Am J Physiol Lung Cell Mol Physiol 310: L328–L336, 2016.
First published December 23, 2015; doi:10.1152/ajplung.00217.2015.

Therapeutic potential of an orally effective small molecule inhibitor of plasminogen activator inhibitor for asthma

Rui-Ming Liu,[1] Stephanie Eldridge,[1] Nobuo Watanabe,[2] Jessy Deshane,[1] Hui-Chien Kuo,[3] Chunsun Jiang,[1] Yong Wang,[1] Gang Liu,[1] Lisa Schwiebert,[4] Toshio Miyata,[2] and Victor J. Thannickal[1]

[1]*Division of Pulmonary, Allergy, and Critical Care, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama;* [2]*United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, Tohoku, Japan;* [3]*Department of Biostatistics, University of Alabama at Birmingham, Birmingham, Alabama; and* [4]*Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, Birmingham, Alabama*

Submitted 2 July 2015; accepted in final form 19 December 2015

Liu RM, Eldridge S, Watanabe N, Deshane J, Kuo HC, Jiang C, Wang Y, Liu G, Schwiebert L, Miyata T, Thannickal VJ. Therapeutic potential of an orally effective small molecule inhibitor of plasminogen activator inhibitor for asthma. *Am J Physiol Lung Cell Mol Physiol* 310: L328–L336, 2016. First published December 23, 2015; doi:10.1152/ajplung.00217.2015.—Asthma is one of the most common respiratory diseases. Although progress has been made in our understanding of airway pathology and many drugs are available to relieve asthma symptoms, there is no cure for chronic asthma. Plasminogen activator inhibitor 1 (PAI-1), a primary inhibitor of tissue-type and urokinase-type plasminogen activators, has pleiotropic functions besides suppression of fibrinolysis. In this study, we show that administration of TM5275, an orally effective small-molecule PAI-1 inhibitor, 25 days after ovalbumin (OVA) sensitization-challenge, significantly ameliorated airway hyperresponsiveness in an OVA-induced chronic asthma model. Furthermore, we show that TM5275 administration significantly attenuated OVA-induced infiltration of inflammatory cells (neutrophils, eosinophils, and monocytes), the increase in the levels of OVA-specific IgE and Th2 cytokines (IL-4 and IL-5), the production of mucin in the airways, and airway subepithelial fibrosis. Together, the results suggest that the PAI-1 inhibitor TM5275 may have therapeutic potential for asthma through suppressing eosinophilic allergic response and ameliorating airway remodeling.

plasminogen activator inhibitor 1; plasminogen activator inhibitor 1 inhibitor; animal model

ASTHMA IS ONE OF THE MOST common respiratory diseases affecting ~5% of the population in the United States. The pathological features of chronic asthmatic airways include infiltration of eosinophils, hyperresponsiveness, mucus hypersecretion, and subepithelial fibrosis. Despite intensive studies, the precise mechanisms underlying the pathogenesis of chronic asthma remain unclear. Most importantly, there are no effective treatments to cure chronic asthma, although anti-inflammatory steroids plus bronchodilators provide temporal relief of the symptoms during asthma exacerbation. Understanding the pathogenesis and developing effective treatments for chronic asthma have been and will continue to be a major challenge for clinicians and basic science researchers.

Plasminogen activator inhibitor 1 (PAI-1) is a primary inhibitor of tissue-type and urokinase-type plasminogen activators (tPA and uPA, respectively). Besides suppressing fibrinolysis through inhibition of tPA and uPA and thereby plasminogen activation, PAI-1 has pleiotropic functions, including inhibition of extracellular protein degradation, modulation of cell attachment and migration, and regulation of cell senescence and apoptosis processes (25). Importantly, it has been reported that PAI-1 protein is elevated in sputum of allergic asthmatic patients (1–3, 21, 22) and in experimental asthma models (23, 31). Individuals carrying polymorphic allele of the PAI-1 gene (4G instead of 5G), which leads to increased plasma PAI-1 level, have higher risk to develop asthma and show more severe asthmatic symptoms than 5G/5G carriers (28, 30, 32). Most importantly, it has been reported that intratracheal administration of small-interfering RNA targeting PAI-1 (29) or the PAI-1 inhibitor tiplaxtinin (24) attenuated allergen-induced pathological changes in experimental asthma models. Together, the data suggest that PAI-1 plays a critical role in the pathogenesis of asthma (3, 4, 28, 37) and therefore may serve as an ideal therapeutic target for the treatment of asthma. Nonetheless, despite strong evidence supporting the role of PAI-1 in asthma pathogenesis, no therapeutic drugs that target PAI-1 for the treatment of chronic asthma have yet been developed.

5-Chloro-2-[{2-[4-(diphenylmethyl)piperazin-1-yl]-2-oxoethoxyl}(28)acetyl]aminobenzoate (TM5275) is an orally effective small-molecule PAI-1 inhibitor. It has potent antithrombotic activity in both rodents and nonhuman primates (cynomolgus monkey) (18). Importantly, TM5275 does not interfere with other serpin/serine protease systems such as α_1-antitrypsin/trypsin and α_2-antiplasmin/plasmin and causes no obvious toxicity to the liver, kidney, hematopoietic system, central nervous system, or cardiovascular system of rats and monkeys when given in doses up to 2,000 $mg \cdot kg^{-1} \cdot day^{-1}$ for 2 wk (18). It has no significant effect on activated partial thromboplastin time, prothrombin time, or bleeding time, the most common side effects of anticoagulation agents (18). These data suggest that TM5275 is relatively specific for PAI-1 with low toxicity. Therefore, we explored, in this study, the therapeutic potential of TM5275 for asthma in an ovalbumin (OVA)-induced chronic asthma model. Our results show that oral administration of TM5275, 25 days after OVA sensitization and challenge, significantly ameliorated OVA-induced airway hyperresponsiveness (AHR), eosinophil and neutrophil infiltration, mucin production, and subepithelial fibrosis. The results suggest that TM5275 may have therapeutic potential for asthma.

Address for reprint requests and other correspondence: R.-M. Liu, Division of Pulmonary, Allergy, and Critical Care, Dept. of Medicine, School of Medicine, Univ. of Alabama at Birmingham, Birmingham, AL (e-mail: rliu@uab.edu).

 1040-0605/16 Copyright © 2016 the American Physiological Society

MATERIALS AND METHODS

Mouse model of chronic allergic asthma. A well-established chronic asthma model induced by ovalbumin (OVA) was used in this project to test the therapeutic value of TM5275 for chronic asthma (10, 11). Specifically, 6- to 8-wk-old female BALB/cJ mice were divided into three groups, 16 mice/group. *Group 1* (nonasthmatic) mice were sensitized and challenged with saline. *Group 2* (asthmatic) mice were sensitized with 50 μg of alum-precipitated chicken egg OVA by intraperitoneal injection two times (*day 0* and *day 14*) and then challenged with OVA by aerosol inhalation, 5 mg/ml, 30 min/day, daily from *day 21* to *day 25*, and 10 min/day every other day from *day 26* to *day 35*. This group of mice was also treated with vehicle (20% DMSO in saline) by oral gavage daily from *day 26* to *day 35*. *Group 3* (asthmatic + TM5275 treatment) mice were sensitized and challenged with OVA as the *group 2* mice and treated with 40 mg/kg TM5275 (dissolved in 20% DMSO) by oral gavage daily from *day 26* to *day 35*. The dose of 40 mg/kg TM5275 was chosen based on our previous study in which we showed that oral administration of 40 mg/kg TM5275 for 10 consecutive days almost completely blocked TGF-β1-induced lung fibrosis with no obvious toxicity (no body weight loss) (13). A schematic diagram with the detailed information about sensitization, challenge, and treatment is presented in Fig. 1. The whole experiment has been repeated one time. Twenty-four hours after the final challenge and TM5275 treatment, eight mice from each group were used for the lung function test using the SCIREQ FlexiVent system, whereas another eight mice from each group were killed for histology and biochemistry analyses. All procedures involving animals were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham (UAB) and conducted at the UAB animal facilities under specific pathogen-free conditions.

