Inhibition of Pim1 Kinase Activation Attenuates Allergen-Induced Airway Hyperresponsiveness and Inflammation

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Pim kinases are a family of serine/threonine kinases whose activity can be induced by cytokines involved in allergy and asthma. These kinases play a role in cell survival and proliferation, but have not been examined, to the best of our knowledge, in the development of allergic disease. This study sought to determine the role of Pim1 kinase in the development of allergic airway responses. Mice were sensitized and challenged with antigen (primary challenge), or were sensitized, challenged, and rechallenged with allergen in a secondary model. To assess the role of Pim1 kinase, a small molecule inhibitor was administered orally after sensitization and during the challenge phase. Airway responsiveness to inhaled methacholine, airway and lung inflammation, cell composition, and cytokine concentrations were assessed. Lung Pim1 kinase concentrations were increased after ovalbumin sensitization and challenge. In the primary allergen challenge model, treatment with the Pim1 kinase inhibitor after sensitization and during airway challenges prevented the development of airway hyperresponsiveness, eosinophilic airway inflammation, and goblet cell metaplasia, and increased Th2 cytokine concentrations in bronchoalveolar fluid in a dose-dependent manner. These effects were also demonstrated after a secondary allergen challenge, where lung allergic disease was established before treatment. After treatment with the inhibitor, a significant reduction was evident in the number of $CD4^+$ and $CD8^+$ T cells and concentrations of cytokines in the airways. The inhibition of Pim1 kinase was effective in preventing the development of airway hyperresponsiveness, airway inflammation, and cytokine production in allergen-sensitized and allergen-challenged mice. These data identify the important role of Pim1 kinase in the full development of allergen-induced airway responses.

Keywords: airway hyperresponsiveness; inflammation; Pim1 kinase; T cells

Asthma is a complex inflammatory disorder, characterized by persistent airway inflammation and airway hyperresponsiveness (AHR) as a result of cellular and molecular responses induced by allergen exposure, infectious pathogens, or chemical agents (1, 2). Several clinical and experimental investigations showed that T cells, and especially Th2-type cells, play a pivotal role in the development of AHR and eosinophilic inflammation through the secretion of a variety of cytokines, including IL-4, IL-5, and IL-13 (3, 4). These cytokines bind to the extracellular Janus kinase (JAK) receptors and subsequently induce the phosphorylation and activation of signal transducers and activators of transcription (STATs), which translocate into the nucleus, where they bind to DNA and affect basic cell functions, including cell growth, differentiation, and death (5).

Pim kinases represent a family of three serine/threonine kinases that control cell survival, proliferation, differentiation, and apoptosis (6–8). Unlike other serine/threonine kinases, these are regulated via JAK/STAT activation–driven transcription of the *Pim* gene, rather than by membrane recruitment and phosphorylation (8). The overexpression of Pim kinase has been demonstrated in various human lymphomas, leukemias, and prostatic cancers (9). The role of Pim-induced oncogenic transformation was extensively studied in hematopoietic tumors (10–13). Despite numerous studies on the role of Pim kinase in the development of tumor cells, studies exploring the role of these kinases in immune cells have been limited. Pim1 kinase was expressed in human eosinophils, and played a major role in the IL-5–induced survival of eosinophils (14, 15). Furthermore, Pim1 expression was increased in eosinophils from bronchoalveolar lavage (BAL) fluid, compared with blood from patients with asthma after an allergen provocation (16). In a recent study, Pim1 kinase was shown to promote cell survival in T cells (17).

$CD4^+$ T cells play a central role in the development of allergic inflammation (18). $CD4^+$ T cells, especially Th2 cells producing IL-4, IL-5, and IL-13, were identified in the BAL fluid and airway tissue in patients with asthma (4). The transfer of Th2 cells, followed by airway allergen challenge in mice, was sufficient to induce airway eosinophilia and AHR (19, 20). Recent studies demonstrated increased numbers of $CD8^+$ T cells in the lung tissue of patients with asthma (21). These studies suggest that not only $CD4^+$ T cells but also $CD8^+$ T cells may be essential in the development of AHR and allergic inflammation (22–25). Subsets of $CD8^+$ T cells that produce IL-4, IL-5, and IL-13, but not IFN-γ, labeled as Tc2 cells, are known to increase AHR and airway inflammation (26–28).

In this study, we determined the role of Pim1 kinase in the development of allergen-induced AHR and airway inflammation *in vivo*, using a Pim1 kinase inhibitor. The inhibitor was examined in two distinct models of allergic airway inflammation. First, the development of allergen-induced airway responses was examined in mice that were systemically sensitized to ovalbumin (OVA), followed by 3 consecutive days of OVA challenge via the airways (primary allergen challenge). Second, effects on established airway disease were examined in mice that were sensitized and challenged with OVA, and when their allergic airway inflammation had resolved, they were exposed to a single provocative airway challenge with allergen (secondary allergen challenge). This condition may more closely resemble allergic asthma, where subjects are already sensitized and repeatedly exposed to allergen. The results demonstrated that the administration of Pim1 kinase inhibitor prevented the development of AHR, airway inflammation, and BAL cytokine production in mice

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sensitized and challenged to allergen. The administration of Pim1 kinase inhibitor also attenuated the consequences of secondary challenge in previously sensitized and challenged mice. These suppressive effects were manifested in both $CD4^+$ and $CD8^+$ T cells, and may help identify a novel role for Pim1 kinase in the development of allergen-induced AHR and airway inflammation.

MATERIALS AND METHODS

Animals

Female BALB/c mice, 8–12 weeks of age and free of pathogens, were purchased from Harlan Laboratory (Indianapolis, IN). The animals were maintained on an OVA-free diet. Experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Sensitization and Challenge with Allergen

Our experimental protocol for the sensitization and primary and secondary challenges to allergen was described previously (29). Briefly, in the primary allergen challenge protocol, mice were sensitized by an intraperitoneal injection of 20 μg OVA (Fisher Scientific, Pittsburgh, PA), emulsified in 2.0 mg of alum (AlumImuject; Pierce, Rockford, IL) on Days 1 and 14, followed by aerosolized OVA challenge (1% in saline for 20 minutes) on Days 28, 29, and 30. Control mice were sensitized with PBS followed by OVA challenge in the same way. In the secondary allergen challenge protocol, mice were sensitized with 10 μg OVA with alum on Days 1 and 7, followed by 0.2% OVA challenge on Days 14–16 (primary allergen challenge). Fourteen days after the final primary allergen challenge, mice were challenged again with 1% OVA for 20 minutes (secondary allergen challenge). A group of mice were sensitized with PBS, followed by primary and secondary challenges with OVA. In all groups, assays were performed 48 hours after the final allergen challenge.

