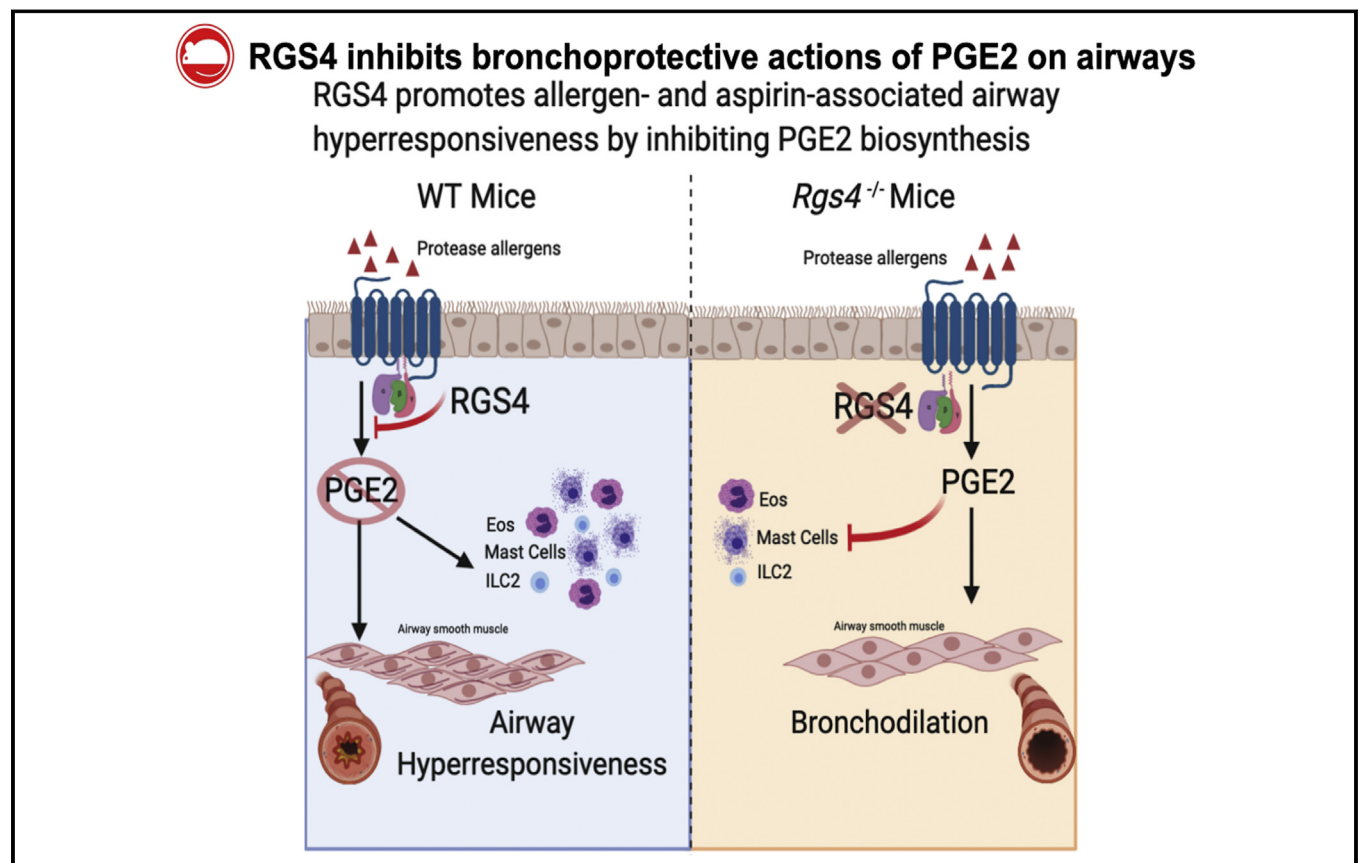


RGS4 promotes allergen- and aspirin-associated airway hyperresponsiveness by inhibiting PGE2 biosynthesis

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GRAPHICAL ABSTRACT



Background: Allergens elicit host production of mediators acting on G-protein-coupled receptors to regulate airway tone. Among these is prostaglandin E2 (PGE2), which, in addition to

its role as a bronchodilator, has anti-inflammatory actions. Some patients with asthma develop bronchospasm after the ingestion of aspirin and other nonsteroidal anti-inflammatory

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Funding for this study was provided in part by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH) (project nos. Z01-AI-000746 and Z01-AI-000943) and by grants

CTSA-UL1TR003017 (CTSA) and 2P01HL114471-06 (PPG) to R.A.P. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does the mention of trade names, commercial products, or organizations imply endorsement by the US government.

Received for publication July 4, 2019; revised February 21, 2020; accepted for publication March 11, 2020.

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<https://doi.org/10.1016/j.jaci.2020.03.004>

drugs, a disorder termed aspirin-exacerbated respiratory disease. This condition may result in part from abnormal dependence on the bronchoprotective actions of PGE₂.

Objective: We sought to understand the functions of regulator of G protein signaling 4 (RGS4), a cytoplasmic protein expressed in airway smooth muscle and bronchial epithelium that regulates the activity of G-protein-coupled receptors, in asthma.

Methods: We examined RGS4 expression in human lung biopsies by immunohistochemistry. We assessed airways hyperresponsiveness (AHR) and lung inflammation in germline and airway smooth muscle-specific *Rgs4*^{-/-} mice and in mice treated with an RGS4 antagonist after challenge with *Aspergillus fumigatus*. We examined the role of RGS4 in nonsteroidal anti-inflammatory drug-associated bronchoconstriction by challenging aspirin-exacerbated respiratory disease-like (*ptges1*^{-/-}) mice with aspirin.

Results: RGS4 expression in respiratory epithelium is increased in subjects with severe asthma. Allergen-induced AHR was unexpectedly diminished in *Rgs4*^{-/-} mice, a finding associated with increased airway PGE₂ levels. RGS4 modulated allergen-induced PGE₂ secretion in human bronchial epithelial cells and prostanoïd-dependent bronchodilation. The RGS4 antagonist CCG203769 attenuated AHR induced by allergen or aspirin challenge of wild-type or *ptges1*^{-/-} mice, respectively, in association with increased airway PGE₂ levels.

