

Janus kinase 1/3 signaling pathways are key initiators of T_H2 differentiation and lung allergic responses

Shigeru Ashino, PhD,^a Katsuyuki Takeda, MD, PhD,^a Hui Li, PhD,^b Vanessa Taylor, PhD,^b Anthony Joetham, BS,^a Polly R. Pine, PhD,^b and Erwin W. Gelfand, MD^a *Denver, Colo, and South San Francisco, Calif*

Background: Janus kinases (JAKs) are regulators of signaling through cytokine receptors. The importance of JAK1/3 signaling on T_H2 differentiation and development of lung allergic responses has not been investigated.

Objective: We sought to examine a selective JAK1/3 inhibitor (R256) on differentiation of T_H subsets *in vitro* and on development of ovalbumin (OVA)-induced airway hyperresponsiveness (AHR) and inflammation in an experimental model of asthma.

Methods: A selective JAK1/3 inhibitor was used to assay the importance of this pathway on induction of T_H1, T_H2, and T_H17 differentiation *in vitro*. *In vivo*, the effects of inhibiting JAK1/3 signaling were examined by administering the inhibitor during the sensitization or allergen challenge phases in the primary challenge model or just before provocative challenge in the secondary challenge model. Airway inflammation and AHR were examined after the last airway challenge.

Results: *In vitro*, R256 inhibited differentiation of T_H2 but not T_H1 or T_H17 cells, which was associated with downregulation of signal transducer and activator of transcription (STAT) 6 and STAT5 phosphorylation. However, once polarized, T_H2 cells were unaffected by the inhibitor. *In vivo*, R256 administered during the OVA sensitization phase prevented the development of AHR, airway eosinophilia, mucus hypersecretion, and T_H2 cytokine production without changes in T_H1 and T_H17 cytokine levels, indicating that selective blockade of T_H2 differentiation was critical. Inhibitor administration after OVA sensitization but during the challenge phases in the primary or secondary challenge models similarly suppressed AHR, airway eosinophilia, and mucus hypersecretion without any reduction in T_H2 cytokine production, suggesting the inhibitory effects were downstream of T_H2 cytokine receptor signaling pathways.

Conclusions: Targeting the T_H2-dependent JAK/STAT activation pathway represents a novel therapeutic approach for the treatment of asthma. (J Allergy Clin Immunol 2013;■■■■:■■■■-■■■■.)

Key words: Janus kinase 1/3, asthma, T_H2

Cell signaling through the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway plays a critical role in the function of cytokines, growth factors, and hormones.¹ The JAK family members are activated after receptor ligation, which leads to phosphorylation of the STATs, an event central to DNA transcription and cell activation.^{2,3} Activation of receptor-associated JAKs by phosphorylation of specific residues leads to the recruitment of specific STATs, which are then tyrosine phosphorylated. The activated STATs are released from the receptor, dimerize, and translocate to the nucleus, where they bind to members of cytokine response elements.^{4,5} Because most proinflammatory cytokines and growth factors use this pathway, the JAK/STAT pathway is linked to various immunodeficiency and autoimmune diseases.^{1,6-8}

There are 4 members of the JAK family: JAK1, JAK2, JAK3, and Tyk2. JAK1 associates with a variety of cytokine and growth factor signaling pathways.¹ JAK3 expression is restricted to hematopoietic cells and colocalizes with JAK1, transmitting signals through the common γ chain of the cytokine receptors for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21.² As a result, JAK1 and JAK3 play critical roles in the initiation of inflammation and have been investigated as potent therapeutic targets in a variety of inflammatory diseases. A number of JAK inhibitors have been developed for clinical use.^{1,9} Ruxolitinib, a selective JAK1 and JAK2 inhibitor, has been used in the treatment of myelofibrosis and shown to impair dendritic cell functions.¹⁰ Tofacitinib, a pan-JAK inhibitor that targets JAK1 and JAK3 and, to a lesser extent, JAK2 has been effective in patients with rheumatoid arthritis,^{11,12} as well as in preliminary studies of other autoimmune diseases, inflammatory bowel disease, transplant rejection, or hematopoietic disorders, such as myelofibrosis and polycythemia vera.^{8,9,13,14}

Asthma is chronic inflammatory respiratory disease associated with T_H2-dominant immune responses.¹⁵ Despite advances in therapy, asthma prevalence remains high in industrial countries, and the socioeconomic burden caused by disease exacerbations or steroid-refractory asthma is significant.¹⁶ Therefore, numerous strategies have been attempted, but to date, they have failed to be sustained in the clinic.¹⁷ Because the JAK/STAT pathways play a critical role in the differentiation of T_H cells, including T_H2 cells, JAK inhibitors were studied in animal models of asthma. Tofacitinib, a pan-JAK inhibitor, was shown to reduce allergen-induced airway eosinophilia and IL-13 levels in mice.¹⁸ Administration of the pan-JAK inhibitor pyridone 6 reduced airway hyperresponsiveness (AHR), airway eosinophilia, and airway mucus hyperproduction when mice were treated only during the challenge phase.¹⁹ Although nonselective JAK inhibitors showed benefit in experimental asthma models, the targeting of JAK2 function with these reagents might be of concern, including alteration of

From ^athe Division of Cell Biology, Department of Pediatrics, National Jewish Health, Denver, and ^bRigel Pharmaceuticals, South San Francisco.

Supported by National Institutes of Health grants HL-36577 and AI-77609. Funding was received from Rigel Pharmaceuticals in support of this study, and E.W.G. was a consultant to Rigel.

Disclosure of potential conflict of interest: V. Taylor is employed by and owns stock/stock options in Rigel Pharmaceuticals. P. R. Pine is employed by Rigel Pharmaceuticals. E. W. Gelfand has been supported by one or more grants from Rigel Pharmaceuticals. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication May 23, 2013; revised October 3, 2013; accepted for publication October 7, 2013.

Corresponding author: Erwin W. Gelfand, MD, Division of Cell Biology, Department of Pediatrics, National Jewish Health, 1400 Jackson St, Denver, CO 80206. E-mail: gelfande@njhealth.org.

0091-6749/\$36.00

© 2013 American Academy of Allergy, Asthma & Immunology
<http://dx.doi.org/10.1016/j.jaci.2013.10.036>

Abbreviations used

AHR:	Airway hyperresponsiveness
BAL:	Bronchoalveolar lavage
BFA:	Brefeldin A
EGF:	Epidermal growth factor
EGFR:	Epidermal growth factor receptor
EPO:	Erythropoietin
FACS:	Fluorescence-activated cell sorting
Foxp3:	Forkhead box protein 3
ICAM:	Intercellular adhesion molecule
JAK:	Janus kinase
NHLF:	Normal human lung fibroblast
OVA:	Ovalbumin
p-STAT:	Phosphorylated STAT
STAT:	Signal transducer and activator of transcription
TCR:	T-cell receptor
Treg:	Regulatory T
VEGFR2:	Vascular endothelial growth factor receptor 2

regulatory T (Treg) cell activity or interference with other important pathways involving JAK2.

R256 is a selective ATP-competitive JAK1/3 inhibitor identified through cell-based assays. In this study we determined the effects of R256 on the differentiation of T_H subsets *in vitro* and the development of allergen-induced AHR, airway inflammation, and cytokine production in the airways. The results demonstrated that selective inhibition of JAK1/3 prevented T_H2 differentiation without altering T_H1 and T_H17 differentiation. When added to differentiated cells, no effects were observed. In an animal model in mice that received R256 during the sensitization phase, the development of AHR, airway eosinophilia, and mucus hypersecretion was prevented. On the other hand, when mice received R256 after allergen sensitization but during either primary allergen challenge or a single provocative secondary allergen challenge, after allergen-induced airway inflammation and AHR were established, AHR, airway eosinophilia, and mucus hypersecretion were reduced, but without any modification of T_H2 cytokine production. These results suggest that R256 has important activities both during the allergen sensitization phase and during the allergen challenge phase, attenuating development of T_H2 -dependent asthma.

METHODS**Animals**

Wild-type female BALB/c, OT-2 T-cell receptor (TCR) transgenic, and C57BL/6 mice aged 6 to 8 weeks were obtained from Jackson Laboratories (Bar Harbor, Me). All mice were maintained under specific pathogen-free conditions. All experiments were conducted under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Cell-based selectivity assays of R256 activities

The activity of R256 (Rigel Pharmaceuticals, South San Francisco, Calif) was assessed in a panel of cell-based assays. R256 is a selective inhibitor of JAK1/3-dependent signaling. Eotaxin production induced by IL-13 (25 ng/mL; PeproTech, Rocky Hill, NJ) or IL-4 (5 ng/mL, PeproTech) in normal human lung fibroblasts (NHLFs; Lonza, Allendale, NJ) was measured by means of ELISA (R&D Systems, Minneapolis, Minn).²⁰⁻²³ STAT6 phosphorylation induced by IL-13 (50 ng/mL) or IL-4 (10 ng/mL) in NHLFs was measured by using intracellular fluorescence-activated cell sorting (FACS; anti-pY641-STAT6 Alexa Fluor 488; BD Biosciences, San Jose, Calif).

IL-2-dependent human primary T-cell proliferation was assessed by using the Promega CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, Wis) in the presence of 40 U/mL IL-2 (R&D Systems).²⁴ STAT5 phosphorylation induced by IL-2 in human primary T cells was measured by using intracellular FACS analysis (anti-pY694-STAT5 Alexa Fluor 488, BD Biosciences). The erythropoietin (EPO; 1 U/mL, R&D Systems)-dependent survival of cultured human erythroid progenitor cells was determined using Promega's CellTiter-Glo Luminescent Cell Viability Assay.^{25,26} Surface intercellular adhesion molecule (ICAM) 1 (anti-ICAM-1-APC, BD Biosciences) expression induced by IFN- γ (10 ng/mL, PeproTech) on U937 cells was measured using FACS.²⁷ Cultured human erythroid progenitor cells were differentiated from CD34⁺ cord blood cells in the presence of IL-3 (10 ng/mL), IL-10 (10 ng/mL), and stem cell factor (25 ng/mL, PeproTech) for 9 days and with addition of EPO for the last day.²⁸ The enzymatic activity of tryptase released by human cultured mast cells on stimulation with IgE was quantified by means of cleavage of the synthetic fluorescent peptide substrate Z Ala Lys-Arg-AMC.2TFA (MP Biomedicals, Solon, Ohio) in tryptase buffer.²⁹ B-cell receptor-dependent extracellular signal-regulated kinase 1/2 phosphorylation was measured in Ramos cells by intracellular FACS (human anti-IgM 5 μ g/mL, Jackson Immunoresearch Labs, West Grove, Pa; anti-pT202/pY204-ERK1/2-Alexa Fluor 488, BD Biosciences). Human primary T-cell activation was assessed by measuring IL-2 production using ELISA (R&D Systems) after plate-bound anti-CD3 (1 μ g/mL) and anti-CD28 (5 μ g/mL) stimulation (anti-human CD3, BD Biosciences; anti-human CD28, Immunotech, Pasadena, Calif). Human umbilical vein endothelial cells (Lonza, Basel, Switzerland) were stimulated with vascular endothelial growth factor, and vascular endothelial growth factor receptor 2 (VEGFR2) phosphorylation was assessed by using ELISA (100 ng/mL VEGF165; R&D Systems; rabbit anti-phospho-VEGFR2 mAb; Cell Signaling Technology, Danvers, Mass).³⁰ Epidermal growth factor receptor (EGFR) phosphorylation was measured in HeLa cells after epidermal growth factor (EGF) stimulation by staining permeabilized cells with a phospho-specific EGFR antibody and quantified by means of chemiluminescence (0.2 μ mol/L EGF, PeproTech; phospho-EGFR Tyr1173, Cell Signaling Technology).