Measurement of AHR. AHR was measured using the FlexiVent system upon challenge with increasing concentrations of methacholine as we described previously (11). Briefly, 48 h after the last OVA challenge and TM5275 treatment, mice were anesthetized with ketamine (450 mg/kg), and a tracheotomy tube (18G) was inserted and connected to the inspiratory and expiratory ports of a ventilator (FlexiVent; SCIREQ, Montreal, PQ, Canada). Mice were mechanically ventilated at a rate of 160 breaths/min at a tidal volume of 0.2 ml with a positive end-expiratory pressure of 2–4 cmH_2O. Methacholine (0, 5, 10, 15, and 20 mg/ml) was administered via aerosolization. From 20 s up to 3 min after each methacholine aerosol challenge, resistance, elastance, tissue damping, and total lung capacity were recorded continuously. The average value of each parameter was taken to express changes in murine airway function.

Zymography analysis of uPA and tPA activities. The activities of tPA and uPA in lung tissue were determined by zymographic analysis as we have described before (13, 39). Briefly, equal amounts of proteins were loaded on 12% polyacrylamide gel containing 2 mg/ml casein in the presence of 5 μg/ml plasminogen. After electrophoresis, the enzyme reaction was initiated by incubating the gel in 0.1 M glycine-NaOH (pH 8.3) at 37°C for 16 h, and the lytic areas were developed by staining the gel with a solution containing 30% methanol, 10% glacial acetic acid, and 0.5% Coomassie blue G250. The gels were destained in the same solution without dye and scanned using a Bio-Rad Fluor-s MultImaging system. To avoid the possible interference of matrix metalloproteinases, EDTA (2 mM) was included in the glycine-NaOH buffer during the incubation period. The tPA and uPA bands were identified based on the mobility of the molecular weight markers. The photonegative images of the gels are presented in Figs. 1–9 and assessed semiquantitatively using the Image J analyzing software (NIH website).

Bronchoalveolar lavage, bronchoalveolar lavage cell counting, and lung tissue processing. Bronchoalveolar lavage (BAL) was performed with 0.8 ml of phosphate-buffered saline (PBS). BAL fluid (BALF) was spun down at 400 *g* for 10 min; the supernatants were collected, and the cells were resuspended uniformly in saline. The BAL cells were centrifuged on a microscope slide using a CytoSpin and stained with a Diff-Quik stain set (B4132-1A; Siemens Healthcare Diagnostics, Newark, DE). Total cell numbers were counted and calculated based on the volume of the BALF; differential cell counts were performed by counting 300–600 cells on each slide using an oil immersion (×100) lens of a Zeiss microscope, and the percentages of monocytes, neutrophils, lymphocytes, and eosinophils were calculated. After lavage, pulmonary artery vascular beds were perfused and then left lung fixed with 10% PBS-buffered formalin for histology and immunochemistry analysis as we have described previously (27). The rest of the lung was frozen immediately in liquid nitrogen for biochemistry analyses.

Measurement of urea concentrations in the plasma and BALF. Concentrations of urea in BALF and in the plasma were measured using a commercially available kit (Teco Diagnostics, Anaheim, CA) following the protocol provided by the manufacturer. The ratio of urea concentrations in the plasma and in BALF (dilution factor) was calculated for each mouse.

Measurement of OVA-specific IgE in plasma and BALF. OVA-specific IgE in mouse plasma and BALF were measured by an anti-OVA mouse-IgE ELISA kit from BioVendor (Gunma, Japan) following the instruction provided by the manufacturer. The results were normalized with urea dilution factors, and the results are expressed as nanograms per milliliter of epithelial lining fluid (ELF).

Measurement of cytokine/chemokines in BALF. The protein levels of cytokines and chemokines in mouse BALF were analyzed using a Bio-Plex multiplex suspension cytokine array (Bio-Rad Laboratories) according to the manufacturer's instructions (26). The data were analyzed using Bio-Plex Manager software (Bio-Rad Laboratories). The results were normalized with urea dilution factors and expressed as nanograms per milliliter of ELF.

Measurement of goblet cell hyperplasia. Lung tissues fixed in 10% paraformaldehyde were stained with periodic acid-Schiff (PAS) to reveal mucin production in goblet cells as described previously (24). Goblet cell hyperplasia was quantified by determining the percentage of PAS-positive cells/length of bronchial basal membrane in 10 sites/mouse by quantitative morphometry techniques using Image J software as we have described previously (13).

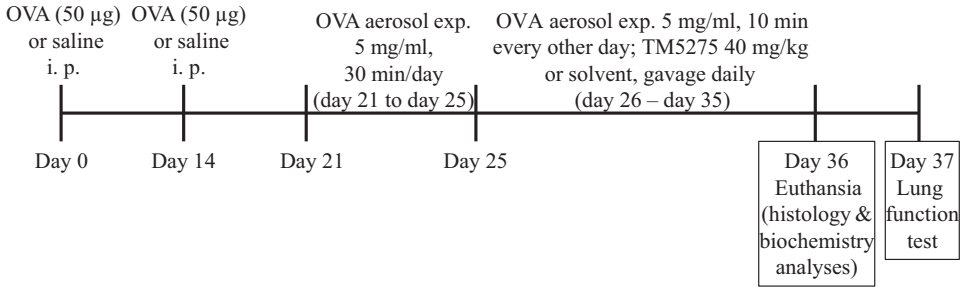

Fig. 1. Schematic diagram of experimental design. OVA, ovalbumin.

Measurement of mucus hypersecretion. The amounts of mucin 5 subtype AC (MUC5AC) in BALF were measured using a mouse Mucin 5 AC ELISA kit (Biomtik, Ontario, Canada) following the instruction provided by the manufacturer. The results were normalized with urea dilution factors and expressed as nanograms per milliliter of ELF.

Collagen staining. Collagen deposition was revealed by Sirius red staining. High-quality images were taken at ×40 magnifications. The collagen deposition around large airways (including the bronchioles and tertiary bronchi, identified by their ciliated pseudostratified columnar epithelium and relative thick smooth muscle layer in the walls with no cartilage) and small airways (including the terminal bronchioles, identified by their low cuboidal epithelium and intact walls, and the respiratory bronchioles, identified by their low cuboidal epithelium and occasional mural alveoli) (19) was quantified by quantitative morphometry techniques using Image J software as we have described previously (13).

Statistical analysis. AHR data (tissue damping, resistance, elastance, and total lung capacity) were analyzed by the two-way analysis of variance (ANOVA) trend test to compare the slopes of the methacholine dose-response curves from the three treatment groups (saline, OVA, and OVA + TM5275). Post hoc analyses were conducted with the Tukey's test. The data presented in other figures were evaluated by one-way ANOVA, and statistical significance was determined post hoc by Fisher's least-significant difference test wherein $P < 0.05$ was considered significant.

