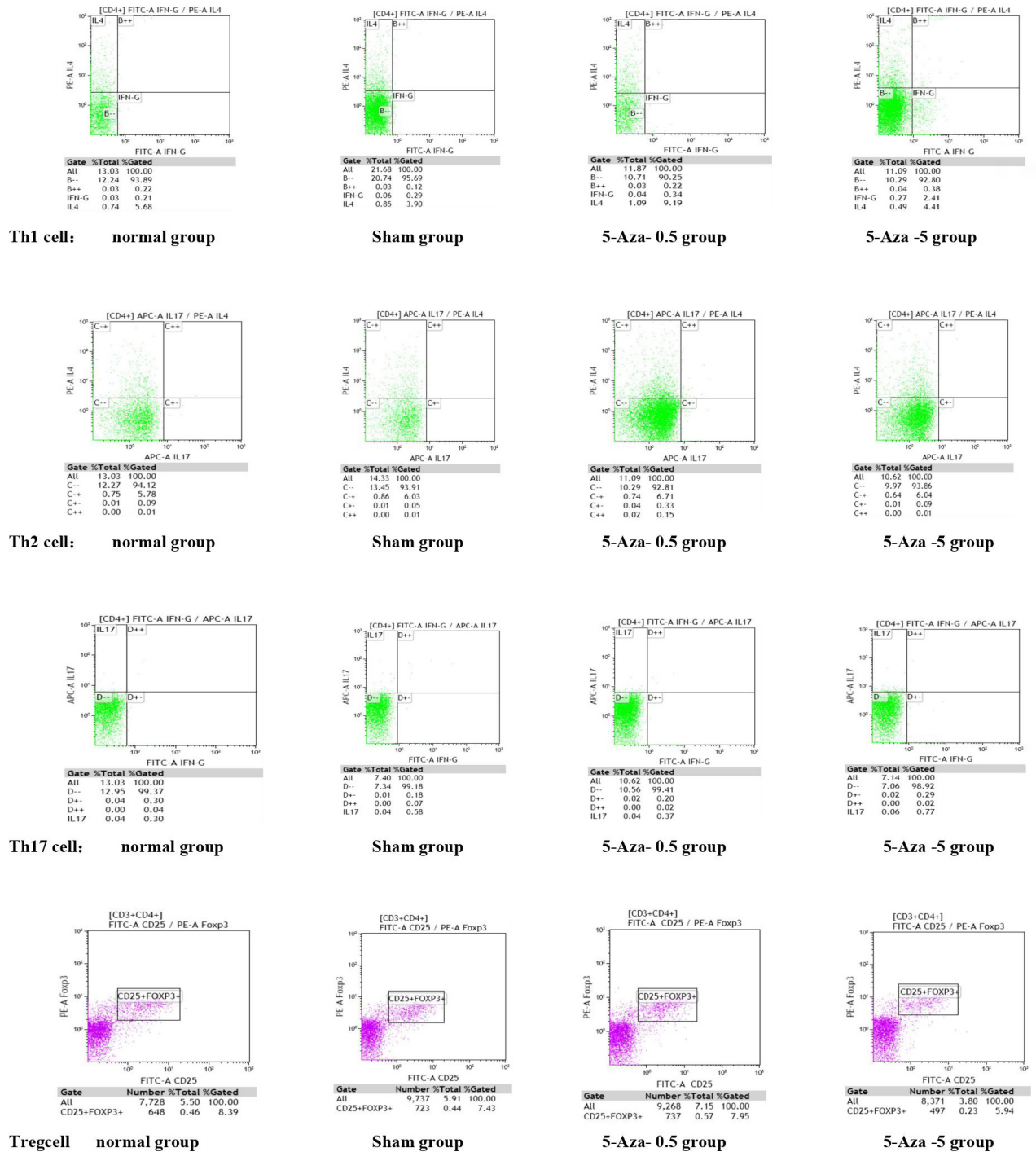
Xiaoying Liang and Xiaofang Jiang contributed equally to this study.