Generation of T_H1 , T_H2 , and T_H17 cells and R256 treatment

CD4⁺CD45RB⁺ naive T_H cells were isolated from OT-2 TCR transgenic mouse spleen cells by flow cytometry (Mo-FLO XDP; Beckman Coulter, Fullerton, Calif). Isolated naive T_H cells were cultured with recombinant murine (rm) IL-2 (20 ng/mL, R&D Systems), rmIL-12 (5 ng/mL, PeproTech), rmIFN- γ (1 ng/mL, PeproTech EC), and anti-IL-4 mAb (10 μ g/mL, eBioscience, San Diego, Calif) for T_H1 differentiation³¹; rmIL-2 (20 ng/mL), rmIL-4 (1 ng/mL, PeproTech), anti-IFN- γ mAb (10 μ g/mL, eBioscience), and anti-IL-12p40 mAb (10 μ g/mL, eBioscience) for T_H2 differentiation³²; or recombinant human (rh) IL-6 (50 ng/mL, PeproTech EC), rhTGF- β_1 (2 ng/mL, PeproTech EC), rmIL-23 (10 ng/mL, R&D Systems), anti-IL-4 mAb (10 μ g/mL), and anti-IFN- γ mAb (10 μ g/mL) for T_H17 differentiation³³ in the presence of mitomycin C-treated splenocytes of wild-type C57BL/6 and 5 μ g/mL ovalbumin (OVA)₃₂₃₋₃₃₉ peptide (AnaSpec, Fremont, Calif). Each T_H subset was restimulated with OVA₃₂₃₋₃₃₉ peptide 48 hours later under the same conditions. Cells were collected and washed after incubation for 6 to 8 days and restimulated with plate-coated anti-CD3 and anti-CD28 (eBioscience) for 6 hours with or without brefeldin A (BFA; Sigma, St Louis, Mo). The supernatants from cultures without BFA were assayed for cytokine production by ELISA, and the cells from cultures with BFA were assayed by means of intracellular cytokine staining. For intracellular staining, 6 hours after stimulation, the cells were fixed in 4% paraformaldehyde and permeabilized with 0.1% saponin buffer before intracellular cytokine staining and flow cytometry using a FACSCalibur with FlowJo software (TreeStar, Ashland, Ore). The following antibodies were used: allophycocyanin-conjugated anti-IL-4 mAb, phycoerythrin-conjugated anti-IL-13 or anti-IL-17 mAbs, and fluorescein isothiocyanate-conjugated IFN- γ mAb (eBioscience).

During differentiation from days 0 to 6 or after polarization was complete from days 6 to 8 of T_H1 , T_H2 , and T_H17 cell cultures, R256 (25, 50, and 100 nmol/L) was added daily. In control cultures dimethyl sulfoxide alone was added.

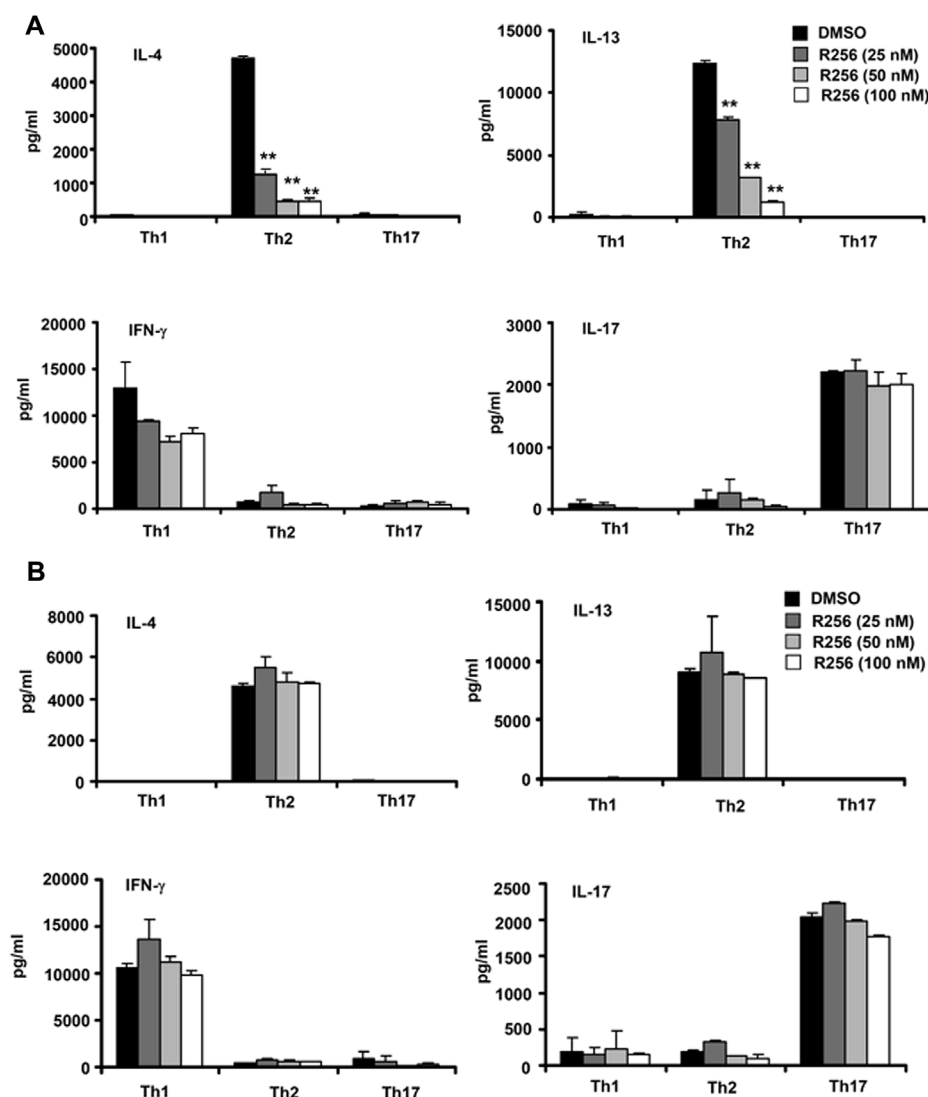


FIG 1. Effects of R256 on *in vitro* differentiated T_H subsets. **A**, Cytokine levels in supernatants from T_H cells. Cells were treated with R256 from days 0 to 6 and restimulated with plate-coated anti-CD3/anti-CD28 mAbs on day 6. **B**, Cytokine levels in supernatants from differentiated T_H cells. Cells were treated with R256 from days 6 to 8 and restimulated with anti-CD3/anti-CD28 mAbs on day 8. **C**, On day 6 of cultures that included R256 from day 0 of T_H subset differentiation, p-STAT3, p-STAT4, p-STAT5, and p-STAT6 in the cells were stained with Alexa Fluor 647-conjugated specific antibodies for flow cytometric analysis. Splenocyte CD4 cells from normal OT-2 mice were used as negative controls for p-STAT staining. Data for each group were expressed as means $\pm$ SEMs. Results are representative of 3 independent experiments carried out in duplicate. ** $P < .01$ compared with the dimethyl sulfoxide (DMSO)-treated groups.

Flow cytometric analyses of STAT phosphorylation

On day 6 of the T_H1 , T_H2 , and T_H17 cell cultures, cells were washed and fixed by the addition of 4% paraformaldehyde for 15 minutes and permeabilized with cold 90% methanol for 30 minutes on ice. Cells were stained with Alexa Fluor 647-conjugated phosphorylated STAT (p-STAT) 1, p-STAT3, p-STAT4, p-STAT5, or p-STAT6 mAb (BD Biosciences). Flow cytometry was carried out with FACSCalibur with FlowJo software.

Development of allergen-induced airway inflammation and treatment with R256

Mice were sensitized intraperitoneally with 20 μ g of OVA (Thermo Fisher, Waltham, Mass) emulsified in 2 mg of alum (Imject Alum; Thermo Fisher) in a total volume of 100 μ L on days 0 and 14. Mice were subsequently challenged

through the airways by means of inhalation exposure to aerosols of OVA (1% in PBS) for 20 minutes on days 28 to 30 (primary allergen challenge model). Sensitized and previously challenged mice received a single provocative allergen challenge on day 56 (secondary allergen challenge) to further investigate the effects of the inhibitor on established allergen-induced airway disease.³⁴ OVA aerosols were produced with an ultrasonic nebulizer (model NE-U07; Omron Healthcare, Kyoto, Japan). Airway function was measured after the last allergen challenge (day 32 or 56), as described below, followed by collection of samples for further analyses.

