



Turn complexity into clarity
with Guava® multichannel, configurable flow cytometers.

EMD Millipore Corp. is a subsidiary of Merck KGaA, Darmstadt, Germany.



Guava easyCyte™ Flow Cytometers

[Request Demo](#)



*The Journal of
Immunology*

This information is current as
of June 7, 2015.

PRMT1 Upregulated by Epithelial Proinflammatory Cytokines Participates in COX2 Expression in Fibroblasts and Chronic Antigen-Induced Pulmonary Inflammation

Qingzhu Sun, Li Liu, Michael Roth, Jia Tian, Qirui He, Bo
Zhong, Ruanjuan Bao, Xi Lan, Congshan Jiang, Jian Sun,
Xudong Yang and Shemin Lu

J Immunol published online 29 May 2015

<http://www.jimmunol.org/content/early/2015/05/29/jimmunol.1402465>

-
- | | |
|----------------------|--|
| Subscriptions | Information about subscribing to <i>The Journal of Immunology</i> is online at:
http://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://jimmunol.org/cgi/alerts/etoc |

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
Copyright © 2015 by The American Association of
Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.



PRMT1 Upregulated by Epithelial Proinflammatory Cytokines Participates in COX2 Expression in Fibroblasts and Chronic Antigen-Induced Pulmonary Inflammation

Qingzhu Sun,^{*,†,‡,1} Li Liu,^{*,†,1} Michael Roth,[‡] Jia Tian,^{*,†} Qirui He,^{*,†} Bo Zhong,^{*,†} Ruanjuan Bao,^{*,†} Xi Lan,^{*,†} Congshan Jiang,^{*,†} Jian Sun,^{*,†} Xudong Yang,^{*,†} and Shemin Lu^{*,†,§}

Protein arginine methyltransferase (PRMT)1, methylating both histones and key cellular proteins, has emerged as a key regulator of various cellular processes. This study aimed to identify the mechanism that regulates PRMT1 in chronic Ag-induced pulmonary inflammation (AIPI) in the E3 rat asthma model. E3 rats were challenged with OVA for 1 or 8 wk to induce acute or chronic AIPI. Expression of mRNAs was detected by real-time quantitative PCR. PRMT1, TGF- β , COX2, and vascular endothelial growth factor protein expression in lung tissues was determined by immunohistochemistry staining and Western blotting. In the *in vitro* study, IL-4-stimulated lung epithelial cell (A549) medium (ISEM) with or without anti-TGF- β Ab was applied to human fibroblasts from lung (HFL1). The proliferation of HFL1 was determined by MTT. AMI-1 (pan-PRMT inhibitor) was administered intranasally to chronic AIPI rats to determine PRMT effects on asthmatic parameters. In lung tissue sections, PRMT1 expression was significantly upregulated, mainly in epithelial cells, in acute AIPI lungs, whereas it was significantly upregulated mainly in fibroblasts in chronic AIPI lungs. The *in vitro* study revealed that ISEM elevates PRMT1, COX2, and vascular endothelial growth factor expressions, and it promoted fibroblast proliferation. The application of anti-TGF- β Ab suppressed COX2 upregulation by ISEM. AMI-1 inhibited the expression of COX2 in TGF- β -stimulated cells. In the *in vivo* experiment, AMI-1 administered to AIPI rats reduced COX2 production and humoral immune response, and it abrogated mucus secretion and collagen generation. These findings suggested that TGF- β -induced PRMT1 expression participates in fibroblast proliferation and chronic airway inflammation in AIPI. *The Journal of Immunology*, 2015, 195: 000–000.

Asthma is a heterogeneous and multifactorial disorder characterized by recurrent episodes of airflow obstruction. The histopathology features of asthma include the dysfunction of the bronchial epithelium, exaggerated bronchoconstrictor response to a wide variety of stimuli (airway hyperresponsiveness),

chronic airway inflammation, and structural changes of the bronchial wall (1).

Protein arginine methylation is performed by a class of enzymes called protein arginine methyltransferases (PRMTs) and is a novel posttranslational protein modification that plays a pivotal role in various intracellular events, such as signal transduction, protein–protein interaction, and transcriptional regulation. PRMT mediates its function either through direct regulation of protein–protein interaction or by arginine methylation, both of which influence NO-dependent processes. A growing body of evidence suggests that both mechanisms are implicated in cardiovascular and pulmonary diseases, including lung cancer, pulmonary fibrosis, pulmonary hypertension, chronic obstructive pulmonary disease, and asthma (2, 3). In our previous study, we have elucidated that IL-4 upregulated PRMT1 in airway epithelial cells, thereby increasing eotaxin-1 expression. Furthermore, pulmonary inflammation in rats waned after inhibition of PRMT activity by AMI-1 (3). However, the distinct regulation of PRMT1 in acute Ag-induced pulmonary inflammation (AIPI) and in chronic AIPI was not investigated.

Fibroblasts, as a major source of interstitial connective tissue extracellular matrix, contribute to the fibrotic changes in asthmatic airways and in Th2 cell-driven inflammation. Various elements of the innate and adaptive immune response participate in the differentiation and activation of subepithelial bronchial fibroblasts in asthma (4). Airway remodeling and inflammation persist in asthma, and the consequences are inappropriate airway function. Damaged bronchial epithelium tissue repairs incompletely and leads to chronic wound repair with increased secretion of a range of secondary growth factors and cytokines that drive fibroblast activation and airway remodeling (5).

^{*}Department of Biochemistry and Molecular Biology, School of Basic Sciences, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi 710061, People's Republic of China; [†]Key Laboratory of Environment and Genes Related to Diseases, Xi'an Jiaotong University, Ministry of Education, Xi'an, Shaanxi 710061, People's Republic of China; [‡]University Hospital Basel, University of Basel, 4031 Basel, Switzerland; and [§]Department of Epidemiology and Health Statistics, School of Public Health, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi 710061, People's Republic of China

¹Q.S. and L.L. contributed equally to this work.

Received for publication October 2, 2014. Accepted for publication May 4, 2015.

This work was supported by National Natural Science Foundation of China Grants 81170017, 81370123, and 81371986, Fundamental Research Funds for the Central Universities Grant DWYXc111000117, and by China Postdoctoral Funds Grant 2012M521754.

Q.S., X.Y., and S.L. conceived and designed the experiments; Q.S. and L.L. performed all experiments, analyzed data, and contributed to the writing of the manuscript; M.R. guided the revision experiments; J.T., Q.H., C.J., R.B., X.L., and J.S. performed part of the experiments and data generation; B.Z. analyzed data and contributed to the writing of the manuscript; and Q.S., X.Y., and S.L. had extensive scientific discussions for this study and wrote the paper. All authors read and approved the final manuscript.

Address correspondence and reprint requests to Prof. Shemin Lu and Dr. Xudong Yang, Department of Biochemistry and Molecular Biology, School of Basic Sciences, Xi'an Jiaotong University Health Science Center, Xi'an, Shaanxi 710061, People's Republic of China. E-mail address: shemin.lu@gmail.com (S.L.) and ewsr1@163.com (X.Y.)

Abbreviations used in this article: AIPI, Ag-induced pulmonary inflammation; HFL, human fibroblast from lung; ISEM, IL-4-stimulated lung epithelial cell (A549) medium; PAS, periodic acid–Schiff; PRMT, protein arginine methyltransferase; RT-qPCR, real-time quantitative PCR; VEGF, vascular endothelial growth factor.

