

regulatory T cells in the draining lymph nodes after caspase-8 blockade (Fig 2, V-Y). These results collectively indicate that ILA inhibition by topical caspase-8 blockade interrupts the transportation of APCs to reduce T_H17 , which subsequently suppresses corneal GR (for the role of HMGB1 and TLR4 in corneal allograft rejection, see the Results sections and Fig E6 in this article's Online Repository at www.jacionline.org).

In summary, this study provides the first evidence that pharmacological inhibition of caspase-8 suppressed lymphangiogenesis and GR; it also identifies an innovative mechanism whereby the caspase-8/NLRP3 inflammasome pathway promotes lymphangiogenesis and GR (see Fig E7 in this article's Online Repository at www.jacionline.org). The CT procedure initially leads to corneal cell damage, resulting in the release of HMGB1. The released HMGB1 bonds with TLR4 to activate caspase-8 signaling in the cornea. Then, the activated caspase-8 signaling mediates macrophage activation and recruitment to induce lymphangiogenesis. Furthermore, caspase-8 regulates NLRP3 inflammasome-mediated HMGB1 secretion in the cornea and macrophage. The secreted HMGB1 may, in turn, interact with TLR4, forming a positive feedback loop to further promote lymphangiogenesis. Finally, new lymphatic vessels transport APCs carrying allograft antigens to activate T cells, which initiate and promote GR. Therefore, the caspase-8/NLRP3 inflammasome pathway functions as an amplification system to promote lymphangiogenesis and GR, whereas the blockage of caspase-8 interrupts this process. These findings provide new insightful views into the pathogenesis of inflammatory lymphangiogenesis and GR. They also support the idea that the pharmacological inhibition of caspase-8 may serve as a new strategy for treating GR and other lymphatic disorders.

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Mast cell-derived plasminogen activator inhibitor type 1 promotes airway inflammation and remodeling in a murine model of asthma



To the Editor:

Asthma is a chronic inflammatory disorder of the airways with airflow obstruction that might initially be reversible but progresses to an irreversible status because of airway remodeling. These structural changes, which occur in both the large and small airways of asthmatic patients, are characterized by subepithelial fibrosis, goblet cell hyperplasia, angiogenesis, and airway smooth muscle cell hypertrophy.¹

Mast cells (MCs) play an important role in tissue fibrosis in asthmatic patients.² MC-deficient mice showed reduced deposition of collagen in the airways in a murine model of asthma, which was restored by MC reconstitution.² However, the mechanisms and mediators of MCs involved in asthmatic airway remodeling remain unclear.

Plasminogen activator inhibitor type 1 (PAI-1) is the primary inhibitor of urokinase-type plasminogen activator and tissue-type plasminogen activator (tPA). Previously, we reported that PAI-1 levels were increased in the airways in a murine model of asthma and that genetic deletion of PAI-1 was associated with diminished airway fibrosis.³ PAI-1 inhibitor treatment also reduced airway inflammation and tissue remodeling in these mice.^{4,5} We have also demonstrated that human MCs are a major source of PAI-1 in the airways of asthmatic patients.^{6,7} In this study we hypothesized that MC-derived PAI-1 plays a major role in airway remodeling.

Experiments using MC-deficient C57BL/6J-Kit^{w-sh/w-sh} mice were performed to verify the role of MC-derived PAI-1 in asthma. MC-deficient C57BL/6J-Kit^{w-sh/w-sh} mice were reconstituted with bone marrow-cultured mast cells (BMCs) from PAI-1^{-/-} and wild-type (WT) mice. The functionality of activated BMCs from PAI-1^{-/-} mice was confirmed by the equal amount of histamine release compared with that seen in activated BMCs from WT mice (see Fig E1 in this article's Online Repository at www.jacionline.org) before reconstitution, and similar numbers of tissue MCs in the lungs from both BMC-reconstituted groups were also observed

