

Original article

A critical role for Pin1 in allergic pulmonary eosinophilia in rats

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Background: Infiltration, accumulation, and degranulation of eosinophils in the lung are hallmarks of active allergic asthma. The pulmonary response to inhaled allergen triggers the secretion of eosinophil chemoattractants and antiapoptotic cytokines, including GM-CSF, IL-3, IL-4, IL-5, and eotaxin, among others. We recently showed that *in vitro* Pin1 regulated eosinophil production of and response to GM-CSF.

Objective: We sought to determine the effect of Pin1 inhibition on pulmonary eosinophilia after allergen challenge.

Methods: The Pin1 inhibitor juglone (5-hydroxy-1,4-naphthoquinone) was administered to allergen-sensitized and allergen-challenged Brown Norway rats. Bronchoalveolar lavage fluid and lungs were assessed for inflammation, cytokine expression, and Pin1 activity.

Results: Juglone-treated rats showed a dramatic reduction (approximately 75%) in bronchoalveolar lavage fluid and pulmonary eosinophilia but no change in lymphocyte, monocyte/macrophage, or neutrophil numbers. GM-CSF and IL-5 expression were also significantly reduced, whereas Pin1-independent cytokines, such as eotaxin or IL-4, as well as housekeeping mRNAs and proteins, including actin, were unaffected by juglone. The eosinophils present in the lung in juglone-treated rats showed significantly greater apoptosis.

Conclusion: These data suggest that *in vivo* Pin1 blockade attenuates GM-CSF and IL-5 production and can selectively reduce eosinophilic allergic inflammation.

Clinical implications: Eosinophils can be selectively reduced by Pin1 blockade, despite allergen challenge. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Eosinophil, asthma, apoptosis, cytokines

Abbreviations used

ARE: Adenosine-uridine-rich elements
BAL: Bronchoalveolar lavage
CyA: Cyclophilin A
FKBP: FK binding protein
NF-κB: Nuclear factor κB
PPIase: Peptidyl-prolyl isomerase
Treg cell: Regulatory T cell
WT: Wild-type

Eosinophils have been implicated in the pathogenesis of numerous inflammatory diseases, including parasitic infections and asthma.¹ Asthma severity and long-term sequelae of subepithelial fibrosis have been positively and highly correlated to the number of airway eosinophils.^{2,3} Despite these findings, the precise contribution of eosinophils to the pathology of asthma remains controversial. Patients treated with anti-IL-5 had no change in lung function, despite a significant decrease in peripheral blood and bronchoalveolar lavage (BAL) fluid eosinophils.⁴ However, eosinophil numbers were reduced by only 50% in the airway wall, suggesting pathogenic eosinophilic inflammation could remain.⁴ Consistent with these observations, 2 distinct transgenic mice lacking eosinophils showed either reduction of hyperresponsiveness, mucus production, and T_H2 cytokines or diminished airway remodeling.^{5,6} Therefore eosinophils likely play an important role in the more chronic phases of asthma.

A variety of proinflammatory cytokines are increased in the blood and lung after allergen exposure. T_H2 CD4⁺ T lymphocytes express IL-5, IL-4, and IL-13.⁷ IL-5 is essential for eosinophil differentiation in the bone marrow.⁸ IL-4 and IL-13 induce expression of vascular cell adhesion molecule 1, which binds to its receptor (very late antigen 4) on the surface of eosinophils and allows trafficking through the vessel walls into sites of inflammation.⁹ Eotaxin, expressed by endothelia, epithelium, and eosinophils, specifically recruits eosinophils by binding to the CCR3 receptor.¹⁰ Finally, GM-CSF, along with IL-3, induces the differentiation, activation, and survival of eosinophils.¹¹⁻¹³ Because pulmonary eosinophils fail to express IL-5 receptor, GM-CSF and IL-3 are likely the primary cytokines that prolong eosinophil survival in the lung.¹⁴