Pim Kinase Inhibitor Treatment

To determine the role of Pim1 kinase in the development of allergen-induced AHR and airway inflammation, we used the Pim1 kinase inhibitor, AR00460770 (Array Pharma, Boulder, CO). To characterize AR00460770 *in vitro*, the cellular half-maximal inhibitory concentration (IC_{50}) and kinase selectivity assays were determined. The cellular IC_{50} of AR00460770 was analyzed by the Ser112 phosphorylation of transiently transfected Bcl-2–associated death promoter (BAD) in HEK-293 cell lines, engineered to express human Pim1 and Pim2 (Millipore, Billerica, MA) and rat Pim3 (Array BioPharma, Boulder, CO) in conjunction with a DNA vector construct directing the expression of the Pim kinase substrate glutathione S-transferase (GST)-BAD (pEBG-mBAD). Cells were treated with serial dilutions of AR00460770 for 1.5 hours, and then labeled with an antibody specific for phospho-BAD (Ser112) and an antibody against GST (Cell Signaling Technology, Danvers, MA) as a normalization control. Immunoreactivity was detected using infrared (IR) fluorophore–conjugated secondary antibodies, and quantified on an imager (Aerius; Li-Cor, Lincoln, NE). The kinase selectivity of AR00460770 was evaluated using the Kinase Profiler service (Millipore) (30–32). The properties and specificity of the inhibitor are described in Tables 1 and 2.

TABLE 1. CELLULAR HALF-MAXIMAL INHIBITORY CONCENTRATIONS FOR PIM INHIBITION WERE DETERMINED FOR AR00460770 (IC_{50}) BY ASSESSING SER112 PHOSPHORYLATION OF TRANSIENTLY TRANSFECTED BAD IN HEK CELL LINES ENGINEERED TO EXPRESS PIM1, PIM2, OR PIM3

	Pim1	Pim2	Pim3
AR00460770	93	9,200	340

Definition of abbreviations: SER, serine.
Cellular half-maximal inhibitory concentration values are reported in nanomole.

Western Blot Analysis

Lung tissue was homogenized, and lysates were cleared of debris and resuspended in an equal volume of 2 × Laemmli buffer. Lysates were loaded onto a 4–10% gradient reducing gel, subjected to electrophoresis, and transferred to nitrocellulose membranes. The membranes were blotted with goat anti-Pim1 (Santa Cruz Biotechnology, Santa Cruz, CA) and rabbit anti-GAPDH (R&D Systems, Minneapolis, MN), anti-goat IgG (Invitrogen, Carlsbad, CA), and anti-rabbit IgG (Rockland, Gilbertson, PA). Images were captured and quantitatively analyzed using an Odyssey infrared imager (Li-Cor).

Assessment of Airway Function

Airway responsiveness was assessed as previously described, by measuring changes in pulmonary resistance (RL) in response to increasing doses of inhaled methacholine (MCh; Sigma-Aldrich, St. Louis, MO) in anesthetized and ventilated mice (33). The values of peak airway responses to inhaled MCh were recorded.

Bronchoalveolar Lavage and Lung Histology

Lungs were lavaged with 1 ml of Hanks' balanced salt solution through the trachea immediately after the assessment of AHR. Numbers of total leukocytes were counted with a hemocytometer, and cell differentiation was performed on cytospin slides prepared with Wright-Giemsa stain. The numbers of inflammatory and mucus-containing cells were quantitated as previously described (34).

Measurement of Cytokines

Cytokine concentrations in BAL fluid and cell culture supernatants were measured by ELISA, as previously described (34).

TABLE 2. CHARACTERIZATION OF PIM INHIBITOR AR00460770

Kinase	Abbreviation	Enzyme IC_{50} (nm)
Proto-oncogene serine/threonine-protein kinase	Pim1	0.300
	Pim2	71
	Pim3	4
Proline–alanine–rich sterile (STE)20–related kinase	PASK	62
Tyrosine kinase, non–receptor 2	TNK2	3,000
Ca^{2+}/calmodulin-dependent protein kinase II–γ	CAMK2γ	6,000
FMS-like tyrosine kinase receptor–3	Flt3	>10,000
Platelet-derived growth factor receptors	PDGFRs	>10,000
MAP/microtubule affinity–regulating kinase 1	MARK1	>1000
Ca^{2+}/calmodulin–dependent protein kinase II–β	CAMK2β	>10,000
5′ AMP-activated protein kinase	AMPK	>100,000
Ribosomal S6 kinase	RSK	>10,000

Definition of abbreviations: IC_{50}, half-maximal inhibitory concentration; MAP, mitogen-activated protein.
AR00460770 was tested at 10 μM against 230 kinases in enzymatic assays (Millipore Kinase Profiler). It was determined to be selective for the three Pim isoforms.

Isolation of Lung Mononuclear Cells and Flow Cytometry

Lung mononuclear cells (MNCs) were isolated as described previously using collagenase digestion, and their cellular composition was identified as described elsewhere (35).

$CD4^+$ and $CD8^+$ T Cell Purification and Cell Proliferation Assay

The purification of $CD4^+$ and $CD8^+$ T cells was conducted as previously described (24). The purity of $CD4^+$ and $CD8^+$ T-cell populations exceeded 95%, as assessed by flow cytometry.