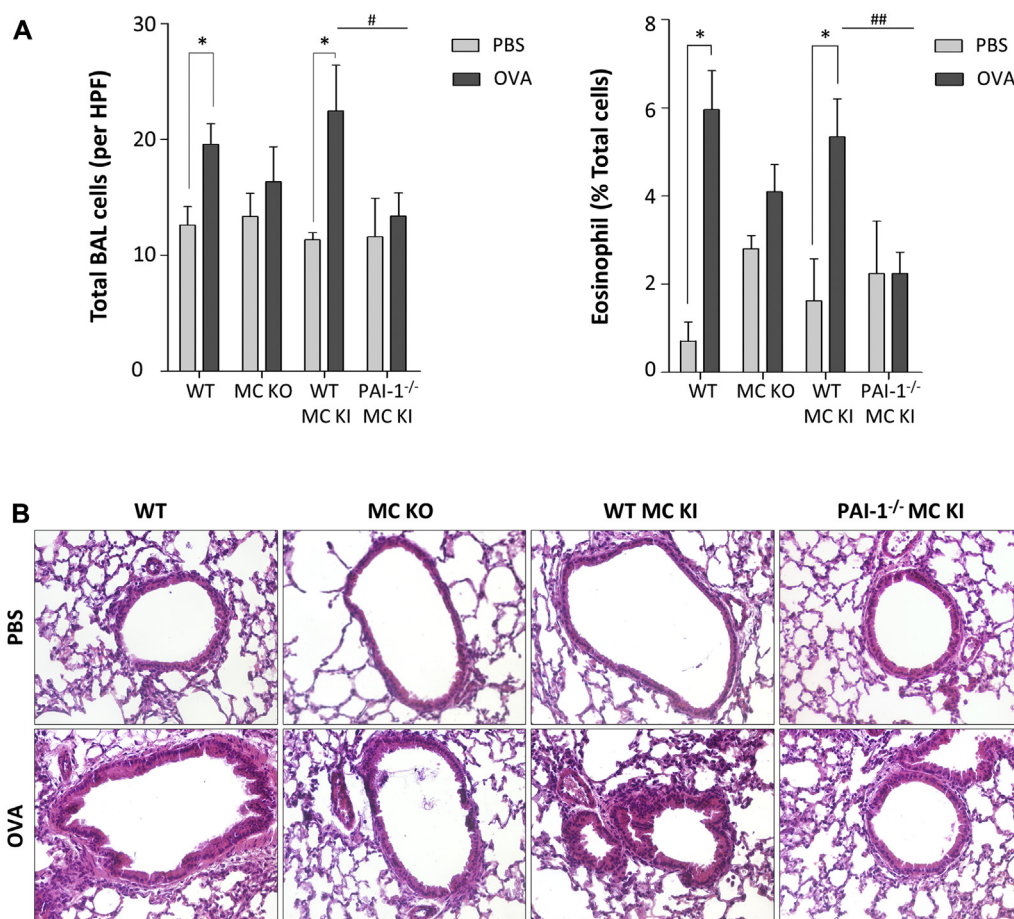


FIG 1. Inflammation in lung tissues of PBS- or OVA-challenged mice. **A**, Number of total inflammatory cells and eosinophils per HPF in BALF from PBS- or OVA-challenged mice. Data are expressed as means $\pm$ SEs. * $P < .05$ PBS versus OVA. $^{##}P < .01$ OVA-challenged WT MC KI versus OVA-challenged PAI-1^{-/-} MC KI. **B**, Representative hematoxylin and eosin staining for histopathologic changes in lung sections of PBS- or OVA-challenged mice. WT, WT mice; MC KO, Kit^{w-sh/w-sh} mice; WT MC KI, WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice; and PAI-1^{-/-} MC KI, PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice. KI, Knock-in; KO, knockout.

after reconstitution (see Fig E2 in this article's Online Repository at www.jacionline.org). Detailed experimental methods and statistical analyses are described in the Methods section in this article's Online Repository at www.jacionline.org.

Numbers of total inflammatory cells and eosinophils in bronchoalveolar lavage fluid (BALF) from Kit^{w-sh/w-sh} mice reconstituted with BMCMCs from PAI-1^{-/-} mice (PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice) were significantly decreased compared with those from Kit^{w-sh/w-sh} mice reconstituted with BMCMCs from WT mice (WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice) after ovalbumin (OVA) challenge (total inflammatory cells: 13.38 ± 5.29 vs 22.46 ± 7.92 cells/high-power field [HPF]; $P < .05$), respectively; eosinophils: $2.24\% \pm 1.28\%$ vs $5.34\% \pm 1.72\%$ [$P < .01$], respectively; Fig 1, A). Hematoxylin and eosin staining showed recruitment of inflammatory cells in the peribronchial and perivascular areas of OVA-challenged WT and WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice. However, OVA-challenged PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice had reduced infiltration of inflammatory cells (Fig 1, B). Collectively, these results show that MC-derived PAI-1 promotes inflammation in this murine model of asthma.

