

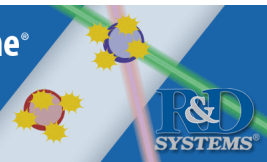


- Select up to 10 analytes from an existing multiplex panel
- Reagents are provided premixed and ready to use

R&D Systems Custom Premix Fluorokine® Multianalyte Profiling Kits

Bead-based Immunoassay Kits for the Luminex® Platform

[Click here for more information](#)



This information is current as of March 11, 2012

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, Xudong Yang, Bo Zhong, Fangfang Jiao, Chenyan Li, Dongmin Li, Xi Lan, Jian Sun and Shemin Lu

J Immunol; Prepublished online 2 March 2012;

doi:10.4049/jimmunol.1102635

<http://www.jimmunol.org/content/early/2012/03/02/jimmunol.1102635>

Supplementary Data	http://www.jimmunol.org/content/suppl/2012/03/02/jimmunol.1102635.DC1.html
Subscriptions	Information about subscribing to <i>The Journal of Immunology</i> is online at http://www.jimmunol.org/subscriptions
Permissions	Submit copyright permission requests at http://www.aai.org/ji/copyright.html
Email Alerts	Receive free email-alerts when new articles cite this article. Sign up at http://www.jimmunol.org/etc/subscriptions.shtml/

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include the digital object identifier (DOIs) and date of initial publication.

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright ©2012 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



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 (AIP), 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 AIP. 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 administered intranasally to AIP rats to observe the effects on inflammatory parameters. The results showed that PRMT1 expression was mainly expressed in bronchus and alveolus epithelium and significantly upregulated in lungs from AIP 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 AIP 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 AIP 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: 000–000.

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 (AIP) 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 methylation (9, 10). Thereinto, 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 AIP in E3 rats and tried to identify the key PRMT and its role in the disease development.

^{*}Department of Genetics and Molecular Biology, Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi 710061, China; [†]Key Laboratory of Environment and Genes Related to Diseases, Xi'an Jiaotong University, Ministry of Education, Xi'an, Shaanxi 710061, China; [‡]Joint Undergraduate and Graduate Program, Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi 710061, China; and [§]Department of Epidemiology and Health Statistics, School of Public Health, Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi 710061, China

¹Q.S. and X.Y. contributed equally to this work.

Received for publication September 12, 2011. Accepted for publication February 2, 2012.

This work was supported by National Natural Science Foundation of China Grants 81070030, 81170017, and 30630058 and Shaanxi Province International Cooperation Foundation of China Grant 2007-KW-06.

Address correspondence and reprint requests to Prof. Shemin Lu, Department of Genetics and Molecular Biology, Xi'an Jiaotong University College of Medicine, Xi'an, Shaanxi 710061, P. R. China. E-mail address: shemin.lu@gmail.com or lushemin@mail.xjtu.edu.cn

The online version of this article contains supplemental material.

Abbreviations used in this article: AIP, Ag-induced pulmonary inflammation; AMI-1, arginine methyltransferase inhibitor 1; BALF, bronchoalveolar lavage fluid; PRMT, protein arginine methyltransferase; RT-qPCR, real-time quantitative PCR; siPRMT, small interfering PRMT; siRNA, small interfering RNA.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/\$16.00

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 administered by 50 μ l 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 immunohistochemical staining, 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 μ M 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'-CCAUCGACCUGGACUUAATT-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.