RESULTS

TM5275 attenuates OVA-induced AHR. Asthmatic airways are highly sensitive to allergens or irritants (AHR). To elucidate whether TM5275 has therapeutic potential for asthma, we first assessed the effect of TM5275 on OVA-induced AHR induced by methacholine using a FlexiVent system. Two-way ANOVA trend tests were conducted to compare the slopes of the methacholine dose-response curves from the three treatment groups (saline, OVA, and OVA + TM5275). The results show that there were statistically significant differences in the slopes among the three groups (Table 1) and between any two groups (Table 2) for the tissue damping, resistance, elastance, or total lung capacities. Post hoc analyses further show that OVA sensitization-challenge significantly increased the resistance, tissue damping, and elastance, and reduced total lung capacity; treatment with TM5275, on the other hand, significantly attenuated OVA-mediated changes in these parameters under the high methacholine concentration condition (Fig. 2). No obvious toxicity, evaluated by body weight loss (data not shown), was observed with TM5275 treatment. The results suggest that PAI-1 inhibitor mitigates allergy-induced AHR. Our results further confirm that TM5275 is relatively safe to use.

OVA suppresses whereas TM5275 restores the activities of uPA and tPA in mouse lung. To confirm the inhibitory effect of TM5275 on PAI-1 in mouse lung, the activities of uPA and tPA in mouse lung homogenates were assessed by zymographic analysis as we have described previously (13). The results show that OVA sensitization-challenge led to decreases in the activities of both tPA and uPA in mouse lung tissue; administration of TM5275, on the other hand, partially protected against OVA-induced inactivation of uPA and tPA in mouse lung (Fig. 3). The results suggest that oral administration of TM5275 is effective.

Table 1. *Two-way ANOVA trend test of the slopes of the methacholine dose-response curves among three treatment groups*

Parameter	Statistical Outcomes	
	$F(2,4)$	P
Tissue damping	23.30	<0.0001
Resistance	23.12	<0.0001
Elastance	30.42	<0.0001
Total lung capacity	32.83	<0.0001

TM5275 suppressed OVA-induced infiltration of inflammatory cells in mouse lung. Inflammatory response in asthmatic airways is characterized by infiltration and activation of eosinophils, which in turn contribute importantly to the pathophysiology of asthma. The OVA-induced chronic asthma model is featured with eosinophilic inflammatory cell infiltration. To determine whether TM5275 can suppress the OVA-induced inflammatory response, we counted the total cell numbers as well as the numbers of neutrophils, lymphocytes, monocytes, and eosinophils in BALF after Diff-Quik staining. The results show that OVA sensitization-challenge significantly increased total cell numbers as well as the numbers of neutrophils, monocytes, and eosinophils in BALF (Fig. 4). Of the total cells found in BALF of OVA-challenged mice, 62% are eosinophils, confirming an eosinophilic inflammatory response in our asthma model. Treatment with TM5275, 25 days after OVA sensitization-challenge, significantly mitigated OVA-induced infiltration of inflammatory cells (Fig. 4). Differential cell count results further show that TM5275 treatment significantly reduced OVA-induced infiltration of neutrophils (reduced by 80%), eosinophils (reduced by 57%), and monocytes (reduced by 43%), although it had no significant effect on OVA-induced lymphocyte infiltration. The results suggest that PAI-1 is involved in inflammatory cell infiltration and that TM5275 has potent anti-inflammatory function.

TM5275 suppressed OVA-induced IgE production. IgE plays a critical role in allergy and is produced mainly by eosinophils. To further determine whether the PAI-1 inhibitor TM5275 suppresses the eosinophilic allergic response, the amounts of OVA-specific IgE in the plasma and BALF were assessed by ELISA. The results show that OVA sensitization-

Table 2. *Two-way ANOVA trend test of the slopes of the methacholine dose-response curves between two treatment groups*

Treatment	Tissue Damping		Resistance		Elastance		Total Lung Capacity	
	$F(1,4)$	P	$F(1,4)$	P	$F(1,4)$	P	$F(1,4)$	P
Saline vs. OVA	23.67	<0.0001	22.78	<0.0001	35.19	<0.0001	44.76	<0.0001
Saline vs. OVA + TM5275	11.69	<0.0001	11.66	<0.0001	13.72	<0.0001	11.14	<0.0001
OVA vs. OVA + TM5275	20.18	<0.0001	20.33	<0.0001	25.72	<0.0001	24.90	<0.0001

OVA, ovalbumin.

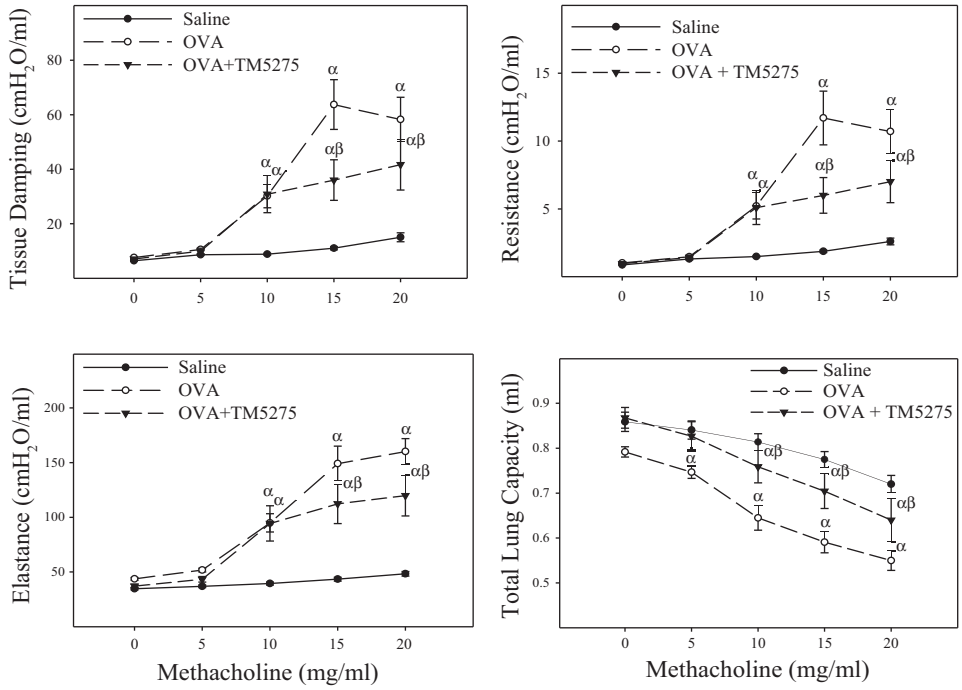

Fig. 2. Effects of TM5275 on OVA-induced airway hyperresponsiveness. Airway hyperresponsiveness was assessed using a FlexiVent system with increasing concentrations of methacholine as described in MATERIALS AND METHODS. The average tissue damping, resistance, elastance, and total lung capacity in each concentration of methacholine were calculated from the record data of 12–14 mice. The statistical results were from Tukey's post hoc analyses. $^{\alpha}$Significantly different from the same concentration of methacholine-stimulated saline control mice; $^{\beta}$significantly different from same concentration of methacholine-stimulated OVA alone-challenged mice ($P < 0.05$, $n = 12$–14).

challenge significantly increased OVA-IgE in the plasma and in BALF. TM5275 treatment, on the other hand, partially blocked the OVA-stimulated increase in OVA-IgE in the BALF, although it had no significant effect on the level of plasma OVA-IgE (Fig. 5). The results further confirm that the PAI-1 inhibitor TM5275 suppressed the OVA-induced eosinophilic inflammatory response in mice.