Copyright © 2015 by The American Association of Immunologists, Inc. 0022-1767/15/\$25.00

The most prominent factors regulating fibroblast function include epithelial-derived growth factor, TGF- β , platelet-derived growth factor, basic fibroblast growth factor, and endothelin, several of which are capable of inducing subepithelial fibroblast proliferation, differentiation, and activation of myofibroblasts. A recent study showed increased levels of TGF- β and asymmetric ADMA, a main PRMT metabolite, in fibrotic tissues in diabetic rats (6). ADMA, as an endothelial NO synthase and inducible NO synthase inhibitor, is involved in proline metabolism and may play an important role in pulmonary fibrosis during airway remodeling (7). In an earlier study we examined PRMT expression in rats with different stages of AIPi. In these animals, PRMT1 was highly expressed in the tissue surrounding the airway, mainly in fibroblasts and airway smooth muscle cells, whereas it was expressed in the bronchial epithelium of rats with acute AIPi. Therefore, the shift of PRMT1 expression from the epithelium to subepithelial mesenchymal cells may be a key event in lung remodeling in chronic AIPi. Consequently, PRMT may be involved in chronic airway remodeling through its function in fibroblasts.

Although the functional characteristics of asthmatic fibroblasts and their interaction with epithelial inflammation on airway wall remodeling is a consistent pathology observation in chronic asthma, very few studies have investigated the role of PRMT1 on fibroblast function during airway remodeling. In this study, we measured the expression and location of PRMT1 in acute and chronic AIPi in E3 rats. Moreover, we studied the effect of IL-4-activated epithelial cells on the proliferation and cytokine production by fibroblasts. Additionally, the inhibition of PRMT1 by AMI-1 was used to assess the role of PRMT in several asthma and airway remodeling parameters. We conclude that IL-4 stimulates epithelial cells, which, in turn, upregulate PRMT1 in subepithelial fibroblasts, thus contributing to the pathogenesis of chronic inflammation in asthma.

Materials and Methods

Induction of AIPi and administration of AMI-1 in E3 rats

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

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 of PRMT1 expression in lungs, 24 rats were divided into a control group, an acute AIPi group (challenged with OVA every day for 1 wk), and a chronic AIPi group (challenged with OVA every other day for 8 wk).

For the AMI-1 treatment experiment, 24 rats were divided into three groups: control group, chronic AIPi group, and AMI-1 group. Two weeks after sensitization, control group rats were sham sensitized and exposed to the same volume of PBS. In the AMI-1 group, rats were administered 50 μ l AMI-1 (Calbiochem, San Diego, CA) at a concentration of 0.1 mg/ml in PBS 2 h before OVA challenge. The asthma index included serum levels of OVA-specific IgG1 and total serum IgE, which were determined by ELISA as described in previous studies (3, 8).

RNA quantitations

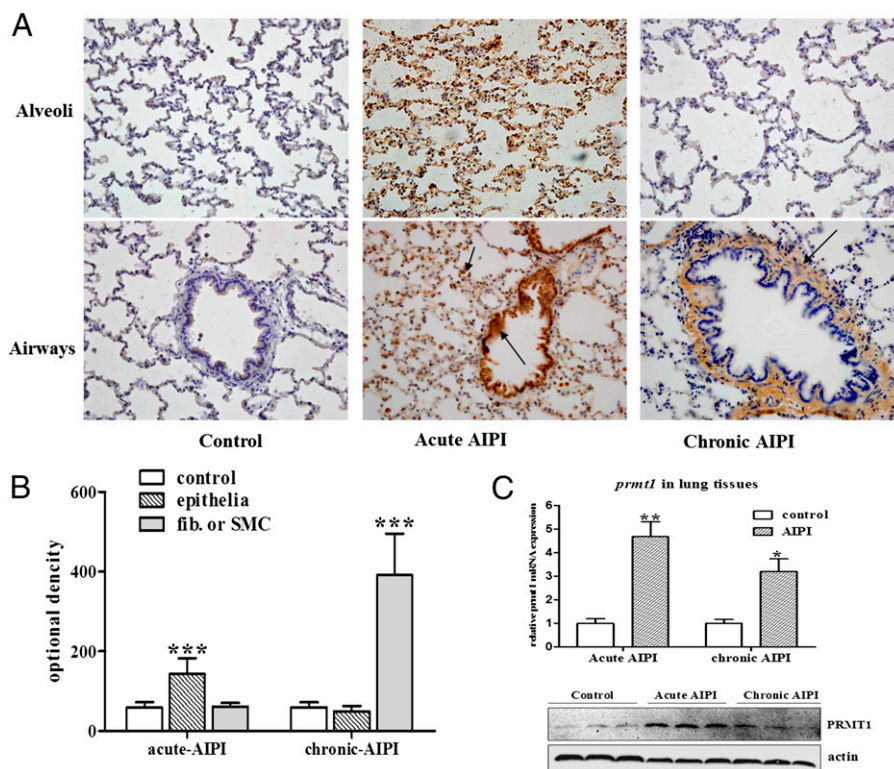
The mRNA expression of PRMT1 and cytokine genes was analyzed by real-time quantitative PCR (RT-qPCR), performed by using a iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) with SYBR Premix Ex Taq II (TaKaRa Bio, Shiga, Japan). The relative gene expression was normalized by GAPDH expression. The information for all primers used in this study is shown in Table I.

Lung histology and immunohistochemistry staining

Lung tissues were stained with H&E for pathological changes, with periodic acid–Schiff (PAS) to detect mucus production, and with Masson staining to determine collagen fibers as previously described (8).

Immunohistological staining tissue sections were incubated with 100-fold diluted anti-PRMT1 Ab or anti-TGF- β Ab (Abcam, Cambridge, U.K.) in blocking solution at 4°C overnight, followed by a two-step plus poly-HRP anti-goat IgG detection kit (ZSGB-Bio, Beijing, China). The intensity of the brown color was determined by using Image-Pro Plus 6.0 software to estimate protein expression in lung tissues.

FIGURE 1. Expression shift of PRMT1 from airway epithelia to fibroblasts in acute AIPi and chronic AIPi. PRMT1 expression of lung tissue (**A**) from E3 rats with AIPi was detected by immunohistological staining. Representative images of PRMT1 protein expression in bronchus and alveolus of control rat lungs (*left panel*), acute (*middle panel*), and chronic AIPi rat lungs (*right panel*) were stained with anti-PRMT1 Ab. Original magnification $\times 200$. Mean density of PRMT1 (**B**) was determined by Image-Pro Plus 6.0 software to estimate the expression of PRMT1 protein in epithelium and subepithelium. The mRNA and protein expression of PRMT1 in lung tissues (**C**) were detected by RT-qPCR and Western blotting. The results were expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between AIPi group (acute or chronic) and control group after Mann–Whitney test ($n = 8$ for each group).