In cell proliferation assays, an anti-mouse CD3e monoclonal antibody (mAb) (5 μg/ml; R&D Systems) was immobilized on 96-well flat-bottom plates overnight at 4°C. Purified $CD4^+$ and $CD8^+$ T cells were incubated the with inhibitor or PBS as vehicle (2×10^5 cells/well), and anti-CD28 mAb (5 μg/ml; R&D Systems) was added to the anti-CD3–precoated plates and incubated at 37°C for 24 hours. After 24 hours, 1 μCi tritium-labeled thymidine per well (Perkin-Elmer, Boston, MA) was added to 96-well plates for 6 hours and harvested with distilled water, followed by counting in a microplate scintillation and luminescence counter (Packard, Meriden, CT). The cell viability of $CD4^+$ and $CD8^+$ T cells was assessed 24 hours after incubation with 10 μM of inhibitor by a vital stain with trypan blue, and determined using an automated cell counter (Countess; Invitrogen).

Statistical Analysis

Results were expressed as means ± SEM. The *t* test was used to determine differences between the two groups. For comparisons between multiple groups, the Tukey-Kramer test was used. Nonparametric analyses, using the Mann-Whitney *U* test or Kruskal-Wallis test, were also applied to confirm that statistical differences remained significant, even if the underlying distribution was uncertain. Differences were regarded as statistically significant when $P < 0.05$.

RESULTS

In Vitro Characterization of AR00460770

The cellular IC_{50} and kinase selectivity of AR00460770 were determined and, as shown in Tables 1 and 2, exhibited strong inhibition specific to Pim1 kinase.

Lung Pim1 Kinase Concentrations Are Increased after Sensitization and Challenge with Allergen

To determine the importance of Pim1 kinase after allergen challenge, we evaluated protein expression levels of the kinase in lung tissue after the OVA challenge of sensitized mice. Pim1 expression levels in OVA-sensitized mice were markedly increased after OVA challenge compared with levels in nonsensitized, challenged-only mice. This up-regulation was detected in OVA-sensitized mice 6 hours after their second OVA challenge, and remained high up to 24 hours after the third OVA challenge (Figure 1).

Pim1 Kinase Inhibitor Treatment Prevents Development of AHR and Airway Inflammation after Primary Allergen Challenge

To determine the effects of Pim1 kinase inhibitor treatment on allergen-induced airway inflammation and AHR, mice were treated with the inhibitor or vehicle during the OVA challenge phase in the primary allergen challenge model. As shown in Figures 2A and 2B, vehicle-treated mice developed greater airway responses to MCh and eosinophil numbers in BAL fluid after sensitization and challenge with OVA, compared with sham-sensitized, OVA-challenged mice. Mice treated with the Pim1 inhibitor at doses of 10, 30, or 100 mg/kg developed significantly lower airway responsiveness to inhaled MCh and lower BAL eosinophil numbers, compared with the vehicle-treated group. Sham-sensitized but OVA-challenged mice were treated with 100 mg/kg of the inhibitor to assess its potential effects on smooth muscle contraction. Treatment with the inhibitor in this way did not alter the development of increasing RL with increasing concentrations of inhaled MCh.

As shown in Figure 2C, the inhibitor treatment of sensitized and challenged mice reduced the concentrations of IL-4, IL-5, IL-13, and IFN-γ in BAL fluid in a dose-dependent manner.

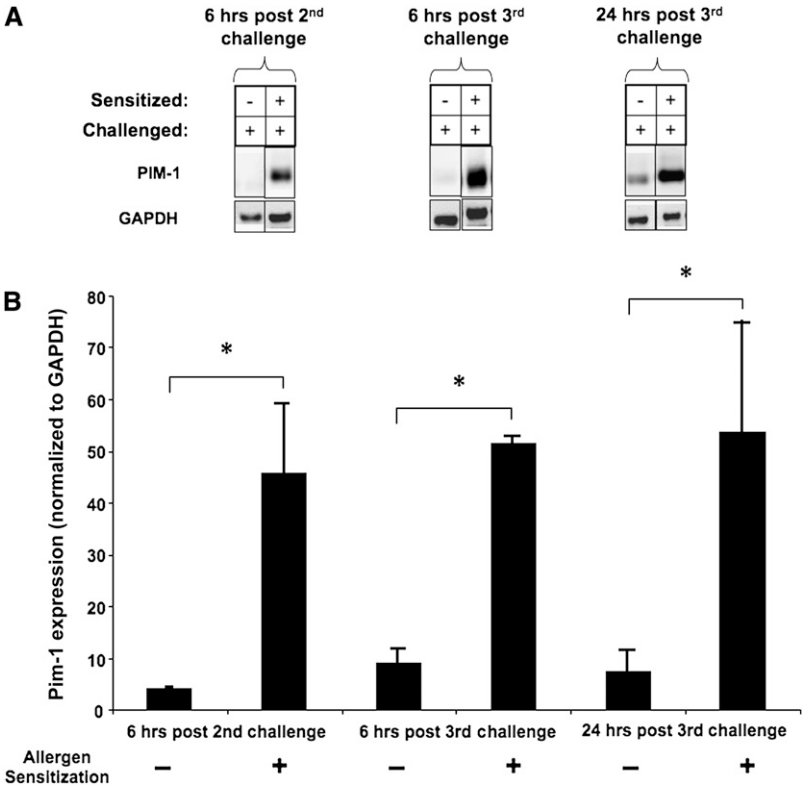

Figure 1. Expression levels of Pim1 kinase in lungs after sensitization and challenge with ovalbumin (OVA). Pim1 kinase concentrations were determined by Western blot analyses in lungs of mice that were sensitized and challenged with OVA, or that received sham sensitization and OVA challenge. Expression levels were examined at three time points: 6 hours after the second OVA challenge, 6 hours after the third OVA challenge, and 24 hours after third OVA challenge. Experiments were repeated at least three times. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control (*A*), and the average optical densitometry was expressed by standardizing to GAPDH (*B*). $*P < 0.05$ compared with the sham-sensitized group.