Conclusions: RGS4 may contribute to the development of AHR by reducing airway PGE₂ biosynthesis in allergen- and aspirin-induced asthma. (J Allergy Clin Immunol 2020;■■■:■■■-■■■.)

Key words: Asthma, aspirin sensitivity, G proteins, PGE₂, aspirin-exacerbated respiratory disease, regulators of G protein signaling protein

Asthma is a heterogeneous syndrome that afflicts more than 300 million people worldwide.¹ The pathological hallmarks of asthma manifest as lung inflammation, excessive mucus production, airway remodeling (epithelial sloughing, subepithelial collagen deposition, airway smooth muscle [ASM] hypertrophy), and bronchoconstriction, which induces reversible airflow obstruction, wheezing, cough, and in severe cases, hypoxemia and respiratory failure.^{2,3} In more than half the patients with asthma, evidence of exuberant type 2 adaptive and innate immune responses is observed in the lungs, including increased eosinophils and elevated levels of type 2 cytokines (eg, IL-4, IL-5, and IL-13).⁴ Allergens and environmental irritants may also directly activate mast cells and respiratory epithelial cells, which release cytokines including IL-25, IL-33, and thymic stromal lymphopoietin, which in turn stimulate lung-resident innate lymphoid type 2 cells (ILC2s).⁵ Other proinflammatory mediators including bradykinin, histamine, and leukotrienes may also affect bronchial tone.⁶⁻⁸

Many of these factors induce ASM shortening by acting on G-protein-coupled receptors (GPCRs) bound to Gαq, a cytoplasmic protein that interacts with guanosine diphosphate in its quiescent state.⁹ Ligand binding to the GPCR promotes exchange of guanosine triphosphate for guanosine diphosphate on Gαq, a response that in turn evokes a downstream signaling cascade in which cytosolic Ca²⁺ flux promotes actin-myosin interactions and force generation.¹⁰ A highly selective antagonist of Gαq elicits profound bronchorelaxation in several species without a substantial impact on allergen-induced inflammation and airway remodeling.¹¹

Abbreviations used

AEC:	Airway epithelial cell
AERD:	Aspirin-exacerbated respiratory disease
AHR:	Airways hyperresponsiveness
ASA:	Aspirin
ASM:	Airway smooth muscle
BALF:	Bronchoalveolar lavage fluid
β-gal:	β-galactosidase
CCh:	Carbachol
GPCR:	G-protein-coupled receptor
MCh:	Methacholine
ILC2:	Innate lymphoid type 2 cell
NSAID:	Nonsteroidal anti-inflammatory drug
PAR2:	Proteinase-activated receptor 2
PCLS:	Precision-cut lung slice
PGE ₂ :	Prostaglandin E ₂
RGS:	Regulators of G protein signaling
RGS4:	Regulator of G protein signaling 4
WT:	Wild-type

Endogenous mechanisms also limit the magnitude of GPCR-mediated responses and modulate bronchial tone. Regulators of G protein signaling (RGS) proteins restrict heterotrimeric G protein activity by accelerating hydrolysis of guanosine triphosphate by Gα subunits ("GAP" activity) and thereby facilitate Gα inactivation.¹² Previously, we detected regulator of G protein signaling 4 (RGS4) immunoreactivity sporadically in cells within the bronchial smooth muscle bundle in lung sections from subjects with asthma and demonstrated that RGS4 knockdown enhanced Ca²⁺ responses of ASM cells from healthy human subjects to histamine *ex vivo*.¹³ Here, we studied the role of RGS4 in airways hyperresponsiveness (AHR) and allergen-associated airway inflammation in mice. We found that *Rgs4*^{-/-} mice were protected from developing AHR after allergen or allergen plus aspirin challenge, both findings that could be explained primarily by modification of airway levels of prostaglandin E₂ (PGE₂).

METHODS

Patients

Human lung specimens and clinical parameters were obtained by the Respiratory Health Network Tissue Bank of the Fonds de Recherche Québécois en Santé, IUCPQ site (www.tissuebank.ca). The IUCPQ Ethics Committee approved the collection of tissue samples by the tissue bank and their use for these studies. All subjects gave informed consent for the tissue sampling and future use of samples for research purposes. Subjects with asthma were classified as having mild to moderate or severe asthma based on criteria established by the American Thoracic Society.¹⁴ All clinical studies and medication dosages were evaluated on the same day or within less than a week from the date of the biopsy.

Allergic airway inflammation model in mice

Six- to 12-week-old male and female mice were sensitized with a mixture of *Aspergillus fumigatus* crude extract (20 μg, Hollister-Stier Allergy, Spokane, Wash) mixed with Imject Alum (20 mg, Pierce, Waltham, Mass) via intraperitoneal injection on days 0 and 14. All mice were then challenged intranasally with either PBS (naïve) or *A. fumigatus* extract (25 μg) 1 week after the last immunization. In certain experiments, RGS4 inhibitor (6 ng/kg intratracheally) or indomethacin (10 mg/kg intraperitoneally) or an equivalent volume of diluent was administered together with or just before the allergen. Studies of respiratory function and organ harvest were conducted 24 hours