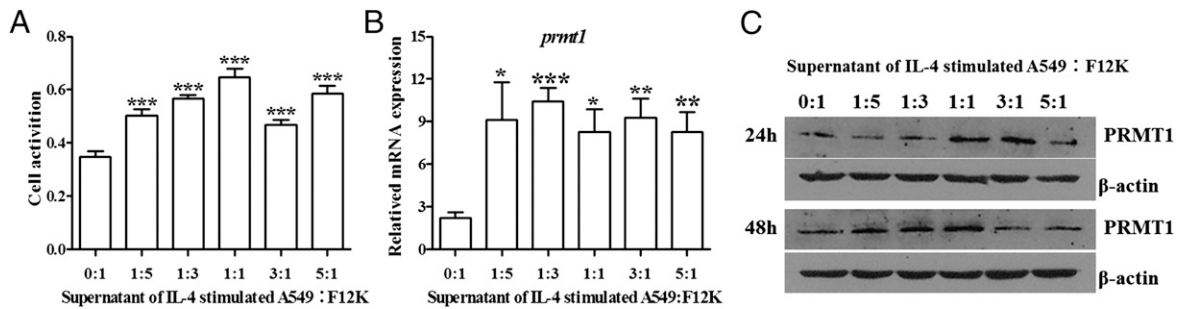


FIGURE 2. Cell activation and PRMT1 expression in fibroblasts after inflammatory epithelium medium stimulation. **(A)** ISEM was collected after 48 h stimulation with IL-4 at the concentration of 100 ng/ml and used to stimulate HFL1 cells at a series of ratios with F12K basic medium (0:1, 1:5, 1:3, 1:1, 3:1, 5:1). The HFL1 proliferation was detected by the MTT method after stimulation with a series of ratios of IL-4-stimulated medium to basic medium for 48 h **(B)**. The expression of PRMT1 in HFL1 cells was determined by RT-qPCR after 48 h stimulation by a series of ratios of IL-4-stimulated medium to basic medium **(C)**. The protein level of PRMT1 was detected by Western blotting after 24 and 48 h stimulation. The results are shown from a representative of three independent experiments. The expression of PRMT1 was determined by RT-qPCR analysis, and GAPDH expression was used to normalize the expression level. The results were expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between indicated groups and control group after Mann-Whitney test.

Cell culture and proliferation analysis

Human fibroblast from lung (HFL1) cell and human A549 alveolar epithelial-like cells (A549) were grown in F12K or RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT). Human rIL-4 (Boster, Wuhan, China) was added into the wells (six-well plates) at 100 ng/ml. The culture supernatant of IL-4-stimulated A549 cell was accumulated into a 15-ml EP tube and centrifuged for 10 min to remove cells and cell debris. FBS (5%) was added to the culture supernatant to replenish the consumption by epithelial cells, and then the IL-4-stimulated epithelial medium was prepared and assigned as IL-4-stimulated lung epithelial cell (A549) medium (ISEM). The ISEM was stored at 4°C for 2 d and at -20°C for 1 mo. The ISEM and basic F12K media were mixed at various ratios of 0:1, 1:5, 1:3, 1:1, 3:1, and 5:1 to stimulate HFL1 cells. TGF- β cytokine (10 ng/ml; PeproTech, Rocky Hill, NJ) or 1 μ g/ml anti-TGF- β Ab (Abcam) was mixed with ISEM to investigate TGF- β effects on the regulation of PRMT1, COX2, and vascular endothelial growth factor (VEGF) expression.

MTT was used to determine HFL1 cell proliferation. HFL1 cells were seeded into 96-well plates at a density of 1×10^4 cells/100 μ l per well. Proliferation was determined 48 h after ISEM treatment by adding 50 μ l MTT reagent and incubating for 4 h at 37°C. After removal of the culture medium, cells were lysed with DMSO to determine the amount of formazan product (9), and the absorption was measured by a microplate reader (Thermo Fisher Scientific, Vantaa, Finland) at 550 nm.

Plasmid construction and transfection of pcDNA3.1-PRMT1

The full-length cDNA of PRMT1 was amplified from HFL1 cell mRNA by RT-PCR with primers (5'-CGGGATCCGATGGCGGCAGCCGAG-3' and 5'-CCCTCGAGGGTCAGCGCATCCGGTAGTCGG-3') and was cloned into a pcDNA3.1(+) vector to construct the pcDNA3.1-PRMT1 recombinant plasmid. With Lipofectamine 2000 (Invitrogen), the recombinant plasmid pcDNA3.1-PRMT1 was transiently transfected into HFL1 cells for 24 or 48 h. The expression of PRMT1, COX2, and VEGF in the HFL1 cells was measured by RT-qPCR.

Western blotting

HFL1 cells (2×10^5 /well) were seeded into six-well plates and grown for 12 h. ISEM and basic F12K medium were mixed at various ratios (0:1, 1:5, 1:3, 1:1, 3:1, 5:1) to stimulate HFL1 cells for 24 or 48 h, before lysis with RIPA buffer (Beyotime, Beijing, China). Cell lysates were centrifuged at 12,000 rpm (15 min) and the protein supernatant was kept. The protein concentration was quantified by the BCA method (Beyotime) and equal amounts of denatured proteins (20 μ g) were separated by SDS-PAGE and subsequently electrotransferred onto polyvinylidene difluoride membranes. The proteins of interest were detected with Abs specific to either PRMT1 (Abcam), COX2 (Cell Signaling Technology, Beverly, MA), or VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized after binding of a secondary Ab conjugated with HRP (Abcam) by ECL reagents (Pierce Biotechnology).

Statistical analysis

Data are expressed as mean $\pm$ SEM. The statistical analysis was performed by a Mann-Whitney U test for the comparison between groups. PRMT1, COX2, and remodeling related cytokine expressions were analyzed by one-way ANOVA for comparison. The null hypothesis was that no treatment had any effect, and a p value < 0.05 was taken as significant.

Results

PRMT1 is expressed in different cell types in different processes of AIPI

In our previous study, we found that in acute AIPI, PRMT1 is expressed only by epithelial cells of the airways and the alveoli. In this study, immunohistochemical staining was performed to visualize PRMT1 protein expression in lung tissue sections obtained from chronic AIPI. The results demonstrated that PRMT1 was expressed in the subepithelial airway structures, mainly in fibroblasts and

Table I. Information on primers for RT-qPCR

Gene	Species	Sequence (5'-3')	Size (bp)	Annealing Temperature (°C)
PRMT1	<i>Rattus norvegicus</i>	F: TTGACTCCTATGCCCACT R: CCACATCCAGCACCACC	126	60
PRMT1	<i>Homo sapiens</i>	F: TTGACTCCTATGCCCACT R: CCACATCCAGCACCACC	126	62
COX2	<i>H. sapiens, R. norvegicus</i>	F: GTGCAACACTTGAGTGGCTAT R: GCAATTTCCTGGTGAATGAT	249	60
VEGF	<i>H. sapiens, R. norvegicus</i>	F: CGGCGAAGAGAAGAGACACATTG R: CGGGAAGGAAGGAAGGAC	224	60
TGF- β	<i>H. sapiens</i>	F: GGACTACTACGCCAAGAAG R: TCAAAGACAGCCACTCAGG	294	63
GAPDH	<i>H. sapiens, R. norvegicus</i>	F: CGGCAAGTTCAACGGCACAG R: GAAGACGCCAGTAGACTCCACGAC	148	65

F, forward; R, reverse.

smooth muscle layers (Fig. 1A, 1B). The analysis of immunohistochemical staining showed that in acute AIPI, epithelial cells expressed PRMT1, and in chronic AIPI, increased PRMT1 was located in the subepithelial airway tissues. Importantly, PRMT1 mRNA and protein expression in acute AIPI and chronic AIPI significantly increased compared with tissue of control rats (Fig. 1C).

The ISEM of inflammatory epithelium induces PRMT1 expression in fibroblasts

To explore the different effects of PRMT1 function in acute AIPI and chronic AIPI, IL-4 (100 ng/ml) was used to stimulate HFL1 cells at the concentration of 100 ng/ml. Against our expectations, PRMT1 expression did not show a significant increase after IL-4 stimulation (data not shown). Considering that epithelial cell inflammation modifies subepithelial fibroblast function in chronic asthma, the supernatant of ISEM was collected after 48 h and then was diluted with F12K basic medium at various ratios (0:1, 1:5, 1:3, 1:1, 3:1, 5:1) to stimulate HFL1 cells. HFL1 cell proliferation increased significantly after stimulation with the mixed medium described

above for 48 h (Fig. 2A). The mRNA expression of PRMT1 (the information on the primers is shown in Table I) in HFL1 cells increased after 48 h of ISEM treatment (Fig. 2B). In parallel, the protein expression of PRMT1 was enhanced after 24 and 48 h of ISEM treatment (Fig. 2C). Additionally, both the supernatant from A549 and BEAS-2B, a normal lung epithelial cell line, had a similar effect on fibroblast proliferation and PRMT1 expression.