The mice were administered R256 orally twice daily at each time point either during the OVA sensitization phase or during the primary or secondary OVA challenge phases. In the sensitization phase R256 (50 mg/kg per time) was administered from days 0 to 4 and days 14 to 18 twice a day. In the primary and secondary challenge phases, mice were treated with R256 (10, 25, and 50 mg/kg per time) on days 28 to 31 or days 56 to 57, respectively, twice a day

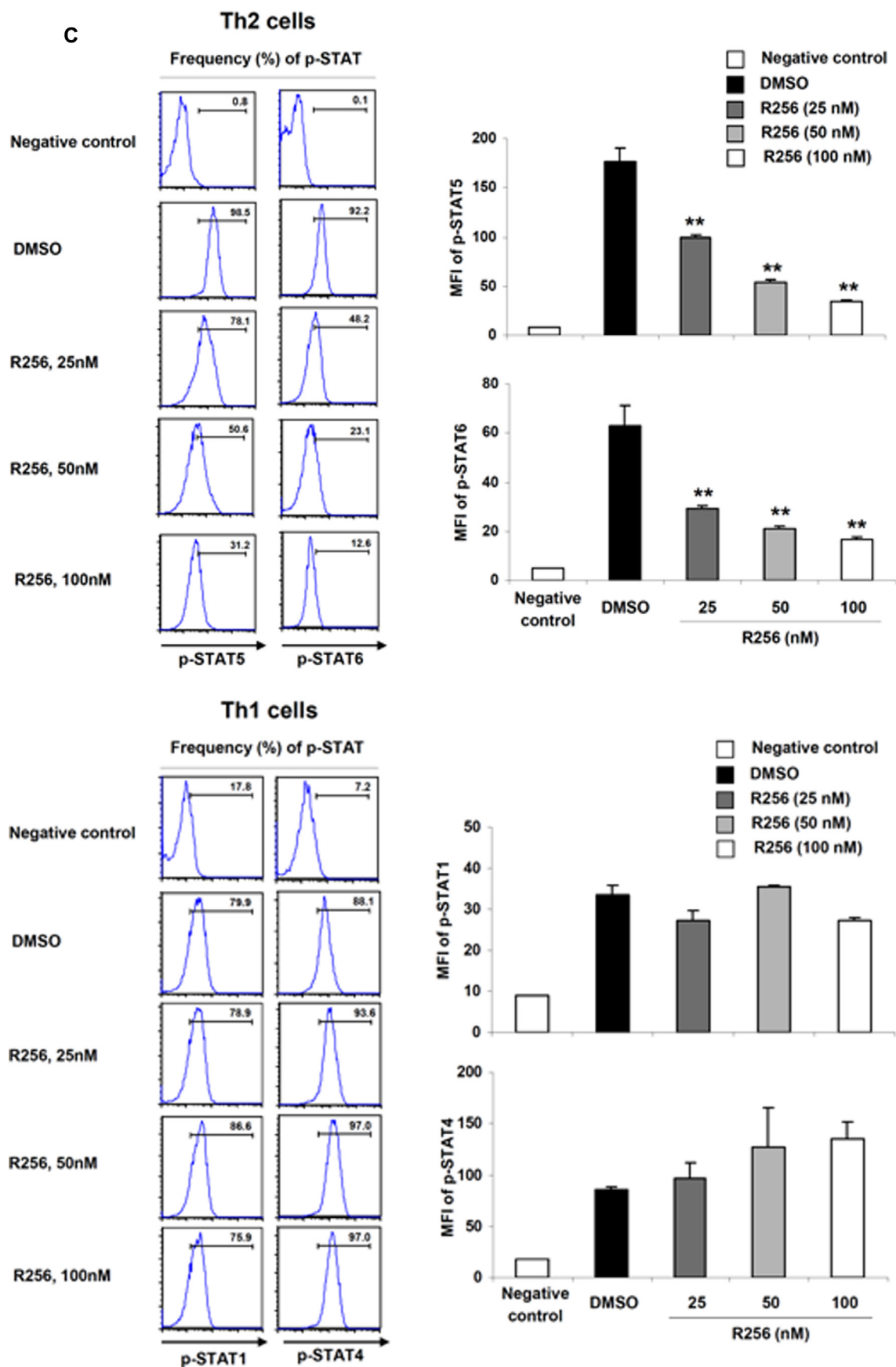


FIG 1. (Continued)

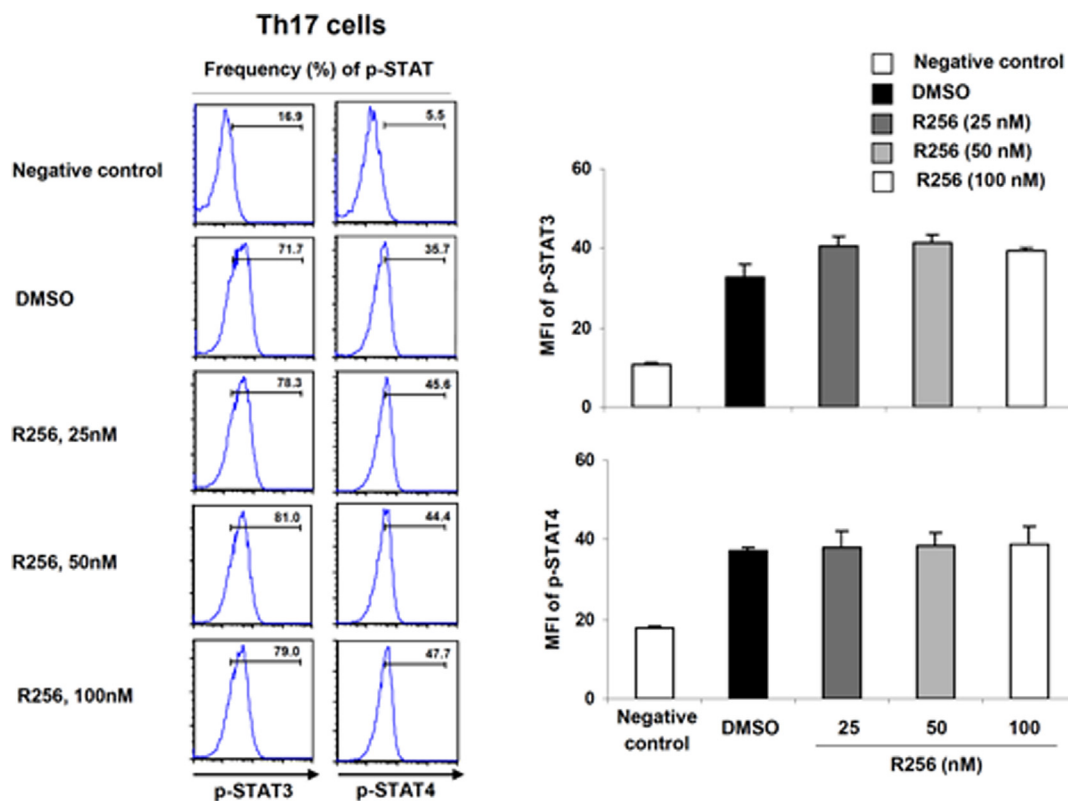


FIG 1. (Continued)

before and subsequent to each OVA challenge. In control groups only vehicle (0.25% hydroxypropyl methylcellulose) was administered.

Assessment of airway function

Airway responsiveness was assessed, as previously described, by measuring changes in airway resistance in response to increasing doses of aerosolized methacholine (Sigma).³⁵ Data are expressed as the percentage change from baseline values obtained after inhalation of saline.

Bronchoalveolar lavage

Immediately after assessment of AHR, lungs were lavaged with 1 mL of Hank's balanced salt solution through the trachea. Total leukocyte and cell differentials were performed, as previously described.³⁵

Measurement of cytokines and chemokines

Cytokine levels in the bronchoalveolar lavage (BAL) fluid and cell-culture supernatants were measured using IL-4, IL-5, IL-10, IL-13, IL-17, and IFN- γ ELISAs (eBioscience), according to the manufacturer's directions. For measurement of eotaxin levels, BAL fluid was collected 6 hours after the final OVA challenge and assayed by using ELISA (eBioscience).

Intracellular cytokine and forkhead box protein 3 staining of lung cells

Lung cells were isolated, as previously described, by collagenase digestion.³⁶ Lung leukocytes were purified by 35% Percoll gradient centrifugation. After 6 hours of stimulation with plate-coated anti-CD3/anti-CD28 (eBioscience) in the presence of BFA, cells were examined for intracellular cytokine staining after cell-surface staining with allophycocyanin-conjugated anti-CD4 mAb (eBioscience), followed by

fixation with 4% paraformaldehyde and permeabilization with 0.1% saponin buffer. For detection of CD4⁺ forkhead box protein 3 (Foxp3)⁺ cells, cells were stained with the anti-CD4 mAb, followed by fixation and permeabilization with the Foxp3 staining buffer set (eBioscience) and then stained with fluorescein isothiocyanate-conjugated Foxp3 mAb (eBioscience).

Treg cell functional assay

CD4⁺CD25⁺ Treg cells were isolated from spleens of naive mice using the Treg isolation kit (Miltenyi Biotec, Auburn, Calif). Isolated Treg cells were treated with dimethyl sulfoxide or R256 (50 nmol/L) for 3 hours. After washing out of drug, Treg cells were cocultured with carboxyfluorescein succinimidyl ester (eBioscience)-labeled CD4⁺CD25⁺ cells, which were isolated from the spleens of naive mice. After 3 days of coculture, division of carboxyfluorescein succinimidyl ester-labeled CD4⁺CD25⁺ cells stimulated with anti-CD3/anti-CD28 was analyzed by flow cytometry.

Histologic studies

After lavage, the lungs were fixed in 10% formalin and processed into paraffin blocks. Tissue sections (5 μ m) were stained with hematoxylin and eosin and periodic acid-Schiff for identification of mucus-containing cells. The slides were transferred to pictures with a Nikon microscope (Melville, NY). Periodic acid-Schiff-positive cells in the tissues were quantified using the NIH Image analysis system (version 1.63; National Institutes of Health, Bethesda, Mass) and counting 6 to 8 different fields per slide in a blinded manner.