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Proinflammatory gene expression depends on the activation of both transcriptional and posttranscriptional regulatory pathways. The former can be antagonized by cyclosporine or tacrolimus, which block calcineurin and prevent nuclear factor of activated T cells activation.¹⁵ These drugs bind to cyclophilin A (CyA) and FK binding protein (FKBP). At a posttranscriptional level, a third peptidyl-prolyl isomerase (PPIase), Pin1, becomes upregulated in activated eosinophils or T cells.^{16,17} Unlike CyA or FKBP, Pin1 only binds to a subset of proteins containing the bipptide motif serine-proline or threonine-proline. Phosphorylation triggers isomerization of the phosphoserine-proline or phosphothreonine-proline peptide bond, often causing substantial changes in the biologic properties of the target proteins.¹⁸ Blockade of Pin1 prevented GM-CSF mRNA stabilization and accumulation in activated T lymphocytes and eosinophils. In addition, the loss of Pin1 activity accelerated eosinophil apoptosis *in vitro*.¹⁶

These data suggested that Pin1 inhibition *in vivo* could have substantial effects on eosinophilic inflammation after allergen challenge. We evaluated this possibility by means of aerosol allergen challenge of Brown Norway rats that had previously been sensitized to ragweed pollen extract. Control rats had substantial and reproducible eosinophilic airway and parenchymal inflammation. Rats who received the Pin1 inhibitor juglone (5-hydroxy-1,4-naphthoquinone) showed markedly reduced eosinophilia without significant reductions in other inflammatory cells. In addition, juglone-treated rats showed significant reductions in proinflammatory and prosurvival cytokine expression and increases in eosinophil apoptosis.

METHODS

Reagents

Juglone was provided by Calbiochem (La Jolla, Calif). Anti-CD3-coated plates, control plates, and the anti-CD28 antibody were provided by BD Biosciences (Bedford, Mass). Recombinant human GM-CSF was from R&D Systems (Minneapolis, Minn). Polyclonal anti-caspase 3 was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif).

Pin1^{-/-} mice

All husbandry, genotype determination, and euthanasia procedures were performed in accordance with the National Institutes of Health and an approved University of Wisconsin–Madison animal-care protocol. Thymocyte suspensions from knockout and wild-type (WT) mice were prepared as previously described.¹⁹

Allergen sensitization and challenges

Brown Norway rats (BN/SsNHsd substrain; Harlan, Indianapolis, Ind) 10 to 13 weeks of age were sensitized and challenged with short ragweed pollen extract, as previously described.²⁰ Briefly, rats were sensitized by means of subcutaneous injection, followed 2 weeks later by aerosol challenge (2% for 20 minutes). On the day before, the day of, and the day after the aerosol challenge, rats received an intraperitoneal injection of juglone (1 mg/kg body weight per day) or vehicle.¹⁹ Two days after the aerosol challenge, at the peak of eosinophilic inflammation,²⁰ BAL of the right lungs, processing of the

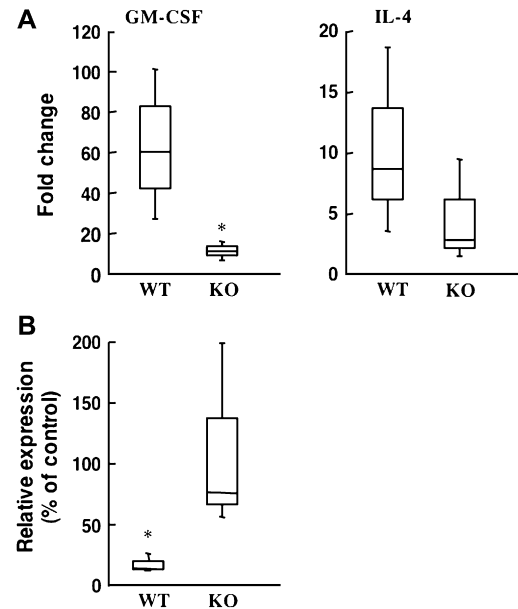


FIG 1. Pin1 controls GM-CSF expression. **A**, Thymocytes from Pin1^{-/-} (KO) or WT mice were activated or not for 3 hours with anti-CD3/anti-CD28. mRNAs are expressed as fold change from resting cells. **B**, Thymocytes were treated as above with or without juglone. GM-CSF mRNA was determined by means of quantitative PCR and expressed as a percentage of control cultures without juglone. **P* < .05 for WT versus KO mice.