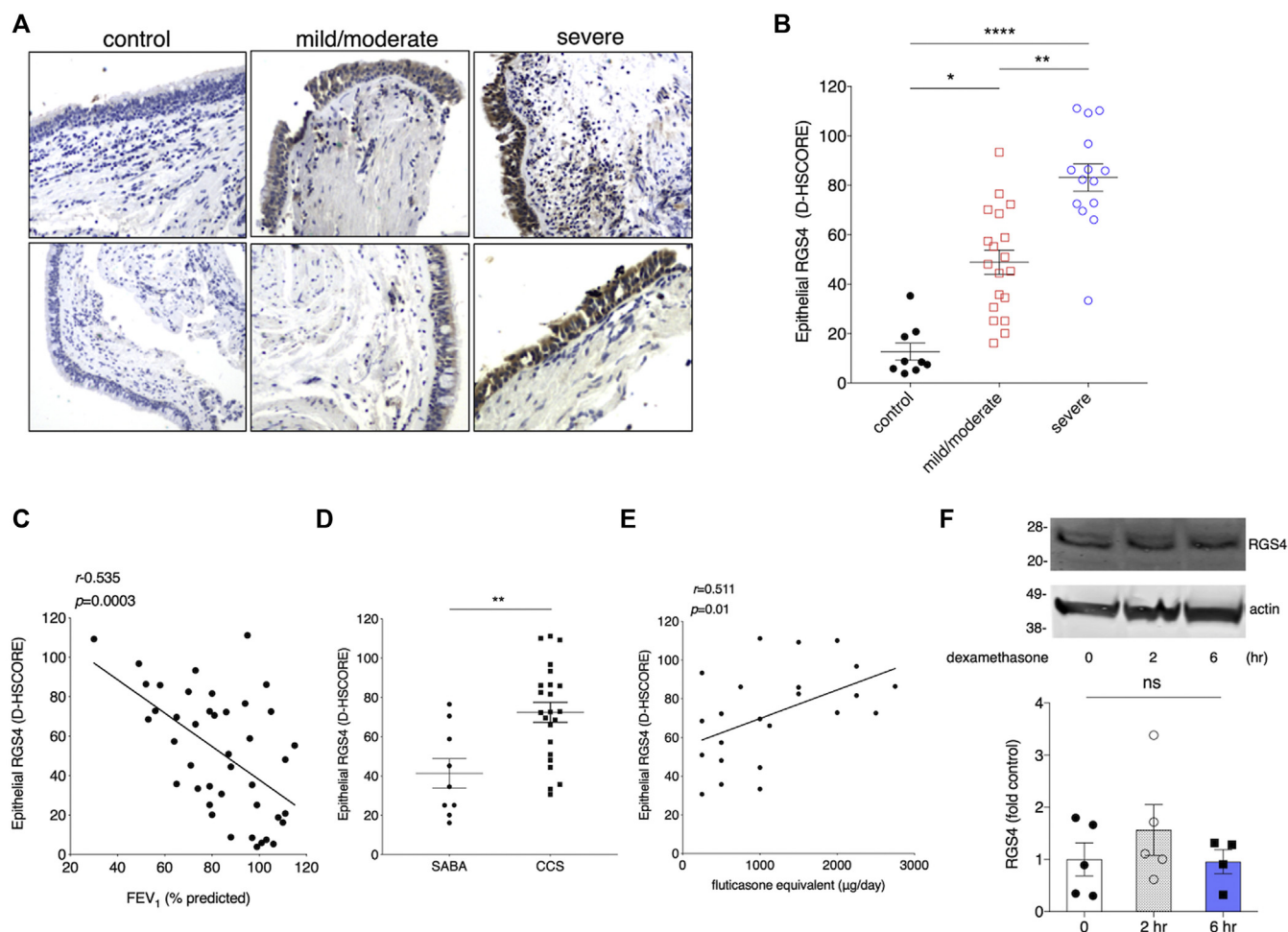


FIG 1. Epithelial RGS4 expression is increased in lungs of patients with asthma. **A** and **B**, Immunohistochemistry of RGS4 in lung epithelium; * $P = .02$, ** $P = .008$, **** $P < .0001$, Kruskal Wallis, Dunn's multiple comparisons. **C**, Negative correlation between RGS4 expression and FEV₁. **D**, RGS4 expression in patients treated with either inhaled corticosteroids (CCSs) or SABA alone; ** $P = .003$, Mann-Whitney U test. **E**, Correlation between RGS4 immunoreactivity and daily dose of inhaled fluticasone or equivalent. **F**, RGS4 expression in AECs treated with dexamethasone (1 μ M) for the indicated times assessed by immunoblotting. Each symbol represents results of a separate experiment. Nonparametric Pearson correlation coefficients were calculated in Fig 1, **C** and **E**. *ns*, Not significant; SABA, short-acting β -adrenergic agonist.

after the last challenge. Lungs were fixed in 10% neutral buffered formalin. Paraffin-embedded sections were analyzed by staining with hematoxylin and eosin and periodic acid-Schiff. Images were obtained at 10 $\times$ original magnification using a Leica DMI400 light microscope equipped with an Infinity3 CCD camera (Teledyne Lumenera, Ottawa, Canada). Periodic acid-Schiff staining in the epithelium was determined using the Color Deconvolution plugin of ImageJ and presented as the area of the stained pixels divided by the area of each airway. For assessment of leukocyte counts, tracheas were injected with ice-cold HBSS and bronchoalveolar lavage fluid (BALF) was collected. Red blood cells were hemolyzed using ACK lysis buffer, followed by an additional wash with PBS. Total cell numbers were determined by hemocytometry, and composition of the cells was ascertained by Diff-Quick stain and microscopy (>250 total cells/sample). Cytokines in BALF supernatants were analyzed using a customized MultiPlex bead array (BioRad, Hercules, Calif) in duplicate per the manufacturer's instructions.

Analysis of lung function in anesthetized mice

Mice were anesthetized with a ketamine (100 mg/kg) and xylazine (20 mg/kg) mixture via intraperitoneal injection followed by transtracheal intubation with a 20-gauge SURFLO Teflon intravenous catheter (Santa Cruz Animal

Health, Ft Worth, Tex). Mice were paralyzed by Santa Cruz Animal Health injection of vecuronium bromide (1 mg/kg) and mechanically ventilated using the FlexiVent FX1 respirator (SCIREQ). Resistance of the conducting airways (R_a) was measured after inhalation of PBS and increasing concentrations of methacholine (MCh) via nebulizer.

Methods for this article can be found in the Online Repository at www.jacionline.org.