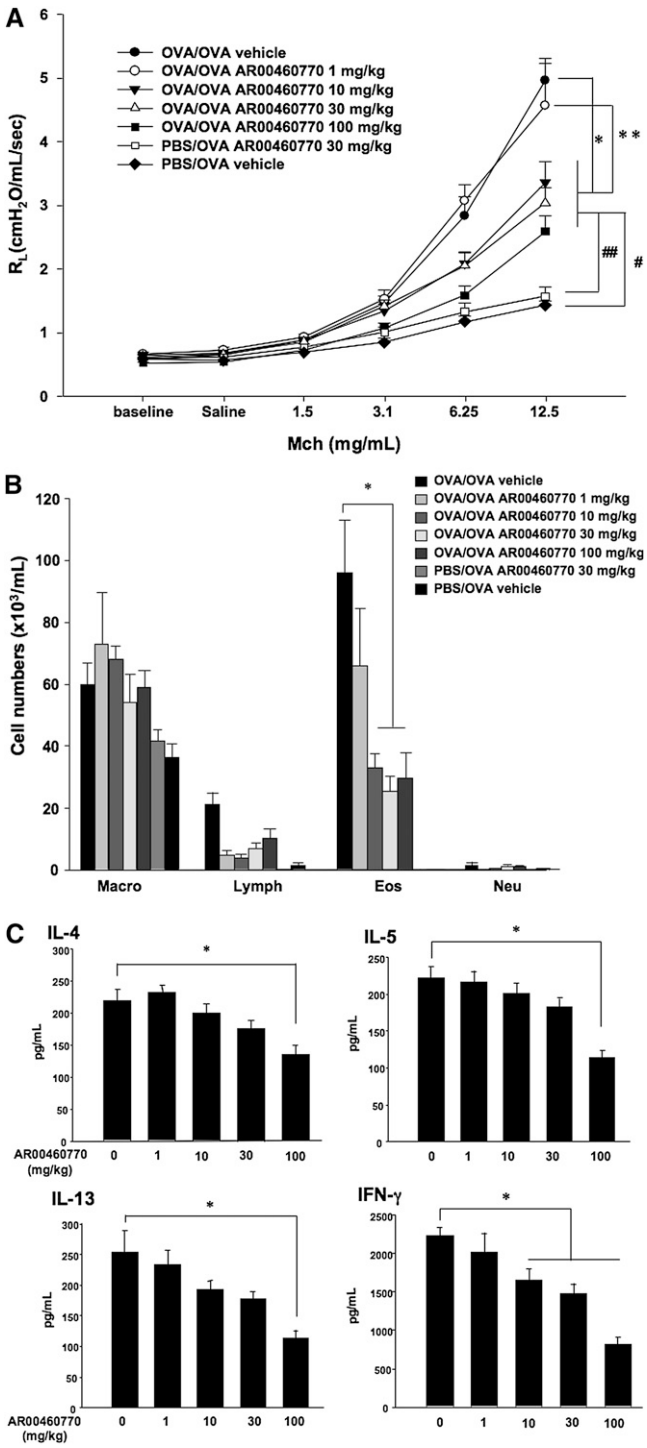

Figure 2. Effect of Pim1 kinase inhibition on airway responses after primary allergen challenge. The effects of the Pim1 kinase inhibitor were determined in the primary allergen challenge model. (*A*) Changes in pulmonary resistance (R_L) in response to increasing doses of methacholine (MCh). (*B*) Cell composition in bronchoalveolar lavage (BAL) fluid. Macro, macrophages; Lympho, lymphocytes; Eos, eosinophils; Neu, neutrophils. (*C*) BAL fluid cytokine concentrations. (*D*) Lung tissue histology after staining with hematoxylin and eosin (H&E). (*E*) Lung tissue histology after staining with periodic acid–Schiff (PAS). Quantitative analyses of inflammatory and PAS$^+$ cells in lung tissue were performed as described in Materials and Methods. Mice were sham-sensitized, followed by OVA challenge (PBS/OVA), or sensitized and challenged with OVA (OVA/OVA). The Pim1 inhibitor, AR00460770, was administered at doses of 1, 10, 30, or 100 mg/kg. $^{\#}P < 0.05$, compared with PBS/OVA vehicle. $^{**}P < 0.05$, compared with OVA/OVA AR00460770 at 1 mg/kg. $^{\#\#}P < 0.05$, compared with PBS/OVA AR00460770 at 30 mg/kg.

Significant changes were evident primarily at the highest administered dose of the inhibitor (100 mg/kg).

Histopathological analyses of lung-tissue sections revealed that the numbers of inflammatory cells, including eosinophils in peribronchial and perivascular areas, were increased in mice after OVA sensitization and challenge, compared with sham-sensitized and challenged mice (Figure 2D). Similarly, the numbers of periodic acid–Schiff–positive (PAS$^+$) mucus-containing goblet cells were increased in the sensitized and challenged mice (Figure 2E). The administration of inhibitor significantly decreased the numbers of inflammatory cells and PAS$^+$ mucus-containing goblet cells in lung tissue in a dose-dependent manner (Figure 2E).

Inhibition of Pim1 Kinase Attenuates the Development of AHR and Airway Inflammation in the Secondary Allergen Challenge Model

The airway responses in the primary allergen challenge model reflect the first immune responses in the lungs, where adaptive immunity is initiated in response to airborne allergen exposure. For the most part, patients with asthma have already developed allergic airway inflammation and airway dysfunction before the initiation of treatment. Immune responses to allergen and tissue remodeling of the airways are generally already established. The secondary allergen challenge model is an approach to examine the response to allergen provocation where allergen-induced airway inflammation is previously established. To determine the effects of Pim1 kinase inhibition in the secondary allergen challenge model, we measured AHR, cell composition, and cytokine concentrations in BAL fluid, 48 hours after a single provocative allergen challenge. As in the primary allergen challenge model, vehicle-treated mice developed significantly higher airway responsiveness to MCh and eosinophils in BAL fluid after OVA sensitization and secondary allergen challenge. Similar to the results observed in the primary allergen challenge model, treatment with the Pim1 kinase inhibitor (at 10, 30, and 100 mg/kg) significantly decreased levels of airway responsiveness and the number of eosinophils in BAL fluid in a dose-dependent manner, compared with vehicle-treated groups (Figures 3A and 3B). Moreover, a significant reduction in numbers of lymphocytes and neutrophils was evident at the higher doses of inhibitor. Assays of BAL cytokine concentrations demonstrated that IL-4, IL-5, IL-13, and IFN-γ were decreased in Pim1 kinase inhibitor (100 mg/kg)–treated mice that had been sensitized and challenged with OVA (Figure 3C). Histopathological analyses revealed that Pim1 kinase inhibition decreased numbers of inflammatory cell in the lungs and goblet cell metaplasia along the airways (Figure 3D).