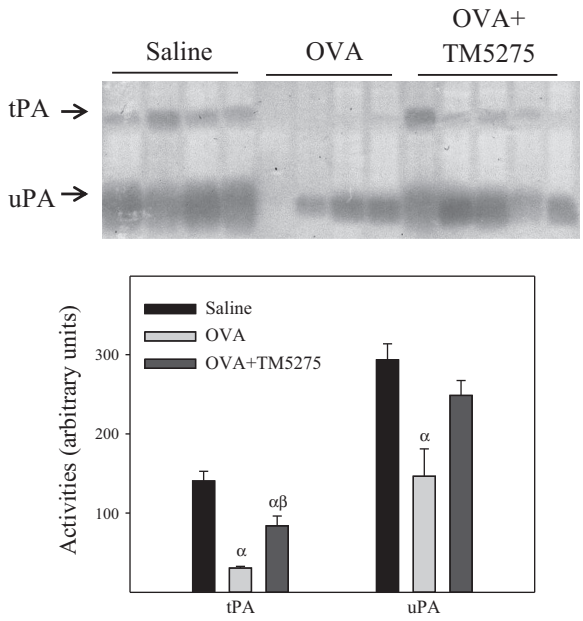

Fig. 3. Effect of TM5275 on the activities of urokinase-type and tissue-type plasminogen activators (uPA and tPA, respectively) in mouse lung. The activities of tPA and uPA in lung tissue were determined by zymographic analysis as described in MATERIALS AND METHODS. The photonegative images of the gels are presented, and the band intensities were semiquantified using the Image J analyzing software. $^{\alpha}$Significantly different from saline control mice; $^{\beta}$significantly different from the OVA alone group ($P < 0.05$, $n = 4$–5).

Effects of TM5275 on OVA-induced cytokine production. Increased Th2 cytokine production is associated with asthma and contributes importantly to asthmatic airway pathology. To further elucidate the mechanism whereby TM5275 mitigates OVA-induced AHR, the amounts of Th1 and Th2 cytokines in BALF were measured by a Bio-Plex mouse cytokine ELISA kit. The results show that OVA sensitization-challenge significantly increased the amounts of IL-4 and IL-5 in BALF, although it had no significant effect on other cytokines, including IL-2, IL-10, IL-12, interferon-γ, tumor necrosis factor-α (TNF-α), and granulocyte macrophage colony-stimulating factor (Fig. 6). TM5275 treatment, on the other hand, reduced OVA-stimulated IL-4 and IL-5 production (Fig. 6).

TM5275 reduced OVA-stimulated mucin production. Mucin overproduction, one of the pathological features of asthmatic airways, was assessed by PAS staining and by measuring MUC5AC, a major form of mucin secreted by airway epithelial cells and associated with asthma, in BALF by ELISA. PAS staining results show that OVA sensitization-challenge significantly increased the amount of mucin in airway epithelial cells, whereas treatment with TM5275 partially blocked the OVA effect (Fig. 7, *A* and *B*). MUC5AC ELISA results further show that OVA sensitization-challenge significantly increased the amount of MUC5AC in BALF; TM5275 administration again reduced the amount of secreted MUC5AC stimulated by OVA (Fig. 7*C*). Together, the results suggest that TM5275 may have therapeutic potential for asthma through reducing allergy-induced airway mucin production.

TM5275 reduced subepithelial collagen deposition in airways. Collagens are the major type of extracellular matrix. Subepithelial collagen deposition (airway remodeling) leads to airway narrowing and airflow obstruction. Because PAI-1 plays a critical role in the regulation of collagen degradation, we further examined whether TM5275 treatment reduces collagen deposition in large and small airways by Sirius red

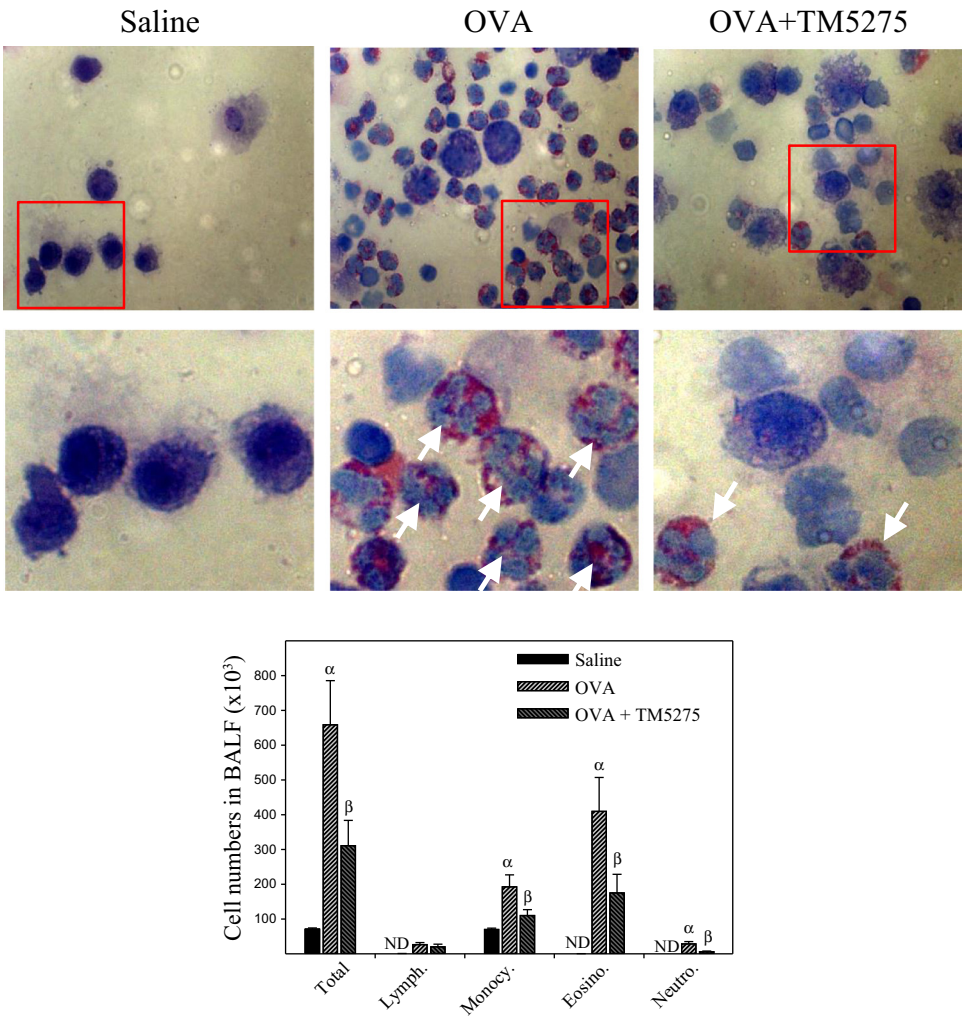

Fig. 4. Effect of TM5275 on OVA-induced inflammatory responses in mouse lung. Total cell numbers as well as differential cell counts in bronchoalveolar lavage fluid (BALF) were determined after the cells were centrifuged on a microscope slide and stained with Diff-Quik as described in MATERIALS AND METHODS. *Top*, representative bronchoalveolar lavage (BAL) cell staining images; *bottom*, quantification data of BAL cells. ND, not detected; $^{\alpha}$significantly different from saline control mice; $^{\beta}$significantly different from the OVA alone group ($P < 0.05$, $n = 5–8$).

staining. The results show that mice sensitized and challenged with OVA had a significantly increased amount of collagen deposition around large airways, although OVA sensitization-challenge had no significant effect on collagen deposition in small airways. Oral administration of TM5275, 25 days after OVA sensitization and challenge, significantly reduced the amount of collagen in the large airways (Fig. 8). The results suggest that TM5275 may have therapeutic potential for chronic asthma by reducing allergy-induced subepithelial collagen deposition.

