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Upregulated Protein Arginine Methyltransferase 1 by IL-4 Increases Eotaxin-1 Expression in Airway Epithelial Cells and Participates in Antigen-Induced Pulmonary Inflammation in Rats

Qingzhu Sun,*†,1 Xudong Yang,*†,1 Bo Zhong,*† Fangfang Jiao,‡ Chenyan Li,*† Dongmin Li,*† Xi Lan,*† Jian Sun,*† and Shemin Lu,*†,§

Protein arginine methyltransferases (PRMTs), catalyzing methylation of both histones and other cellular proteins, have emerged as key regulators of various cellular processes. This study aimed to identify key PRMTs involved in Ag-induced pulmonary inflammation (APIP), a rat model for asthma, and to explore the role of PRMT1 in the IL-4-induced eosinophil infiltration process. E3 rats were i.p. sensitized with OVA/alum and intranasally challenged with OVA to induce APIP. The expressions of PRMT1–6, eotaxin-1, and CCR3 in lungs were screened by real-time quantitative PCR. Arginine methyltransferase inhibitor 1 (AMI-1, a pan-PRMT inhibitor) and small interfering RNA–PRMT1 were used to interrupt the function of PRMT1 in A549 cells. In addition, AMI-1 was administrated intranasally to APIPI rats to observe the effects on inflammatory parameters. The results showed that PRMT1 expression was mainly expressed in bronchus and alveolar epithelium and significantly upregulated in lungs from APIPI rats. The inhibition of PRMTs by AMI-1 and the knockdown of PRMT1 expression were able to downregulate the expressions of eotaxin-1 and CCR3 with the IL-4 stimulation in the epithelial cells. Furthermore, AMI-1 administration to APIPI rats can also ameliorate pulmonary inflammation, reduce IL-4 production and humoral immune response, and abrogate eosinophil infiltration into the lungs. In summary, PRMT1 expression is upregulated in APIPI rat lungs and can be stimulated by IL-4. Intervention of PRMT1 activity can abrogate IL-4–dependent eotaxin-1 production to influence the pulmonary inflammation with eosinophil infiltration. The findings may provide experimental evidence that PRMT1 plays an important role in asthma pathogenesis. The Journal of Immunology, 2012, 188: 3506–3512.

Airway inflammation in asthma is a multicellular process involving mainly macrophages, neutrophils, T lymphocytes, and mast cells, with eosinophilic infiltration being the most striking feature (1). Eosinophils, which play an important role in asthma, are recruited into the lung during both acute and chronic pulmonary inflammation (2, 3). Accumulating evidence has demonstrated that the eosinophil infiltration into lungs both in animal models and human diseases can be attributed to the high expression of eotaxins (4, 5), and increased IL-4 production is associated with eosinophilia and promotes eosinophilic inflammation through induction of eotaxin-1 (6, 7).

Despite increasing evidence to support the effector role of eosinophils in asthma and other allergic diseases, the mechanism underlying the recruitment of eosinophils into airways by IL-4 is not completely understood. In our previous study, we found that there are significant differences in the gene expressions of protein arginine methyltransferases (PRMTs) between Ag-induced pulmonary inflammation (APIPI) model rats and control rats by RT-PCR (8), suggesting that PRMTs may play an important role in the regulation of asthma-related genes.

PRMTs are evolutionarily conserved from yeast to humans and classified as type I, II, and III based on the nature of methyltransfer (9, 10). Therefore, PRMT1, belonging to type I enzymes, participates in a variety of cellular processes including signal transduction, epigenetic regulation, and DNA repair pathways (11, 12). PRMT1, on the one hand, can catalyze the methylation of histones H4 at arginine 3 (H4R3), which acts as a part of the histone code to regulate gene expression (11–13); on the other hand, recent studies have revealed that a plethora of proteins with methylated arginine residues catalyzed by PRMT1 implicate in a variety of cellular processes. For example, PRMT1 enhances gene transcription by methylating N-terminal arginine residues in NFAT-interacting protein 45, which heterodimerizes with NFAT and then enhances NFAT, driving cytokine production upon TCR signaling of T cells (14, 15).