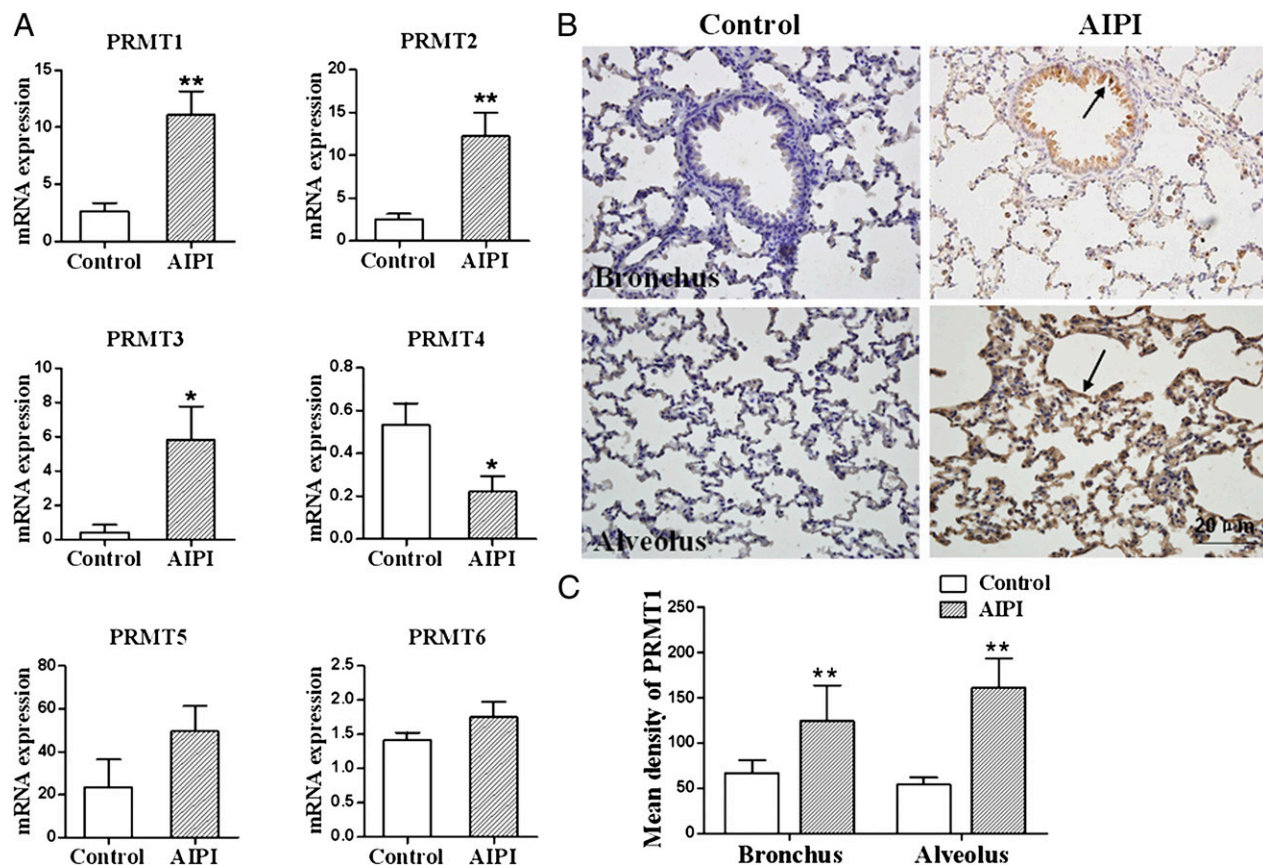


FIGURE 1. Expression changes of PRMT1–6 mRNA and PRMT1 protein in E3 rats with AIPI. PRMT1–6 expression in rat lungs (**A**) was determined by RT-qPCR analysis, and GAPDH expression was used to normalize the expression level. Representative images of PRMT1 protein expression in bronchus and alveolus of control rat lung (left panel) and AIPI rat lung tissues (right panel) (**B**) were from the tissue sections stained with anti-PRMT1 Ab by immunohistochemistry. Mean density of PRMT1 (**C**) was determined by Image-Pro Plus 6.0 software to estimate the expression of PRMT1 protein. The results were expressed as means $\pm$ SEM; * p < 0.05 and ** p < 0.01, between AIPI group and control group after Mann–Whitney test (n = 8 for each group).

Western blot of PRMT1

A549 cells at 2×10^5 per well were seeded in 6-well plates and precultured 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 μ g) was separated by SDS-PAGE, and subsequently proteins were electrottransferred 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 H_2SO_4 , and read at 450 nm by ELISA reader (Thermo Electron).

Statistical analysis

Data were expressed as mean $\pm$ SEM. The statistical analysis was performed by Mann-Whitney *U* test for the comparison between groups. The change of PRMT1, eotaxin-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 I. Information of primers for RT-qPCR