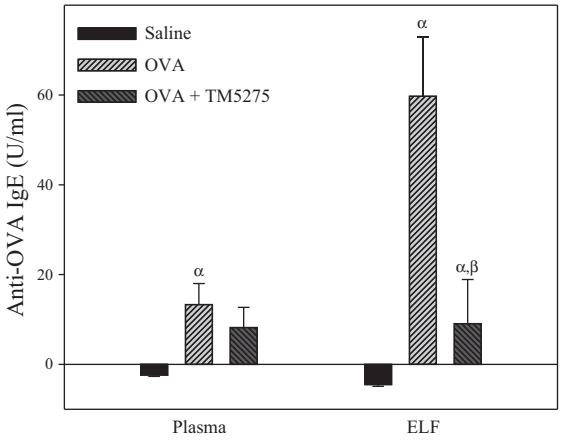

Fig. 5. Effect of TM5275 on OVA-induced IgE in the plasma and BALF. OVA-specific IgE in the plasma and BALF was measured using an anti-OVA mouse-IgE ELISA kit, and the results were normalized by urea dilution factors as described in MATERIALS AND METHODS. $^{\alpha}$Significantly different from saline control mice; $^{\beta}$significantly different from the OVA alone group ($P < 0.05$, $n = 7$).

DISCUSSION

PAI-1 expression is increased in the sputum of asthmatic patients and in experimental asthma models, and has been shown to play a critical role in the pathogenesis of asthma (1–3, 21–24, 28–32). Although PAI-1 small-interfering RNA (siRNA) and the small-molecule PAI-1 inhibitor tiplaxtinin have been tested in animal models, no therapeutics that target PAI-1 for the treatment of asthma have been yet developed (24, 29). TM5275 is an orally effective small-molecule PAI-1 inhibitor. It has potent antithrombotic activity in rat and monkey thrombosis models (18). Most importantly, it does not interfere with other serpin/serine protease systems nor prolong bleeding time or cause obvious toxicity in rats or monkeys when administrated with up to 2,000 mg/kg for 2 wk (18), suggesting that TM5275 is relatively specific for PAI-1 and safe to use (low toxicity). In this study, we show that oral administration of 40 mg/kg of TM5275, a dose that is much

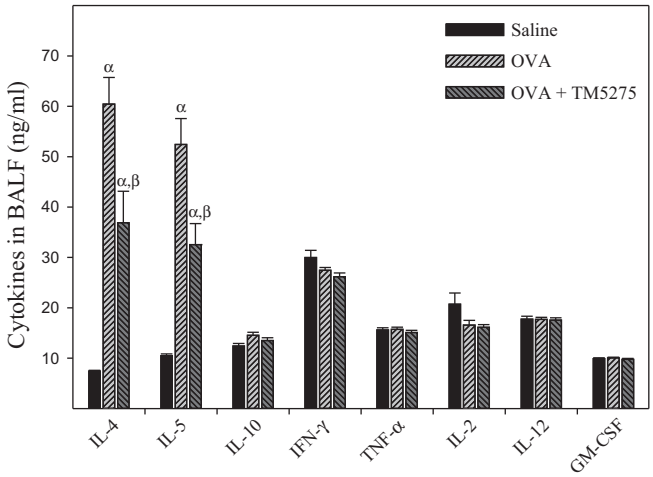

Fig. 6. Effect of TM5275 on OVA-stimulated production of cytokine/chemokines in BALF. The protein levels of cytokines/chemokines in mouse BALF were analyzed using a Bio-Plex multiplex suspension cytokine array, and the data were analyzed using Bio-Plex Manager software as described in MATERIALS AND METHODS. IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte macrophage colony-stimulating factor. $^{\alpha}$Significantly different from saline control mice; $^{\beta}$significantly different from OVA alone-treated mice ($P < 0.05$, $n = 11–13$).

lower than its no observed adverse effect level (NOAEL, 2,000 mg/kg), 25 days after OVA sensitization and challenge, ameliorated OVA-induced AHR and suppressed the OVA-induced eosinophilic inflammatory response, mucin production, and airway remodeling. The results suggest that TM5275 may have therapeutic potential for chronic asthma. It should be pointed out that, although tiplaxtinin, another small-molecule PAI-1 inhibitor, has been shown previously to ameliorate airway pathological changes when given simultaneously with OVA in a similar asthma model (24), TM5275 may be a better candidate drug, since its toxicity is lower (NOAEL of TM5275 in monkey is >2,000 mg/kg, whereas NOAEL of tiplaxtinin in dogs is 100 mg/kg) and the bioavailability is higher (96% in monkeys for TM5275 vs. 36% in dogs for tiplaxtinin) compared with tiplaxtinin (9, 18).

One of the pathological features of asthmatic airways is inflammation. Eosinophils play an important role in allergic response and in asthma pathology. In this study, we show that challenge with OVA dramatically increased the number of eosinophils and the amount of OVA-IgE in BALF, confirming the nature of the allergic response in our OVA-induced asthma model. Treatment with TM5275 significantly reduced the number of eosinophils (by 57%) and the amount of OVA-IgE in BALF of OVA-challenged mice, indicating that PAI-1 is involved in the eosinophilic allergic response in asthma. Besides eosinophils, TM5275 administration also significantly reduced the numbers of neutrophils and monocytes in BALF of OVA-challenged mice. It should be pointed out that, although the percentages of cell types in BALF are similar between OVA and OVA plus TM5275 groups (4, 29.3, 62.4, and 4.3% in OVA-challenged mice vs. 6.4, 35.4, 56.4, and 1.8% in OVA + TM5275-treated mice for lymphocytes, monocytes, eosinophils, and neutrophils, respectively), TM5275 had a more dramatic effect on BALF cell counts of neutrophils (reduced by 80%), eosinophils (reduced by 57%), and monocytes (reduced by 43%) than on lymphocytes (no significant decrease). The mechanism and biological significance underlying the selective effect of TM5275 on BAL cells is unclear and warrants further investigation. Associated with suppression of inflammatory cell infiltration, TM5275 reduced the production of OVA-IgE as well as IL-4 and IL-5, two Th2 cytokines that promote eosinophil response and IgE production. The results suggest