It is plausible that PRMTs may, in part, play a potential role in the disease process of asthma. Hitherto, there is no detailed study to characterize the roles of PRMTs in the asthma animal model. So, in the current study, we induced APIPI in E3 rats and tried to identify the key PRMT and its role in the disease development.
Materials and Methods

Rats

E3 rats were bred in a specific pathogen-free animal house. Age- and sex-matched rats were used within the experiments, and each group contained eight rats at age of 8–10 wk. The experiments were approved by the Institutional Animal Ethics Committee of Xi’an Jiaotong University.

Induction of AIPI and administration of arginine methyltransferase inhibitor 1 in rats

AIPI was induced, as previously described (16). Briefly, the rats were immunized by i.p. injection with 1 ml emulsion solution containing 1 mg OVA (Sigma-Aldrich, St. Louis, MO) and 50 mg Al (OH)3 (Pierce Biotechnology, Rockford, IL). For screening the expression of PRMTs in lungs, 16 rats were divided into control group and AIPI group. For arginine methyltransferase inhibitor 1 (AMI-1) treatment experiment, 24 rats were divided into three groups, as follows: control group, AIPI group, and AMI-1 group. Two weeks after the sensitization, control group rats were sham sensitized and exposed to the same volume of solvent. AIPI group rats were subjected to intranasal challenge of OVA solution (1 mg/ml in PBS). In AMI-1 group, the rats were administrated by 50 μl AMI-1 (Calbiochem) at a concentration of 0.1 mg/ml in PBS at 2 h before OVA challenge.

RNA quantitation

The mRNA expressions of PRMT, chemokine, and cytokine genes were tested by real-time quantitative PCR (RT-qPCR), which was performed on iQ5 real-time PCR detection system (Bio-Rad) with SYBR Premix Ex TaqTM II (TaKaRa). The relative gene expression is normalized by GAPDH. The information of primers is shown in Table I.

Lung histology and immunohistochemistry staining

The scoring of lung histology (H&E staining) was performed in a blind fashion, and leukocyte infiltration around bronchus was performed as the scoring system, as follows: 0, no cells; 1, a few cells; 2, a ring of cells 1 cell layer deep; 3, a ring of cells 2–4 cells deep; 4, a ring of cells higher than 4 cells deep.

For immunohistostaining, the common protocol was used. The sections were incubated with 100-fold diluted anti-PRMT1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution at 4°C overnight. Then 2-Step Plus poly-HRP anti-goat IgG detection kit (ZSGB-BIO, Beijing, China) was used. The gradation of the brown color was determined by using the Image-Pro Plus 6.0 soft to estimate the protein expression in lungs.

IL-4 stimulation in A549 cells and coincubating with PRMT inhibitor

Human A549 alveolar epithelial-like cells (A549) were cultured in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% FCS (HyClone, Logan, UT). Human rIL-4 (Boster, Wuhan, China) was added into the wells (6-well plates) at a series of concentrations of 0, 25, 50, 100, and 200 ng/ml. The working concentration of human rTNF-α (PeproTech, Rocky Hill, NJ) to treat the cells was 100 ng/ml. AMI-1 was used at the concentration of 8.44 μM as suggested in a previous study (17).

PRMT1 intervention with RNA interference

Three small interfering RNAs (siRNA) were designed and synthesized by Genechem (Shanghai, China). A549 cells were transfected with siRNA according to manufacturer’s protocols. The third of the three different sequences of small interference RNA for PRMT1 (siPRMT1–3) was found as the most efficient to knockdown the gene expression, and its sequence is as follows: sense, 5′-CCAUCGACCUGGACUUCAATT-3′; antisense, 5′-UUGAAGUCCAGGUCGAUGGTT-3′. This siRNA and mock sequence at the final concentration of 50 nM was transfected into A549 cells, respectively, with Lipofectamine 2000 (Invitrogen) for 24 h. The gene expression was determined with RT-qPCR.
Western blot of PRMT1

A549 cells at 2 × 10⁶ per well were seeded in 6-well plates and preincubated for 12 h. After treated with siRNA-PRMT1 and stimulated with or without IL-4 for 24 h, the cells were lysed with RIPA lysis buffer (Beyotime, Beijing, China). The lysates were centrifuged at 12,000 rpm for 15 min, and the supernatant was kept. The protein concentration in the supernatant was quantified by using bicinchoninic acid method (Beyotime). An equal amount of the denatured protein (20 μg) was separated by SDS-PAGE, and subsequently proteins were electrophoresed to polyvinylidene difluoride membranes. The proteins were detected with PRMT1-specific Ab and the secondary Ab conjugated with HRP (Santa Cruz), and visualized by using ECL reagents (Pierce).