RESULTS

RGS4 expression in airway epithelium correlates with asthma severity and respiratory dysfunction

To explore the patterns of RGS4 expression in human lung, we examined RGS4 immunoreactivity in lung biopsies from a cohort of subjects diagnosed with asthma of varying severities. The patient characteristics are described in Table E2 in this article's Online Repository at www.jacionline.org. RGS4 immunoreactivity was much more extensive in the bronchial epithelium of lungs from subjects with asthma compared with healthy controls, and the extent of coverage increased proportionally with disease

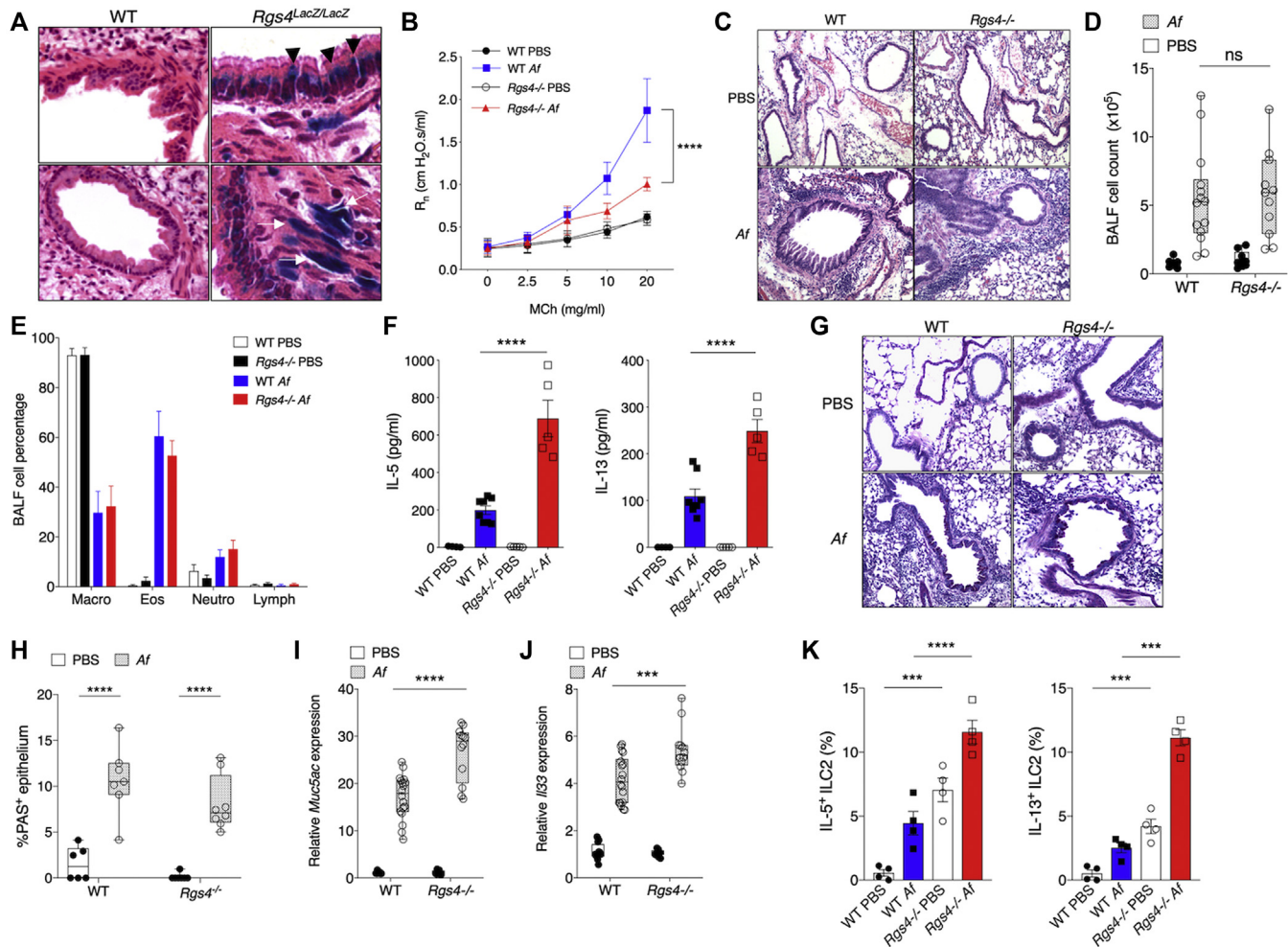


FIG 2. Diminished allergen-induced AHR of *Rgs4^{-/-}* mice. **A**, β -Gal activity in lungs of naive WT or *Rgs4^{LacZ/LacZ}* mice. Epithelium (black arrowheads) and ASM cells (white arrows) are marked. **B–J**, Analysis of lungs from WT and *Rgs4^{-/-}* mice as follows: **Fig 2, B**, airway resistance (mean $\pm$ SEM of 6–12 mice/group analyzed in 2 independent experiments; **** $P < .0001$, 2-way ANOVA); **Fig 2, C**, airway inflammation (hematoxylin and eosin staining); **Fig 2, D**, BALF total leukocyte counts; **Fig 2, E**, Leukocyte composition; **Fig 2, F**, BALF type 2 cytokines IL-5 and IL-13 (mean $\pm$ SEM, **** $P < .0001$, 1-way ANOVA, Tukey multiple comparisons); **Fig 2, G–H**, airway mucous (PAS staining) (mean $\pm$ SEM of 7–8 mice/group, **** $P < .0001$, 2-way ANOVA, Sidak multiple comparisons); relative expression of *Muc5ac* (**Fig 2, I**) or *Il33* (**Fig 2, J**) (mean $\pm$ SEM, *** $P = .0001$, **** $P < .0001$, 2-way ANOVA, Sidak multiple comparisons). **K**, Percentages of cytokine⁺ ILC2s (CD45⁺Lin⁻IL-7R⁺GATA3⁺) in lung cells determined by flow cytometry (mean $\pm$ SEM, of 4 mice/group; **** $P < .0008$, **** $P < .00001$, 1-way ANOVA, Tukey multiple comparisons). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Macro*, macrophages; *Neutro*, neutrophils; *ns*, not significant; *PAS*, Periodic acid-Schiff.