Statistical analysis

Values for all measurements were expressed as means $\pm$ SEMs. For comparisons between multiple groups, the Tukey-Kramer test was used. Nonparametric analyses using the Mann-Whitney *U* or Kruskal-Wallis tests were also applied to confirm that statistical differences remained significant,

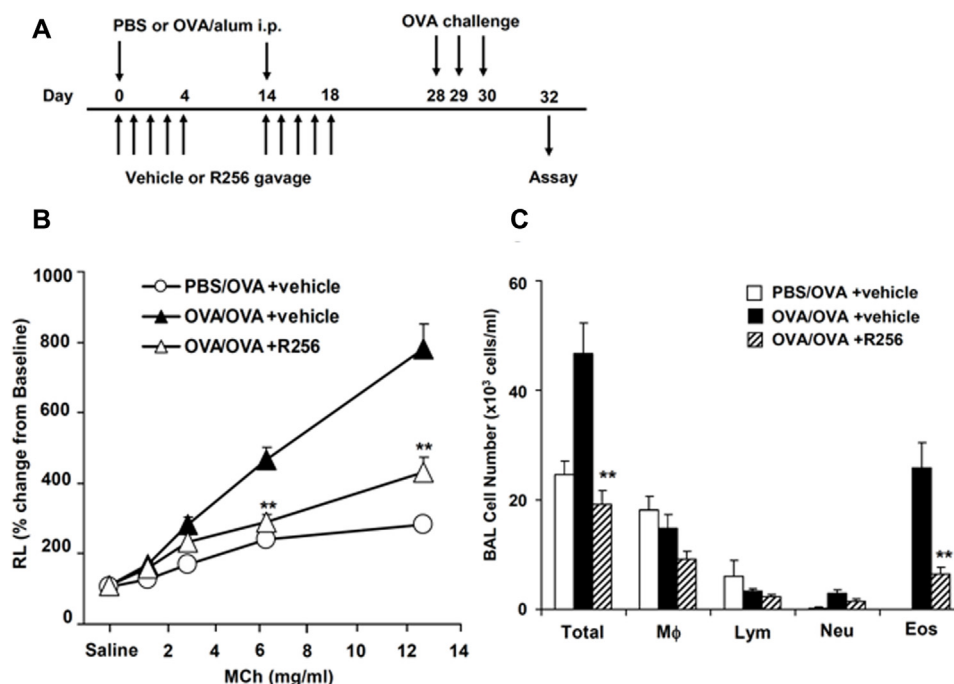


FIG 2. Effects of R256 treatment during allergen sensitization on development of allergen-induced AHR and airway inflammation. **A**, R256 was orally administered during the sensitization phase, as described in the Methods section. Two weeks after the last allergen sensitization, mice were exposed to 3 consecutive days of allergen challenge, followed by assessments of airway responsiveness to aerosolized methacholine and BAL/lung tissue sampling 48 hours after the last OVA challenge. **B**, Lung resistance (*RL*). **C** and **D**, BAL cell composition (Fig 2, *C*) and goblet cell metaplasia (Fig 2, *D*). *PAS*, Periodic acid–Schiff. **E**, Representative pictures show PBS/OVA plus vehicle (*a*), OVA/OVA plus vehicle (*b*), and OVA/OVA plus R256 (*c*). **F**, Cytokine levels in BAL fluid. Mice were sham sensitized and challenged to OVA (PBS/OVA) or sensitized and challenged to OVA (OVA/OVA). *Eos*, Eosinophil; *Lym*, lymphocyte; *MCh*, methacholine; *Mφ*, macrophage; *Neu*, neutrophil. Data for each group were expressed as means $\pm$ SEMs. Results are from 2 independent experiments with 4 mice per group ($n = 8$). * $P < .05$ and ** $P < .01$ compared with the OVA/OVA plus vehicle–treated group.

even if the underlying distribution was uncertain. The P value for significance was set at less than .05.

RESULTS

R256 is a selective JAK1/3 inhibitor identified through cell-based screening

R256 is a selective JAK1/3 inhibitor in cell-based assays (Table I). R256 potently suppressed JAK1/JAK2/Tyk2-dependent STAT6 phosphorylation and eotaxin production in NHLFs in response to IL-13, with median effective concentration values of 37 and 11 nmol/L, respectively. R256 inhibited equally effectively JAK1/3-dependent STAT6 phosphorylation and eotaxin production in response to IL-4 in the same cells. Furthermore, R256 inhibited JAK1/3-dependent T-cell proliferation and STAT5 phosphorylation in response to IL-2 with a median effective concentration value of less than 50 nmol/L. In contrast, R256 showed limited inhibition of JAK2-dependent primary erythroblast cell survival in response to EPO and JAK1/2-dependent ICAM-1 expression on the monocytic cell line U937 in response to IFN- γ .

In a panel of cell-based assays to assess activity against a range of cytoplasmic and receptor tyrosine kinase–dependent pathways, including Syk-dependent signaling, Lck/ZAP70-dependent signaling, and VEGFR2 activation, R256 showed little effect.

Effects of R256 on differentiating T_H cells

The effects of the selective JAK1/3 inhibitor on the differentiation of naive CD4 cells into T_H1, T_H2, and T_H17 cells were determined. R256 was added to the culture medium from day 0 of the differentiation protocol, followed at the end of the culture period (day 6) by analysis of cytokine production levels after TCR stimulation with anti-CD3/anti-CD28. As shown in Fig 1, A, IL-4 and IL-13 production from cells cultured under the T_H2 polarization protocol were reduced in a dose-dependent manner in parallel to the decrease in their frequency (see Fig E1 in this article's Online Repository at www.jacionline.org). In contrast, IFN- γ production from T_H1 cells and IL-17 production from T_H17 cells were not altered nor was the frequency of IFN- γ – or IL-17–producing cells (see Fig E1). However, when cells were treated with R256 from days 6 to 8 of culture, after completion of T_H cell differentiation, cytokine production levels from all of the T_H subsets after TCR stimulation were not altered (Fig 1, B).

Because T_H2 differentiation requires IL-4–induced STAT6 and, to some extent, IL-2–induced STAT5 activation,^{37,38} phosphorylation levels of STAT5 and STAT6 were determined by flow cytometry. As shown in Fig 1, C, the JAK1/3 inhibitor blocked phosphorylation of both STAT6 and STAT5 in T_H2 cells. By comparison, phosphorylation of STAT1 and STAT4 in T_H1 cells and STAT3 and STAT4 in

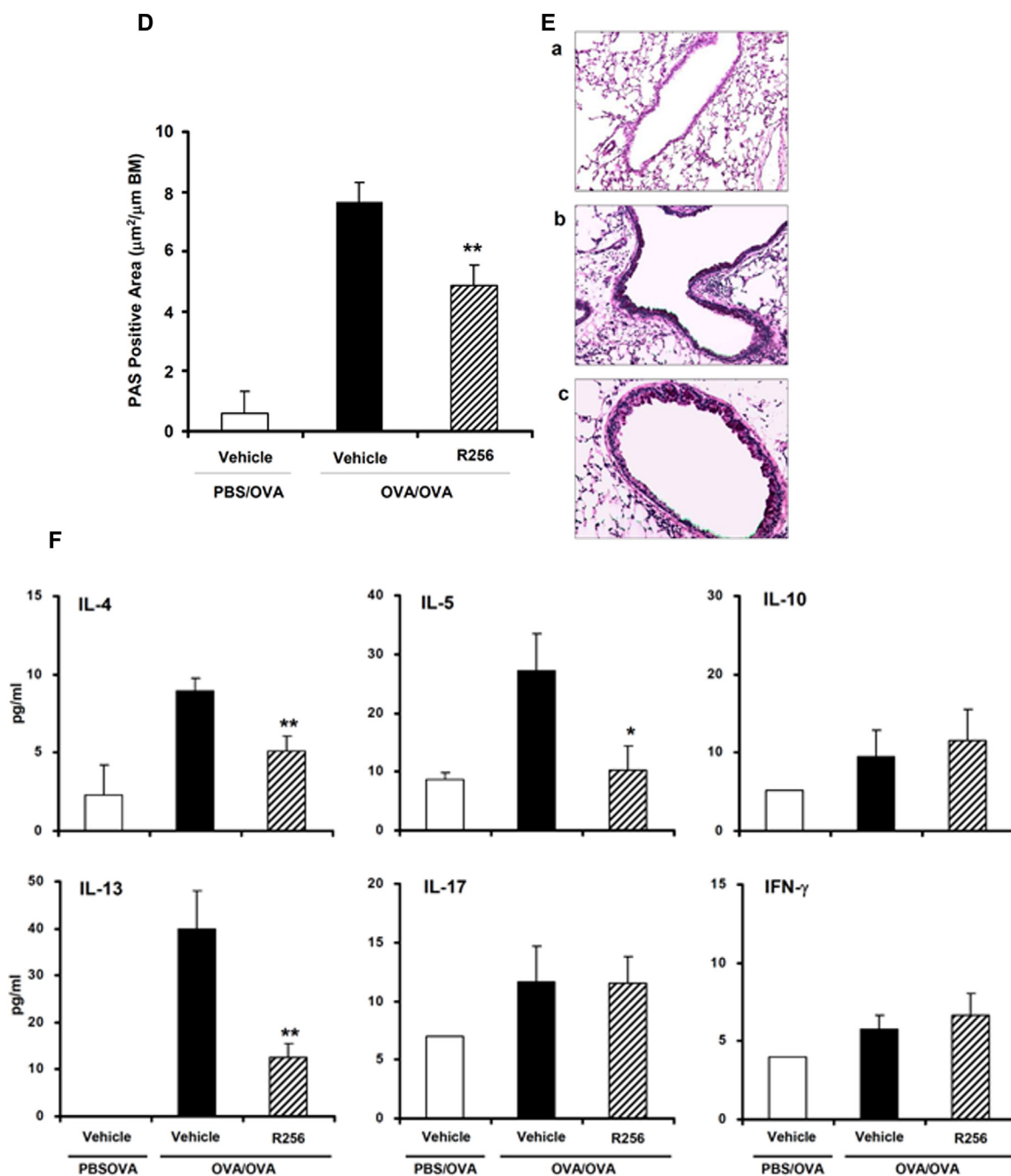


FIG 2. (Continued)

T_H17 cells, which is essential in the polarization of T_H1 cells in the presence of IFN- γ and IL-12^{39,40} and T_H17 cells in the presence of IL-6 and IL-23,^{41,42} respectively, was not altered by the drug. The phosphorylation levels of STAT5 in T_H1 and T_H17 cells were not altered by the drug (data not shown).

These data identify 2 important aspects related to JAK1/3 inhibition by R256. The first is that the pathway is essential for the differentiation of T_H2 cells, but once T_H2 cell differentiation has been completed, the inhibitor was no longer effective in blocking or inhibiting T_H2 cytokine production. The second outcome of inhibiting JAK1/3 signaling by R256 was that the effects were

restricted to T_H2 differentiation without affecting T_H1 or T_H17 differentiation.