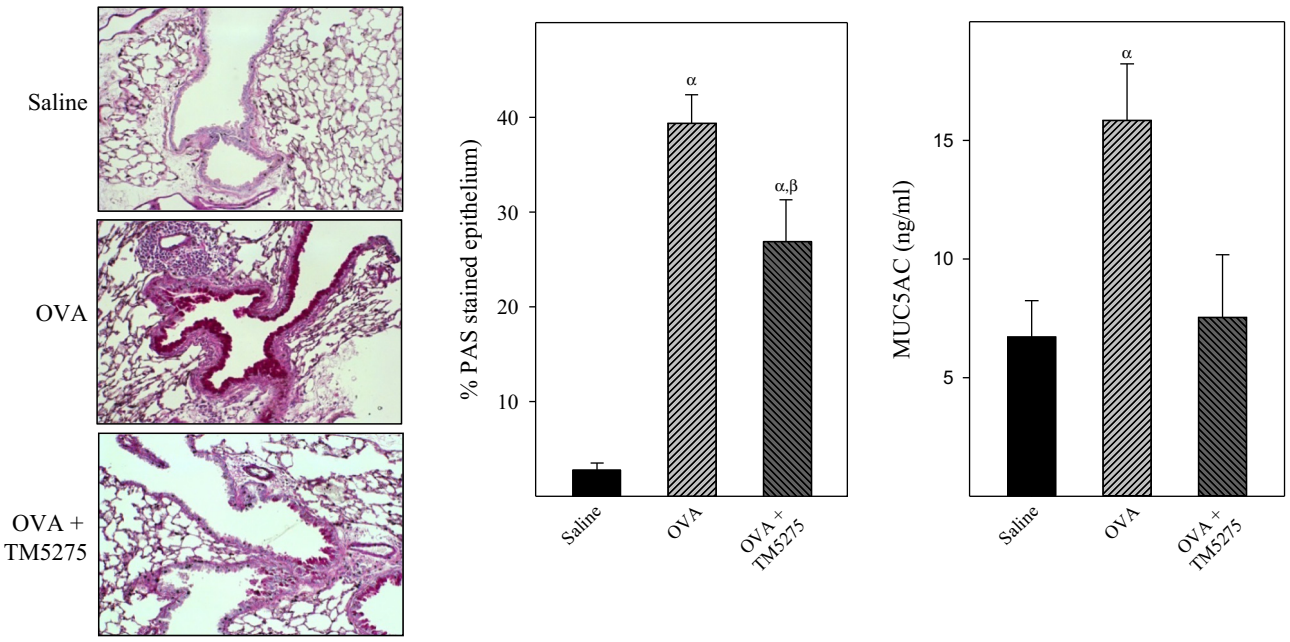

Fig. 7. Effect of TM5275 on OVA-induced mucin production. Mucin production in airway epithelial cells was revealed by periodic acid-Schiff (PAS) staining and by measuring mucin 5 subtype AC (MUC5AC) in BALF using an ELISA kit as described in MATERIALS AND METHODS. *Left*, representative images of PAS staining; *middle*, semiquantified results of PAS staining; *right*, ELISA results of MUC5AC in BALF. $^{\alpha}$Significantly different from saline control mice; $^{\beta}$significantly different from the OVA alone group ($P < 0.05$, $n = 4–7$).

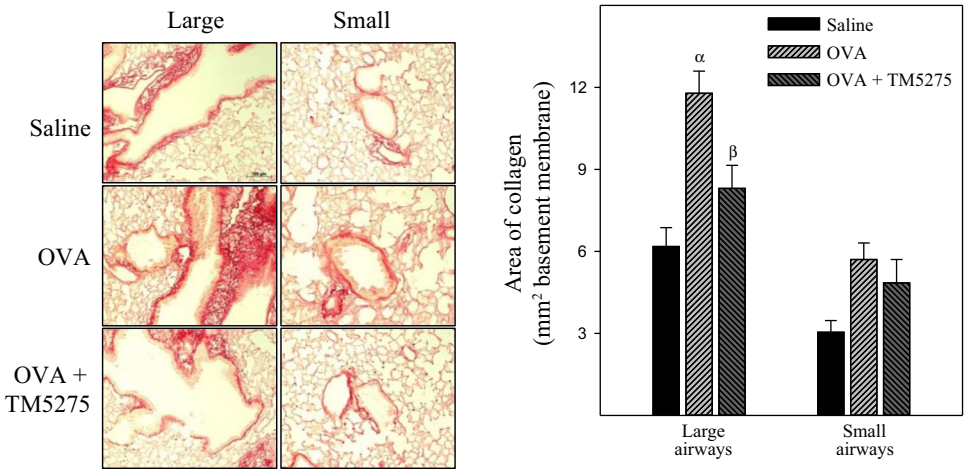

Fig. 8. Effect of TM5275 on OVA-induced collagen deposition in small and large airways. Collagen deposition in the large and small airways in mouse lung was revealed by Sirius red staining and quantified by morphometric techniques using Image J software as described in MATERIALS AND METHODS. The results are expressed as area of collagen/mm^2 basement membrane. $^{\alpha}$Significantly different from saline control mice; $^{\beta}$significantly different from the OVA alone group ($P < 0.05$, $n = 11$–13).

that TM5275 has a potent anti-inflammatory effect. Similar effects on inflammatory cell infiltration have been reported by other groups with another PAI-1 inhibitor, tiplaxtinin (24), and PAI-1 siRNA (29). Together, the results suggest that PAI-1 inhibitors may have therapeutic potential for asthma through suppressing the inflammatory response, specifically the eosinophilic response (Fig. 9).

The mechanism whereby TM5275 reduces the numbers of neutrophils, eosinophils, and monocytes in BALF is unknown at the moment. PAI-1 has pleiotropic functions besides inhibiting the activities of uPA and tPA. It binds to the extracellular matrix protein vitronectin and thereby blocks the binding of vitronectin to integrins and uPA receptor (uPAR) on the cell surface, leading to cell detachment/migration (6, 36, 38). PAI-1 can also affect cell migration and function through binding to uPA-uPAR and then low-density lipoprotein receptor-related protein (LRP) on the cell surface (7, 16). Interestingly, it has been reported that macrophage migration depends on the interaction between PAI-1 and LRP, and that TM5275 inhibits macrophage migration through interfering in such interaction (16). Therefore, one of the potential mechanisms whereby TM5275 reduces the numbers of neutrophils, eosinophils, and monocytes in BALF is to block PAI-1-mediated cell migration

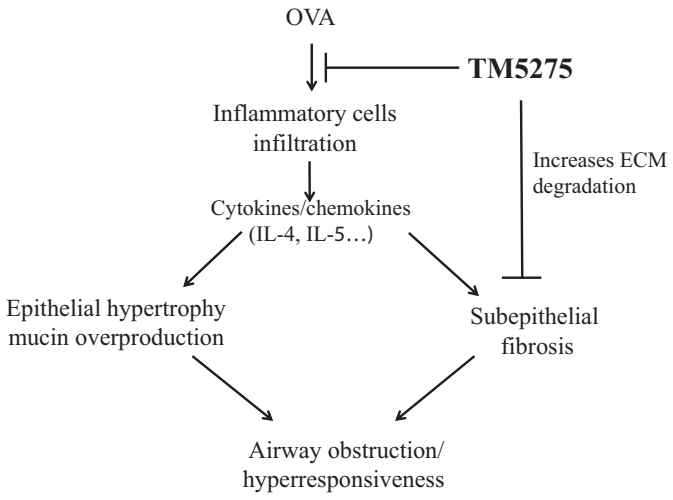

Fig. 9. Potential mechanisms whereby TM5275 ameliorates OVA-induced airway pathophysiological changes. ECM, extracellular matrix.

(infiltration) through interfering with the interaction between PAI-1 and vitronectin and/or LRP. Emerging evidence also shows that PAI-1 modulates the survival/apoptosis process in different types of cells (12, 13, 34, 41). Previous study from this lab showed that TM5275 induces p53, a master apoptosis inducer, and apoptosis in primary human (13) and mouse (12) lung fibroblasts. A previous study from this group also showed that treatment with PAI-1 protein inhibited spontaneous and TNF-α-induced apoptosis of neutrophils, whereas knockout of the PAI-1 gene (PAI-1$^{-/-}$ mice) promoted neutrophil apoptosis induced by lipopolysaccharide (41). Therefore, another potential mechanism whereby TM5275 reduced the numbers of eosinophil, neutrophils, and/or monocytes in BALF of OVA-challenged mice is to induce apoptosis of these inflammatory cells. More studies are needed to decipher the mechanisms whereby TM5275 reduces inflammatory cell numbers in BALF.