Decrease of CD4$^+$ and CD8$^+$ T Cells in the Lungs of Sensitized and Challenged Mice after Treatment with the Pim1 Kinase Inhibitor

Because both CD4$^+$ and CD8$^+$ T cells are potent effector cells in the development of allergic inflammation, their numbers were examined after treatment with inhibitor in sensitized and challenged mice. Lungs from OVA-sensitized and OVA-challenged mice that received either inhibitor or vehicle were excised, and lung MNCs were purified. Numbers of CD4$^+$ and CD8$^+$ T cells were determined by flow cytometry. As shown in Figure 4, the overall number of CD4$^+$ T cells was significantly lower in the

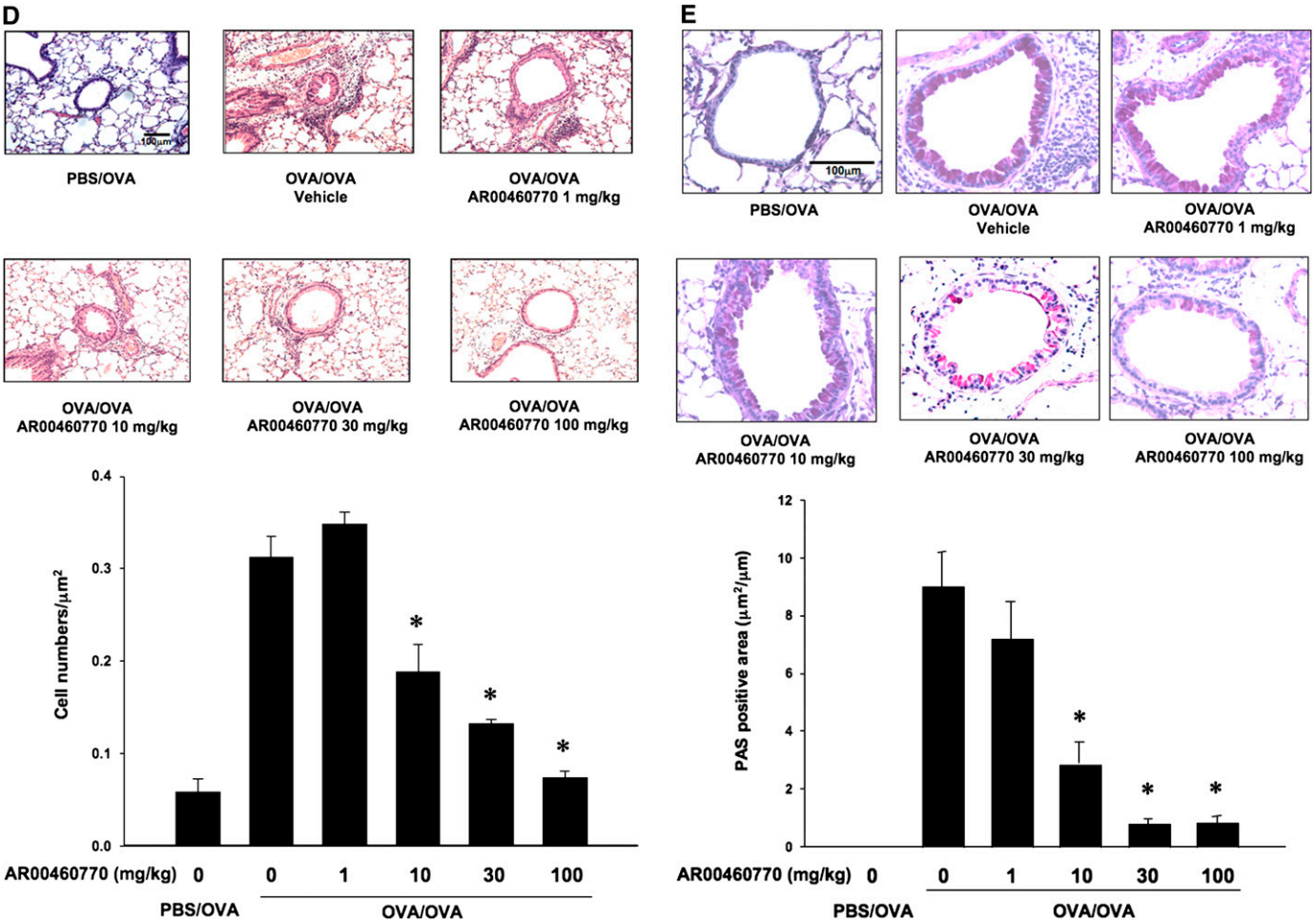

Figure 2. (*Continued*)

inhibitor-treated mice ($1.48 \pm 0.26 \times 10^6$ cells/lung, versus $3.09 \pm 0.35 \times 10^6$ cells/lung in vehicle-treated mice). $CD8^+$ T cells were also significantly decreased after Pim1 kinase inhibition, from $0.57 \pm 0.21 \times 10^6$ cells/lung to $0.29 \pm 0.06 \times 10^6$ cells/lung. These results demonstrate that Pim1 kinase inhibition *in vivo* reduces the numbers of $CD4^+$ and $CD8^+$ T cells that accumulate in the lungs of sensitized and challenged mice.

Reduction of $CD4^+$ and $CD8^+$ T-Cell Proliferation and Cytokine Production *In Vitro* after Pim1 Kinase Inhibitor Treatment

To examine the proliferative capacity of T cells after the inhibition of Pim1 kinase, CD4 and CD8 T cells were isolated and purified from spleens and incubated with a combination of anti-CD3 and anti-CD28 for 24 hours. The cell viabilities of $CD4^+$ or $CD8^+$ T cells were determined in the presence of 10 μM of the inhibitor. After 24 hours, inhibitor treatment did not show significant effects on cell viabilities compared with vehicle control samples (from 90.0–90.3% in $CD4^+$ T cells, and from 80.2–82.8% in $CD8^+$ T cells, respectively). In a dose-dependent manner, the Pim1 kinase inhibitor reduced the $CD4^+$ and $CD8^+$ T-cell proliferation triggered by the combination of anti-CD3 and anti-CD28. In stimulated cell cultures, increased concentrations of IL-4, IL-5, IL-13, and IFN-γ were detected. Treatment with the inhibitor decreased the concentrations of all of these cytokines in a dose-dependent fashion (Figure 5).