Gene Name	Species	Sequence	Size (bp)	Annealing Temperature ($^{\circ}C$)
PRMT1	<i>Rattus norvegicus</i>	Forward, 5'-TTGACTCCTATGCCCACT-3'	126	60
		Reverse, 5'-CCACATCCAGCACCACC-3'		
PRMT2	<i>Rattus norvegicus</i>	Forward, 5'-ATTACACCGCCACCACGAC-3'	216	60
		Reverse, 5'-GAAGTTTCAGAGTCCCATAGC-3'		
PRMT3	<i>Rattus norvegicus</i>	Forward, 5'-TGCTCCCTTCACATACCC-3'	234	60
		Reverse, 5'-CTGAAGTAGACGCCATCCT-3'		
PRMT4	<i>Rattus norvegicus</i>	Forward, 5'-ATGCCTACTGCCTACGACCT-3'	398	60
		Reverse, 5'-CGGGTGTTCACGCTTCTC-3'		
PRMT5	<i>Rattus norvegicus</i>	Forward, 5'-GCCATCCCAACCGAGA-3'	247	60
		Reverse, 5'-CGCCAGAAACGCACA-3'		
PRMT6	<i>Rattus norvegicus</i>	Forward, 5'-TATGTGGATTGGGACTGG-3'	100	60
		Reverse, 5'-TGAGGGAGGTAGGTGGG-3'		
Eotaxin-1	<i>Rattus norvegicus</i>	Forward, 5'-GAGAATATCGGCACCCATCCC-3'	214	60
		Reverse, 5'-AGTCTCCTATCATCCTCAGTTACC-3'		
CCR3	<i>Rattus norvegicus</i>	Forward, 5'-CTACTATCACCAGCATCATCACTTG-3'	207	60
		Reverse, 5'-AGCAGATAACCATAATGAGGAGAGG-3'		
TARC	<i>Rattus norvegicus</i>	Forward, 5'-GTGCTGCCTGGACTACTTCAAG-3'	109	56
		Reverse, 5'-CTGGACAGTCTCAACACGATGG-3'		
CCR4	<i>Rattus norvegicus</i>	Forward, 5'-GCACCAAGGAAGGCATCAAGG-3'	109	60
		Reverse, 5'-ACAGCACCAGAACCAACAG-3'		
IL-4	<i>Rattus norvegicus</i>	Forward, 5'-TGACCGAGATGTTTGTACCAGA-3'	92	63
		Reverse, 5'-TTGCGAAGCACCTGGAAG-3'		
PRMT1	<i>Homo sapiens</i>	Forward, 5'-TTGACTCCTATGCCCACT-3'	126	62
		Reverse, 5'-CCACATCCAGCACCACC-3'		
PRMT2	<i>Homo sapiens</i>	Forward, 5'-GCAGACCAGCCACGAACAAC-3'	254	62.8
		Reverse, 5'-AGCACCACATCCTCCACCTTC-3'		
PRMT3	<i>Homo sapiens</i>	Forward, 5'-GCCTTGGGAGAAAGAAGATATTG-3'	274	63
		Reverse, 5'-TGACGAGCAGTTCTGACATC-3'		
PRMT4	<i>Homo sapiens</i>	Forward, 5'-CGGCTCCCACTACACATCTCC-3'	103	65
		Reverse, 5'-GTCATAGGCGGTGCGCATCC-3'		
PRMT5	<i>Homo sapiens</i>	Forward, 5'-GCCATCCCAACCGAGA-3'	247	60
		Reverse, 5'-CGCCAGAAACGCACA-3'		
PRMT6	<i>Homo sapiens</i>	Forward, 5'-TGCTGTCCACCTCGCCTTTTC-3'	190	63
		Reverse, 5'-TTGGTCTTCTCCTCCTGGTCTCC-3'		
Eotaxin-1	<i>Homo sapiens</i>	Forward, 5'-TTCTGTGGCTGCTGCTCATAG-3'	125	63
		Reverse, 5'-GCTCTCTAGTCGTGAAGGG-3'		
CCR3	<i>Homo sapiens</i>	Forward, 5'-GGCAATGTGGTGGTGGTGATG-3'	113	62
		Reverse, 5'-GGAAGGGTGACGAGGAAGAGC-3'		
TARC	<i>Homo sapiens</i>	Forward, 5'-CCCTGGTCAACCTCCTCTTG-3'	229	62
		Reverse, 5'-GCATTCTTCACTCTTGTGTGTGG-3'		
CCR4	<i>Homo sapiens</i>	Forward, 5'-GAGAAGAAGAACAAGGCGGTGAAG-3'	204	62
		Reverse, 5'-GATGGGATTAAGGCAGCAGTGAAC-3'		
GAPDH	<i>Homo sapiens, Rattus norvegicus</i>	Forward, 5'-CGGCAAGTTCAACGCACAG-3'	148	65
		Reverse, 5'-GAAGACGCCAGTAGACTCCACGAC-3'		

PRMT1 and eotaxin 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 eotaxin-1 and its receptor was also determined in epithelial cells after the stimulation of IL-4. The results showed that the expressions of eotaxin-1 and CCR3 were upregulated after IL-4 stimulation. Meanwhile, the expression of eotaxin-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 eotaxin-1 expression in alveolar epithelial cells

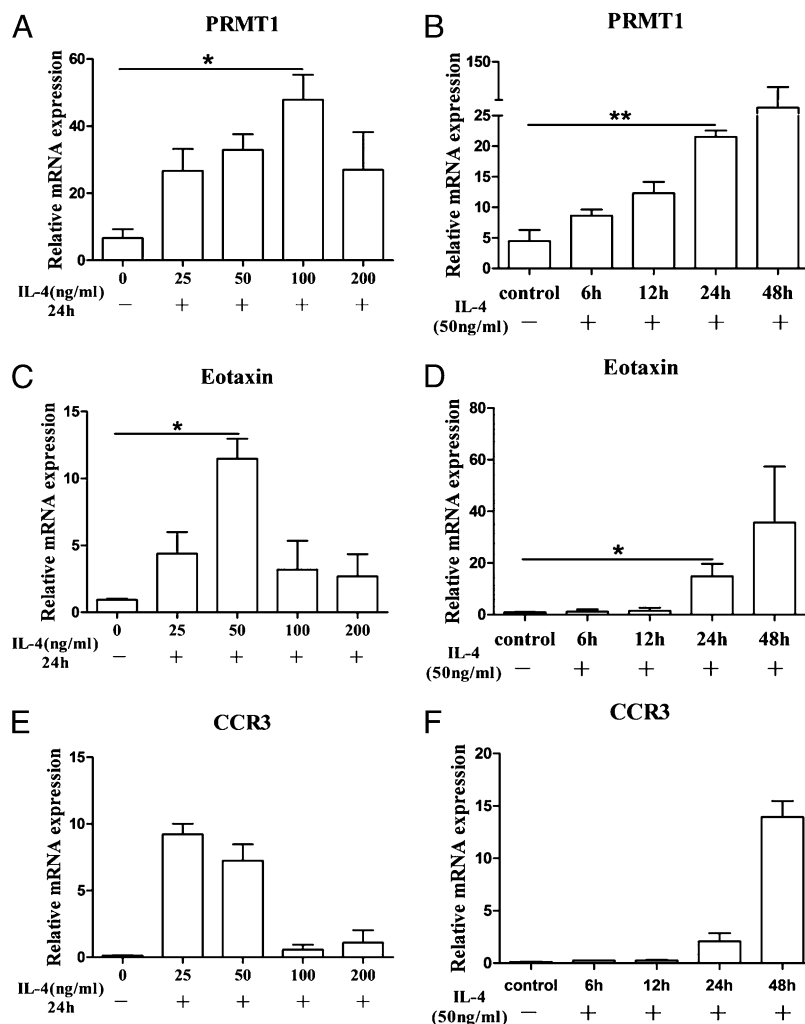
To explore the effects of PRMT function on the expression of eotaxin-1, AMI-1, the first inhibitor of PRMTs, was used to treat on A549 cells with and without IL-4 stimulation. The results with AMI-1 and IL-4 coinubation demonstrated a decrease in eotaxin-1 expression compared with the cells stimulated in the absence of PRMT inhibitor (Fig. 3A, 3B). Meanwhile, TNF- α had no influence on the expression of eotaxin-1 and CCR3 compared with control. The expressions of TARC and CCR4 did not show the same changes as eotaxin-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 eotaxin-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 eotaxin-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 eotaxin-1, CCR3 expression, and eosinophil infiltration in the rats with AIP1