severity (**Fig 1, A and B**). No immunostaining was observed in lung sections probed with rabbit IgG or antirabbit IgG controls (see **Fig E1** in this article's Online Repository at www.jacionline.org). RGS4 immunostaining correlated inversely with lung function (FEV₁) (**Fig 1, C**) and was also higher in patients treated with inhaled corticosteroids than in those treated with short-acting β -adrenergic agonists alone (**Fig 1, D**). Among those patients treated with steroids, RGS4 immunostaining correlated with dosage requirements for symptom management (**Fig 1, E**), suggesting a linkage between corticosteroids and RGS4 expression. In a previous study, corticosteroid treatment (dexamethasone) increased RGS2 expression in ASM.¹⁵ To examine the impact of dexamethasone on RGS4 quantities in airway epithelial cells (AECs), we applied dexamethasone at concentrations

equivalent to those detected in the airways of patients and likewise previously shown to induce RGS2 expression.¹⁵ As shown in **Fig 1, F**, addition of dexamethasone had no impact on RGS4 expression in AECs. This finding suggests that corticosteroids are unlikely to promote the expression of RGS4 in the bronchial epithelium. There was also a significant correlation between the age of the subjects and RGS4 expression (see **Fig E2, A**, in this article's Online Repository at www.jacionline.org). However, as shown in **Fig 1, B**, RGS4 expression was elevated in subjects with severe asthma compared with those with mild/moderate disease in the absence of any age discrepancies between these 2 groups. These results indicate that age alone cannot account for the increased RGS4 immunoreactivity detected in respiratory epithelium in severe asthma.