DISCUSSION

In these initial studies using an experimental model of asthma, we demonstrated that concentrations of Pim1 kinase were elevated in the lungs of OVA-sensitized and OVA-challenged mice, compared with concentrations in the lungs of nonsensitized, challenged-only mice. Pim kinases are among the few to be up-regulated during cell activation through increased transcription (8). There are three subtypes of Pim kinases. Pim1 and Pim2 are primarily restricted to hematopoietic cells, and Pim3 is expressed in brain, kidney, and mammary tissue (36). To date, only a single study demonstrated that the expression of Pim1 was induced in human immune cells, and that was in eosinophils from either whole blood or BAL fluid after stimulation with IL-5 (16). Concentrations of Pim1 kinase in BAL eosinophils, compared with blood eosinophils, were further increased after segmental allergen challenge in patients with asthma (16). Linking the increases in Pim1 kinase concentrations in sensitized and challenged mice to function, we demonstrated that sensitized and challenged mice treated with a Pim1 kinase inhibitor developed significantly lower levels of AHR, cytokines, eosinophilic airway inflammation, lung inflammatory cell accumulation, and goblet cell metaplasia. The effects of the kinase inhibitor *in vivo* on the development of AHR, BAL eosinophilia, airway inflammation, and goblet cell metaplasia were seen at doses (10–30 mg/kg) that, based on the cellular activity of AR460770 (Table 1), were specific to Pim1 kinase inhibition. The suppressive effects associated with Pim1 kinase inhibition were observed in both the

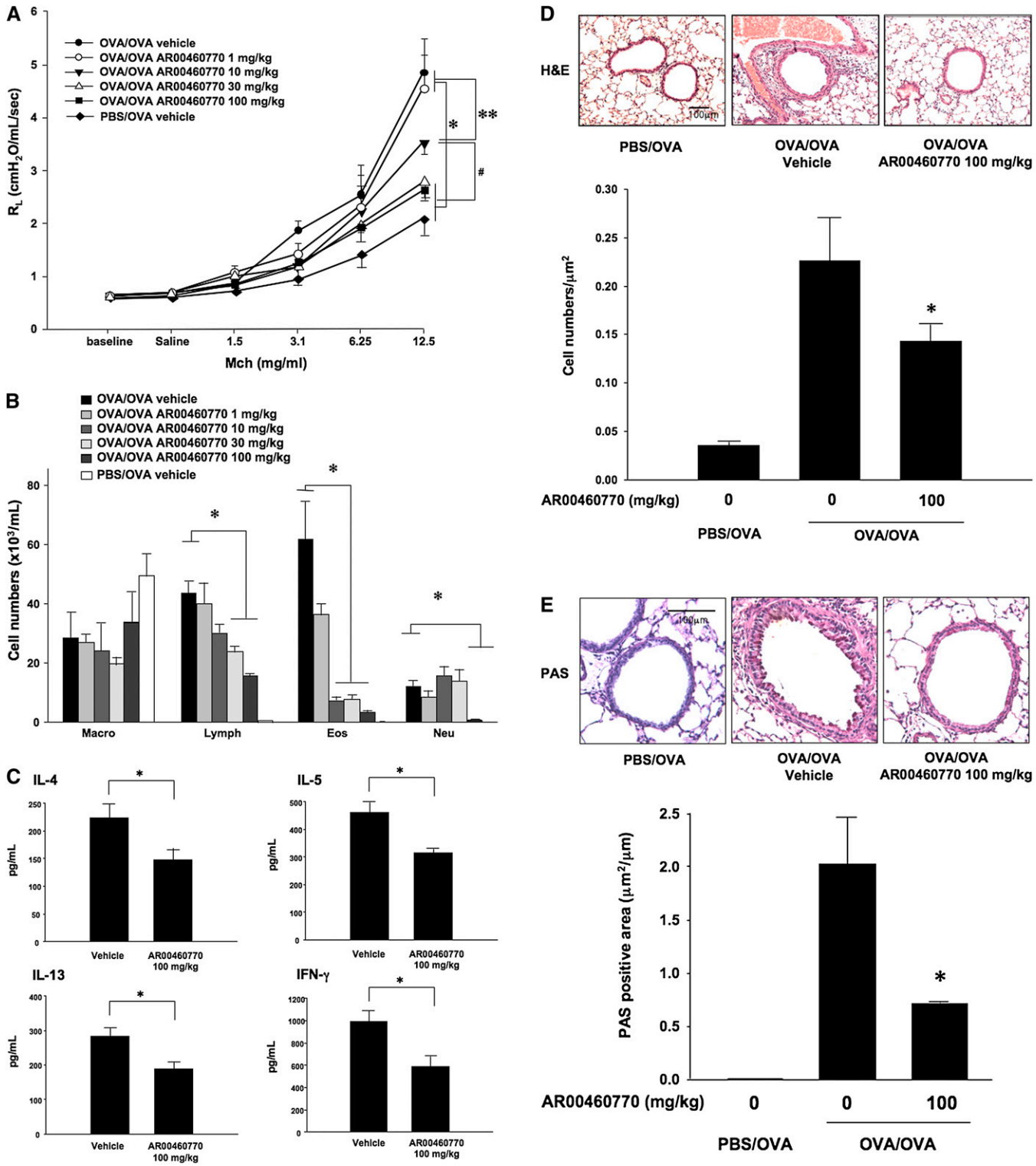

Figure 3. Effects of Pim1 kinase inhibition on airway responses in the secondary allergen challenge model. The effects of Pim1 kinase inhibition were determined in the secondary allergen challenge model. (*A*) Changes in R_L in response to increased doses of MCh. (*B*) Cell composition in BAL fluid. (*C*) BAL fluid cytokine concentrations. (*D*) Lung tissue histology after staining with H&E. (*E*) Lung tissue histology after staining with PAS. Quantitative analyses of inflammatory and goblet cells were performed as described in Materials and Methods. Mice were sham-sensitized, followed by OVA challenge (PBS/OVA), or sensitized and challenged with OVA (OVA/OVA). The Pim1 inhibitor was administered at doses of 1, 10, 30, or 100 mg/kg. Control groups received vehicle ($n = 8$). *$P < 0.05$, compared with OVA/OVA vehicle or OVA/OVA AR00460770 at 1 mg/kg. #$P < 0.05$, compared with OVA/OVA AR00460770 10 mg/kg. **$P < 0.05$, compared with OVA/OVA vehicle or OVA/OVA AR00460770 at 1 mg/kg.