BAL fluid cells, and formalin fixation of the left lungs were performed as described previously.²⁰ All animal procedures conformed to the “Guide for the care and use of laboratory animals” and were approved by the University of Wisconsin Animal Care and Use Committee.

Lung eosinophils

Paraffin sections of the left lung were prepared and stained for eosinophil major basic protein, as previously described.²¹ Eosinophils were quantified with Stereo Investigator (MicroBrightField, Wiliston, Vt). Tracing around the lung section was done at 2.5×. Then the Fractionator was used at 20× with a counting frame of 200/200 μm and a systematic random scan grid of 2500/2000 μm. Forty-six frames per lung section were counted, and then the number of eosinophils per square millimeter in the whole section was calculated.

Cytokine mRNA analysis

For the measurement of mRNA steady-state levels, cells were processed as previously described.^{13,19} For GM-CSF mRNA analysis in BAL fluid cells, custom-designed primer/probe sets for rat GM-CSF were purchased from Applied Biosystems (Foster City, Calif). All reactions were run in duplicate, and GM-CSF cycle thresholds were normalized to the endogenous control, β-actin. Thymocyte GM-CSF and IL-4 mRNA levels were measured by using the ΔΔCt method¹⁷ and expressed in arbitrary units as activated relative to resting (Fig 1, A).

ELISA

Eotaxin was measured in BAL fluid by using a mouse eotaxin ELISA kit (R&D Systems) that also recognizes rat eotaxin. The sensitivity of this ELISA kit is 3 pg/mL. BAL fluids were 15× concentrated, as previously described.²²

Eosinophil apoptosis

BAL fluid cells pooled from 2 rats were density fractionated over discontinuous Percoll gradients (1.075, 1.085, and 1.100 g/mL) for 20 minutes at 700g at room temperature. BAL fluid cells at the 1.075/1.085 g/mL interface were collected and ranged from 40% to 80% pure eosinophils (mean $\pm$ SEM: $64.7\% \pm 3.8\%$ [range, 40% to 80%], $n = 6$). To determine eosinophil apoptosis, 0.5×10^6 fractionated BAL fluid cells were labeled with 5 μ L of annexin–fluorescein isothiocyanate for 15 minutes at 4°C. The apoptotic cells were then assessed by means of flow cytometry. Each group represents 6 independent cell preparations.

Bone marrow

Two days after allergen challenge, bone marrow was flushed from the femur with PBS. Differential cell counts (200 cells) on cytospin slides stained with eosin–methylene blue stain were determined by means of microscopy.

Human eosinophil preparation

Peripheral blood was obtained by means of venipuncture from normal donors. All participants have a clinical record at the University of Wisconsin Hospital, and informed consent was obtained according to an approved University of Wisconsin Hospital Institutional Review Board protocol. Peripheral blood eosinophils were purified with a negative immunomagnetic procedure, as described previously.^{13,23}

Immunoblot

After culture, eosinophils were snap-frozen at -80°C , and cytoplasmic lysates and immunoblots were processed as previously described.²⁴ Actin was used as an internal loading control. For Fig 5, anti-caspase 3 detects both the inactive (32 kd) and the cleaved active (17 kd) forms.

Statistical analysis

Data that did not conform to parametric assumptions were analyzed by using Mann-Whitney tests (thymocyte data, Fig 1) or Kruskal-Wallis tests, followed by planned pairwise comparisons with the Mann-Whitney test (tissue eosinophil, BAL cell, BAL cell IL-4 mRNA, and eotaxin data). For data that conformed to parametric assumptions (BAL cell GM-CSF mRNA data), ANOVA (general linear model) was performed, and the Fisher least significant difference test was used for planned post-hoc pairwise comparisons. For the BAL cell IL-5 mRNA and eosinophil apoptosis data, which conformed to parametric assumptions after square root and log transformations, respectively, a randomized block ANOVA (general linear model), where each block represented an individual batch of animals, was performed, and the Fisher least significant difference test was used for planned post-hoc pairwise comparisons. A residual analysis was used to test the adequacy of the ANOVA models. Box plots depict the median and the interquartile range between the 25th and 75th percentiles. Whiskers show the range of values that fall within 1.5 times the interquartile range. Systat version 11 software (Systat Software, Inc, San Jose, Calif) was used for analyses.