PRMT1 regulates the COX2 and VEGF expression in fibroblasts

To confirm that ISEM induces remodeling driving cytokines in fibroblasts, we determined the expressions of *cox2*, *veg*, *tgf- α* , *tgf- β* , *egfr*, and *b-fgf* mRNAs (data not shown). Additionally, the mRNAs (the information on the primers is shown in Table I) and proteins expression of COX2 and VEGF increased in HFL1 cells after the stimulation with ISEM (Fig. 3A–C). To explore the effects of PRMT1 on the expression of *cox2* and *veg*, a recombinant plasmid pcDNA3.1-PRMT1 and plasmid pcDNA3.1+ as mock were used to transfect HFL1 cells and grow them for 24 or

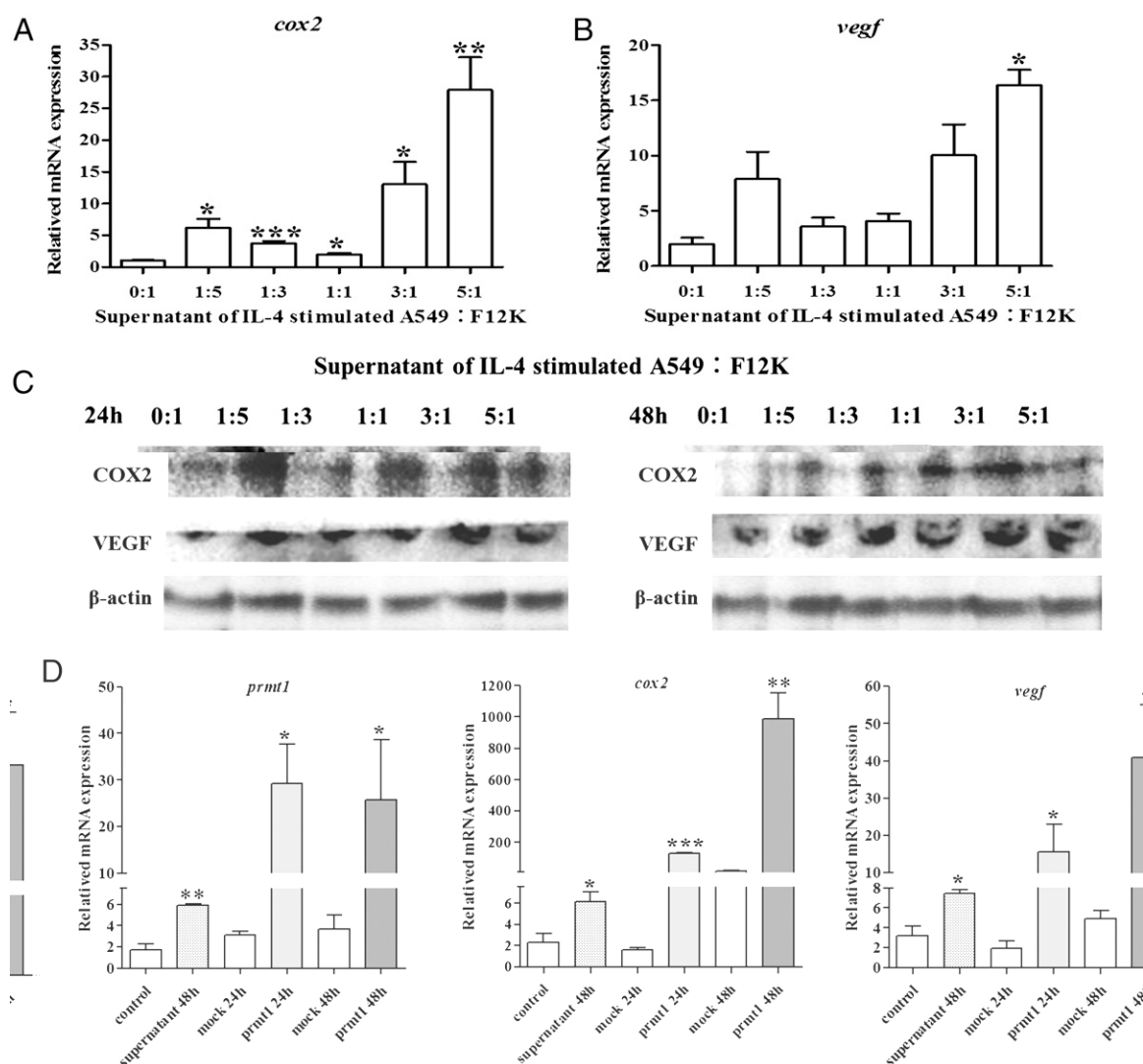


FIGURE 3. Expression of *cox2* and *veg* in fibroblasts after ISEM treatment and upregulation of PRMT1. HFL1 cells were stimulated by IL-4-stimulated epithelium medium and mixed with basic medium at a series of ratios. The mRNA expression of *cox2* and *veg* in HFL1 cells was determined by RT-qPCR after 48 h stimulation (A and B). The protein level of COX2 and VEGF were detected by Western blotting after 48h stimulation (C). The recombinant plasmid pcDNA3.1-PRMT1 or plasmid pcDNA3.1+ as mock was used to transfect HFL1 cells for 24 and 48 h, and HFL1 cells were stimulated by ISEM mixed with basic medium at a 1:1 ratio as positive control for 48 h. The expression of PRMT1, *cox2*, and *veg* in the HFL1 cells was measured by qRT-PCR (D). The results are shown from a representative of three different experiments. GAPDH expression was used to normalize the expression level. The results were expressed as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between indicated groups after Mann-Whitney test.

48 h. After the transfection of plasmid pcDNA3.1-PRMT1, the expression of PRMT1 was significantly upregulated in HFL1 cells; upregulated *cox2* and *veg* mRNA expressions were detected together with PRMT1 upregulation (Fig. 3D).

TGF- β in inflammatory epithelium culture supernatant induces PRMT1 and COX2 elevation

To identify which component contained in ISEM induced the PRMT1 and COX2 expression in fibroblasts, the immunohistology was performed in lung tissues from control, acute, and chronic AIPi rats. In the lung tissue of acute AIPi rats, TGF- β was mainly expressed in airway epithelial cells, whereas in the lung tissue of chronic AIPi rats, TGF- β was mainly expressed in subepithelial structure as smooth muscle cells and fibroblasts (Fig. 4A). Additionally, IL-4 was used to stimulate A549 epithelial cells for 3, 6, 12, 24, or 48 h. As shown in Fig. 4B, the TGF- β expression by epithelial cells was significantly increased by IL-4 stimulation. Then, different concentrations of TGF- β (1, 5, or 10 ng/ml) were used to stimulate HFL1 for 48 h and the expression of PRMT1 and COX2 was detected by Western blotting. The expression of PRMT1 and of COX2 was dose-dependently upregulated by TGF- β (5 or 10 ng/ml after 48 h), whereas the expression of VEGF was enhanced by all concentrations of TGF- β (1, 5, or 10 ng/ml) as depicted (Fig. 4C).