JAK1/3 inhibition during the sensitization phase prevents allergen-induced airway inflammation and AHR

In light of the *in vitro* findings, we determined the effects of R256 *in vivo* in an animal model of allergen-induced airway inflammation and AHR during 2 distinct phases of the response. The initial sensitization phase is thought to be IL-4 dependent, and the latter or challenge phase was IL-13

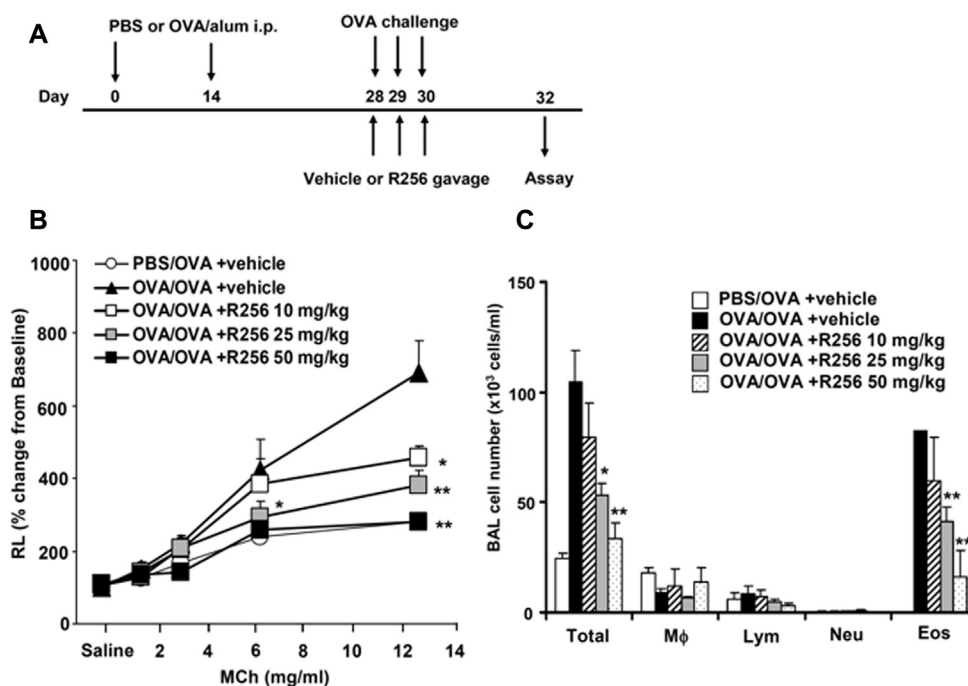


FIG 3. Effects of R256 treatment during primary allergen challenge on development of allergen-induced AHR and airway inflammation. **A**, Indicated doses of R256 were orally administered during the allergen challenge phase, as described in the Methods section. Two weeks after the last allergen sensitization, mice were exposed to 3 consecutive days of allergen challenge, followed by assessments of airway responsiveness and BAL/lung tissue sampling 48 hours after the last OVA challenge. **B**, Lung resistance (RL). **C** and **D**, BAL cell composition (Fig 3, C) and goblet cell metaplasia (Fig 3, D). PAS, Periodic acid–Schiff. **E**, Representative images show PBS/OVA plus vehicle (a), OVA/OVA plus vehicle (b), OVA/OVA plus R256 (10 mg/kg; c), OVA/OVA plus R256 (25 mg/kg; d), and OVA/OVA plus R256 (50 mg/kg; e). **F** and **G**, Cytokine levels (Fig 3, F) and eotaxin levels (Fig 3, G) in BAL fluid collected at 48 and 6 hours after final OVA challenge. *Eos*, Eosinophil; *i.p.*, intraperitoneal; *Lym*, lymphocyte; *MCh*, methacholine; *Mφ*, macrophage; *Neu*, neutrophil. Data for each group were expressed as means $\pm$ SEMs. Results are from 3 independent experiments with 3 mice per group ($n = 9$). * $P < .05$ and ** $P < .01$ compared with the OVA/OVA plus vehicle–treated group.

dependent.⁴³ In the first protocol animals were treated with R256 only during the sensitization phase, followed 2 weeks later by allergen challenge (Fig 2, A). As shown in Fig 2, B and C, R256 administered only during sensitization prevented the development of AHR and BAL eosinophilia. Histologic analysis revealed marked reductions in airway inflammation and goblet cell metaplasia (Fig 2, D and E). In addition, TH2 cytokine levels (IL-4, IL-5, and IL-13) in BAL fluid after allergen challenge were also reduced after R256 administration during sensitization (Fig 2, F), paralleling the *in vitro* results when the inhibitor was added during TH2 induction (Fig 1, A). Under these *in vivo* conditions, no significant differences were observed in IFN- γ , IL-17, and IL-10 levels in the vehicle- and R256-treated groups.

JAK1/3 inhibition during the primary challenge phase prevents allergen-induced airway inflammation and AHR

We next determined the effects of R256 when administered after allergen sensitization but solely during the first allergen challenge phase (Fig 3, A). As shown in Fig 3, B, when the inhibitor was administered during the allergen challenge phase, the development of AHR was prevented and BAL eosinophilia (Fig 3, C) and goblet cell metaplasia (Fig 3, D and E) were

significantly reduced. Surprisingly, unlike administration during the sensitization phase (Fig 2, F), levels of the TH2 cytokines IL-4, IL-5, and IL-13 were not reduced (Fig 3, F); levels of IFN- γ , IL-17, and IL-10 were unaltered, which were similar to the *in vitro* results when the inhibitor was added to differentiated T cells (Fig 1, B). This was also confirmed by means of flow cytometric analyses, which demonstrated that the frequencies of IFN- γ - or IL-17-expressing CD4⁺ T cells in lung tissue were not altered by R256 treatment (see Fig E2 in this article's Online Repository at www.jacionline.org). The frequencies of Treg cells in lung tissues were not affected by R256 treatment (see Fig E3 in this article's Online Repository at www.jacionline.org). Moreover, R256 did not affect Treg cell-suppressive activity when assayed against the proliferative activity of CD4⁺CD25⁻ T cells (see Fig E4 in this article's Online Repository at www.jacionline.org). When the inhibitor was administered during challenge, eotaxin levels in BAL fluid were significantly reduced when compared with those in nontreated mice (Fig 3, G).

JAK1/3 inhibition during the secondary challenge phase prevents allergen-induced airway inflammation and AHR

Mice received the inhibitor during secondary allergen challenge to determine the effects of R256 in the airway, in which

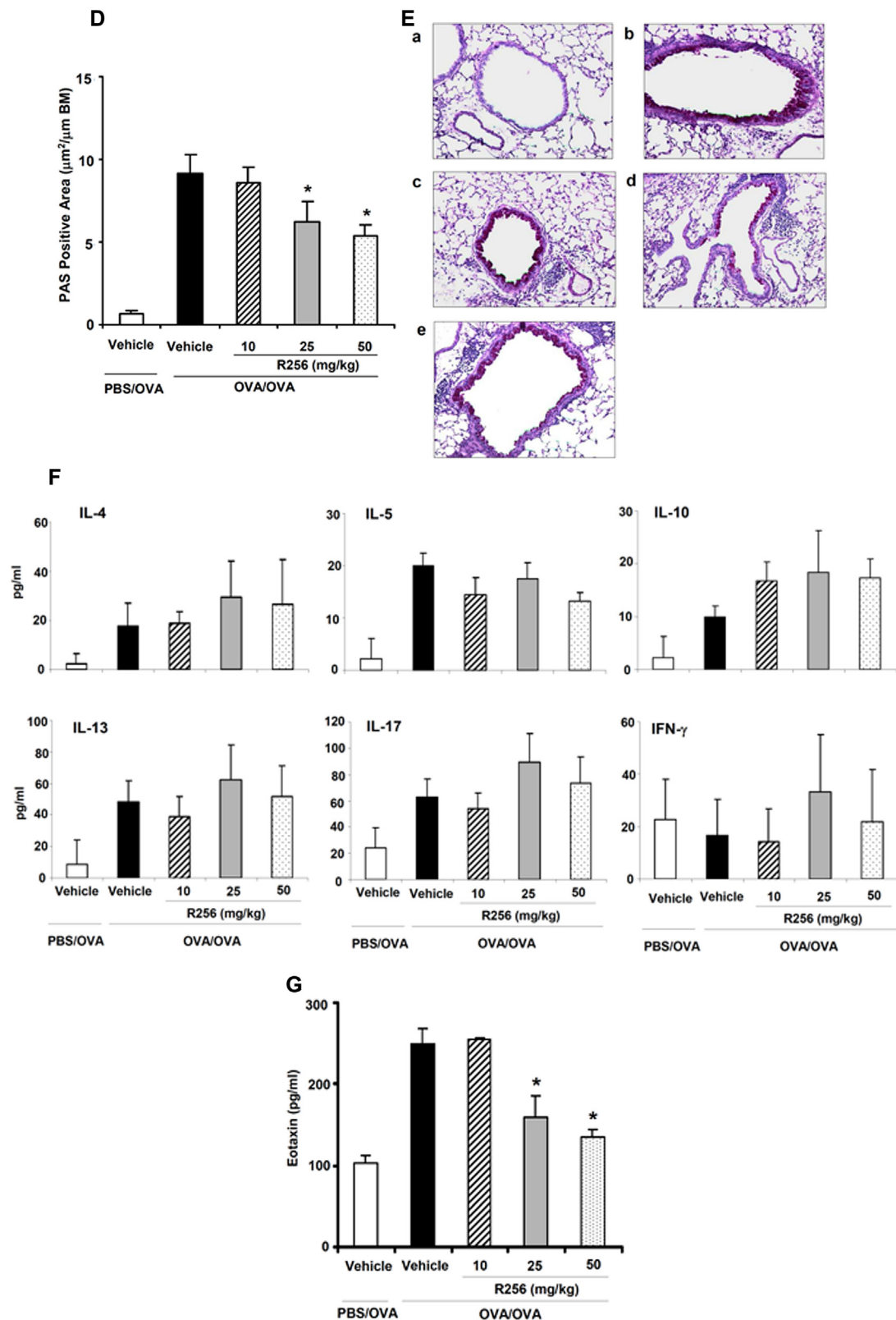


FIG 3. (Continued)