As shown in Fig 2, A, PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice displayed markedly suppressed goblet cell hyperplasia compared with WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice after OVA challenge ($0.13\% \pm 0.22\%$ vs $24.52\% \pm 12.75\%$, $P < .01$). Accumulation of collagen in lung tissues was markedly increased in WT and WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice after OVA challenge compared with that seen in PBS-challenged control mice (WT mice: $18.26\% \pm 5.94\%$ vs $8.40\% \pm 1.43\%$ [$P < .01$], respectively; WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice: $23.06\% \pm 10.37\%$ vs $7.75\% \pm 3.54\%$ [$P < .01$], respectively). In contrast, PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice had remarkably decreased collagen deposition compared with WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice after OVA challenge ($10.21\% \pm 2.17\%$ and $23.06\% \pm 10.37\%$ [$P < .01$]; Fig 2, B). Our results implicate the importance of MC-derived PAI-1 in OVA-induced goblet cell hyperplasia and collagen deposition.

Furthermore, we measured the enhanced minute pause (Penh) to determine the physiologic effect on airway hyperresponsiveness (AHR). The Penh value of PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice was significantly decreased compared with values in WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice after OVA challenge ($355.6\% \pm 60.4\%$ vs $629.2\% \pm 76.1\%$ Penh [$P < .05$]; Fig 2, C).