Consequently, human recombination TGF- β and a neutralizing anti-TGF- β Ab were mixed with ISEM and the expression of *prmt1*, *cox2*, and *veg* was determined (Fig. 5A–C). The data showed that the abrogation of TGF- β by a specific neutralizing Ab attenuated the expression of *prmt1*, *cox2*, and *veg* with ISEM stimulation, whereas supplementation of TGF- β increased expression of these genes. Meanwhile, the protein expressions of

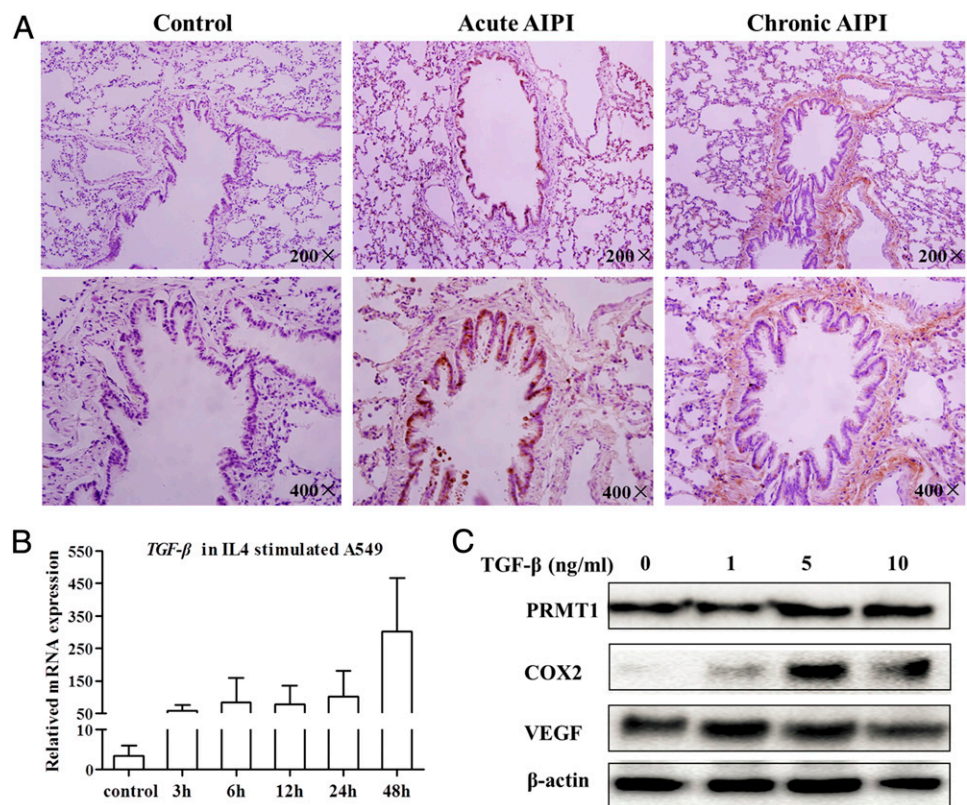
PRMT1 and COX2 were elevated by ISEM, which can be neutralized by anti-TGF- β Ab (Fig. 5D, 5E). Additionally, it is noteworthy that the anti-TGF- β Ab did not abrogate the increase of PRMT1 expression by ISEM completely, suggesting that additional components upregulate the expression of PRMT1. COX2 expression by HFL1 cells was also detected by Western blotting after stimulation with 5 ng/ml TGF- β with or without AMI-1 (5 and 10 μ M). The results showed that 10 μ M AMI-1 counteracted the stimulatory effect of TGF- β on COX2 expression (Fig. 5F).

Inhibition of PRMT ameliorates COX2 expression and asthmatic indexes in chronic AIPi of E3 rats

We determined the effects of AMI-1 in chronic AIPi rats. The mRNA expressions of *cox2* (Fig. 6A) and *veg* (Fig. 6B) were detected by RT-qPCR in lung tissues from control rats, chronic AIPi rats, and chronic AIPi rats with administration of AMI-1. *Cox2* expression in lung tissues returned to normal levels in the AMI-1-treated rats; however, the *veg* expression did not fully reverse with administration of AMI-1. Serum IgE concentrations (Fig. 6C) returned to baseline after AMI-1 administration, but in either case no difference in serum NO concentration was observed (Fig. 6D).

Next, histopathological analysis of airway remodeling in chronic AIPi rats with and without the administration of AMI-1 was performed. In the chronic AIPi group, the lesions of airways and alveoli were serious. Extensive pulmonary emphysema emerged, and alveolar ectasia, disintegration, and narrowing of alveolar septum were presented when compared with control rat lungs (Fig. 7A). The secretion of mucus by using PAS staining was obvious in chronic AIPi rats (Fig. 7B). Additionally, Masson staining indicated a significant increase of collagen around air-

FIGURE 4. TGF- β is produced by lung epithelial cells in vivo and in vitro. The expressed location in lung of TGF- β in control rats and acute and chronic AIPi was detected by immunohistological staining (A). In the lung tissue of acute AIPi rats, TGF- β was mainly expressed in airway epithelial cells, whereas in the lung tissue of chronic AIPi rats, TGF- β was mainly expressed in subepithelial structure as smooth muscle cells and fibroblasts. IL-4 was used to stimulate A549 epithelial cells for 3, 6, 12, 24, and 48 h, and TGF- β mRNA expression was detected by RT-qPCR (B). Different concentrations of TGF- β (1, 5, and 10 ng/ml) were used to stimulate HFL1, and the expression of PRMT1 and COX2 was detected by Western blot (C).



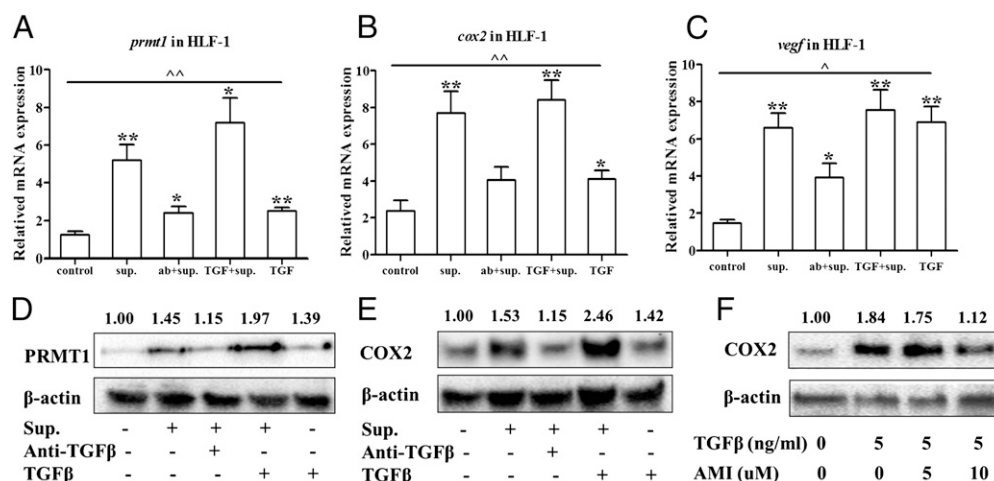


FIGURE 5. TGF- β is the main component in ISEM that induced PRMT1 upregulation in fibroblasts. ISEM from epithelial cells, TGF- β Ab, and TGF- β Ab were used to stimulate HFL1 cells, and the expressions of *prmt1*, *cox2*, and *veg* were detected by RT-qPCR (A–C). The expressions of PRMT1 and COX2 protein were detected by Western blot (D and E). TGF- β with or without AMI-1 (5 and 10 μ M), the pan PRMT enzymatic activity inhibitor, was used to stimulate HFL1, and COX2 expression was detected by Western blot (F). The results are shown from a representative of three different experiments. Band density was measured (ImageJ software) and normalized to β -actin. The results were expressed as mean $\pm$ SEM and analyzed by one-way ANOVA. $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ in all groups; $^*p < 0.05$, $^{**}p < 0.01$ between indicated groups and control group.

ways in the AIPI group (Fig. 7D). Importantly, the nasal administration of AMI-1 decreased the airway and alveoli lesions, mucus secretion, and collagen deposition in chronic AIPI lungs. We scored the lung tissue of individual rats and found that the diameter of alveoli, mucus secretion, and collagen deposition were attenuated after AMI-1 administration (Fig. 7C, 7E).