primary and secondary allergen challenge protocols. The primary challenge model reflects the initial immune responses of the airways to airborne allergen exposure, and best defines the development of lung allergic responses. In contrast, the secondary allergen challenge model detects airway secondary immune responses in lungs on a background of previously established

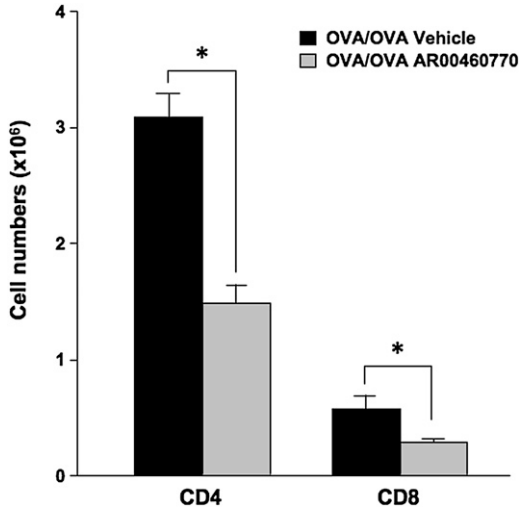

Figure 4. The effects of Pim kinase inhibition on numbers of $CD4^+$ and $CD8^+$ T cells. In OVA-sensitized and OVA-challenged mice, the numbers of $CD4^+$ T cells (CD4) and $CD8^+$ T cells (CD8) in the lungs of mice treated with the Pim1 kinase inhibitor (OVA/OVA AR00460770) or vehicle (OVA/OVA vehicle) were determined. Mononuclear cells isolated from lungs were stained with anti-CD3, anti-CD4, and anti-CD8 for flow cytometry analysis, as described in MATERIALS AND METHODS. The data shown are representative of three independent experiments. $*P < 0.05$, compared with vehicle.

allergic airway inflammation. In both models, the reduction of eosinophils in the BAL appeared at lower doses of inhibitor, and these doses did not alter BAL cytokine production levels. Together with the data on human eosinophils (16), our findings suggest that the inhibition of Pim1 kinase directly affects eosinophil function. The comparable suppressive effects of Pim1 kinase inhibition after both primary and secondary allergen challenge imply that the activation of this kinase plays a critical role in both the initiation and recall responses induced by allergen challenges of sensitized mice.

Pim1 kinase controls cellular survival, and the inhibition of this kinase can cause cell death. The antibody targeting of Pim kinase synergistically enhanced the cytotoxicity of anticancer agents, and the inhibition of Pim1 kinase triggered the death of leukemic cells (37, 38). To address the possibility that the suppressive effects of Pim1 kinase inhibition on allergen-induced airway responses were attributable to its toxic effects on immune cells, an evaluation of cell viability was performed. At concentrations up to 10 μM, the inhibitor had no effect on cell number or viability, suggesting its effects on airway responses were not attributable to drug-mediated cellular toxicity. These findings are similar to those described in Pim-deficient mice, in which the rate of apoptosis of T cells was not different than in wild-type mice (36). Furthermore, treatment with Pim1 kinase inhibitor in sham-sensitized but OVA-challenged mice did not alter airway responsiveness to MCh, indicating that the inhibitor less likely exhibited toxic effects on lung resident cells, including airway smooth muscle. Microarray data indicate that Pim1 is expressed in airway epithelial cells, indicating the potential for additional suppressive effects of the inhibitor on airway epithelial cell function, as well as eosinophil and T-cell function in the development of AHR and allergic airway inflammation (39). Although the specificity of the inhibitor was demonstrable *in vitro*, the effects of the inhibitor on other cells cannot be completely ruled out at this time.

The pathogenesis of asthma is complex, and the role of Th2 $CD4^+$ T cells secreting proallergic cytokines such as IL-4, IL-5, and