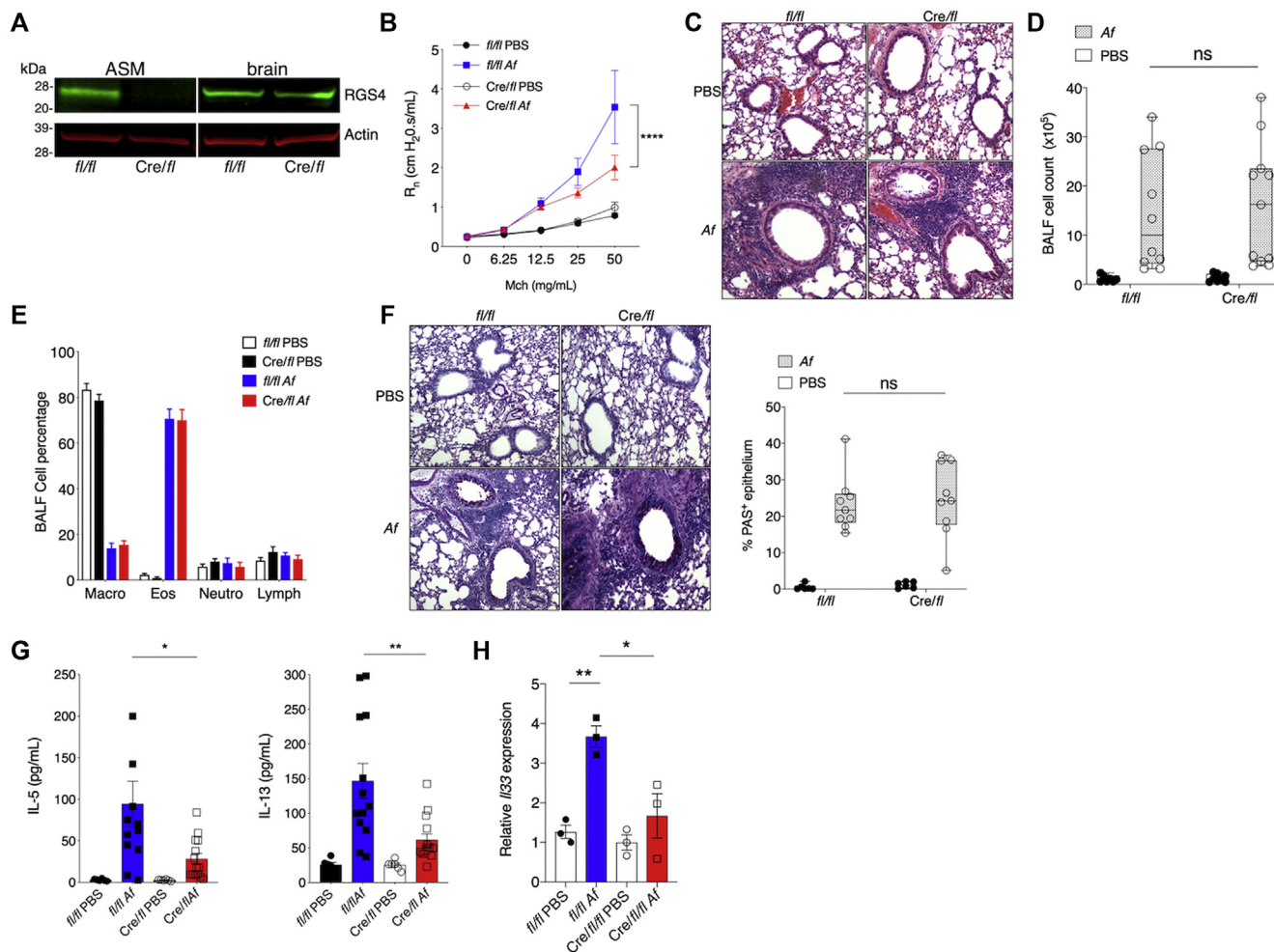


FIG 3. ASM-specific deletion of *Rgs4* phenocopies global RGS4 deficiency. **A**, RGS4 expression in brain tissue or ASM cells from *Rgs4*^{fl/fl} or *Rgs4*^{Cre/fl} mice (ASM cells pooled from 4–5 mice/group or brains from 2 mice). **B**, Airway resistance (mean ± SEM of 6–12 mice/group analyzed in 2 separate experiments; *****P* < .0001, 2-way ANOVA). **C–G**, airway inflammation (hematoxylin and eosin staining) (Fig 3, C), total leukocyte counts (Fig 3, D), and leukocyte composition (Fig 3, E) in BALF; airway mucus assessed by PAS staining (Fig 3, F); type 2 cytokines IL-5 and IL-13 in BALF (Fig 3, G) (mean ± SEM; **P* = .02, ***P* = .002, 1-way ANOVA, Tukey multiple comparisons) in *Rgs4*^{fl/fl} or *Rgs4*^{Cre/fl} mice. Images are representative of 6–9 mice/group. **H**, Lung *I33* expression (mean ± SEM, **P* = .01, ***P* = .002, 1-way ANOVA, Tukey multiple comparisons). *Eos*, Eosinophils; *Lymph*, lymphocytes; *Macro*, macrophages; *Neutro*, neutrophils; *ns*, not significant; *PAS*, Periodic acid-Schiff.

RGS4 immunoreactivity was similar in patients with allergic and nonallergic asthma (Fig E2, B). To further examine the relationship of RGS4 to allergic inflammation, we assessed the expression of MUC5AC, a critical glycoprotein component of airway mucin that is increased in human airways after allergen challenge and required for AHR in mice.^{16,17} MUC5AC immunoreactivity in lung epithelium was significantly higher in the group with severe asthma than in subjects with mild/moderate disease or healthy controls (Fig E2, C and D) and correlated strongly with RGS4 expression (Fig E2, E). From these findings we conclude that RGS4 immunostaining in the respiratory epithelium may be a biomarker of asthma severity.

***Rgs4*^{-/-} mice have diminished allergen-induced bronchospasm.** We localized RGS4 protein expression in the mouse lower respiratory tract by evaluating β-galactosidase (β-gal) activity in lung sections from mice harboring alleles in which

Rgs4 was replaced by an in-frame *LacZ* insertion. As expected, we detected no β-gal activity in naive wild-type (WT) mice; however, we detected prominent β-gal staining in ciliated respiratory epithelial cells and intermittent staining of ASM cells in lung sections from naive *Rgs4*^{LacZ/LacZ} mice (hereafter referred to as *Rgs4*^{-/-}) (Fig 2, A). To determine the mode of action of RGS4 in murine airway inflammation, we subjected WT and *Rgs4*^{-/-} mice to sensitization and challenge with extracts derived from the ubiquitous mold *A fumigatus*, a respiratory allergen linked to severe asthma in humans.¹⁸ Although total β-gal activity in lungs of *A fumigatus*-challenged mice was similar to that of naive controls, we detected less activity within the epithelium but increased numbers of β-gal⁺ cells in ASM bundles of allergen-challenged mice (see Fig E3 in this article's Online Repository at www.jacionline.org). We next assessed airway tone by invasive measurement of airway resistance in