Determination of inflammatory cells in bronchoalveolar lavage fluid

Lungs were lavaged by instillation and withdrawal of 2 ml ice-cold PBS through the tracheal route. Bronchoalveolar lavage fluid (BALF) was collected and centrifuged (1000 rpm, 10 min), and the cellular pellet was resuspended in 1 ml PBS. Total cell numbers were determined with crystal violet staining by using a hemocytometer. For counting differential cells, such as eosinophils, macrophages, and lymphocytes, cytocentrifuged preparations were fixed, stained with Wright–Giemsa staining, and counted according to differentiated morphology.

Determination of OVA-specific IgG1 and total serum IgE

The serum levels of OVA-specific IgG1 and total serum IgE were measured by ELISA, as previously described (18). Total serum IgE and OVA-specific IgG1 were measured with HRP-conjugated mouse anti-rat κ/λ-chain (AbD, Serotec). The reaction was terminated with 1 M H₂SO₄, and read at 450 nm by ELISA reader (Thermo Electron).

Statistical analysis

Data were expressed as mean ± SEM. The statistical analysis was performed by Mann-Whitney U test for the comparison between groups. The change of PRMT1, etoxin-1, and CCR3 expression between different dose and timing of IL-4 stimulation was analyzed by one-way ANOVA for comparison. The p value <0.05 was considered as statistically significant.

Results

PRMT1 expression of lung exhibits a remarkable increase in AIPI

The screen of PRMT expression by RT-qPCR showed that PRMT1 (p = 0.0017), PRMT2 (p = 0.0045), and PRMT3 (p = 0.0292) from AIPI lungs significantly increased, and PRMT1 showed the most significant upregulation (Fig. 1A). All of the primer information is shown in Table I.

Next, we performed immunohistochemical staining to visualize PRMT1 protein expression in the lungs. The results demonstrated that PRMT1 was expressed in both airway and alveolar epithelial cells of the rats (Fig. 1B). The protein expression in bronchi and alveoli of AIPI rats significantly increased compared with the control rats (Fig. 1C).

Table 1. Information of primers for RT-qPCR

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<tr>
<th>Gene Name</th>
<th>Species</th>
<th>Sequence</th>
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<td>Reverse, 5'-GGAGACCGCCAGAGAATCTCCACAGC-3'</td>
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**PRMT1 and eotxin expression significantly increases after IL-4 stimulation in dose- and time-dependent manner**

A549 cells were stimulated with IL-4 at a different concentration and time. The results showed that the expression of PRMT1 increased with IL-4 dose and culturing time (Fig. 2A, 2B). However, other PRMTs, including PRMT2, 3, 5, and 6, did not show this feature. Meanwhile, the expressions of PRMTs did not show any change after the stimulation of TNF-α (Supplemental Fig. 1).

The expression of eotxin-1 and its receptor was also determined in epithelial cells after the stimulation of IL-4. The results showed that the expressions of eotxin-1 and CCR3 were upregulated after IL-4 stimulation. Meanwhile, the expression of eotxin-1 showed dose- and time-dependent manner with IL-4 stimulation similar as observed in PRMT1 expression (Fig. 2C–F).

**Inhibition of PRMT1 function regulates eotxin-1 expression in alveolar epithelial cells**

To explore the effects of PRMT function on the expression of eotxin-1, AMI-1, the first inhibitor of PRMTs, was used to treat on A549 cells with and without IL-4 stimulation. The results with AMI-1 and IL-4 coinoculation demonstrated a decrease in eotxin-1 expression compared with the cells stimulated in the absence of PRMT inhibitor (Fig. 3A, 3B). Meanwhile, TNF-α had no influence on the expression of eotxin-1 and CCR3 compared with control. The expressions of TARC and CCR4 did not show the same changes as eotxin-1 after the stimulation of IL-4, AMI-1, and TNF-α (Supplemental Fig. 2).