Taken together, these results indicated that PRMT inhibition in chronic AIPI rats reduced several asthma parameters and ameliorated the disease severity.

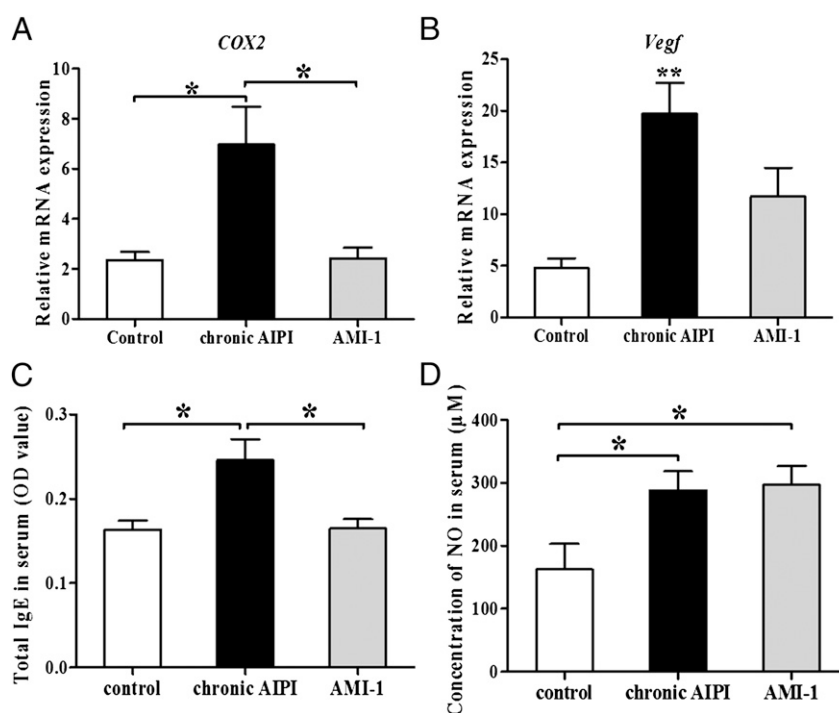
Discussion

In this study we observed that IL-4-stimulated epithelial cells produced TGF- β , which affected the proliferation of fibroblasts and elevated the production of PRMT1, COX2, and VEGF by

fibroblasts. Additionally, TGF- β also increased the expressions of COX2 and VEGF through PRMT1. Our in vitro findings may explain that the mechanism PRMT1 is regulated by TGF- β in the epithelium in acute AIPI, whereas PRMT1 is expressed in sub-epithelial mesenchymal cells in chronic AIPI. Furthermore, we provide evidence that the inhibition of PRMT in vivo dampened most remodeling-related asthma indices, such as collagen deposition, mucus secretion, serum IgE, and the expression of remodeling-related cytokines. Thus, we conclude that PRMT1 plays an important role in chronic asthma through the regulation of remodeling relevant cytokines.

Asthma is characterized by variable degrees of chronic inflammation and structural alterations in the airway walls. The most prominent pathologies include epithelial denudation, subepithelial

FIGURE 6. Expressions of *cox2* and *veg* and concentrations of IgE and NO in serum from chronic AIPI rats with or without AMI-1 administration. The expressions of *cox2* (A) and *veg* (B) were detected by RT-qPCR in lung tissues from control rats, AIPI rats, and AIPI rats with administration of AMI-1. Total IgE (C) and NO concentrations (D) in serum were detected by ELISA and the Griess method. The results were expressed as mean $\pm$ SEM. $^*p < 0.05$ and $^{**}p < 0.01$, respectively, between control and chronic AIPI groups and AIPI and AMI-1 groups after a Mann-Whitney test ($n = 8$ for each group).



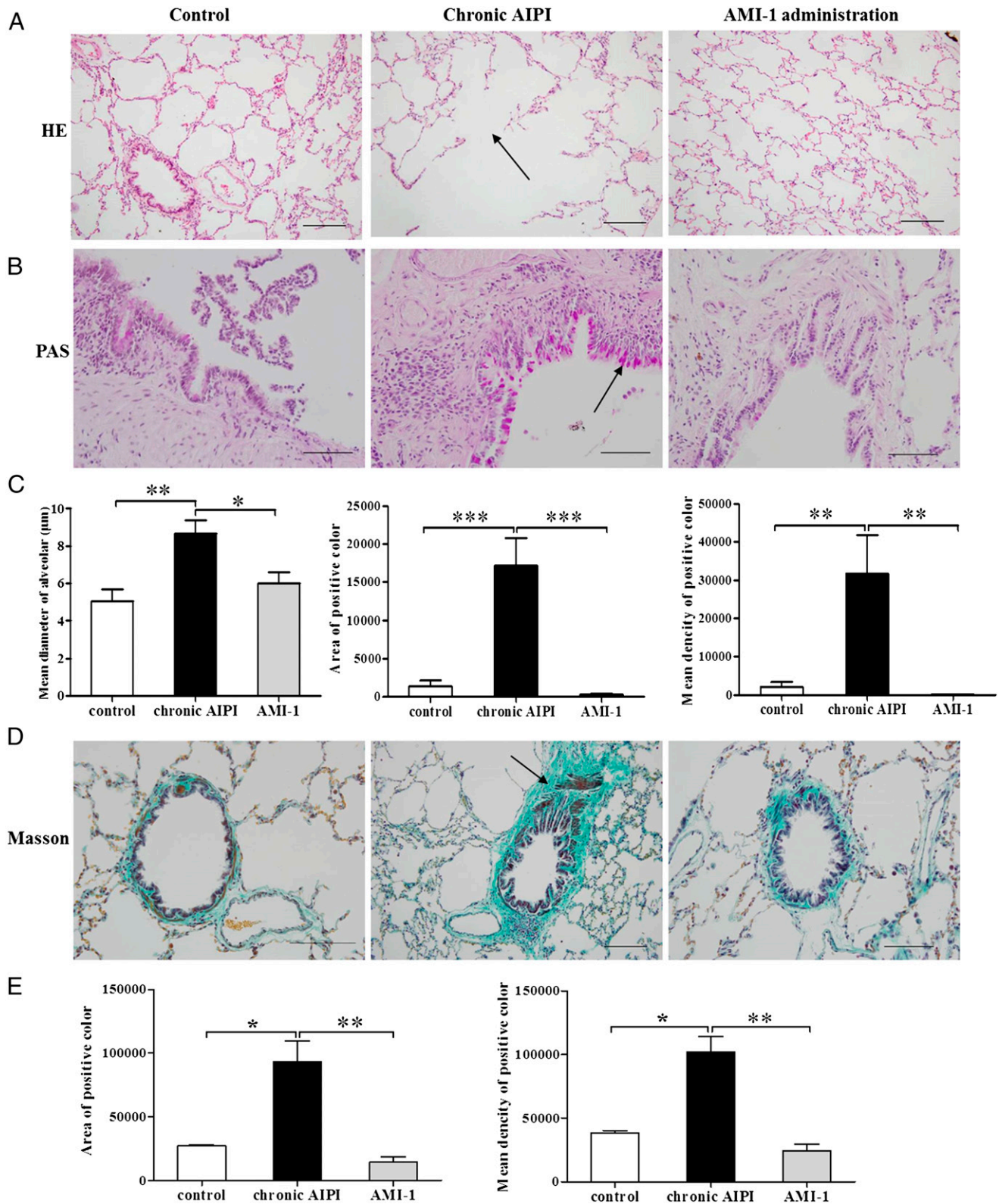


FIGURE 7. Histopathological changes and remodeling in chronic AIPI rats with and without the administration of AMI-1. Representative images of the histopathological changes by H&E staining (A), PAS staining (B), and Masson staining (D) were from lung sections of E3 rats without Ag challenge, with Ag challenge for 8 wk, and with both Ag challenge and AMI-1 administration for 8 wk, respectively. Image-Pro Plus 6.0 software was used to estimate the mean diameter of alveoli, gradation of the color in PAS, and Masson staining in lungs (C and E). Scale bars in (A), (B), and (D), 20 μm. The results were expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between control and AIPI groups and between AIPI and AMI-1 groups after Mann-Whitney test ($n = 8$ for each group).