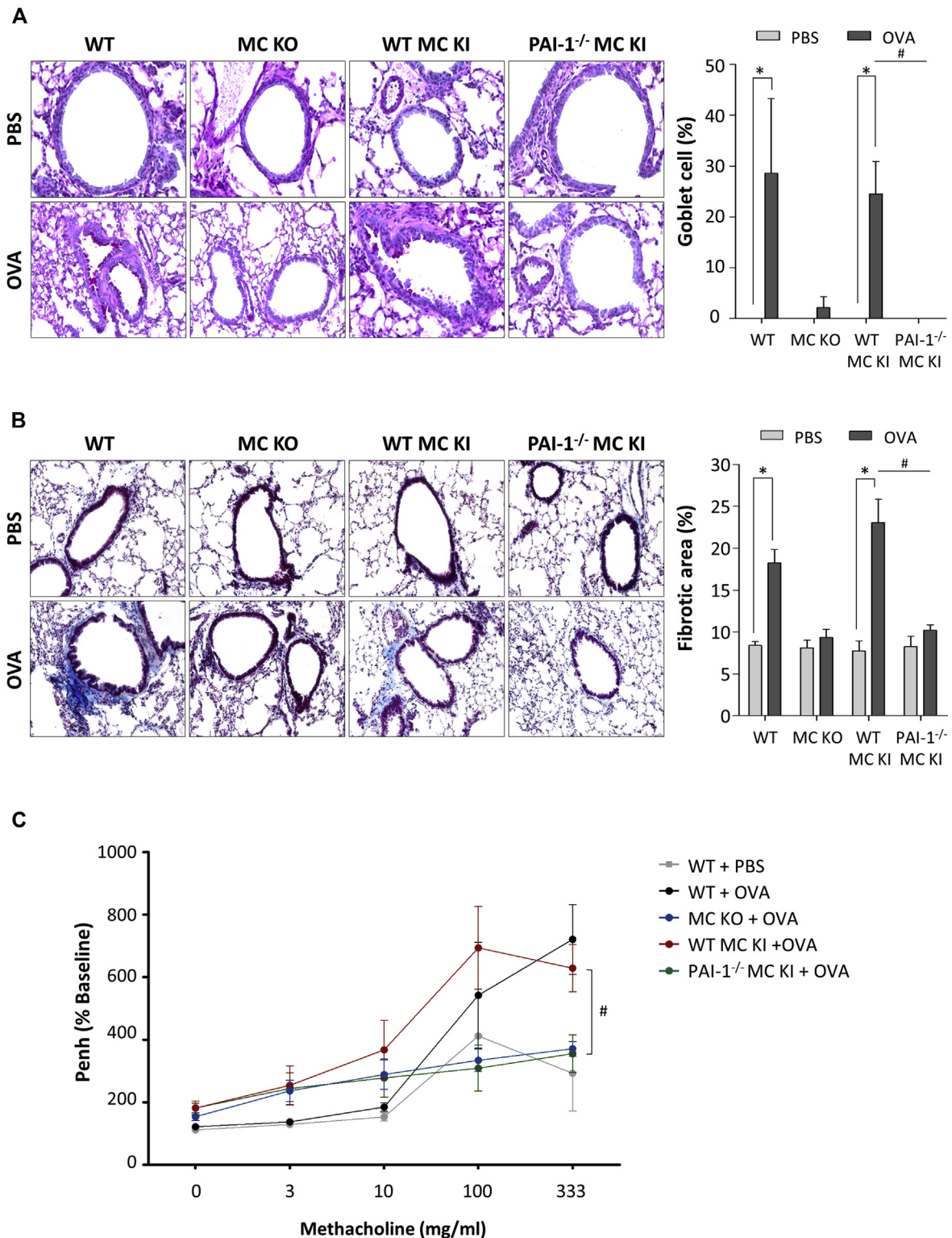


FIG 2. Airway remodeling in lung tissues of PBS- or OVA-challenged mice. **A**, Lung sections were stained with periodic acid-Schiff to assess goblet cell hyperplasia. Original magnification $\times 400$. The number of periodic acid-Schiff-positive cells per 100 μm of basement membrane is visualized graphically with ImageJ software (right panel). * $P < .05$ PBS versus OVA. # $P < .05$ OVA-challenged WT MC KI versus OVA-challenged PAI-1^{-/-} MC KI. ## $P < .01$ OVA-challenged WT MC KI versus OVA-challenged PAI-1^{-/-} MC KI. **B**, Masson trichrome staining was conducted to evaluate collagen deposition (blue). Original magnification $\times 200$. * $P < .05$ PBS versus OVA. # $P < .05$ OVA-challenged WT MC KI versus OVA-challenged PAI-1^{-/-} MC KI. ## $P < .01$ OVA-challenged WT MC KI versus OVA-challenged PAI-1^{-/-} MC KI. **C**, AHR to increasing concentrations of methacholine (0–333 mg/mL) was measured by using the Buxco apparatus and was reported as percentage change of Penh per baseline value. # $P < .05$ OVA-challenged WT MC KI versus OVA-challenged PAI-1^{-/-} MC KI. All data are expressed as means $\pm$ SEs. WT, WT mice; MC KO, Kit^{w-sh/w-sh} mice; WT MC KI, WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice; and PAI-1^{-/-} MC KI, PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh} mice.

Lastly, we examined expression of PAI-1 in each group by using immunohistochemical analysis. As expected, overall PAI-1 protein expression was significantly lower in the lungs of $\text{Kit}^{\text{w-sh/w-sh}}$ and $\text{PAI-1}^{-/-}$ BMCMC $\rightarrow \text{Kit}^{\text{w-sh/w-sh}}$ mice compared with WT and WT BMCMC $\rightarrow \text{Kit}^{\text{w-sh/w-sh}}$ mice after OVA challenge (see Fig E3, A, in this article's Online Repository at www.jacionline.org). MCs were strongly positive for PAI-1 staining, and monocytes/macrophages were another source of strong PAI-1 expression (see Fig E3, B).

Yu et al² demonstrated previously that MCs play a critical role in airway remodeling in asthma using a MC knockout and reconstitution approach, which is similar to our current experimental approach. Emerging evidence from our laboratory and those of others shows that PAI-1 plays an important role in the pathogenesis of asthma and that MCs are a major source of PAI-1.⁵⁻⁷ Therefore we further investigated the causative role of MC-derived PAI-1 in allergen-induced asthma by using an MC knockout and reconstitution model. This study confirmed that MC-specific PAI-1 promotes airway inflammation, goblet cell hyperplasia, collagen deposition, and AHR.

Activated MCs in the airways of asthmatic patients are important not only in initiating allergic inflammation after allergen challenge but also in maintaining inflammation through interaction with resident cells and migratory inflammatory cells in the airways. Endothelial cells, macrophages, and other inflammatory cells tend to produce both PAI-1 and tPA simultaneously.^{7,8} However, MCs release tPA constitutively in their resting state and PAI-1 only on activation.⁶ Consequently, activated MC-derived PAI-1 converts a fibrinolytic environment to a fibrogenic environment by overcoming tPA activity, which is dominant during the resting state.⁶ Previously, we reported that MC-derived PAI-1 is expressed at high levels in human lung samples from fatal asthmatic patients.⁶ Taken together, allergen-induced MC activation results in increased levels of PAI-1, which in turn converts fibrinolytic environment to fibrogenic environment.