Hypertrophy and hyperplasia of goblet cells, which leads to overproduction of mucin, is another important pathological feature of asthmatic airways. Using the PAS staining technique, Lee et al. showed that treatment with the PAI-1 inhibitor tiplaxtinin reduced OVA-induced mucin overproduction, although the mechanism by which tiplaxtinin reduces mucin overproduction was not explored (24). In this study, we show that OVA challenge dramatically increased, whereas TM5275 administration significantly reduced, OVA-stimulated mucin production in airways by PAS staining and by measuring the amount of MUC5AC in BALF. We further show that TM5275 administration attenuated the OVA-mediated increase in IL-4 and IL-5, two Th2 cytokines that have been shown to stimulate mucin production in goblet cells (17, 20, 35). Together, our results suggest that TM5275 administration ameliorated OVA-induced airway mucin production/accumulation probably through inhibition of the inflammatory response and Th2 cytokine production.

Airway remodeling or subepithelial fibrosis is another important pathological feature of chronic asthmatic airways. Collagens are the major types of extracellular matrix proteins and the major component of fibrotic tissue. Previous study from this lab and others has shown that PAI-1 inhibited collagen degradation in cultured fibroblasts (39) and plays an important role in the development of lung fibrosis (5, 8, 13, 33, 40). In this study, we show that OVA sensitization-challenge signifi-

cantly increased collagen deposition in large airways, whereas treatment with TM5275 partially blocked the OVA effect. These results are consistent with our previous findings, although in a different disease model (13). In that study, we showed that treatment of mice with TM5275 almost completely blocked collagen and hydroxyproline accumulation in the lung in a mouse lung fibrosis model induced by AdTGF-$\beta1^{223/225}$, an adenovirus expressing constitutively active TGF-β1, when given 4 days after AdTGF-$\beta1^{223/225}$ instillation (13). It is unclear at this moment whether TM5275 reduces collagen deposition/accumulation by stimulating collagen degradation and/or by suppressing collagen synthesis. Because previous studies from this lab and others have shown that PAI-1 suppresses collagen degradation (14, 15, 39), it is speculated that TM5275 reduces collagen accumulation in large airways in part by increasing collagen degradation.

In summary, our data suggest that the orally effective small-molecule PAI-1 inhibitor TM5275 attenuates OVA-induced pathophysiological changes in mouse airways and may have therapeutic potential for chronic asthma.

ACKNOWLEDGMENTS

We thank Dr. Chad Steele for assistance in the measurement of cytokines/chemokines in BAL fluid.

GRANTS

This work was supported by grants from the National Heart, Lung, and Blood Institute to R.-M. Liu (5R01-HL-088141) and to V. J. Thannickal (P01-HL-114470).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.-M.L., S.E., N.W., J.S.D., G.L., L.M.S., T.M., and V.J.T. conception and design of research; R.-M.L., S.E., N.W., C.J., and Y.W. performed experiments; R.-M.L., S.E., N.W., J.S.D., H.-C.K., C.J., and Y.W. analyzed data; R.-M.L., S.E., N.W., J.S.D., H.-C.K., G.L., L.M.S., and T.M. interpreted results of experiments; R.-M.L., S.E., and C.J. prepared figures; R.-M.L. drafted manuscript; R.-M.L., N.W., J.S.D., Y.W., G.L., L.M.S., T.M., and V.J.T. edited and revised manuscript; R.-M.L., S.E., N.W., J.S.D., H.-C.K., C.J., Y.W., G.L., L.M.S., T.M., and V.J.T. approved final version of manuscript.

REFERENCES

1. **Brims FJ, Chauhan AJ, Higgins B, Shute JK.** Up-regulation of the extrinsic coagulation pathway in acute asthma–a case study. *J Asthma* 47: 695–698, 2010.
2. **Cho S, Kang J, Lyttle C, Harris K, Daley B, Grammer L, Avila P, Kumar R, Schleimer R.** Association of elevated plasminogen activator inhibitor 1 levels with diminished lung function in patients with asthma. *Ann Allergy Asthma Immunol* 106: 371–377, 2011.
3. **Cho SH, Hong SJ, Chen H, Habib A, Cho D, Lee SH, Kang J, Ward T, Boushey HA, Schleimer RP, Avila PC.** Plasminogen activator inhibitor-1 in sputum and nasal lavage fluids increases in asthmatic patients during common colds. *J Allergy Clin Immunol* 133: 1465–1467, 2014.
4. **Cho SH, Ryu CH, Oh CK.** Plasminogen activator inhibitor-1 in the pathogenesis of asthma. *Exp Biol Med (Maywood)* 229: 138–146, 2004.
5. **Chuang-Tsai S, Sisson TH, Hattori N, Tsai CG, Subbotina NM, Hanson KE, Simon RH.** Reduction in fibrotic tissue formation in mice genetically deficient in plasminogen activator inhibitor-1. *Am J Pathol* 163: 445–452, 2003.
6. **Czekay RP, Aertgeerts K, Curriden SA, Loskutoff DJ.** Plasminogen activator inhibitor-1 detaches cells from extracellular matrices by inactivating integrins. *J Cell Biol* 160: 781–791, 2003.
7. **Czekay RP, Loskutoff DJ.** Plasminogen activator inhibitors regulate cell adhesion through a uPAR-dependent mechanism. *J Cell Physiol* 220: 655–663, 2009.
8. **Eitzman DT, McCoy RD, Zheng X, Fay WP, Shen T, Ginsburg D, Simon RH.** Bleomycin-induced pulmonary fibrosis in transgenic mice that either lack or overexpress the murine plasminogen activator inhibitor-1 gene. *J Clin Invest* 97: 232–237, 1996.
9. **Elokdah H, Abou-Gharbia M, Hennan JK, McFarlane G, Mugford CP, Krishnamurthy G, Crandall DL.** Tiplaxtinin, a novel, orally efficacious inhibitor of plasminogen activator inhibitor-1: design, synthesis, and preclinical characterization. *J Med Chem* 47: 3491–3494, 2004.
10. **Hewitt M, Creel A, Estell K, Davis IC, Schwiebert LM.** Acute exercise decreases airway inflammation, but not responsiveness, in an allergic asthma model. *Am J Respir Cell Mol Biol* 40: 83–89, 2009.
11. **Hewitt M, Estell K, Davis IC, Schwiebert LM.** Repeated bouts of moderate-intensity aerobic exercise reduce airway reactivity in a murine asthma model. *Am J Respir Cell Mol Biol* 42: 243–249, 2010.
12. **Huang WT, Akhter H, Jiang C, MacEwen M, Ding Q, Antony V, Thannickal VJ, Liu RM.** Plasminogen activator inhibitor 1, fibroblast apoptosis resistance, and aging-related susceptibility to lung fibrosis. *Exp Gerontol* 61: 62–75, 2015.
13. **Huang WT, Vayalil PK, Miyata T, Hagood J, Liu RM.** Therapeutic value of small molecule inhibitor to plasminogen activator inhibitor-1 for lung fibrosis. *Am J Respir Cell Mol Biol* 46: 87–95, 2012.
14. **Huang Y, Border WA, Lawrence DA, Noble NA.** Mechanisms underlying the antifibrotic properties of noninhibitory PAI-1 (PAI-1R) in experimental nephritis. *Am J Physiol Renal Physiol* 297: F1045–F1054, 2009.
15. **Huang Y, Border WA, Yu L, Zhang J, Lawrence DA, Noble NA.** A PAI-1 mutant, PAI-1R, slows progression of diabetic nephropathy. *J Am Soc Nephrol* 19: 329–338, 2008.
16. **Ichimura A, Matsumoto S, Suzuki S, Dan T, Yamaki S, Sato Y, Kiyomoto H, Ishii N, Okada K, Matsuo O, Hou FF, Vaughan DE, van Ypersele de Strihou C, Miyata T.** A small molecule inhibitor to plasminogen activator inhibitor 1 inhibits macrophage migration. *Arterioscler Thromb Vasc Biol* 33: 935–942, 2013.
17. **Izuhara K, Ohta S, Shiraishi H, Suzuki S, Taniguchi K, Toda S, Tanabe T, Yasuo M, Kubo K, Hoshino T, Aizawa H.** The mechanism of mucus production in bronchial asthma. *Curr Med Chem* 16: 2867–2875, 2009.
18. **Izuhara Y, Yamaoka N, Kodama H, Dan T, Takizawa S, Hirayama N, Meguro K, van Ypersele de Strihou C, Miyata T.** A novel inhibitor of plasminogen activator inhibitor-1 provides antithrombotic benefits devoid of bleeding effect in nonhuman primates. *J Cereb Blood Flow Metab* 30: 904–912, 2010.
19. **Jiang XB, Huang M, Cui XF, Zhu Y, Yin KS, Yao K.** Respiratory syncytial virus infection differentiates airway dysfunction in the central and peripheral airways in OVA-sensitized mice. *Exp Lung Res* 38: 453–462, 2012.
20. **Kasaian MT, Marquette K, Fish S, DeClercq C, Agostinelli R, Cook TA, Brennan A, Lee J, Fitz L, Brooks J, Vugmeyster Y, Williams CM, Lofquist A, Tchistiakova L.** An IL-4/IL-13 dual antagonist reduces lung inflammation, airway hyperresponsiveness, and IgE production in mice. *Am J Respir Cell Mol Biol* 49: 37–46, 2013.
21. **Kowal K, Bodzenta-Lukaszyk A, Pampuch A, Szmitkowski M, Donati MB, Iacoviello L.** Plasminogen activator inhibitor-1 plasma concentration in allergic asthma patients during allergen challenge. *Int Arch Allergy Immunol* 144: 240–246, 2007.
22. **Kowal K, Moniuszko M, Zukowski S, Bodzenta-Lukaszyk A.** Concentrations of plasminogen activator inhibitor-1 (PAI-1) and urokinase plasminogen activator (uPA) in induced sputum of asthma patients after allergen challenge. *Folia Histochem Cytobiol* 48: 518–523, 2010.
23. **Kucharewicz I, Mogielnicki A, Kasacka I, Buczko W, Bodzenta-Lukaszyk A.** Plasmin system regulation in an ovalbumin-induced rat model of asthma. *Int Arch Allergy Immunol* 147: 190–196, 2008.
24. **Lee SH, Eren M, Vaughan DE, Schleimer RP, Cho SH.** A plasminogen activator inhibitor-1 inhibitor reduces airway remodeling in a murine model of chronic asthma. *Am J Respir Cell Mol Biol* 46: 842–846, 2012.
25. **Lijnen HR.** Pleiotropic functions of plasminogen activator inhibitor-1. *J Thromb Haemost* 3: 35–45, 2005.
26. **Lilly LM, Scopel M, Nelson MP, Burg AR, Dunaway CW, Steele C.** Eosinophil deficiency compromises lung defense against Aspergillus fumigatus. *Infect Immun* 82: 1315–1325, 2014.