RESULTS

Pin1, but not CyA or FKBP, can be selectively blocked by juglone, which covalently binds to and irreversibly inhibits Pin1 PPIase activity.²⁵ Pin1 knockout mice also exist but are infertile, display accelerated neurodegeneration, and show both T_H1 and T_H2 abnormalities (data not

shown).^{19,26} The ability of these mice to be sensitized and challenged remains unclear. Thus we elected to use pharmacologic approaches to block Pin1 in a well-established Brown Norway rat model of allergic airway inflammation.²⁰ However, before juglone use *in vivo*, thymocytes from Pin1 WT and knockout mice were activated *in vitro* with anti-CD3/anti-CD28 with or without juglone for 3 hours (Fig 1). GM-CSF mRNA expression was dramatically increased (approximately 55-fold) in activated WT thymocytes, which was reduced by approximately 80% by juglone. Pin1 PPIase activity was reduced to basal unstimulated levels by juglone, as expected (not shown). Compared with WT cells, Pin1 knockout cells showed a markedly attenuated response to mitogenic antibodies (approximately 13-fold increase in GM-CSF mRNA) but were unaffected by juglone (Fig 1). Unlike GM-CSF, IL-4 expression was not significantly affected in knockout cells compared with that seen in WT cells, suggesting variable sensitivity of cytokine mRNAs to lack of Pin1 activity (Fig 1). In all cases 18S rRNA and actin mRNA levels were unchanged by inhibition of Pin1. These data suggest that genetic or pharmacologic inhibition of Pin1 prevents the accumulation of GM-CSF, but not IL-4, mRNA by activated thymocytes. At the juglone concentrations used here, neither CyA nor FKBP12 PPIase activities are affected.²⁵

To characterize Pin1 function *in vivo* during eosinophilic inflammation, we used a well-established Brown Norway rat allergic airway inflammation model.²⁰ Some sensitized rats were sham challenged and received only aerosolized PBS. The remainder, which received ragweed challenge, were split and either received diluent (ragweed group) or juglone (1 mg/kg/d; ragweed plus juglone group) intraperitoneal injection. Major basic protein-stained sections of lung tissue (Fig 2, A) showed large numbers of eosinophils in the airspaces and walls of alveoli and small-to-medium airways in the ragweed-challenged group. Rats who received juglone showed significantly fewer total eosinophils, which were reduced in all compartments. Quantification revealed juglone treatment reduced eosinophilia by approximately 75% (Fig 2, B). Rats that were sham challenged showed very few eosinophils (Fig 2, B).

We next evaluated inflammation in the BAL fluid. Typically, eosinophils comprise 50% to 60% of the airway inflammation in the challenged Brown Norway rat.²⁰ Challenged and untreated rats showed similar eosinophilia, with the remainder of BAL cells predominantly monocytic with smaller and roughly even numbers of lymphocytes and neutrophils (Fig 3). However, in the juglone-treated rats BAL fluid eosinophils were selectively reduced by more than 75%, whereas none of the other inflammatory cell numbers were affected (Fig 3). These data confirm that the allergen challenge elicited lymphocyte and neutrophil recruitment despite treatment with juglone but that eosinophil recruitment was selectively inhibited. Therefore both BAL fluid and parenchyma showed significant and selective reductions in eosinophils. Indeed, the Spearman rank correlation coefficient was highly