Because AMI-1 is a pan-PRMT inhibitor, we used siPRMT1 transfection to clarify whether the PRMT1 plays the role of elevated expression of eotxin-1 after IL-4 stimulation. The results showed that siPRMT1–3 downregulated to 9.24% of PRMT1 mRNA expression after the transfection (Supplemental Fig. 2C). Then we used this siPRMT1–3 for further study. A549 cells treated with siPRMT1–3 reduced both mRNA and protein expressions of PRMT1 effectively, and the expression of PRMT1 showed no change with both IL-4 stimulation and siPRMT1 compared with control (Fig. 3C). Meanwhile, the siPRMT1 invalidated the elevation of eotxin-1 after IL-4 stimulation, but not significantly for CCR3 (Fig. 3D, 3E). The results indicated that in epithelial cells, IL-4 certainly orchestrated high expression of PRMT1, which activated the downstream chemokine production.

**AMI-1 suppresses eotxin-1, CCR3 expression, and eosinophil infiltration in the rats with AIPI**

To confirm whether upregulation of PRMTs plays an important role in the development of AIPI in vivo, we administrated AMI-1 to AIPI E3 rats. CCR3 and eotxin-1 expressions increased in the AIPI lung tissues compared with lungs from control group. After administration of AMI-1, the expression of CCR3 and eotxin-1 decreased significantly in AIPI rats (Fig. 4A, 4B). Additionally, eosinophil infiltration was abated in the BALF from AMI-1–administrated rats compared with control AIPI rats (Fig. 4C). The results indicated that the inhibition of PRMT can downregulate eotxin-1, CCR3 expressions, and eosinophil infiltration in AIPI rats.
AMI-1 treatment can ameliorate the pulmonary inflammation of AIPI rats

Finally, we observed the effects of AMI-1 treatment on the inflammation of AIPI rats. Pathological changes indicated that in the AIPI group, the inflammation infiltration is strikingly serious, no matter in airway or in alveoli. However, in the AMI-1 group, the inflammation cells significantly decreased after the drug administration (Fig. 5A–C). In the AMI-1 group, the inflammation score dramatically decreased compared with the AIPI group (Fig. 5D). The expression of IL-4 also decreased after AMI-1 administration, and the inhibition of PRMT activity also decreased total IgE and OVA-specific IgG1 in serum significantly (Fig. 5E–G). However, AMI-1 administration did not influence all asthmatic indices, for example, the total cells in lung infiltration, delayed-type hypersensitivity, concentration of NO in serum, and the TGF-β expression in lung tissue did not return to normal level (Supplemental Fig. 3). Taken together, these results indicated that the administration of PRMT inhibitor to AIPI rats influenced the inflammatory parameters and ameliorated the disease severity.

Discussion

In this study, we observed an upregulated expression of PRMTs in AIPI, a canonical animal model for asthma. Particularly, upregulated PRMT1 by IL-4 can elicit the increased eotaxin-1 expression in epithelial cells. Meanwhile, intervention of PRMT1 with an inhibitor, AMI-1, and specific RNA interference were able to prevent the observed increase of eotaxin-1 from stimulating by IL-4 in vitro. Administration of AMI-1 to AIPI rats can reduce the inflammation score of lung tissue, the production of IgE, and OVA-specific Ab in serum, especially the eosinophil infiltration in BALF.

It has been known that PRMTs, as transcription factor regulators, are able to regulate gene expression. Furthermore, the degradation of asymmetrically dimethylated proteins catalyzed by PRMTs produces ADMA, which shows increased circulating levels in numerous diseases (19–21). Accumulating evidence shows that the deregulated levels of PRMT and its product ADMA may participate in pathogenesis of cancer, viral infections, chronic pulmonary diseases (22), and cardiovascular diseases (23). This is the first finding, to our knowledge, to show that PRMT1 is involved in
the pathogenesis of asthmatic disease. The AIPI model in E3 rats shows most similarities to human asthma in pulmonary pathology (18). We screened PRMT expression profile in lungs of the AIPI and control rats, and PRMT1 is upregulated the most significantly. The epithelial cells express the PRMT1 protein in both cytoplasm and nucleus. The findings suggested that PRMTs, in particular PRMT1, indeed participate in the pathogenesis of allergic inflammation.