To confirm whether upregulation of PRMTs plays an important role in the development of AIP1 in vivo, we administrated AMI-1 to AIP1 E3 rats. CCR3 and eotaxin-1 expressions increased in the AIP1 lung tissues compared with lungs from control group. After administration of AMI-1, the expression of CCR3 and eotaxin-1 decreased significantly in AIP1 rats (Fig. 4A, 4B). Additionally, eosinophil infiltration was abated in the BALF from AMI-1-administrated rats compared with control AIP1 rats (Fig. 4C). The results indicated that the inhibition of PRMT can downregulate eotaxin-1, CCR3 expressions, and eosinophil infiltration in AIP1 rats.

FIGURE 2. Relative expression of PRMT1, eotaxin-1, and CCR3 after the stimulation of IL-4 with different timings and doses on A549 cells. A549 cells were stimulated with IL-4 at a series of concentrations of 25, 50, 100, and 200 ng/ml, and PRMT1, eotaxin-1, and CCR3 mRNA expression was detected at 24 h after IL-4 stimulation (A, C, E). With 50 ng/ml IL-4 stimulation, PRMT1, eotaxin-1, and CCR3 mRNA expression was measured at 0, 6, 12, 24, and 48 h after the stimulation (B, D, F). The results are shown from a representative of three different experiments. All the expressions were determined by RT-qPCR analysis, and GAPDH expression was used to normalize the expression level. Statistical analysis was performed using one-way ANOVA for comparison. * $p < 0.05$, ** $p < 0.01$.