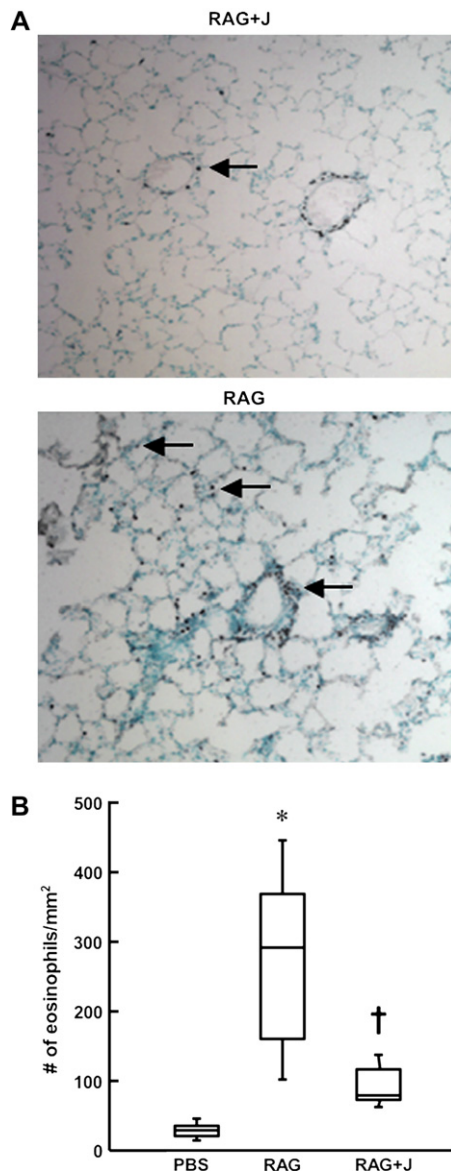


FIG 2. Pin1 blockade reduces eosinophilia in lung tissue. Lung sections from ragweed-challenged rats with (RAG+J) or without (RAG) juglone were stained with anti-major basic protein. **A**, Representative lung section. Arrows denote eosinophils ($\times 100$ magnification). **B**, Numbers of eosinophils in lung tissue ($n = 6$ in each group). $*P < .05$ between RAG and PBS groups. $\dagger P < .05$ between RAG+J and RAG groups.

significant ($r = 0.91$, $P = .0002$, $n = 18$) between parenchymal and BAL fluid eosinophilia. These data show evidence of a critical role for Pin1 activity in pulmonary eosinophilia after allergen challenge.

To establish that Pin1 was inhibited by juglone, as previously described *in vitro* in human eosinophils and lymphocytes,^{16,17} total BAL fluid cell lysate was prepared, and its ability to isomerize a model, Pin1-specific penta-peptide substrate, was determined.^{16,17,25} We observed that the decrease of eosinophil numbers after juglone treatment (see Fig E1 in this article's Online Repository at

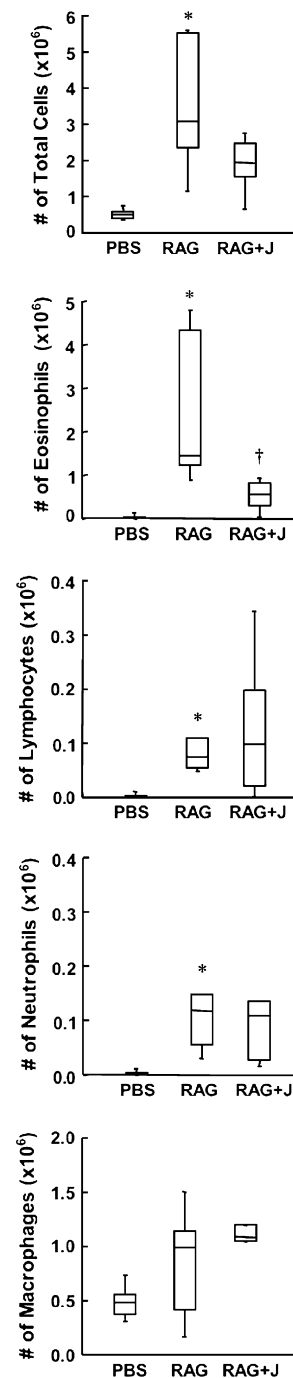


FIG 3. The number of BAL fluid eosinophils is selectively reduced by Pin1 blockade. Numbers of total cells, eosinophils, lymphocytes, neutrophils, and macrophages obtained by means of BAL from PBS- or ragweed-challenged rats with (RAG+J) or without juglone (RAG) are shown. $*P < .05$ between RAG and PBS groups. $\dagger P < .05$ between RAG+J and RAG groups. $N = 6$ in each group.

www.jacionline.org) was accompanied by a substantial reduction of Pin1 activity (Fig 3), which was not further inhibited by addition of juglone to the cell lysates *ex vivo*. This suggests that juglone treatment *in vivo* was effective to block Pin1.