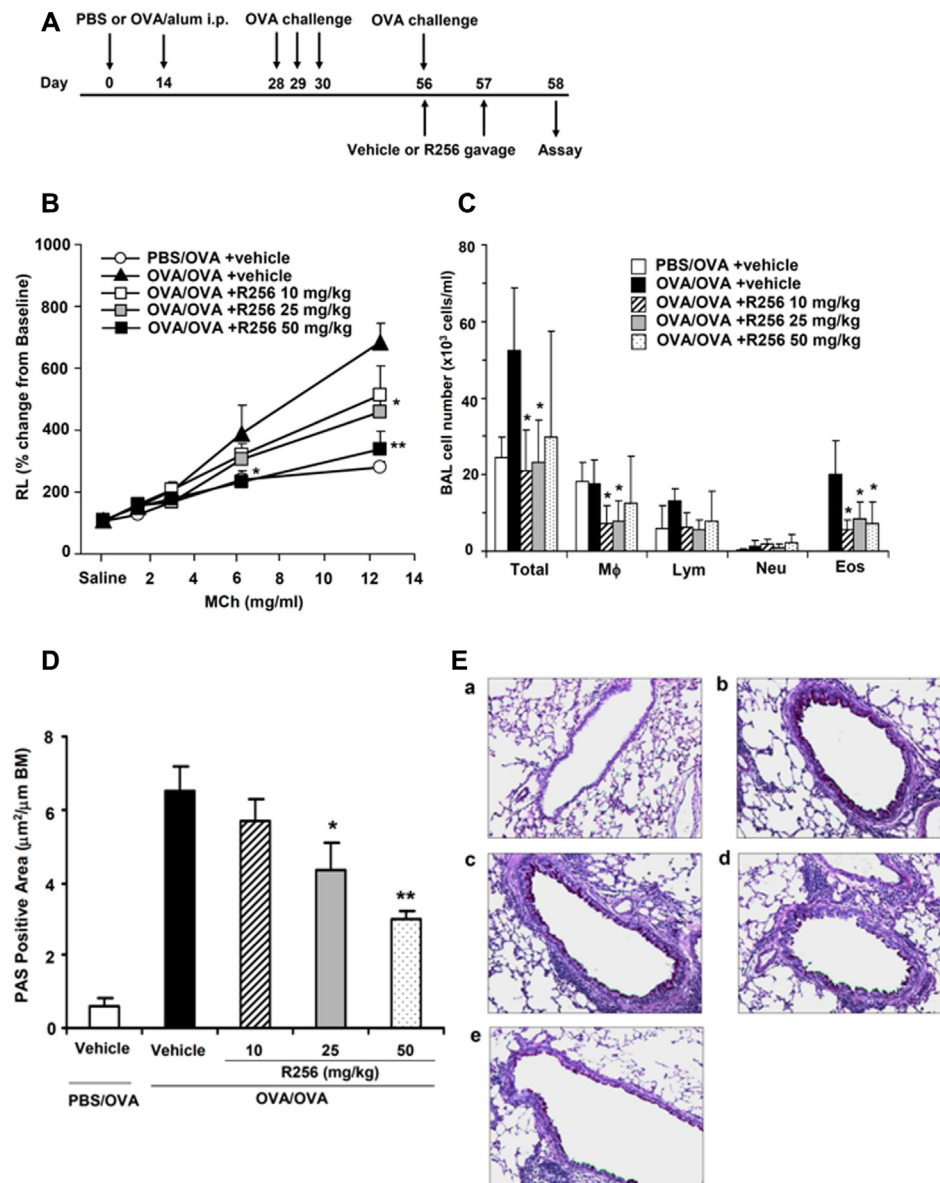


FIG 4. Effects of R256 treatment during secondary allergen challenge on development of allergen-induced AHR and airway inflammation. **A**, Indicated doses of R256 were orally administered during and after secondary allergen challenge, as described in the Methods section. Four weeks after primary allergen challenge, mice were exposed to a single allergen challenge, followed by assessments of airway responsiveness and BAL/lung tissue sampling 48 hours after secondary OVA challenge. **B**, Lung resistance (RL). **C** and **D**, BAL cell composition (Fig 4, C) and goblet cell metaplasia (Fig 4, D). **E**, Representative images show PBS/OVA plus vehicle (a), OVA/OVA plus vehicle (b), OVA/OVA plus R256 (10 mg/kg; c), OVA/OVA plus R256 (25 mg/kg; d), and OVA/OVA plus R256 (50 mg/kg; e). *Eos*, Eosinophil; *Lym*, lymphocyte; *MCh*, methacholine; *Mφ*, macrophage; *Neu*, neutrophil. Data for each group were expressed as means $\pm$ SEMs. Results are from 3 independent experiments with 4 mice per group ($n = 12$). * $P < .05$ and ** $P < .01$ compared with OVA/OVA plus vehicle-treated group.

allergen-induced airway inflammation and AHR were previously established. As shown in Fig 4, the development of AHR (Fig 4, B), airway eosinophilia (Fig 4, C), and goblet cell metaplasia (Fig 4, D and E) was significantly reduced by R256 in a dose-dependent manner, which was similar to the results observed in the primary allergen challenge model. In addition, administration of the inhibitor during secondary challenge did not alter cytokine levels in BAL fluid (data not shown), despite changes in the other lung allergic response outcomes (Fig 4).

DISCUSSION

JAK/STAT pathways play a critical role in the functional differentiation of immune cells.⁶ As a result, this pathway has been targeted in the treatment of various immune disorders.⁸ To date, there have been few studies investigating the effects of JAK pathway inhibition in asthmatic patients in whom T_H2 responses predominate. In this study we first demonstrated in cell-based assays that R256 inactivated JAK1 and JAK3 with 100-fold or greater selectivity over JAK2. R256 was shown to

TABLE I. Activity of R256 in a cell-based assay panel assessing selectivity

Assay	Stimulation	Upstream signals	R256 ($\mu\text{mol/L}$), $\text{EC}_{50} \pm \text{SD}$
NHLF eotaxin production	IL-13	JAK1/JAK2/Tyk2	0.011 ± 0.003
NHLF p-Stat6	IL-13	JAK1/JAK2/Tyk2	0.037 ± 0.021
NHLF eotaxin production	IL-4	JAK1/JAK3	0.020 ± 0.007
NHLF p-Stat6	IL-4	JAK1/JAK3	0.037 ± 0.016
Primary T-cell proliferation	IL-2	JAK1/JAK3	0.017 ± 0.005
Primary T-cell p-STAT5	IL-2	JAK1/JAK3	0.044 ± 0.016
U937 ICAM-1	IFN- γ	JAK1/JAK2	6.52 ± 0.788
Primary erythroblast survival	EPO	JAK2	1.35 ± 0.493
CHMC tryptase	IgE	Syk	0.409 ± 0.227
Ramos p-ERK	IgM	Syk	0.911 ± 0.015
Primary T-cell IL-2 production	TCR/CD28	Lck/ZAP70	>4
HUVEC p-VEGFR2	VEGF	VEGFR2	>6
HeLa p-Akt	EGF	EGFR	Inactive @ $10 \mu\text{mol/L}$

CHMC, Cultured human mast cell; EC_{50} , median effective concentration of R256 at which response is inhibited by 50%; ERK, extracellular signal-regulated kinase; HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor.

have limited activity on other kinase-signaling pathways, as shown in Table I. We determined the effects of a selective JAK1/3 inhibitor on the differentiation of T_H cells *in vitro* and the development of allergen-induced AHR, airway inflammation, and cytokine/chemokine production in the airways. *In vitro* experiments demonstrated that selective inhibition of JAK1/3 targeted T_H2 differentiation without altering T_H1 and T_H17 differentiation. In T_H2 cells p-STAT6 and p-STAT5 expression was reduced in a dose-dependent manner without affecting the STAT phosphorylation necessary for T_H1 (p-STAT1 and p-STAT4) or T_H17 (p-STAT3 and p-STAT4) differentiation. When the inhibitor was added to cultures after differentiation of the cells was completed, no changes in cytokine production were detected. These results suggested that the outcome of selective inhibition of JAK1/3 was restricted to T_H2 cells and did not affect T_H1 or T_H17 cells. In addition, the inhibitory effects of R256 were limited to the initiation of T_H2 polarization. When administered *in vivo* at 2 different time points, the outcomes, although similar, appeared dictated by different events. When R256 was administered during the allergen sensitization phase, AHR and other lung allergic responses, including increases in BAL fluid T_H2 levels, were markedly reduced, indicating that selective blockade of T_H2 differentiation during allergen sensitization was critical. However, when administered during the primary challenge phase after sensitization was completed although development of AHR, airway eosinophilia, and goblet cell metaplasia were prevented, this was not associated with any reduction in T_H2 cytokine levels. In a confirmatory manner administration of the inhibitor during secondary challenge, after sensitization and primary challenge were completed, also prevented development of AHR, eosinophilia, and goblet cell metaplasia without any alterations in T_H2 cytokine levels, supporting the notion that

during the challenge phases, the inhibitory effects were downstream of T_H2 cytokine receptor signaling pathways.

Specific JAK/STAT pathways have been shown to be essential for the differentiation of distinct T_H subsets: T_H2 differentiation requires activation of the IL-2/STAT5 and IL-4/STAT6 signaling pathways,^{37,38} T_H1 differentiation depends on IFN- γ /STAT1 and IL-12/STAT4 signaling,^{39,40} whereas differentiation of T_H17 cells primarily requires STAT3 and STAT4 activated by IL-6 or IL-23.^{41,42} Our results confirmed that the selective inhibition of the JAK1/3 pathway by R256 was critical in the differentiation of T_H2 cells through downregulation of STAT5 and STAT6 phosphorylation. Furthermore, this was extended to observations in *in vivo* experiments; R256 administered during the allergen sensitization phase but before allergen challenge prevented the development of allergen-induced AHR, eosinophilic airway inflammation, and mucus metaplasia and was associated with reduced T_H2 cytokine levels in the airways. In contrast, it appeared that activation of JAK1/3 was not required after completion of T_H2 differentiation *in vivo* because R256 did not alter the function of differentiated T_H2 cells *in vitro*. This dichotomy was confirmed *in vivo* because R256 administered during the primary allergen challenge but after sensitization did not alter T_H2 cytokine levels in BAL fluid, suggesting that sensitization initiated T_H2 differentiation, subsequently rendering the cells resistant to the effects of the inhibitor. Regardless of sustained T_H2 cytokine levels when administered during the primary challenge phase, development of AHR, eosinophilia, and goblet cell metaplasia were reduced on allergen challenge. These same outcomes were observed in the secondary allergen challenge model in which allergic airway inflammation and altered airway function were previously established and provoked by a single allergen challenge, perhaps similar to clinical asthma. The likely explanation was that these results were the consequence of the inhibition of downstream signaling pathways initiated by the unimpaired production of the T_H2 cytokines. In previous studies a critical role for JAK1 was identified because STAT6 was activated by IL-4 or IL-13 through JAK1 in rat alveolar type II cells and airway epithelial cells were shown to require IL-4 signaling after allergen exposure.^{44,45} Although a predominant role for JAK3 over JAK1 was suggested from small interfering RNA knockdown experiments,⁴⁶ intracellular enzymatic levels of JAK1 were shown to be higher than levels of JAK3.⁴⁷ This dissociation might reflect differences in individual JAK kinase binding capabilities to substrates or ligands versus enzymatic activities. Activation of the JAK1-STAT6 pathway after IL-13 stimulation was shown in both rat and human bronchial smooth muscle cells, and this pathway was associated with activation of RhoA, which induces smooth muscle contraction.^{48,49} When the inhibitor was administered in the primary challenge phase, a significant reduction in BAL fluid eotaxin levels was demonstrated, which is similar to the inhibition of eotaxin triggered by IL-13 in lung fibroblasts.^{20,22} Thus in the challenge phase, despite abundant T_H2 cytokine levels in the airways, where the effects of IL-13 and, to some degree, IL-4 are prominent,⁴³ lower levels of eotaxin might be at the core of reduced airway eosinophilia, whereas the reduced mucus production and AHR were the result of R256-mediated impairment of IL-13 signaling in goblet cells and airway smooth muscle. Inhibition of IL-13 using a receptor antagonist resulted in similar effects.⁵⁰ Taken together, the data indicate that the timing of inhibitor administration has a profound effect on effector responses, which contributed to similar outcomes.