tissue thickening, increased airway smooth muscle mass, and alterations of extracellular matrix components (10). Chronic inflammation is thought to initiate and perpetuate cycles of tissue injury and repair in asthma, although remodeling may also occur

in parallel with inflammation. In recent years, a significant body of information on a close relationship between epithelial inflammation and airway remodeling has emerged showing that chronic injury or defective repair of the bronchial and alveolar epithelium

results in its persistent activation, with consequent chronic secretion of a variety of proinflammatory cytokines and growth factors that further drive chronic inflammation and remodeling in the subepithelial compartments (11).

Our data clearly demonstrated that PRMT1 participates in both the inflammation and remodeling process in asthma. At the beginning of inflammation, increased PRMT1 occurs mainly in epithelial cells attracting eosinophil infiltration and it exacerbates inflammation in acute AIPI through the upregulation of eotaxin-1. However, in chronic AIPI, PRMT1 expression is observed mainly in subepithelial fibroblasts. Interestingly, IL-4-stimulated inflammatory epithelial cells produced TGF- β , thereby inducing fibroblast proliferation and elevated PRMT1 expression. This, in turn, upregulated its downstream target genes, *cox2* and *vegf*. Our data suggest that PRMT1 is involved in both early epithelium inflammation and later subepithelial remodeling.

Recently it was reported that inflammation can be viewed as a response of lung tissue to injury, and thereby the lung aims to repair the injury. Among the large variety of cytokines and chemokines produced by bronchial epithelial cells, TGF- β is increasingly recognized as an important factor that induces proliferation of subepithelial fibroblasts and as a driving force of differentiation and activation of myofibroblasts. Redington et al. (12) also reported that TGF- β levels were increased in bronchoalveolar lavage fluid of asthma patients after segmental allergen challenge. Similarly, other studies confirmed that epithelial cells from asthma patients released higher levels of TGF- β than did epithelial cells obtained from nonasthma subjects (13, 14). Furthermore, TGF- β expression correlated with the degree of subepithelial fibrosis and was significantly increased in subjects with severe asthma and associated eosinophilia (15). It has also been shown that mechanical or chemical damage to the epithelium leads to increased release of TGF- β 1 and TGF- β 2 (16). In this regard, our in vivo study showed that TGF- β production was significantly increased by lung epithelial cells in acute AIPI lungs. In lungs with chronic AIPI, TGF- β was mainly expressed by subepithelial smooth muscle cells and fibroblasts, suggesting its important role in the maintenance of growth and proliferation of fibroblasts, as well as its participation in airway remodeling in chronic asthma. Additionally, the in vitro study proved that IL-4-stimulated inflammatory epithelium created an active TGF- β milieu that regulated the proliferation and differentiation of subepithelial fibroblasts. Therefore, our novel observation supports the hypothesis that the bronchial epithelium has a key role in modulating the bronchial wall structure and inflammation in asthma.

Our in vitro study proved that increased production of TGF- β by inflamed A549 epithelium induced the expression of PRMT1 in fibroblasts, whereas the blockade of TGF- β partially inhibited the expression of PRMT1 by ISEM. Additionally, both A549 and BEAS-2B, a normal epithelial cell line, had a similar effect on fibroblast proliferation and PRMT1 upregulation. These findings indicate that TGF- β is a very important inducer of PRMT1 in inflammation. Although there is no direct evidence to prove the relationship between TGF- β and PRMT1, a study on renal fibrosis was found that ADMA, as an endogenous endothelial NO synthase and inducible NO synthase competitive inhibitor (17, 18), can reduce the NO concentration in serum in asthmatic animals (7); furthermore, ADMA is also involved in proline metabolism, which may play an important role in the pulmonary remodeling process through raising collagen synthesis (19). In our study, the supplementation of TGF- β to basic medium increased the expression of PRMT1 in fibroblasts. However, our data also indicated that TGF- β is not the only cytokine regulating PRMT1 expression because

blocking TGF- β activity in ISEM did not abolish all PRMT1 expression with ISEM stimulation in fibroblasts.

In chronic inflammation, COX2 immunoreactivity was reported in the bronchial mucosa of normal and asthmatic lungs (20), as well as in the bronchial epithelium (21). The observed overexpression of COX2 in normal human lung fibroblasts is important, as it may drive inflammation and fibrotic conditions in lung (22). The increase of COX2 expression is often accompanied by NF- κ B activation (23, 24) in human fibroblasts. However, when mice were treated with pyrrolidine dithiocarbamate, a nonspecific NF- κ B inhibitor inhibiting COX2 transcription, decreased COX2 mRNA synthesis and protein were reported in epithelial cells (25). NF- κ B is an important transcription factor for IL-1 β -induced COX2 gene expression, and it is involved in inducing COX2 gene transcription (26). Our results demonstrated that COX2 is a target gene of PRMT1, and the upregulation of PRMT1 by plasmid transfection in fibroblasts increased COX2 expression, whereas PRMT enzyme activity inhibited by AMI-1 can reduce the TGF- β -induced COX2 upregulation. Others have demonstrated that a cooperative action of PRMT1 with CARM1 is required for NF- κ B-dependent gene expression (27). Therefore, we speculate that PRMT1 regulates COX2 expression through the activation of NF- κ B.

Our previous in vitro experiments in acute AIPI rats proved that AMI-1 ameliorates pulmonary inflammation through down-regulating eotaxin-1 expression, eosinophil infiltration, and epithelial cell exfoliation (3). Eosinophils and injured epithelium have been confirmed as important sources of TGF- β as well as of other important cytokines that activate the epithelium and subepithelial mesenchymal cells, thus driving airway remodeling. Interestingly, we have illustrated in chronic AIPI that AMI-1 reduced COX2 expression in lung tissue, IgE concentrations in serum, and ameliorated histopathological remodeling.

In conclusion, TGF- β produced by IL-4-stimulated epithelium elevates PRMT1 expression, which plays a crucial role in chronic AIPI through its regulation of COX2. The findings, summarized in Fig. 8, may offer novel insights into the pathogenesis of asthmatic airway remodeling and suggest a new target to intervene in asthma.