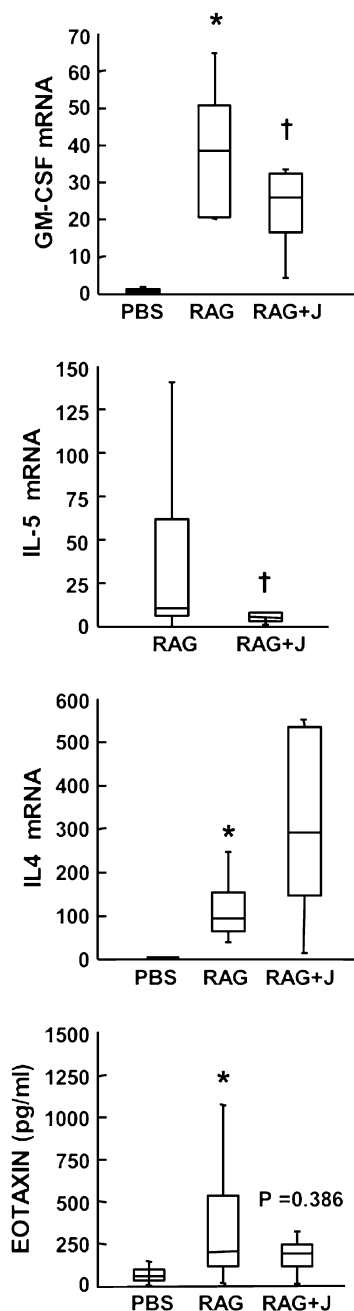


FIG 4. GM-CSF and IL-5 expression are reduced by Pin1 blockade in BAL cells. BAL cells were analyzed by means of quantitative PCR for the cytokine mRNAs shown along the *x-axis* (GM-CSF, *n* = 6; IL-5, *n* = 12; and IL-4, *n* = 6). Eotaxin was measured by means of ELISA in BAL fluid (*n* = 12). **P* < .05 between PBS and ragweed-challenged without juglone (RAG) groups. †*P* < .05 between ragweed-challenged with juglone (RAG+J) and RAG groups.

The attenuation in pulmonary eosinophils after Pin1 blockade might be due to reductions in chemoattractants or increased cell death. To better understand the observed changes, we measured BAL fluid expression of critical cytokines, including GM-CSF, IL-3, IL-4, IL-5, or eotaxin, by means of quantitative PCR or ELISA. GM-CSF and IL-5 mRNA levels were significantly decreased

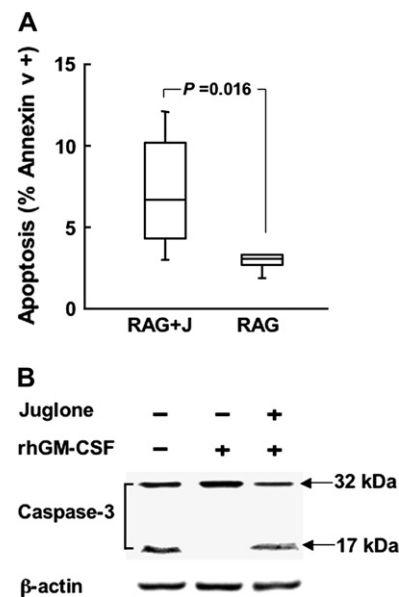


FIG 5. Pin1 regulates eosinophil apoptosis. **A**, Challenged rats received juglone (RAG+J) or not (RAG). BAL fluid eosinophils were stained with anti-annexin V. **P* < .05 between RAG+J and RAG groups. **B**, Immunoblot with antibodies shown of human blood eosinophils after culture for 24 hours with (+) or without (–) recombinant human GM-CSF (rhGM-CSF), juglone, or both.