It is interesting that $\text{PAI-1}^{-/-}$ BMCMC $\rightarrow \text{Kit}^{\text{w-sh/w-sh}}$ mice had reduced eosinophilic inflammation. Our previous study showed that PAI-1 inhibition by tiplaxtinin decreased the degree of eosinophilic airway inflammation and AHR in an OVA challenge model of allergic asthma.⁴ Miyamoto et al⁵ also reported that intra-airway administration of small interfering RNA against PAI-1 reduced eosinophilic airway inflammation and AHR in a murine model of acute asthma. They suggest that increased levels of hepatocyte growth factor caused by PAI-1 inhibition might be a mechanism of attenuation in airway inflammation and AHR in their model of acute asthma. We also measured hepatocyte growth factor and other growth factors, such as TGF- β 1 and vascular endothelial growth factor, but could not find any significant difference between the groups. Interestingly, however, our *in vitro* study showed that PAI-1 enhanced IL-4/double-stranded RNA-induced thymic stromal lymphopoietin (TSLP) production from airway epithelial cells (see Fig E4 in this article's Online Repository at www.jacionline.org), suggesting a possible feedback loop of IgE-PAI-1-TSLP-IL-4/IL-5-IgE/eosinophil inflammation. Based on this result, we speculate that deletion of MC-derived PAI-1 can reduce eosinophilic airway inflammation at least in part through attenuation of the PAI-1-TSLP feedback pathway.

In conclusion, our experiments demonstrated that MCs are an important source of PAI-1 and that MC-derived PAI-1 contributes to airway inflammation, goblet cell hyperplasia, collagen deposition, and AHR. These data suggest that MC-derived PAI-1 can be a novel therapeutic target for airway remodeling.

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Recurrent wheezing in children following human metapneumovirus infection



To the Editor:

Human metapneumovirus (HMPV) is a paramyxovirus responsible for a significant portion (5%-10%) of respiratory tract infections.¹ HMPV causes infection ranging from self-limiting acute upper respiratory tract infection to severe lower respiratory

METHODS

MC reconstitution

MC-deficient C57BL/6J-Kit^{w-sh/w-sh} mice (Jackson Laboratory, Bar Harbor, Me) were reconstituted with BMCMCs from PAI-1^{-/-} and WT mice on the C57BL/6J background. Briefly, suspended bone marrow cells from PAI-1^{-/-} and WT mice were obtained from 4- to 6-week-old PAI-1^{-/-} and WT C57BL/6J mice and then cultured in conditioned medium for 4 to 5 weeks, at which time the cell populations were composed of greater than 95% MCs. Histamine release from activated MCs through IgE-receptor cross-linking was measured with an ELISA (LifeSpan BioSciences, Seattle, Wash). Ten million BMCMCs were infused through the tail vein into MC-deficient Kit^{w-sh/w-sh} mice, and the recipients were studied at least 18 weeks later. Animal experiments were conducted according to the protocol approved by the Animal Care and Ethics Committee at Northwestern University.

Mouse model of asthma

Mice were injected intraperitoneally with alum-precipitated OVA (grade V) in PBS. After 21 days, mice were challenged intranasally with 20 μ g of OVA in 30 μ L of PBS 3 times weekly for 4 weeks. Control mice received PBS intraperitoneally and intranasally on the same schedule. Twenty-four hours after the last allergen challenge, mice were killed, and lung tissues were collected for histologic and immunohistochemical analysis, whereas BALF was collected for inflammatory cell counts.

Inflammatory cell counts in BALF

BALF was collected by cannulating the upper part of the trachea, followed by lavage 3 times with 0.5 mL of 1% BSA in PBS. BALF from each mouse was centrifuged. Cells were resuspended in 1 mL of PBS/1% BSA. Total and differential cell counts were determined with Wright-Giemsa-stained cytospin preparations.

Histologic and immunohistochemical analysis of animal models

Lungs were fixed in 4% paraformaldehyde and embedded in paraffin. Routine hematoxylin and eosin-stained sections were examined to ensure

lung inflammation of the tissue by using a microscope. Tissue sections were stained with periodic acid-Schiff for enumeration of goblet cells. Goblet cell hyperplasia was assessed by determining the percentage of periodic acid-Schiff-positive cells per bronchial basal lamina in 10 random fields, as measured with ImageJ analysis software (National Institutes of Health, Bethesda, Md). Masson trichrome staining was used to evaluate collagen deposition. Deposition of collagen was quantified as a percentage of surface area stained with ImageJ software.