The selectivity of R256 for JAK1/3 and T_H2 differentiation in patients with allergen-driven diseases might have advantages over pan-JAK inhibitors. Despite reported efficacy in reducing airway eosinophilia and AHR to some extent, both tofacitinib and pyridone 6, unlike R256, might alter Treg, T_H1, and T_H17 responses,^{19,51} potentially limiting efficacy in some diseases or increasing the likelihood of adverse events under some conditions. Selective targeting of JAK1/3 might provide important therapeutic advances in the treatment of T_H2-mediated allergic diseases, such as asthma.

We thank Diana Nabighian for her assistance in the preparation of the manuscript.

Clinical implications: Targeting JAK1/3 signaling pathways provides a novel intervention for preventing allergen-induced alterations in lung function.

REFERENCES

- Casanova JL, Holland SM, Notarangelo LD. Inborn errors of human JAKs and STATs. *Immunol Rev* 2012;36:515-28.
- Ghoreschi K, Laurence A, O'Shea JJ. Janus kinases in immune cell signaling. *Immunol Rev* 2009;228:273-87.
- Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. *Nat Rev Mol Cell Biol* 2002;3:651-62.
- Shuai K, Liu B. Regulation of JAK-STAT signaling in the immune system. *Nat Rev Immunol* 2003;3:900-11.
- Schindler CW. JAK-STAT signaling in human disease. *J Clin Invest* 2002;109:1133-7.
- O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. *Immunity* 2008;28:477-87.
- Pesu M, Laurence A, Kishore N, Zwillich SH, Chan G, O'Shea JJ. Therapeutic targeting of Janus kinases. *Immunol Rev* 2008;223:132-42.
- O'Shea JJ, Plenge R. JAK and STAT signaling molecules in immune regulation and immune-mediated disease. *Immunity* 2012;36:542-50.
- Kontzias A, Kotlyar A, Laurence A, Changelian P, O'Shea JJ. Jakinibs: a new class of kinase inhibitors in cancer and autoimmune disease. *Curr Opin Pharmacol* 2012;12:464-70.
- Heine A, Held SA, Daecke SN, Wallner S, Jayanarayana SP, Kurts C, et al. The JAK-inhibitor ruxolitinib impairs dendritic cell function in vitro and in vivo. *Blood* 2013;122:1192-202.
- Fleischmann R, Kremer J, Cush J, Schulze-Koops H, Connell CA, Bradley JD, et al. Placebo-controlled trial of tofacitinib monotherapy in rheumatoid arthritis. *N Engl J Med* 2012;367:495-507.
- van Vollenhoven RF, Fleischmann R, Cohen S, Lee EB, García Mejjide JA, Wagner S, et al. Tofacitinib or adalimumab versus placebo in rheumatoid arthritis. *N Engl J Med* 2012;367:508-19.
- Ghoreschi K, Laurence A, O'Shea JJ. Selectivity and therapeutic inhibition of kinases: to be or not to be? *Nat Immunol* 2009;10:356-60.
- Fox DA. Kinase inhibition—a new approach to the treatment of rheumatoid arthritis. *N Engl J Med* 2012;367:565-7.
- Bosnjak B, Stelzmueller B, Erb KJ, Epstein MM. Treatment of allergic asthma: modulation of Th2 cells and their responses. *Respir Res* 2011;12:114.
- Szeffer SJ, Zeiger RS, Haselkorn T, Mink DR, Kamath TV, Fish JE, et al. Economic burden of impairment in children with severe or difficult-to-treat asthma. *Ann Allergy Asthma Immunol* 2011;107:110-9.e1.
- Walsh GM. An update on emerging drugs for asthma. *Expert Opin Emerg Drugs* 2012;17:37-42.
- Kudlacz E, Conklyn M, Andresen C, Whitney-Pickett C, Changelian P. The JAK-3 inhibitor CP-690550 is a potent anti-inflammatory agent in a murine model of pulmonary eosinophilia. *Eur J Pharmacol* 2008;582:154-61.
- Matsunaga Y, Inoue H, Fukuyama S, Yoshida H, Moriwaki A, Matsumoto T, et al. Effects of a Janus kinase inhibitor, pyridone 6, on airway responses in a murine model of asthma. *Biochem Biophys Res Commun* 2011;404:261-7.
- May RD, Monk PD, Cohen ES, Manuel D, Dempsey F, Davis NHE, et al. Preclinical development of CAT-354, an IL-13 antibody for the treatment of severe uncontrolled asthma. *Br J Pharmacol* 2012;166:177-93.
- Nakano T, Inoue H, Fukuyama S, Matsumoto K, Matsumura M, Tsuda T, et al. Niflumic acid suppresses interleukin-13-induced asthma phenotypes. *Am J Respir Crit Care Med* 2006;173:1216-21.
- Hershey GK. IL-13 receptors and signaling pathways: an evolving web. *J Allergy Clin Immunol* 2003;111:677-90.
- Wenzel SE, Trudeau JB, Barnes S, Zhou X, Cundall M, Westcott JY, et al. TGF-beta and IL-13 synergistically increase eotaxin-1 production in human airway fibroblasts. *J Immunol* 2002;169:4613-9.
- Liao W, Lin JX, Leonard WJ. IL-2 family cytokines: new insights into the complex roles of IL-2 as a broad regulator of T helper cell differentiation. *Curr Opin Immunol* 2011;23:598-604.
- Oda A, Sawada K, Druker BJ, Ozaki K, Takano H, Koizumi K, et al. Erythropoietin induces tyrosine phosphorylation of Jak2, STAT5A, and STAT5B in primary cultured human erythroid precursors. *Blood* 1998;92:443-51.
- Tong W, Zhang J, Lodish HF. Lnk inhibits erythropoiesis and Epo-dependent JAK2 activation and downstream signaling pathways. *Blood* 2005;105:4604-12.
- Chang YJ, Holtzman MJ, Chen CC. Interferon-gamma-induced epithelial ICAM-1 expression and monocyte adhesion. Involvement of protein kinase C-dependent c-Src tyrosine kinase activation pathway. *J Biol Chem* 2002;277:7118-26.
- Scicchitano MS, McFarland DC, Tierney LA, Narayanan PK, Schwartz LW. In vitro expansion of human cord blood CD36+ erythroid progenitors: temporal changes in gene and protein expression. *Exp Hematol* 2003;31:760-9.
- Rossi AB, Herlaar E, Braselmann S, Huynh S, Taylor V, Frances R, et al. Identification of the Syk kinase inhibitor R112 by a human mast cell screen. *J Allergy Clin Immunol* 2006;118:749-55.
- Holland SJ, Pan A, Franci C, Hu Y, Chang B, Li W, et al. R428, a selective small molecule inhibitor of Axl kinase, blocks tumor spread and prolongs survival in models of metastatic breast cancer. *Cancer Res* 2010;70:1544-54.
- Kobayashi M, Ashino S, Shiohama Y, Wakita D, Kitamura H, Nishimura T. IFN-gamma elevates airway hyper-responsiveness via up-regulation of neurokinin A/neurokinin-2 receptor signaling in a severe asthma model. *Eur J Immunol* 2012;42:393-402.
- Ashino S, Wakita D, Zhang Y, Chamato K, Kitamura H, Nishimura T. CpG-ODN inhibits airway inflammation at effector phase through down-regulation of antigen-specific Th2-cell migration into lung. *Int Immunol* 2008;20:259-66.
- Ashino S, Wakita D, Shiohama Y, Iwakura Y, Chamato K, Ohkuri T, et al. A T(h)17-polarized cell population that has infiltrated the lung requires cells that convert to IFN-gamma production in order to induce airway hyperresponsiveness. *Int Immunol* 2010;22:503-13.
- Kanehiro A, Ikemura T, Makela MJ, Lahn M, Joetham A, Dakhama A, et al. Inhibition of phosphodiesterase 4 attenuates airway hyperresponsiveness and airway inflammation in a model of secondary allergen challenge. *Am J Respir Crit Care Med* 2001;163:173-84.
- Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. *J Exp Med* 1997;186:449-54.
- Oshiba A, Hamelmann E, Takeda K, Bradley KL, Loader JE, Larsen GL, et al. Passive transfer of immediate hypersensitivity and airway hyperresponsiveness by allergen-specific immunoglobulin (Ig) E and IgG1 in mice. *J Clin Invest* 1996;97:1398-408.
- Zhu J, Guo L, Watson CJ, Hu-Li J, Paul WE. Stat6 is necessary and sufficient for IL-4's role in Th2 differentiation and cell expansion. *J Immunol* 2001;166:7276-81.
- Zhu J, Cote-Sierra J, Guo L, Paul WE. Stat5 activation plays a critical role in Th2 differentiation. *Immunity* 2003;19:739-48.
- Lighvani AA, Frucht DM, Jankovic D, Yamane H, Aliberti J, Hissong BD, et al. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A* 2001;98:15137-42.
- Afkarian M, Sedy JR, Yang J, Jacobson NG, Cereb N, Yang SY, et al. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol* 2002;3:549-57.
- Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 2007;8:967-74.
- Mathur AN, Chang HC, Zisoulis DG, Stritesky GL, Yu Q, O'Malley JT, et al. Stat3 and Stat4 direct development of IL-17-secreting Th cells. *J Immunol* 2007;178:4901-7.
- Takeda K, Dakhama A, Gelfand EW. Allergic asthma: what have we learned from the mouse model? *Allergol Int* 2005;54:263-71.
- Liu T, Jin H, Ullenbruch M, Hu B, Hashimoto N, Moore B, et al. Regulation of found in inflammatory zone 1 expression in bleomycin-induced lung fibrosis: role of IL-4/IL-13 and mediation via STAT-6. *J Immunol* 2004;173:3425-31.
- Kuperman DA, Huang X, Nguyen L, Holscher C, Brombacher F, Erle DJ. IL-4 receptor signaling in Clara cells is required for allergen-induced mucus production. *J Immunol* 2005;175:3746-52.