by Pin1 inhibition, whereas IL-4 and eotaxin levels were unchanged (Fig 4). The amount of IL-5 mRNA was undetectable in BAL fluid from PBS-challenged rats (not shown). Unlike eotaxin, GM-CSF was undetectable in BAL fluid, even after 15-fold concentration. In addition, IL-3, IFN- γ , and FoxP3 mRNA levels were also increased in BAL fluid cells after allergen challenge, but none were affected by Pin1 blockade (*P* > .26, Mann-Whitney *U* Test, data not shown). Moreover, GM-CSF and IL-5 mRNA expression were highly correlated with eosinophils in BAL fluid (*r* = 0.65, *P* = .03 and *r* = 0.85, *P* = .0068, respectively; Spearman rank test). No correlation between any other cytokines and any other cell types was observed (not shown). These data suggest that Pin1 blockade modulates eosinophilia by influencing GM-CSF and IL-5 production.

Bone marrow differential counts showed a slight increase in eosinophil numbers after Pin1 blockade (3% in challenged but untreated control animals versus 8% in juglone-treated animals, data not shown), suggesting eosinophil production remained intact or eosinophils are confined to the bone marrow and are not recruited to the lung.

In vitro GM-CSF withdrawal induces eosinophil apoptosis and death.¹⁶ Therefore BAL fluid eosinophils were purified on a Percoll gradient, stained with anti-annexin V and analyzed by means of flow cytometry for apoptosis. There was a significant increase in apoptotic BAL fluid eosinophils after Pin1 inhibition (Fig 5, A). These data suggest that reductions in GM-CSF levels, IL-5 levels, or both led to increased apoptosis,

culminating in fewer airway and parenchymal eosinophils. We modeled this situation *in vitro* by comparing caspase 3 activation in juglone-treated peripheral blood eosinophils exposed to exogenous GM-CSF. Pin1 inhibition prevented GM-CSF pro-survival signaling, resulting in caspase 3 activation (Fig 5, B). Therefore Pin1 likely participates in GM-CSF production, as well as its pro-survival signaling.

DISCUSSION

Asthma severity is highly correlated with pulmonary eosinophilic inflammation. As such, a variety of agents targeted at eosinophils have been evaluated. Clinical trials with anti-IL-5 successfully reduced airway, peripheral blood, and bone marrow eosinophilia but showed no effect on clinical disease.⁴ Here we show that Pin1 blockade selectively reduced airway and parenchymal eosinophilia by approximately 75% after allergen challenge in a model of allergic airway inflammation. Total GM-CSF and IL-5 mRNA expression was significantly reduced, likely contributing to attenuated eosinophilic pulmonary entry, as well as enhanced apoptosis.

A variety of inflammatory, as well as parenchymal, cells produce proinflammatory cytokines in asthma. Activated infiltrating T lymphocytes, macrophages, and eosinophils, as well as endothelia and fibroblasts, produce GM-CSF, whereas IL-5 is mainly produced by T lymphocytes.²⁷ Whether attenuated GM-CSF expression in the BAL fluid reflects decreased production per eosinophil or fewer total eosinophils cannot be unambiguously determined at this time. However, because T-cell airway inflammation was unaffected by juglone, these data suggest that IL-5 mRNA expression per cell was decreased by *in vivo* Pin1 blockade. Purification of individual cell types by means of negative selection and subsequent analysis of mRNA content will answer this question.

The selectivity of cytokine suppression observed in juglone-treated rats was somewhat unexpected. Juglone suppressed IFN- γ expression and secretion *in vivo* after MHC-mismatched lung transplantation, as well as in isolated T cells activated *in vitro*.¹⁹ In the former, T cells are the predominant inflammatory cells, especially 1 to 2 weeks after transplantation. The results observed here suggest that IFN- γ produced after allergen challenge is from neutrophils or macrophages. The latter are essentially insensitive to juglone but do contain immunoreactive Pin1.¹⁷ The expression of IL-4 was Pin1 independent, as seen previously *in vivo* after transplantation¹⁹ or *in vitro* with activated T cells from human blood or Pin1 WT or knockout mice.^{17,19} In aggregate, these results suggest that Pin1 regulates the production and accumulation of a subset of cytokine mRNAs produced by T cells and eosinophils. The data also suggest that juglone displays relatively modest off-target effects.