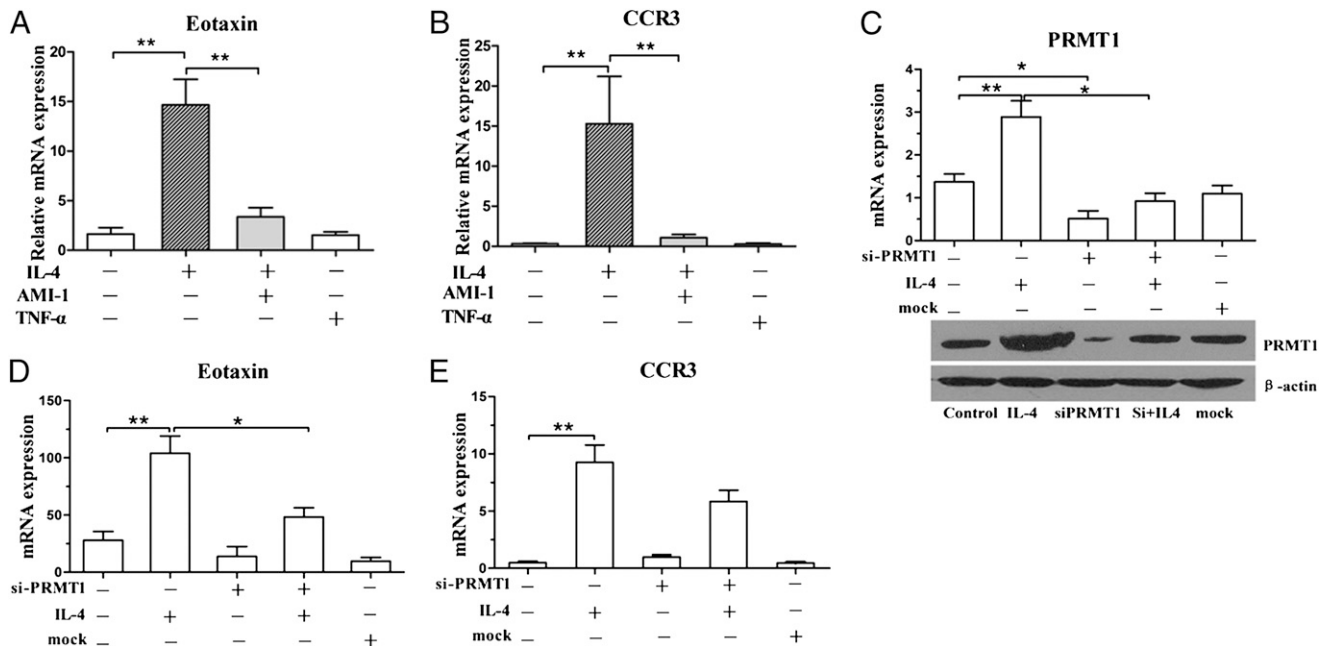


FIGURE 3. The effects of IL-4, AMI-1 (PRMT inhibitor), TNF- α , and siPRMT1 on the expression of PRMT1, eotaxin-1, and CCR3 on A549 cells. The eotaxin-1 and CCR3 expression was determined after 24-h stimulation with 50 ng/ml IL-4, 5 μ g/ml AMI-1, and 100 ng/ml TNF- α (A, B). Epithelial cells were transfected with 50 nM siPRMT1 or mock. IL-4 was added to stimulate the cells. The mRNA and protein expression of PRMT1 and the expression of eotaxin-1 and ccr3 were detected after siPRMT1 and mock transfection with or without IL-4 stimulation (C, D, E). The results are shown from a representative of three different experiments. All the expressions were determined by RT-qPCR analysis, and GAPDH expression was used to normalize the expression level. The results were expressed as means $\pm$ SEM; * p < 0.05 and ** p < 0.01, between indicated groups after Mann-Whitney test (n = 3).

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.

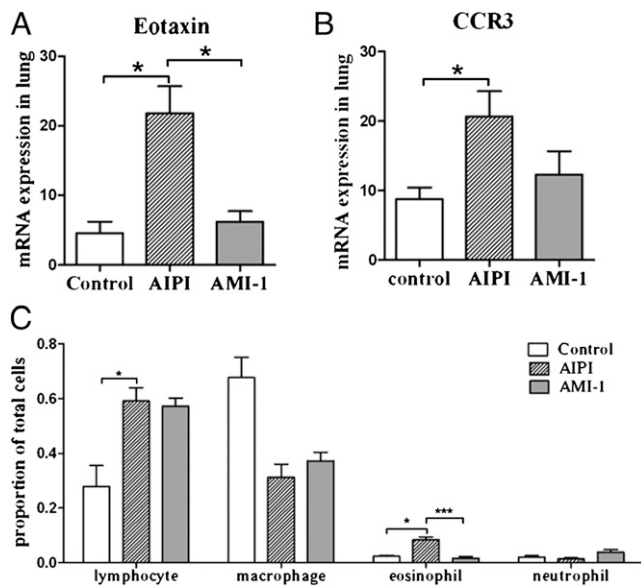


FIGURE 4. Eotaxin-1 and CCR3 expressions and proportion of inflammatory cells in BALF from AIPi rats with and without AMI-1 administration. The expressions of eotaxin-1 (A) and CCR3 (B) were detected by RT-qPCR in lung tissues from control rats, AIPi rats, and AIPi rats with administration of AMI-1. The proportion of lymphocyte, macrophage, eosinophil, and neutrophil in the BALF was numerated by relying on differentiated morphology after Wright-Giemsa staining (C). The results were expressed as means $\pm$ SEM; * p < 0.05, ** p < 0.01, and *** p < 0.001, between control and AIPi groups, and AIPi and AMI-1 groups after Mann-Whitney test (n = 8 for each group).

Discussion

In this study, we observed an upregulated expression of PRMTs in AIPi, a canonical animal model for asthma. Particularly, up-regulated 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