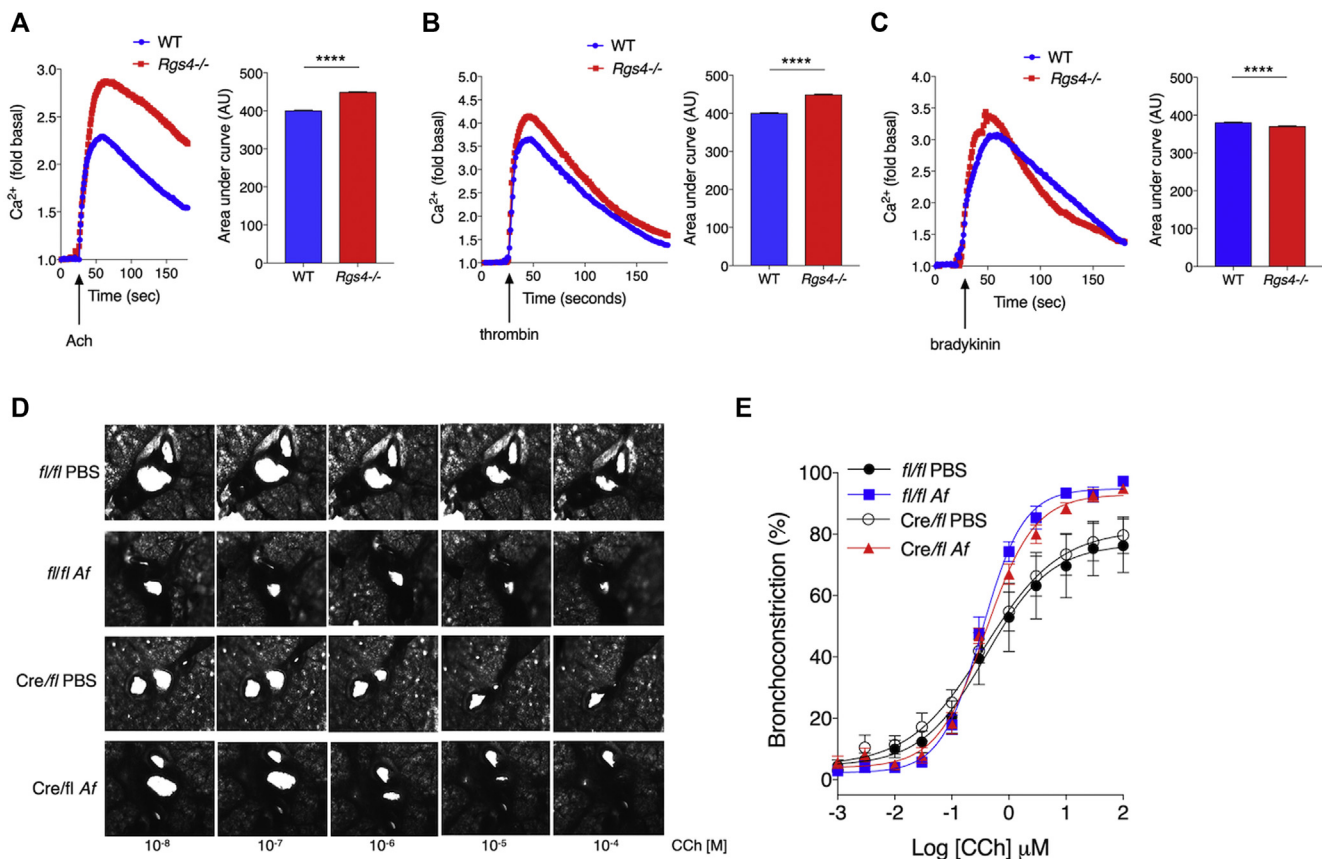


FIG 4. RGS4 enhances contraction signaling in ASM. **A-C**, Intracellular Ca^{2+} in ASM cells treated with acetylcholine (Ach, 1 mM) (Fig 4, A), thrombin (3 units/mL) (Fig 4, B), or bradykinin (500 μ M) (Fig 4, C). Results compiled from at least 60 cells/condition analyzed in 2-3 independent experiments each using cells pooled from 4-5 mice/group; time of agonist addition is noted by the arrow (mean $\pm$ SEM, AU = arbitrary units, **** $P < .0001$, t test). **D** and **E**, Airway contraction in PCLSs from PBS- or allergen-challenged *Rgs4*^{fl/fl} or *Rgs4*^{Cre/fl} mice treated with the indicated doses of CCh (mean $\pm$ SEM of 8-9 airways from 2-3 mice/group analyzed in 2 independent experiments).

response to the bronchoconstrictor MCh. Although *Rgs4* deletion had no effect on airway resistance in PBS-treated mice, *A fumigatus*-challenged *Rgs4*^{-/-} mice displayed markedly reduced responses to MCh when compared with responses of *A fumigatus*-challenged WT mice (Fig 2, B).

Lungs of WT and *Rgs4*^{-/-} mice had a similar appearance at homeostasis and likewise after *A fumigatus* allergen challenge (Fig 2, C). Total cells and leukocyte differentials in bronchoalveolar lavage fluid (BALF) in WT and *Rgs4*^{-/-} mice were also indistinguishable from one another (Fig 2, D and E). In contrast, type 2 cytokines (IL-5, IL-13) were significantly increased in BALF from *Rgs4*^{-/-} mice compared with WT controls (Fig 2, F). Other asthma-associated cytokines (IL-4, IL-9, IL-10, and IL-17A) were similar in BALF of mice of either genotype (see Fig E4 in this article's Online Repository at www.jacionline.org). We also observed significantly more mucin in the airways of allergen-challenged mice compared with PBS controls (Fig 2, G), but the magnitude of increase was equivalent in WT and *Rgs4*^{-/-} mice (Fig 2, H). Surprisingly, we detected more *Muc5ac* and *Ii33* expression in lungs of allergen-challenged *Rgs4*^{-/-} mice relative to those from WT counterparts (Fig 2, I and J).

To determine the source of allergic cytokines in this model, we immunophenotyped lung cells by flow cytometry. Percentages of

lung CD4⁺ and ILC2 cells were equivalent in naive and *A fumigatus*-challenged mice, and likewise did not differ between WT and *Rgs4*^{-/-} mice (see Fig E5, A and B, in this article's Online Repository at www.jacionline.org). IL-5⁺ and IL-13⁺ ILC2 cells were detected with greater frequency in allergen-challenged mice compared with PBS-treated controls and were increased in *Rgs4*^{-/-} mice compared with WT regardless of allergen exposure (Fig 2, K, and Fig E5, C and D). In contrast, CD4⁺IL-5⁺ or CD4⁺IL-13⁺ cells were nearly undetectable in PBS- or *A fumigatus*-challenged mice of either genotype (Fig E5, E). Finally, expression of airway remodeling-associated genes (*Col1a1*, *Col3a1*, *Il25*, *Pstn*) was similar in *A fumigatus*-challenged WT and *Rgs4*^{-/-} mice (see Fig E6 in this article's Online Repository at www.jacionline.org). These findings favor the interpretation that absence of RGS4 reduces susceptibility to bronchospasm through a mechanism that is independent of allergic inflammation and airway remodeling.

Depletion of RGS4 in ASM recapitulates the phenotype of *Rgs4*^{-/-} mice

To investigate further how RGS4 controls AHR, we evaluated mice in which RGS4 was specifically eliminated in