46. Gomez-Valades AG, Llamas M, Blanch S, Perales JC, Roman J, Gomez-Casajus, et al. Specific Jak3 downregulation in lymphocytes impairs γ c cytokine signal transduction and alleviates antigen-driven inflammation in vivo. *Mol Ther Nucleic Acids* 2012;1:e42.
47. Haan C, Rolvering C, Raulf F, Kapp M, Drückes P, Thoma G, et al. Jak1 has a dominant role over Jak3 in signal transduction through γ c-containing cytokine receptors. *Chem Biol* 2011;18:314-23.
48. Chiba Y, Goto K, Matsusue K, Kimura S, Misawa M. Identification and characterization of rat RhoA gene promoter. *J Pharmacol Sci* 2010;112:467-72.
49. Chiba Y, Goto K, Misawa M. Interleukin-13-induced activation of signal transducer and activator of transcription 6 is mediated by an activation of Janus kinase 1 in cultured human bronchial smooth muscle cells. *Pharmacol Rep* 2012;64:454-8.
50. Taube C, Duez C, Chi ZH, Takeda K, Rha YH, Park JW, et al. The role of IL-13 in established allergic airway disease. *J Immunol* 2002;169:6482-9.
51. Ghoreschi K, Jesson MJ, Li X, Lee JL, Ghosh S, Alsup JW, et al. Modulation of innate and adaptive immune responses by tofacitinib (CP-690,550). *J Immunol* 2011;186:4234-43.

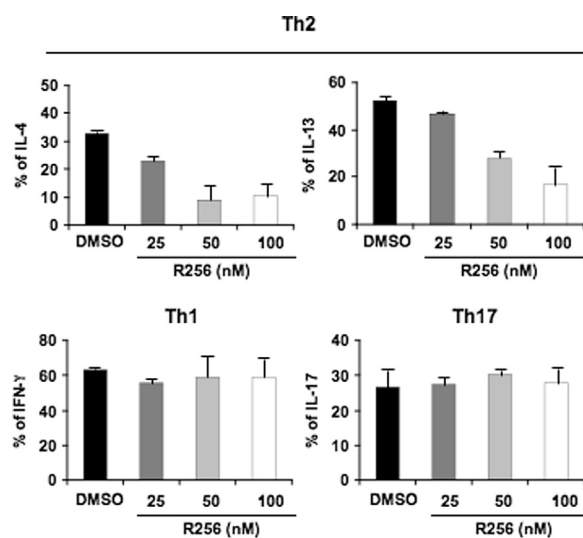


FIG E1. Effects of R256 on frequency of T_H subsets *in vitro*. **A**, On day 6, T_H subsets were restimulated with plate-coated anti-CD3/anti-CD28 mAbs for 6 hours. Cells were collected and analyzed by using intracellular staining. R256 or dimethyl sulfoxide (*DMSO*) as a control was added from days 0 to 6. Results are from 2 independent experiments ($n = 6$).

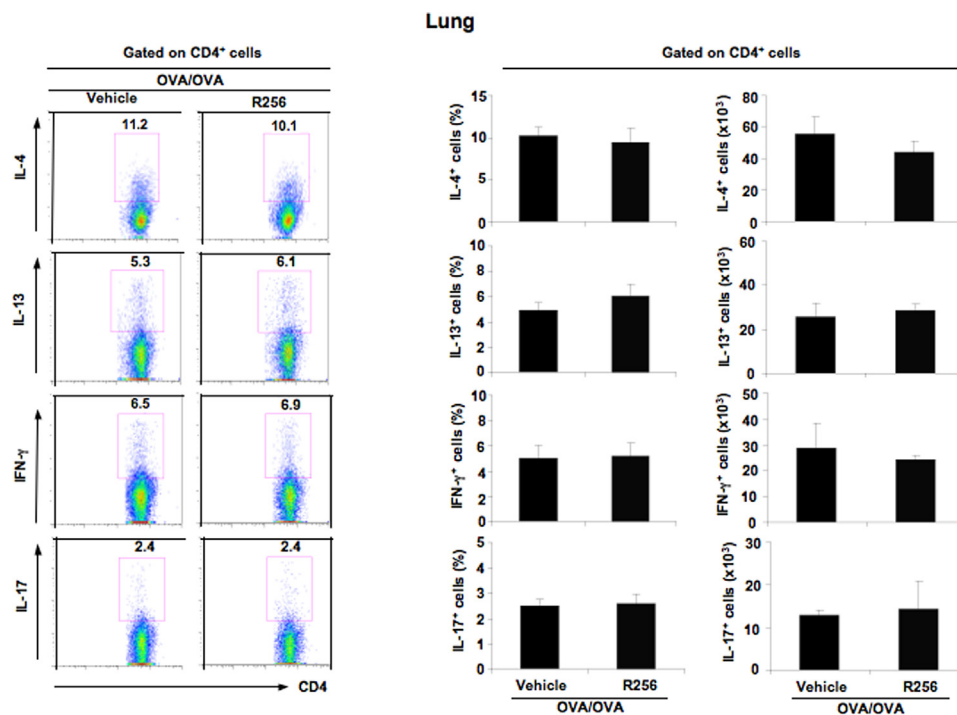


FIG E2. Effects of R256 treatment during primary allergen challenge on frequency of cytokine-expressing cells in lungs. Cells were collected from lung tissues of mice after vehicle or R256 (50 mg/kg) treatment during primary OVA challenge. Cells were stimulated with anti-CD3 and anti-CD28 for 6 hours in the presence of BFA and then analyzed by means of intracellular staining, as described in the Methods section. A representative experiment is shown, as well as results from 2 independent experiments with 3 mice per group ($n = 6$).

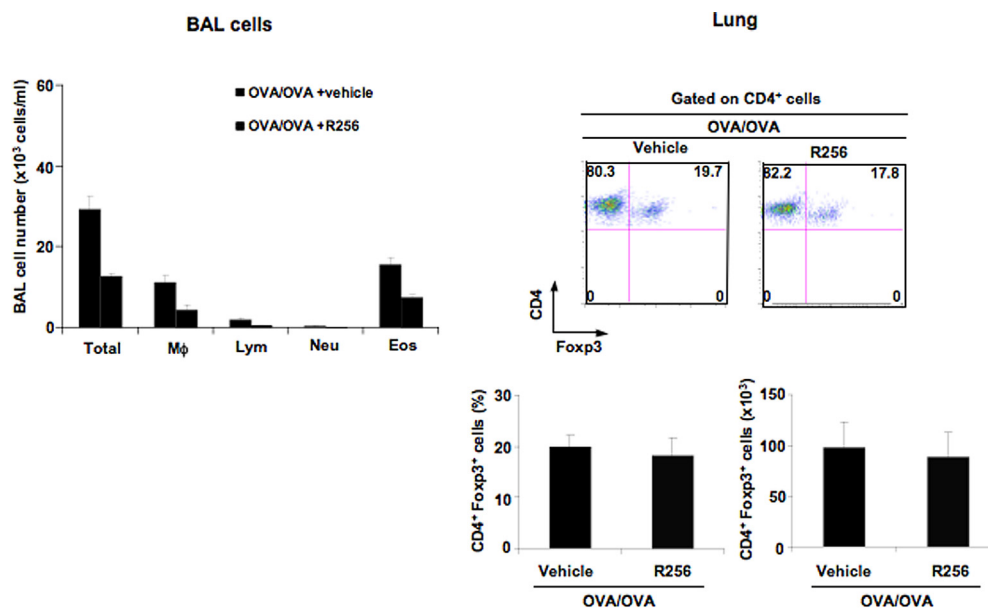


FIG E3. Effects of R256 treatment during primary allergen challenge on frequency and numbers of Treg cells in the lungs. Cells were collected from lung tissues of mice after vehicle or R256 (50 mg/kg) treatment during primary OVA challenge and were stained with allophycocyanin-conjugated anti-CD4 and fluorescein isothiocyanate-conjugated Foxp3 mAb, followed by flow cytometric analyses. Shown is a representative analysis and results from 2 independent experiments with 3 mice per group ($n = 6$). *Eos*, Eosinophil; *Lym*, lymphocyte; *MCh*, methacholine; *Mφ*, macrophage; *Neu*, neutrophil.

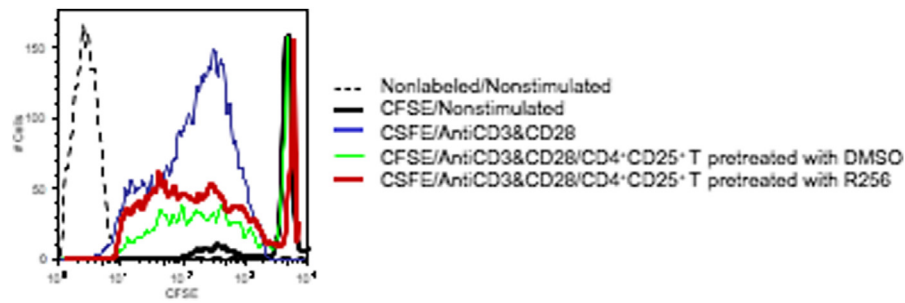


FIG E4. Effects of R256 on functions of Treg cells. $CD4^{+}CD25^{+}$ Treg cells isolated from naive mice were treated with dimethyl sulfoxide (*DMSO*) or R256 (50 nmol/L) for 3 hours. Drug-pretreated Treg cells were cocultured with anti-CD3/anti-CD28–prestimulated and carboxyfluorescein succinimidyl ester–labeled $CD4^{+}CD25^{-}$ cells. After 72 hours of coculture, carboxyfluorescein succinimidyl ester fluorescence intensities were monitored by using flow cytometry. A representative experiment is shown.