The mechanisms by which Pin1 controls select cytokine gene expression in T cells or eosinophils remain largely unknown. These mRNAs are often unstable in

resting cells because of 3' untranslated region adenosine-uridine-rich elements (AREs). The AREs are interaction sites for tristetraproline, Krl-type splicing regulatory protein, or heterogeneous nuclear ribonucleoprotein D, a Pin1 target,¹⁶ which target these mRNAs to the exosome for rapid decay.¹⁶ We have shown that Pin1 was necessary for the activation-dependent stabilization of GM-CSF mRNAs by activated eosinophils¹⁶ and T cells.¹⁷ Anti-CD3 plus anti-CD28-activated splenocytes from Pin1 knockout versus WT mice showed a selective inability to stabilize the most ARE-positive and hence most labile cytokine mRNAs (GM-CSF, TNF- α , IL-2, IL-5, and IFN- γ), whereas cytokine mRNAs with few AREs were normally regulated (IL-4, CXCL-10, and TGF- β ; data not shown).¹⁹ Therefore the *in vitro* and *in vivo* results suggest that juglone reduced GM-CSF and IL-5 mRNA expression by preventing activation-induced stabilization. However, it is important to note that these results reflect regulation in T lymphocytes or eosinophils, which might differ in other cells. In addition, the involvement of Pin1 in nuclear events, such as transcription, is certainly possible because nuclear factor κ B and nuclear factor of activated T cells are possible targets for Pin1 as well.^{28,29}

The attenuation in eosinophilia after Pin1 blockade was likely due to reduced recruitment and enhanced apoptosis. IL-5 is necessary for eosinophil proliferation, differentiation, and release from the bone marrow.³⁰ Regulatory T (Treg) cells can influence eosinophil production and the allergic response through IL-5 production.³¹ We did not analyze Treg cell function, but there were no changes in FoxP3 expression despite Pin1 blockade. Total T cells were unchanged here, whereas CD4/CD8 ratios and total $\gamma\delta$ T cells in blood and BAL fluid were also unchanged after Pin1 blockade after lung transplantation.¹⁹ These results suggest that neither increased Treg cells nor suppressor activity were directly responsible for reduced eosinophil inflammation after juglone. In addition, we and others^{16,32} have shown that Pin1 might be involved in the regulation of apoptosis. Therefore loss of Pin1 likely accelerates apoptosis by attenuating the production of IL-5 and GM-CSF and interfering with their pro-survival antiapoptotic signaling.

It is worth noting that rats receiving juglone for 2 weeks had normal red cell mass, white cell counts, serum chemistry results, liver function test results, or renal function (not shown). Therefore juglone administration appeared to be safe and nontoxic. *In vitro* 5.7 μ M/L juglone eliminated essentially 100% of Pin1 and highly related yeast and *Escherichia coli* parvulin isomerase activity but had no effect on CyA or FKBP12.²⁵ Assuming complete distribution throughout all body water, the 1 mg/kg dose of juglone used here should generate a maximal concentration of approximately 10 μ M/L, of which only 0.01% to 0.4% was found in the lung 24 hours after administration.³³ Therefore it is very unlikely that CyA or FKBP12 were affected by juglone treatment. Although juglone has been shown to affect other enzymes, including RNA polymerase II,³⁴ the inhibitory concentration of 50% was approximately 10-fold that used here. Therefore

we conclude that the immunosuppressive effects of juglone *in vivo* reflect Pin1 blockade.

In conclusion, we have shown that Pin1 selectively controls pulmonary eosinophilia *in vivo* in a well-established rat model of acute pulmonary inflammation. The effects are predominantly through the regulation of antiapoptotic cytokine production and their signaling rather than modulation of T_H2 or Treg cells. A number of anti-Pin1 agents have been developed for cancer chemotherapy³⁵ but, to our knowledge, have not been applied to the treatment of asthma. Given our results, Pin1 should be considered as a possible target to control eosinophilic inflammation in asthma.

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