Deparaffinized and rehydrated sections were incubated overnight at 4°C with a polyclonal PAI-1 antibody at 1:100 dilutions. Immunostained sections were visualized with the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif) with a liquid DAB-plus substrate kit (Invitrogen, Carlsbad, Calif). Toluidine blue was used to detect MCs (purple).

Measurement of airway hyperresponsiveness

Airway hyperresponsiveness was measured with whole-body plethysmography (Buxco, Wilmington, NC). Mice were exposed to nebulized PBS for 2 minutes and then to increasing doses of nebulized methacholine (3, 10, 100, and 333 mg/mL) by using an ultrasonic nebulizer. Penh measurements were averaged and expressed as absolute Penh values.

Airway epithelial cell culture and TSLP measurement

Primary cultured normal bronchial epithelial cells were obtained from ATCC (Manassas, Vass) and cultured according to the protocol. Cells were stimulated with PAI-1 with or without a Toll-like receptor 3 agonist, double-stranded RNA (polyinosinic:polycytidylic acid), and IL-4 for 6 hours, and the production of TSLP mRNA and protein was measured by using RT-PCR and ELISA (R&D Systems, Minneapolis, Minn), respectively.

Statistical analysis

Statistical analyses were performed with GraphPad Prism software 6.0 (GraphPad Software, La Jolla, Calif). All results were expressed as means $\pm$ SEMs for groups of individual mice. The difference between 2 groups was determined by using the 2-tailed unpaired Student *t* test, and a *P* value of less than .05 was considered significant.

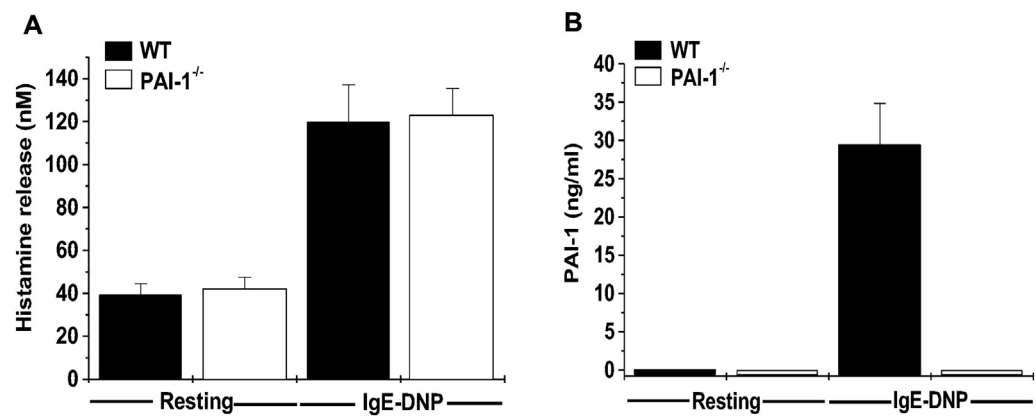


FIG E1. A, Histamine release from BMCMCs derived from WT and PAI-1^{-/-} mice. **A,** BMCMCs derived from WT and PAI-1^{-/-} mice were stimulated by using IgE receptor cross-linking, and histamine levels were measured in supernatants from the cells by using ELISA (n = 3). **B,** PAI-1 levels were measured in cell supernatants by means of ELISA (n = 3). *DNP*, 2,4-dinitrophenol.