Regarding the upstream mechanism of the PRMT upregulation, we conjectured that Th2 cytokines probably play a crucial role because T cells may control PRMT activity through increasing cytokine production (15, 24). IL-4 is one of the most important players in airway inflammation and can enhance the expression of eotaxin-1 in lung stable cells (25). We found in this study that the expression of only PRMT1 was significantly increased in a time- and dose-dependent manner under the stimulation of IL-4, but TNF-α stimulation did not have any effect on the expression of PRMTs in epithelial cells.

To find the exact interaction between IL-4 and PRMTs, we stimulated epithelial cells with IL-4 and added AMI-1, an inhibitor of PRMT, and the in vitro results proved that PRMT1 may play an important role in the classic IL-4/eotaxin-1 pathway. AMI-1 is a potent inhibitor of PRMT1 function, but can also inhibit the SET domain of histone lysine methyltransferases (26, 27). To investigate the specific role of PRMT1, we used siRNA-mediated knockdown and studied the effect of IL-4 on eotaxin expression.

Airway epithelial cells express IL-4R constitutively, and the integration of IL-4 and IL-4R is known to have pleiotropic effects on development of AHR, eosinophil infiltration, airway inflammation, and mucus hypersecretion (28, 29). IL-4 leads to the activation of multiple signaling pathways, including Stat6, CREB, NF-κB, and GATA3 (30–32), which induce eotaxin production. We used the software, TFSEARCH (version 1.3, Web site: http://www.cbrc.jp/research/db/TFSEARCH.html), to predict the promoter region of PRMT1 and found that STATs, CREB, NF-κB, and GATA3 may combine with the promoter region of PRMT1. Thereby, IL-4 can integrate with IL-4R on epithelial cells and activate multiple transcription factors, which may bind with the promoter region of PRMT1 to upregulate PRMT1 expression.

It has been known that PRMTs can catalyze arginine methylation of several proteins, including STAT family (33). Transcription factors of the STAT family are important in signal transduction of cytokines. They are subject to posttranslational modification by phosphorylation on tyrosine and serine residues. Recent evidence suggested that STATs are methylated on a conserved arginine residue within the N-terminal region. STAT arginine methylation has been described to be important for STAT function, and loss of arginine methylation was discussed to be involved in IFN resistance of cancer cells (34, 35). IL-4 and IL-13 share receptor
The authors have no financial conflicts of interest.

Components and activate similar signal transduction pathways (30). It has been reported that IL-13 upregulates eotaxin expression in airway epithelial cells by a mechanism involving activation of STAT6 (36). We proved that the stimulation of IL-4 in epithelial cells can induce the upregulation of PRMT1, which may upregulate the transcription of eotaxin-1 by activating the STAT pathway. AMI-1 has been applied to inhibit PRMT activity only in in vitro experiments as yet (11, 17), and no in vivo experiment has been reported. After the AMI-1 rats were given AMI-1 treatment, we found that eotaxin infiltration was the most striking change. In vivo studies of allergic inflammation have shed much light upon the role of eotaxin-1 and its receptor in disease pathology (37). Eotaxin-1 may only attract eosinophils to the site of allergic inflammation and activate them on arrival, but may also be important in promoting conditions for a positive feedback loop that produces continued commitment to Th2 cytokine-driven allergic inflammation in vivo (25, 38), which is coincident to our findings that the expression of IL-4 also decreased after AMI-1 administration. Eotaxin-1 level is associated with the severity of eosinophilic airway inflammation (39), and the eosinophil migration into inflammatory tissues induces other aberrations of asthma (40). After the inhibition of PRMTs, the inflammation score in lung tissue, total IgE, and OVA-specific IgG1 in serum decreased significantly in this study.

In summary, we document that IL-4–induced eotaxin-1 upregulation in epithelial cells of OVA rats can be mediated by PRMTs, especially PRMT1 (Fig. 6). Additionally, suppression of PRMTs is closely associated with the decreased release of eotaxin-1, eosinophil infiltration, and other asthmatic indices in vivo. On the whole, PRMT1 plays a crucial role in AMI-1 through its regulation on eotaxin-1, and these findings may provide an important clue for further research in asthma pathogenesis and suggest a new remedy for asthma treatment.

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References