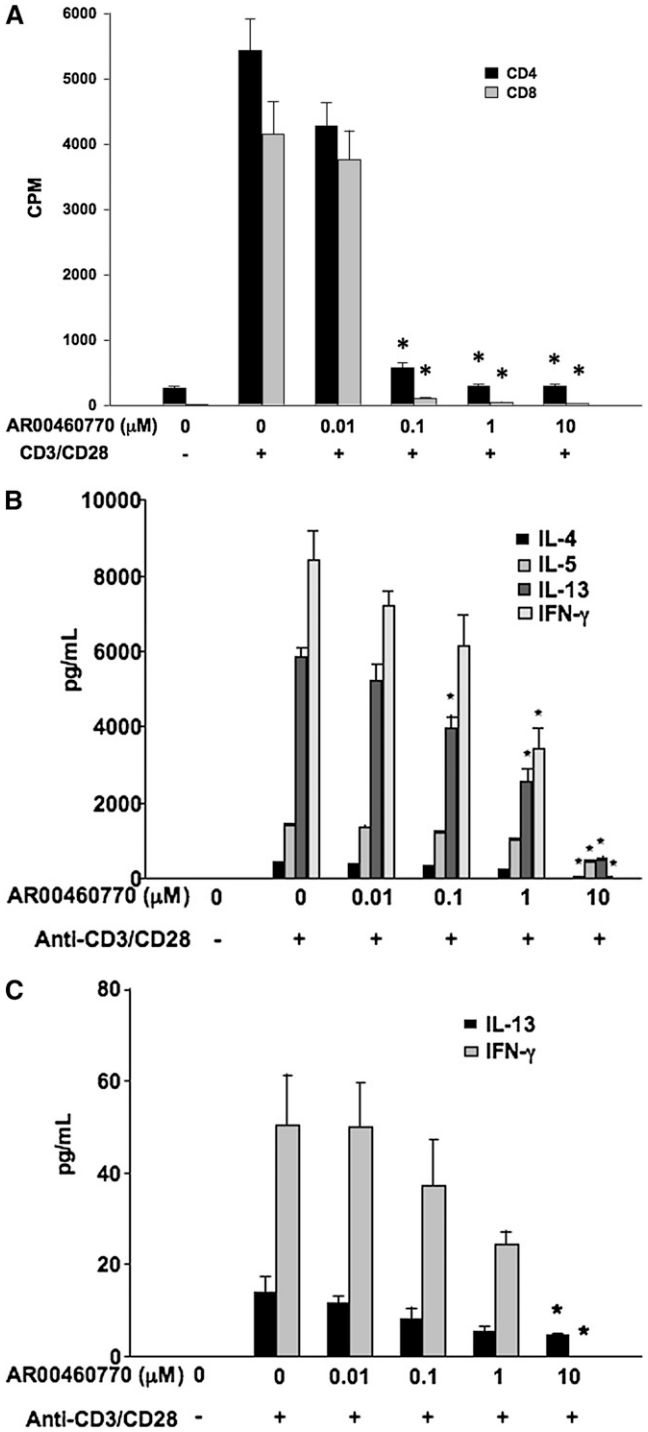

Figure 5. Effects of Pim1 kinase inhibition on cell proliferative responses and cytokine production from $CD4^+$ and $CD8^+$ T cells. Purified spleen $CD4^+$ and $CD8^+$ T cells were preincubated with the Pim1 kinase inhibitor, followed by anti-CD3 and anti-CD28 stimulation. (*A*) Cell proliferation assays were performed 24 hours after anti-CD3/anti-CD28 stimulation, and calculated from the uptake of tritium-labeled thymidine ($n = 8$). CPM, counts per minute. (*B*) Quantitation of cytokine concentrations in supernates from anti-CD3/anti-CD28–stimulated $CD4^+$ cells. (*C*) Quantitation of cytokine concentrations in supernates from anti-CD3/anti-CD28–stimulated $CD8^+$ T cells. CPM, counts per minute. $*P < 0.05$, compared with vehicle-treated cells.

IL-13 has been emphasized (1, 3, 19). The role of IFN-γ may be more complex. In several studies, IFN-γ was shown to be a potent suppressor of allergen-induced AHR, lung pathology, and Th2 cytokine production (40–42). In other studies, IFN-γ was shown to be essential in the development of AHR (43), and the administration of IFN-γ was shown to be ineffective in preventing AHR or airway eosinophilia (40). In patients with asthma, elevations of IFN-γ were shown to correlate with asthma severity (44) and bronchial hyperresponsiveness (45). Given the distribution of Pim1 kinase, several points of action likely explain the attenuation of AHR, BAL cytokine concentrations, and lung inflammation in primary or secondary allergen challenge models. Effector $CD4^+$ and $CD8^+$ T cells play a role in the pathogenesis of asthma through cell proliferation to specific allergens, followed by prolonged survival (25, 45). T cells are also required for the development or initiation of airway eosinophilia and goblet cell metaplasia through the release of specific cytokines (46, 47). The inhibition of Pim1 kinase altered the activities of these effector cells in the airways, because both the numbers of $CD4^+$ and $CD8^+$ T cells in the lungs and BAL cytokine concentrations were decreased in inhibitor-treated, sensitized, and challenged mice. Further, according to *in vitro* experiments, inhibitor treatment demonstrated suppressive effects on the proliferation of $CD4^+$ and $CD8^+$ T cells induced in response to stimulation with anti-CD3 and anti-CD28. In parallel, concentrations of cytokines, IL-4, IL-5, IL-13, and IFN-γ, in stimulated cell culture supernates were also reduced in a dose-dependent manner. Of interest here, Pim-deficient T cells were shown to display normal proliferative responses to high concentrations of anti-CD3 and IL-2, but Pim was required for normal proliferation at lower levels of the stimuli (36). Together, the results suggest that under experimental conditions *in vivo* and *in vitro*, the inhibition of Pim1 kinase limits responses through interference with the expansion and activities of critical effector cells, $CD4^+$ and $CD8^+$ T cells in the airways, and possibly eosinophils, as reported elsewhere (18–25, 48, 49).

Although survival kinases, such as the Pim kinases, are known to regulate common substrates such as BAD or 4EBP1 (50), the downstream activities of individual Pim kinases may be different. Targeting Pim1 kinase may account for effects on allergic lung responses in other ways. The suppressor of cytokine signaling-1, c-myc, Pim-associated protein-1, protein tyrosine phosphatase U2S, and heterochromatin protein 1 are all potential downstream targets of Pim1 kinase (51–56). Recently, the nuclear factor of activated T cells (NFAT) was reported to be a potential downstream substrate of Pim1 kinase (57). The regulation of NFAT activity was shown to be important for the normal selection of thymocytes and may play a role in the functional development of T cells. The down-regulation of NFAT may play a role in the suppression of both $CD4^+$ and $CD8^+$ T-cell proliferation and T-cell cytokine production as a downstream substrate of the kinase.

Asthma is a chronic airway inflammatory disease, as demonstrated by the infiltration and proliferation of inflammatory cells in the airways (48, 59). As a result, interference with inflammatory cell accumulation in the airways and cell expansion represent potential strategies in the treatment of inflammatory diseases such as asthma. Despite the successful introduction of anti-inflammatory drugs and immunomodulators in the treatment of autoimmune diseases, few have produced similar benefits in asthma (60, 61). This finding may suggest that the inflammatory pathways in asthma differ from those in other diseases, and novel strategies are required. To date, compelling evidence for the involvement of Pim1 kinase in the development and progression of several different diseases has made it a potential pharmaceutical target. Limited numbers of Pim kinase inhibitors have been available for study. One recent study using a Pim1 kinase inhibitor demonstrated therapeutic effects in chronic lymphocytic leukemia (37). The data reported here demonstrate for the first time, to the best of our knowledge, that targeting Pim1 kinase effectively reduces the development of the full spectrum of allergen-induced lung inflammatory responses, at least in part through limiting the expansion and activities of effector $CD4^+$ and $CD8^+$ T cells. As such, the inhibition of Pim1 kinase represents a novel therapeutic target in the treatment of asthma.

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