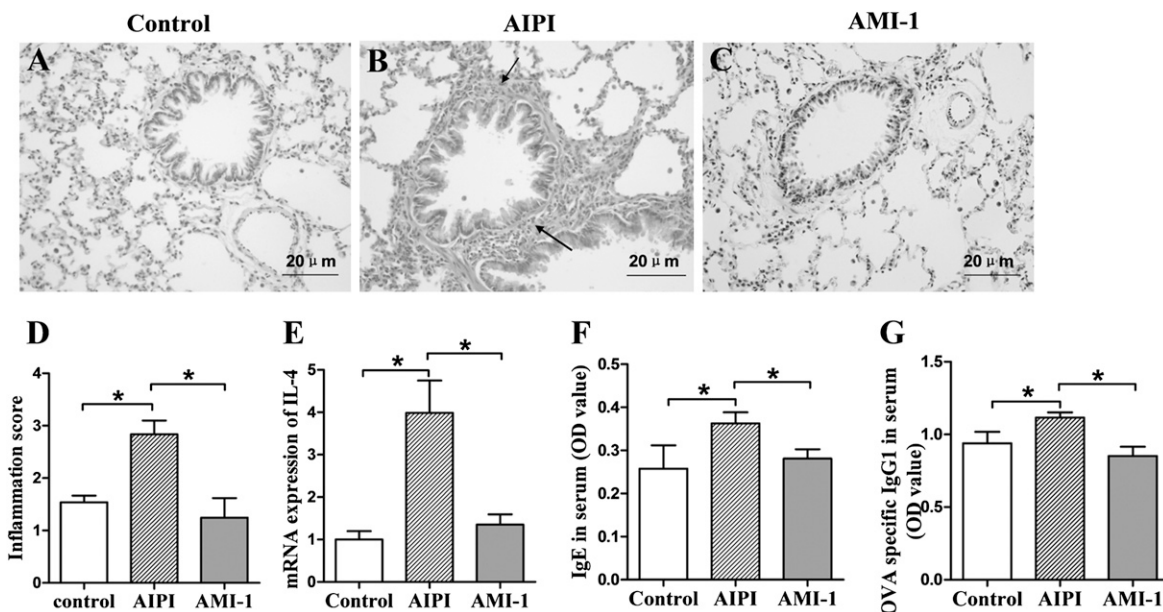


FIGURE 5. Histopathological changes and inflammatory reactions in AIPI rats with and without the administration of AMI-1. Representative images of the histopathological changes were from lung sections of E3 rats without Ag challenge (A), with Ag challenge (B), and with both Ag challenge and AMI-1 administration (C), respectively. The arrows indicate the eosinophil infiltration around bronchus. Scoring of inflammatory infiltration in the lung tissues was performed, as described in *Materials and Methods* (D). The expression of IL-4 was determined by RT-qPCR (E), and the total IgE and OVA-specific IgG1 concentrations in serum were detected by ELISA methods (F, G). The results were expressed as means $\pm$ SEM; * p < 0.05, between control and AIPI groups, and AIPI and AMI-1 groups after Mann-Whitney test (n = 8 for each group).

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 PMRT1 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- κ B, 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- κ B, 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

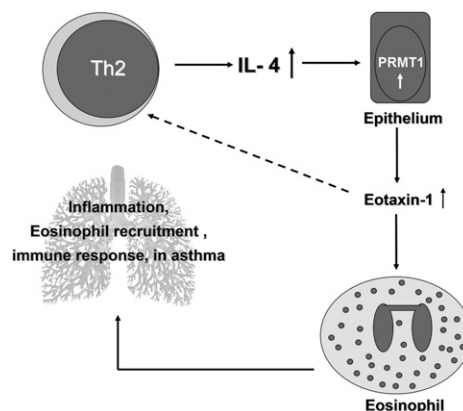


FIGURE 6. The schematic diagram of PRMT1 roles in pathogenesis of AIPI. In AIPI, IL-4 produced by Th2 cells induces intracellular PRMT1 production and activation in epithelial cells. Overexpression of eotaxin-1 regulated by PRMT1 in epithelial cells expedites eosinophil recruitment into the lungs and also increases IL-4 production of Th2 cells in a positive feedback style. As a consequence, the pulmonary inflammation occurs or deteriorates. Therefore, PRMT1 may play a crucial role in the pathogenetic pathway of asthmatic diseases.

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 vitro experiments as yet (11, 17), and no in vivo experiment has been reported. After the AAPI rats were given AMI-1 treatment, we found that eosinophil 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 not 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 AAPI 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 AAPI 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.

Acknowledgments

We are grateful to Yan Han, Qilan Ning, and Fujun Zhang for expert assistance and Liesu Meng and Wenhua Zhu for helpful discussions and productive critiques.

Disclosures

The authors have no financial conflicts of interest.