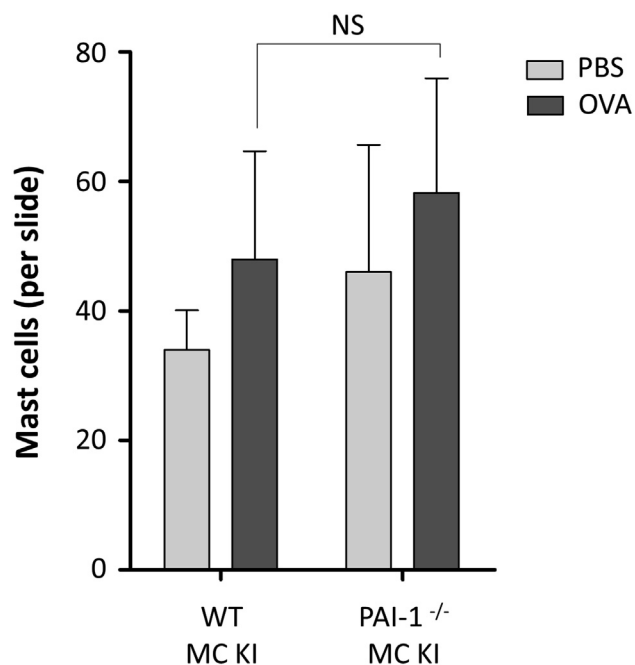


FIG E2. Numbers of tissue MCs in lungs of MC knock-in mice reconstituted with WT or PAI-1-deficient MCs. Tissue MCs were stained with toluidine blue, and numbers were measured in lung tissue sections from MC knock-in mice reconstituted with WT or PAI-1-deficient MCs. *C*, WT BMC MC $\rightarrow$ Kit^{w-sh/w-sh} mice; *D*, PAI-1^{-/-} BMC MC $\rightarrow$ Kit^{w-sh/w-sh} mice. There was no significant (*NS*) difference between groups *C* and *D* (PBS or OVA challenge).

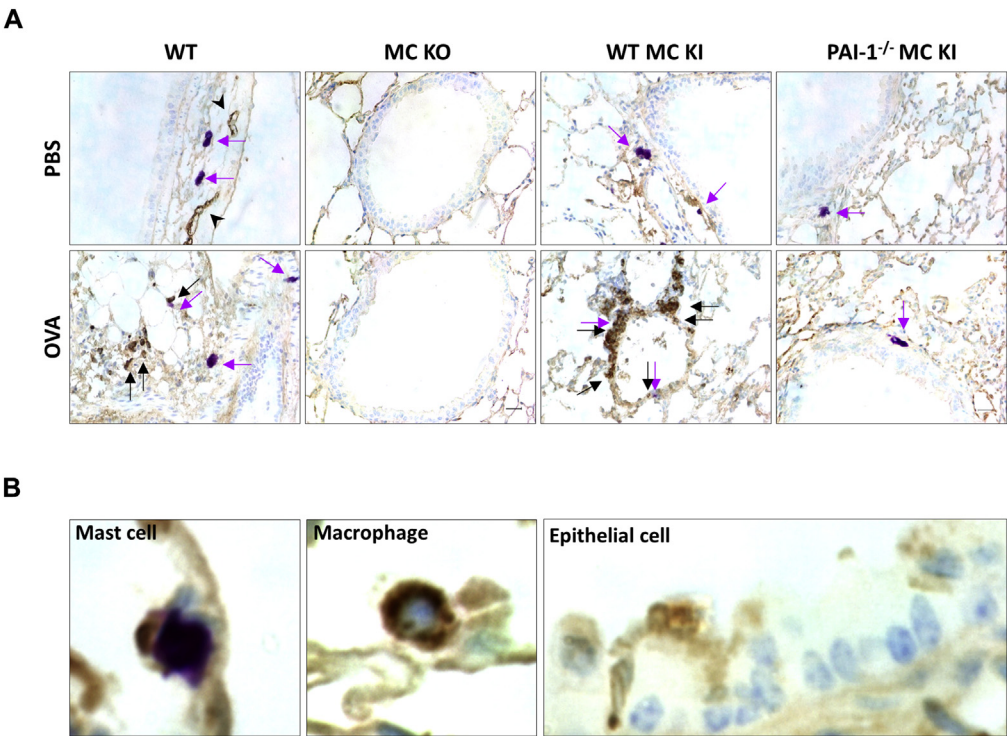


FIG E3. Immunohistochemistry of PAI-1 and toluidine blue staining for MCs in lung tissue. **A**, Data show a representative image of PAI-1 (brown, *black arrow*) and toluidine blue (purple, *purple arrow*) staining. *Black arrowhead* indicates normal positive PAI-1 staining of the blood vessel. Original magnification $\times 400$. *MC KO*, *Kit^{w-sh/w-sh}* mice; *PAI1^{-/-} MC KI*, *PAI-1^{-/-} BMCMC $\rightarrow$ Kit^{w-sh/w-sh}* mice; *WT*, *WT* mice; *WT MC KI*, *WT BMCMC $\rightarrow$ Kit^{w-sh/w-sh}* mice. **B**, Cellular sources of PAI-1: mast cell, macrophage, and epithelial cells. Original magnification $\times 600$.