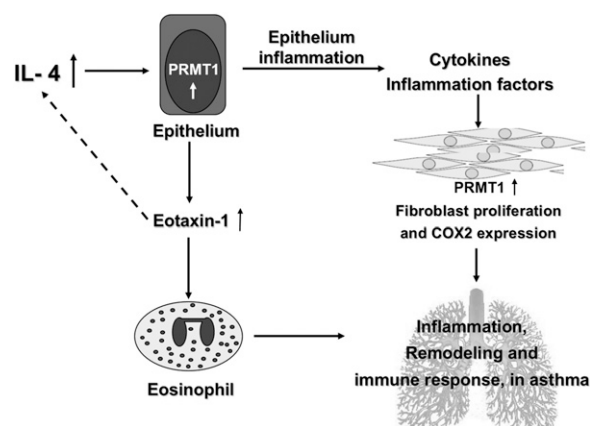


FIGURE 8. Schematic diagram of PRMT1 roles in different AIPI process. In the acute process of asthma disease, IL-4-stimulated epithelial cells induce epithelium inflammation, and the epithelium can produce a series of inflammatory cytokines, including TGF- β , which are involved in the proliferation of fibroblasts and induce elevated production of PRMT1, COX2, and VEGF in fibroblasts; additionally, expressions of COX2 and VEGF are regulated by PRMT1 directly. In conclusion, PRMT1 plays an important role in chronic asthma through the regulation of remodeling related cytokines and orchestrates epithelial inflammation and airway remodeling in the asthmatic process.

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

- Jeffery, P. K. 2001. Remodeling in asthma and chronic obstructive lung disease. *Am. J. Respir. Crit. Care Med.* 164: S28–S38.
- Zakrzewicz, D., A. Zakrzewicz, K. T. Preissner, P. Markart, and M. Wygrecka. 2012. Protein arginine methyltransferases (PRMTs): promising targets for the treatment of pulmonary disorders. *Int. J. Mol. Sci.* 13: 12383–12400.
- Sun, Q., X. Yang, B. Zhong, F. Jiao, C. Li, D. Li, X. Lan, J. Sun, and S. Lu. 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. *J. Immunol.* 188: 3506–3512.
- Wynn, T. A., and T. R. Ramalingam. 2012. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. *Nat. Med.* 18: 1028–1040.
- Royce, S. G., L. Tan, A. A. Koek, and M. L. Tang. 2009. Effect of extracellular matrix composition on airway epithelial cell and fibroblast structure: implications for airway remodeling in asthma. *Ann. Allergy Asthma Immunol.* 102: 238–246.
- Shibata, R., S. Ueda, S. Yamagishi, Y. Kaida, Y. Matsumoto, K. Fukami, A. Hayashida, H. Matsuoka, S. Kato, M. Kimoto, and S. Okuda. 2009. Involvement of asymmetric dimethylarginine (ADMA) in tubulointerstitial ischaemia in the early phase of diabetic nephropathy. *Nephrol. Dial. Transplant.* 24: 1162–1169.
- 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.
- Sun, Q., X. 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.
- Hansen, M. B., S. E. Nielsen, and K. Berg. 1989. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J. Immunol. Methods* 119: 203–210.
- Georas, S. N., and F. Rezaee. 2014. Epithelial barrier function: at the front line of asthma immunology and allergic airway inflammation. *J. Allergy Clin. Immunol.* 134: 509–520.
- Lambrecht, B. N., and H. Hammad. 2013. Death at the airway epithelium in asthma. *Cell Res.* 23: 588–589.
- Redington, A. E., J. Madden, A. J. Frew, R. Djukanovic, W. R. Roche, S. T. Holgate, and P. H. Howarth. 1997. Transforming growth factor- β 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am. J. Respir. Crit. Care Med.* 156: 642–647.
- Kato, M., T. Fujisawa, D. Hashimoto, M. Kono, N. Enomoto, Y. Nakamura, N. Inui, E. Hamada, O. Miyazaki, S. Kuraishi, et al. 2014. Plasma connective tissue growth factor levels as potential biomarkers of airway obstruction in patients with asthma. *Ann. Allergy Asthma Immunol.* 113: 295–300.
- Movahedi, M., S. A. Mahdavian, N. Rezaei, B. Moradi, S. Dorkhosh, and A. A. Amirzargar. 2008. IL-10, TGF- β , IL-2, IL-12, and IFN- γ cytokine gene polymorphisms in asthma. *J. Asthma* 45: 790–794.
- Minshall, E. M., D. Y. Leung, R. J. Martin, Y. L. Song, L. Cameron, P. Ernst, and Q. Hamid. 1997. Eosinophil-associated TGF- β 1 mRNA expression and airways fibrosis in bronchial asthma. *Am. J. Respir. Cell Mol. Biol.* 17: 326–333.
- Howat, W. J., S. T. Holgate, and P. M. Lackie. 2002. TGF- β isoform release and activation during in vitro bronchial epithelial wound repair. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 282: L115–L123.
- Wang, L., D. Zhang, J. Zheng, Y. Feng, Y. Zhang, and W. Liu. 2012. Actin cytoskeleton-dependent pathways for ADMA-induced NF- κ B activation and TGF- β high expression in human renal glomerular endothelial cells. *Acta Biochim. Biophys. Sin. (Shanghai)* 44: 918–923.
- Mihout, F., N. Shweke, N. Bigé, C. Jouanneau, J. C. Dussault, P. Ronco, C. Chatziantoniou, and J. J. Boffa. 2011. Asymmetric dimethylarginine (ADMA) induces chronic kidney disease through a mechanism involving collagen and TGF- β 1 synthesis. *J. Pathol.* 223: 37–45.
- Yildirim, A. O., P. Bulau, D. Zakrzewicz, K. E. Kitowska, N. Weissmann, F. Grimminger, R. E. Morty, and O. Eickelberg. 2006. Increased protein arginine methylation in chronic hypoxia: role of protein arginine methyltransferases. *Am. J. Respir. Cell Mol. Biol.* 35: 436–443.
- Demoly, P., D. Jaffuel, N. Lequeux, B. Weksler, C. Crémion, F. B. Michel, P. Godard, and J. Bousquet. 1997. Prostaglandin H synthase 1 and 2 immunoreactivities in the bronchial mucosa of asthmatics. *Am. J. Respir. Crit. Care Med.* 155: 670–675.
- Taha, R., R. Olivenstein, T. Utsumi, P. Ernst, P. J. Barnes, I. W. Rodger, and A. Giaid. 2000. Prostaglandin H synthase 2 expression in airway cells from patients with asthma and chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 161: 636–640.
- Ryu, J. H., T. V. Colby, T. E. Hartman, and R. Vassallo. 2001. Smoking-related interstitial lung diseases: a concise review. *Eur. Respir. J.* 17: 122–132.
- Yang, G., H. J. Im, and J. H. Wang. 2005. Repetitive mechanical stretching modulates IL-1 β induced COX-2, MMP-1 expression, and PGE₂ production in human patellar tendon fibroblasts. *Gene* 363: 166–172.
- Martey, C. A., S. J. Pollock, C. K. Turner, K. M. O'Reilly, C. J. Baglioni, R. P. Phipps, and P. J. Sime. 2004. Cigarette smoke induces cyclooxygenase-2 and microsomal prostaglandin E₂ synthase in human lung fibroblasts: implications for lung inflammation and cancer. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 287: L981–L991.
- Wardlaw, S. A., T. H. March, and S. A. Belinsky. 2000. Cyclooxygenase-2 expression is abundant in alveolar type II cells in lung cancer-sensitive mouse strains and in premalignant lesions. *Carcinogenesis* 21: 1371–1377.
- Nakao, S., Y. Ogata, E. Shimizu-Sasaki, M. Yamazaki, S. Furuyama, and H. Sugiya. 2000. Activation of NF- κ B is necessary for IL-1 β -induced cyclooxygenase-2 (COX-2) expression in human gingival fibroblasts. *Mol. Cell. Biochem.* 209: 113–118.
- Hassa, P. O., M. Covic, M. T. Bedford, and M. O. Hottiger. 2008. Protein arginine methyltransferase 1 coactivates NF- κ B-dependent gene expression synergistically with CARM1 and PARP1. *J. Mol. Biol.* 377: 668–678.