References

- Kay, A. B. 2005. The role of eosinophils in the pathogenesis of asthma. *Trends Mol. Med.* 11: 148–152.
- Kay, A. B. 1991. Asthma and inflammation. *J. Allergy Clin. Immunol.* 87: 893–910.
- Janson, C., and M. Heral. 1992. Blood eosinophil count as risk factor for relapse in acute asthma. *Respir. Med.* 86: 101–104.
- Conroy, D. M., and T. J. Williams. 2001. Eotaxin and the attraction of eosinophils to the asthmatic lung. *Respir. Res.* 2: 150–156.
- Richter, A., S. M. Puddicombe, J. L. Lordan, F. Bucchieri, S. J. Wilson, R. Djukanovic, G. Dent, S. T. Holgate, and D. E. Davies. 2001. The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am. J. Respir. Cell Mol. Biol.* 25: 385–391.
- Banwell, M. E., N. S. Tolley, T. J. Williams, and T. J. Mitchell. 2002. Regulation of human eotaxin-3/CCL26 expression: modulation by cytokines and glucocorticoids. *Cytokine* 17: 317–323.
- Fallon, P. G., H. E. Jolin, P. Smith, C. L. Emson, M. J. Townsend, R. Fallon, P. Smith, and A. N. J. McKenzie. 2002. IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity* 17: 7–17.
- Sun, Q. Z., F. F. Jiao, X. D. Yang, B. Zhong, M. H. Jiang, G. L. Li, B. Lu, Y. Han, Q. L. Ning, F. J. Zhang, et al. 2010. Expression of protein arginine N-methyltransferases in E3 rat models of acute asthma. *Journal of Southern Medical University* 30: 716–719.
- Kakimoto, Y. 1971. Methylation of arginine and lysine residues of cerebral proteins. *Biochim. Biophys. Acta* 243: 31–37.
- Bedford, M. T., and S. Richard. 2005. Arginine methylation an emerging regulator of protein function. *Mol. Cell* 18: 263–272.
- Huang, S. M., M. Litt, and G. Felsenfeld. 2005. Methylation of histone H4 by arginine methyltransferase PRMT1 is essential in vivo for many subsequent histone modifications. *Genes Dev.* 19: 1885–1893.
- Strahl, B. D., S. D. Briggs, C. J. Brame, J. A. Caldwell, S. S. Koh, H. Ma, R. G. Cook, J. Shabanowitz, D. F. Hunt, M. R. Stallcup, and C. D. Allis. 2001. Methylation of histone H4 at arginine 3 occurs in vivo and is mediated by the nuclear receptor coactivator PRMT1. *Curr. Biol.* 11: 996–1000.
- Wang, H. B., Z. Q. Huang, L. Xia, Q. Feng, H. Erdjument-Bromage, B. D. Strahl, S. D. Briggs, C. D. Allis, J. M. Wong, P. Tempst, and Y. Zhang. 2001. Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science* 293: 853–857.
- Mowen, K. A., B. T. Schurter, J. W. Fathman, M. David, and L. H. Glimcher. 2004. Arginine methylation of NIP45 modulates cytokine gene expression in effector T lymphocytes. *Mol. Cell* 15: 559–571.
- Parry, R. V., and S. G. Ward. 2010. Protein arginine methylation: a new handle on T lymphocytes? *Trends Immunol.* 31: 164–169.
- Yang, X. D., Q. Z. Sun, M. B. R. Asim, X. G. Jiang, B. Zhong, M. Shahzad, F. J. Zhang, Y. Han, and S. M. Lu. 2010. Nitric oxide in both bronchoalveolar lavage fluid and serum is associated with pathogenesis and severity of antigen-induced pulmonary inflammation in rats. *J. Asthma* 47: 135–144.
- Cheng, D. H., N. Yadav, R. W. King, M. S. Swanson, E. J. Weinstein, and M. T. Bedford. 2004. Small molecule regulators of protein arginine methyltransferases. *J. Biol. Chem.* 279: 23892–23899.
- Sun, Q. X., Y. Yang, M. B. Asim, F. Jiao, X. He, B. Zhong, D. Li, and S. Lu. 2011. Different challenge terms determine disease patterns of antigen-induced pulmonary inflammation in E3 rats. *APMIS* 119: 229–238.
- Chen, Y., X. Xu, M. Sheng, X. Zhang, Q. Gu, and Z. Zheng. 2009. PRMT-1 and DDAHs-induced ADMA upregulation is involved in ROS- and RAS-mediated diabetic retinopathy. *Exp. Eye Res.* 89: 1028–1034.
- Pope, A. J., K. Karupiah, and A. J. Cardouel. 2009. Role of the PRMT-DAH-ADMA axis in the regulation of endothelial nitric oxide production. *Pharmacol. Res.* 60: 461–465.
- Liu, H., X. Qu, Z. Liang, W. Chen, W. Xia, and Y. Song. 2008. Variance of DDAH/PRMT/ADMA pathway in atrial fibrillation dogs. *Biochem. Biophys. Res. Commun.* 377: 884–888.
- Zakrzewicz, D., and O. Eickelberg. 2009. From arginine methylation to ADMA: a novel mechanism with therapeutic potential in chronic lung diseases. *BMC Pulm. Med.* 9: 5.
- Lee, D. Y., C. Teyssier, B. D. Strahl, and M. R. Stallcup. 2005. Role of protein methylation in regulation of transcription. *Endocr. Rev.* 26: 147–170.
- Glimcher, L. H., and K. M. Murphy. 2000. Lineage commitment in the immune system: the T helper lymphocyte grows up. *Genes Dev.* 14: 1693–1711.
- Gangur, V., and J. J. Oppenheim. 2000. Are chemokines essential or secondary participants in allergic responses? *Ann. Allergy Asthma Immunol.* 84: 569–579, quiz 579–581.
- Nicholson, T. B., T. Chen, and S. Richard. 2009. The physiological and pathophysiological role of PRMT1-mediated protein arginine methylation. *Pharmacol. Res.* 60: 466–474.
- Bonham, K., S. Hemmers, Y. H. Lim, D. M. Hill, M. G. Finn, and K. A. Mowen. 2010. Effects of a novel arginine methyltransferase inhibitor on T-helper cell cytokine production. *FEBS J.* 277: 2096–2108.
- Webb, D. C., A. N. J. McKenzie, A. M. L. Koskinen, M. Yang, J. Mattes, and P. S. Foster. 2000. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperreactivity. *J. Immunol.* 165: 108–113.
- Dabbagh, K., K. Takeyama, H. M. Lee, I. F. Ueki, J. A. Lausier, and J. A. Nadel. 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *J. Immunol.* 162: 6233–6237.
- Nelms, K., A. D. Keegan, J. Zamorano, J. J. Ryan, and W. E. Paul. 1999. The IL-4 receptor: signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* 17: 701–738.
- Perkins, C., N. Yanase, G. Smulian, L. Gildea, T. Orekov, C. Potter, F. Brombacher, B. Aronow, M. Wills-Karp, and F. D. Finkelman. 2011. Selective stimulation of IL-4 receptor on smooth muscle induces airway hyper-responsiveness in mice. *J. Exp. Med.* 208: 853–867.
- White, S. R., L. D. Martin, R. Stern, B. Laxman, and B. A. Marroquin. 2010. Expression of IL-4/IL-13 receptors in differentiating human airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 299: L681–L693.
- Igarashi, H., K. Kuwahara, M. Yoshida, Y. Xing, K. Maeda, K. Nakajima, and N. Sakaguchi. 2009. GANP suppresses the arginine methyltransferase PRMT5 regulating IL-4-mediated STAT6-signaling to IgE production in B cells. *Mol. Immunol.* 46: 1031–1041.
- Mowen, K. A., J. Tang, W. Zhu, B. T. Schurter, K. Shuai, H. R. Herschman, and M. David. 2001. Arginine methylation of STAT1 modulates IFN α /beta-induced transcription. *Cell* 104: 731–741.
- Komyod, W., U. M. Bauer, P. C. Heinrich, S. Haan, and I. Behrmann. 2005. Are STATs arginine-methylated? *J. Biol. Chem.* 280: 21700–21705.
- Matsukura, S., C. Stellato, S. N. Georas, V. Casolaro, J. R. Plitt, K. Miura, S. Kurosawa, U. Schindler, and R. P. Schleimer. 2001. Interleukin-13 upregulates eotaxin expression in airway epithelial cells by a STAT6-dependent mechanism. *Am. J. Respir. Cell Mol. Biol.* 24: 755–761.
- Rothenberg, M. E., J. A. MacLean, E. Pearlman, A. D. Luster, and P. Leder. 1997. Targeted disruption of the chemokine eotaxin partially reduces antigen-induced tissue eosinophilia. *J. Exp. Med.* 185: 785–790.
- Dixon, H., C. Blanchard, M. L. Deschoolmeester, N. C. Yuill, J. W. Christie, M. E. Rothenberg, and K. J. Elise. 2006. The role of Th2 cytokines, chemokines and parasite products in eosinophil recruitment to the gastrointestinal mucosa during helminth infection. *Eur. J. Immunol.* 36: 1753–1763.
- Rankin, S. M., D. M. Conroy, and T. J. Williams. 2000. Eotaxin and eosinophil recruitment: implications for human disease. *Mol. Med. Today* 6: 20–27.
- Kodali, R. B., W. J. Kim, I. I. Galaria, C. Miller, A. D. Schecter, S. A. Lira, and M. B. Taubman. 2004. CCL11 (Eotaxin) induces CCR3-dependent smooth muscle cell migration. *Arterioscler. Thromb. Vasc. Biol.* 24: 1211–1216.