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0030-9923/2022/0001-0001 \$ 9.00/0

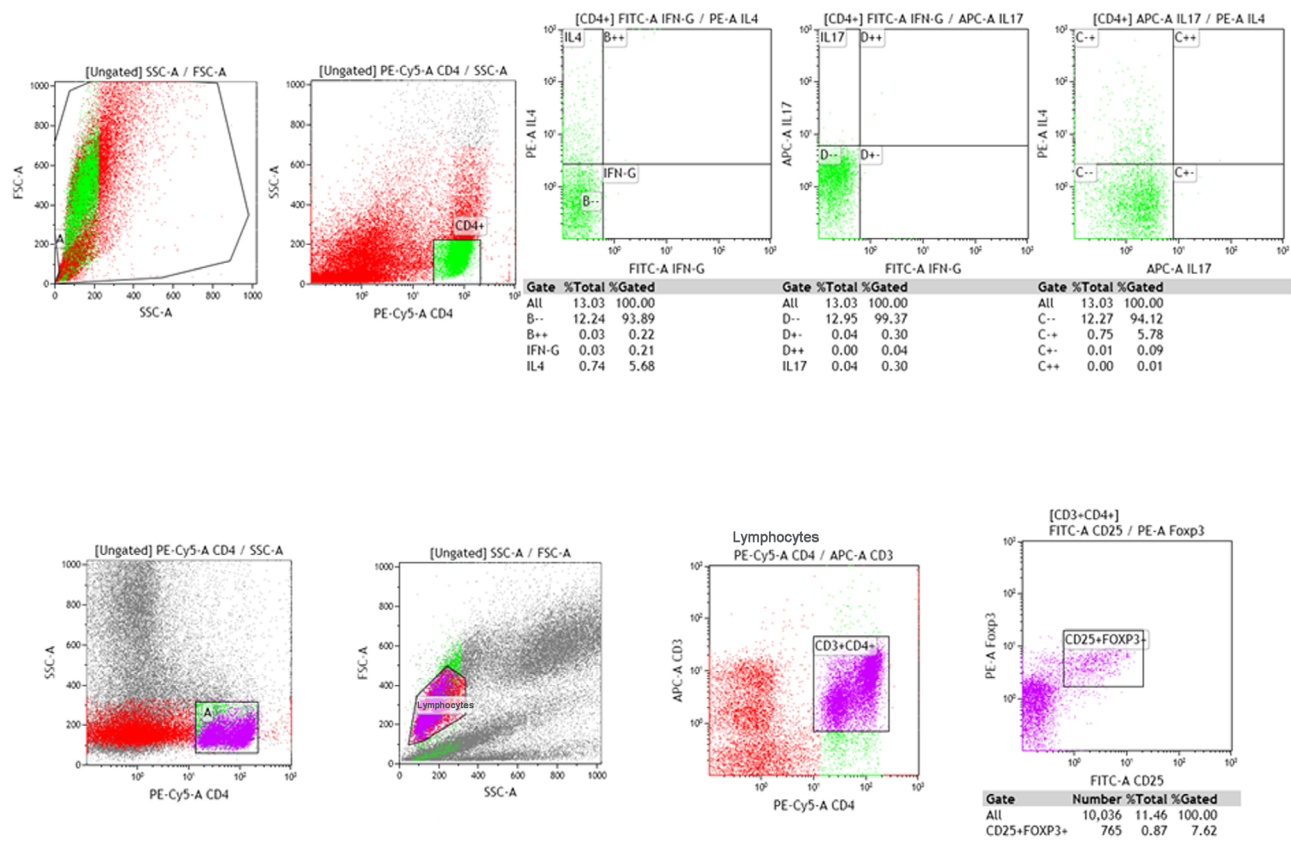


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Supplement Fig. 1. Distribution of flow cytometry.



Supplement Fig. 2. Gating in Cytometry.