27. **Liu RM, Vayalil PK, Ballinger C, Dickinson DA, Huang WT, Wang S, Kavanagh TJ, Matthews QL, Postlethwait EM.** Transforming growth factor beta suppresses glutamate-cysteine ligase gene expression and induces oxidative stress in a lung fibrosis model. *Free Radic Biol Med* 53: 554–563, 2012.
28. **Ma Z, Paek D, Oh CK.** Plasminogen activator inhibitor-1 and asthma: role in the pathogenesis and molecular regulation. *Clin Exp Allergy* 39: 1136–1144, 2009.
29. **Miyamoto S, Hattori N, Senoo T, Onari Y, Iwamoto H, Kanehara M, Ishikawa N, Fujitaka K, Haruta Y, Murai H, Yokoyama A, Kohno N.** Intra-airway administration of small interfering RNA targeting plasminogen activator inhibitor-1 attenuates allergic asthma in mice. *Am J Physiol Lung Cell Mol Physiol* 301: L908–L916, 2011.
30. **Nie W, Li B, Xiu QY.** The -675 4G/5G polymorphism in plasminogen activator inhibitor-1 gene is associated with risk of asthma: a meta-analysis. *PloS one* 7: e34385, 2012.
31. **Oh CK, Ariue B, Alban RF, Shaw B, Cho SH.** PAI-1 promotes extracellular matrix deposition in the airways of a murine asthma model. *Biochem Biophys Res Commun* 294: 1155–1160, 2002.
32. **Ozbek OY, Atac FB, Ogus E, Ozbek N.** Plasminogen activator inhibitor-1 gene 4G/5G polymorphism in Turkish children with asthma and allergic rhinitis. *Allergy Asthma Proc* 30: 41–46, 2009.
33. **Senoo T, Hattori N, Tanimoto T, Furonaka M, Ishikawa N, Fujitaka K, Haruta Y, Murai H, Yokoyama A, Kohno N.** Suppression of plasminogen activator inhibitor-1 by RNA interference attenuates pulmonary fibrosis. *Thorax* 65: 334–340, 2010.
34. **Shetty SK, Bhandary YP, Marudamuthu AS, Abernathy D, Velusamy T, Starcher B, Shetty S.** Regulation of airway and alveolar epithelial cell apoptosis by p53-Induced plasminogen activator inhibitor-1 during cigarette smoke exposure injury. *Am J Respir Cell Mol Biol* 47: 474–483, 2012.
35. **Shirasaki H, Kanaizumi E, Seki N, Himi T.** Leukotriene E4 induces MUC5AC release from human airway epithelial NCI-H292 cells. *Allergol Int* 64: 169–174, 2015.
36. **Stefansson S, Su EJ, Ishigami S, Cale JM, Gao Y, Gorlatova N, Lawrence DA.** The contributions of integrin affinity and integrin-cytoskeletal engagement in endothelial and smooth muscle cell adhesion to vitronectin. *J Biol Chem* 282: 15679–15689, 2007.
37. **Stevens PT, Kicic A, Sutanto EN, Knight DA, Stick SM.** Dysregulated repair in asthmatic paediatric airway epithelial cells: the role of plasminogen activator inhibitor-1. *Clin Exp Allergy* 38: 1901–1910, 2008.
38. **Takahashi T, Suzuki K, Ihara H, Mogami H, Kazui T, Urano T.** Plasminogen activator inhibitor type 1 promotes fibrosarcoma cell migration by modifying cellular attachment to vitronectin via alpha(v)beta(5) integrin. *Semin Thromb Hemost* 31: 356–363, 2005.
39. **Vayalil PK, Olman M, Murphy-Ullrich JE, Postlethwait EM, Liu RM.** Glutathione restores collagen degradation in TGF-beta-treated fibroblasts by blocking plasminogen activator inhibitor-1 expression and activating plasminogen. *Am J Physiol Lung Cell Mol Physiol* 289: L937–L945, 2005.
40. **Zhang YP, Li WB, Wang WL, Liu J, Song SX, Bai LL, Hu YY, Yuan YD, Zhang M.** siRNA against plasminogen activator inhibitor-1 ameliorates bleomycin-induced lung fibrosis in rats. *Acta Pharmacol Sin* 33: 897–908, 2012.
41. **Zmijewski JW, Bae HB, Deshane JS, Peterson CB, Chaplin DD, Abraham E.** Inhibition of neutrophil apoptosis by PAI-1. *Am J Physiol Lung Cell Mol Physiol* 301: L247–L254, 2011.