Supplementary figure legends

Figure S1. Relative expression of PRMTs after the stimulation of IL-4 or TNF α on epithelial cells. A549 cells were stimulated with IL4 at a series of concentrations of 25, 50, 100, 200 ng/ml, and PRMTs mRNA expression was detected at 24 hours after IL4 stimulation (A). With 50ng/ml IL-4 stimulation, PRMTs mRNA expression was measured at 0, 6, 12, 24, and 48 hours after the stimulation (B). The Th1 cytokine, TNF α was used to stimulate A549 on the concentration of 100ng/ml for 24h or 48h to detect PRMTs expressions (C). The results are shown from a representative of three different experiments. All the expressions were determined by RT-qPCR analysis and GAPDH expression was used to normalize the expression level.

Figure S2. The effects IL-4, AMI-1 (PRMT inhibitor), TNF- α on the expression of TARC and CCR4 on A549 cells, and the detection of effective sequence of siPRMT1. The TARC and CCR4 expressions were determined after the 24h stimulation with 50 ng/ml IL-4, 5 μ g/ml AMI-1 and 100ng/ml TNF- α (A, B). The company supported 3 different sequences to knockdown the PRMT1 expression, and the most efficacious sequence was identified by qRT-PCR (C). The results are shown from a representative of three different experiments. All the expressions were determined by RT-qPCR analysis and GAPDH expression was used to normalize the expression level. * represents $P < 0.05$ between indicated groups after Mann-Witney test (n=3).

Figure S3. Total cells in BALF, DTH, concentration of NO in serum and the

expression of TGF- β from AIPI rats with and without AMI-1 administration. Total cell numbers were determined by using a hemocytometer (A). The weight difference between two ear pieces represented the magnitude of DTH (B). NO concentration in the serum was measured by the Griess method (C). The expression of TGF- β of lung tissues was detected by RT-qPCR (D). The results were expressed as means $\pm$ S.E.M. *, ** and *** represent $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively between control and AIPI groups, and AIPI and AMI-1 groups after Mann-Witney test (n=8 for each group).