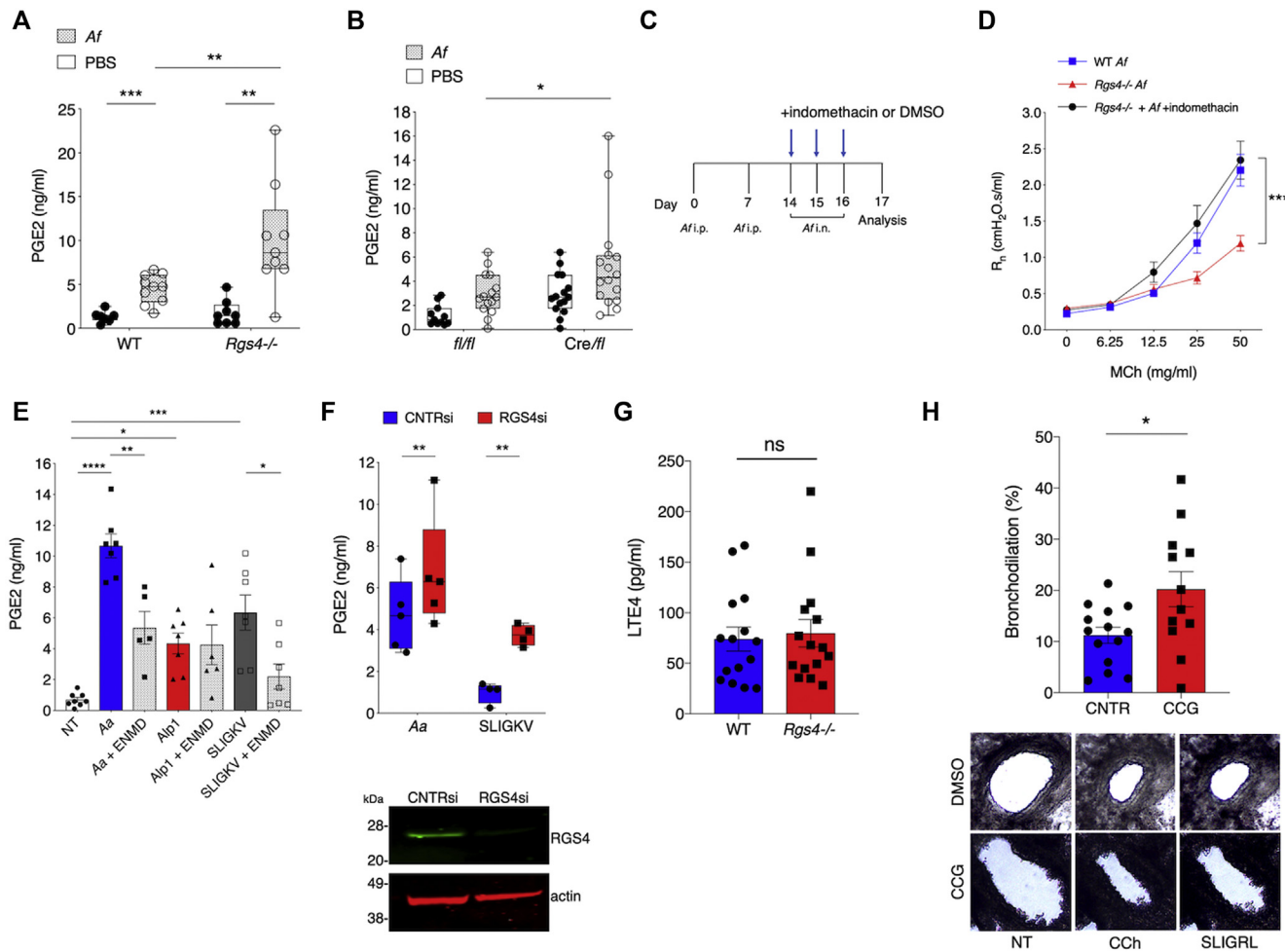


FIG 5. RGS4 regulates AHR through prostaglandin-dependent mechanisms. **A** and **B**, PGE2 levels in BALF from WT and *Rgs4*^{-/-} (Fig 5, A) or *Rgs4*^{fl/fl} and *Rgs4*^{Cre/fl} mice (Fig 5, B) (mean ± SEM, ***P* = .002, ****P* = .0002, Holm-Sidak corrected *t* tests, PBS vs *A. fumigatus*; **P* = .01 *Rgs4*^{fl/fl} vs *Rgs4*^{Cre/fl}, ***P* = .002, WT vs *Rgs4*^{-/-} 2-way ANOVA, Sidak multiple comparisons). **C**, Schematic of inoculation strategy for indomethacin experiments (i.p. = intraperitoneal; i.n. = intranasal). **D**, Lung resistance in WT or *Rgs4*^{-/-} mice challenged with *A. fumigatus* together with diluent or indomethacin (mean ± SEM of 7-17 mice/group analyzed in 4 independent experiments; ****P* = .001, 2-way ANOVA, Tukey multiple comparisons). **E**, PGE2 levels in supernatants from human AEC cells incubated as indicated (NT = not treated, Aa [200 μg/mL], Alp1 [10 μg/mL], SLIGKV [300 μM]) for 6 hours (mean ± SEM of 4-7 independent experiments performed in duplicate; **P* < .03, ***P* < .004, ****P* = .0003, *****P* < .0001, 1-way ANOVA, Holm-Sidak multiple comparisons). **F**, PGE2 levels (graph) in AEC supernatants treated as indicated; blot shows RGS4 expression in siRNA-treated AECs (mean ± SEM of 4-5 independent experiments performed in duplicate, ***P* < .006, 2-way ANOVA, Sidak multiple comparisons). **G**, LTE4 levels in BALF of *A. fumigatus*-challenged WT or *Rgs4*^{-/-} mice. **H**, Bronchodilation of PCLS airways pretreated with RGS4 inhibitor (CCG) or diluent (DMSO) followed by sequential treatment with CCh and PAR2 peptide SLIGRL (100 μM) (mean ± SEM of 12-14 airways/group analyzed in 2-4 independent experiments; **P* = .04, Mann-Whitney). Alp1, Alkaline protease 1; DMSO, dimethyl sulfoxide; LTE4, leukotriene E4; ns, not significant; siRNA, small-interfering RNA.

ASM (*Rgs4*^{fl/fl} × smooth muscle α actin-Cre).¹⁹ RGS4 was virtually undetectable in lysates of ASM cells from *Rgs4*^{Cre/fl} mice compared with those from *Rgs4*^{fl/fl} mice, whereas RGS4 expression in brain tissue of *Rgs4*^{Cre/fl} mice was comparable to that in controls (Fig 3, A). Although this genetic manipulation had no impact on airway resistance in PBS-challenged mice, *Rgs4*^{Cre/fl} mice had significantly reduced AHR after *A. fumigatus* challenge compared with *fl/fl* controls (Fig 3, B). *A. fumigatus*-induced inflammation was comparable in lungs of *Rgs4*^{Cre/fl} and *Rgs4*^{fl/fl} mice including peribronchial leukocyte infiltration (Fig 3, C),

leukocyte counts and composition of BALF (Fig 3, D and E), and mucin staining within the bronchial epithelium (Fig 3, F). Although IL-5 and IL-13 were significantly increased in BALF from allergen-challenged mice of both strains compared with PBS-challenged controls, levels were lower in allergen-challenged *Rgs4*^{Cre/fl} mice than in *Rgs4*^{fl/fl} mice (Fig 3, G). Lung *Il33* expression was also significantly lower in allergen-challenged *Rgs4*^{Cre/fl} mice than in *fl/fl* controls (Fig 3, H). These findings suggest that RGS4 expressed in ASM regulates bronchial responsiveness through mechanism(s) unrelated to leukocyte

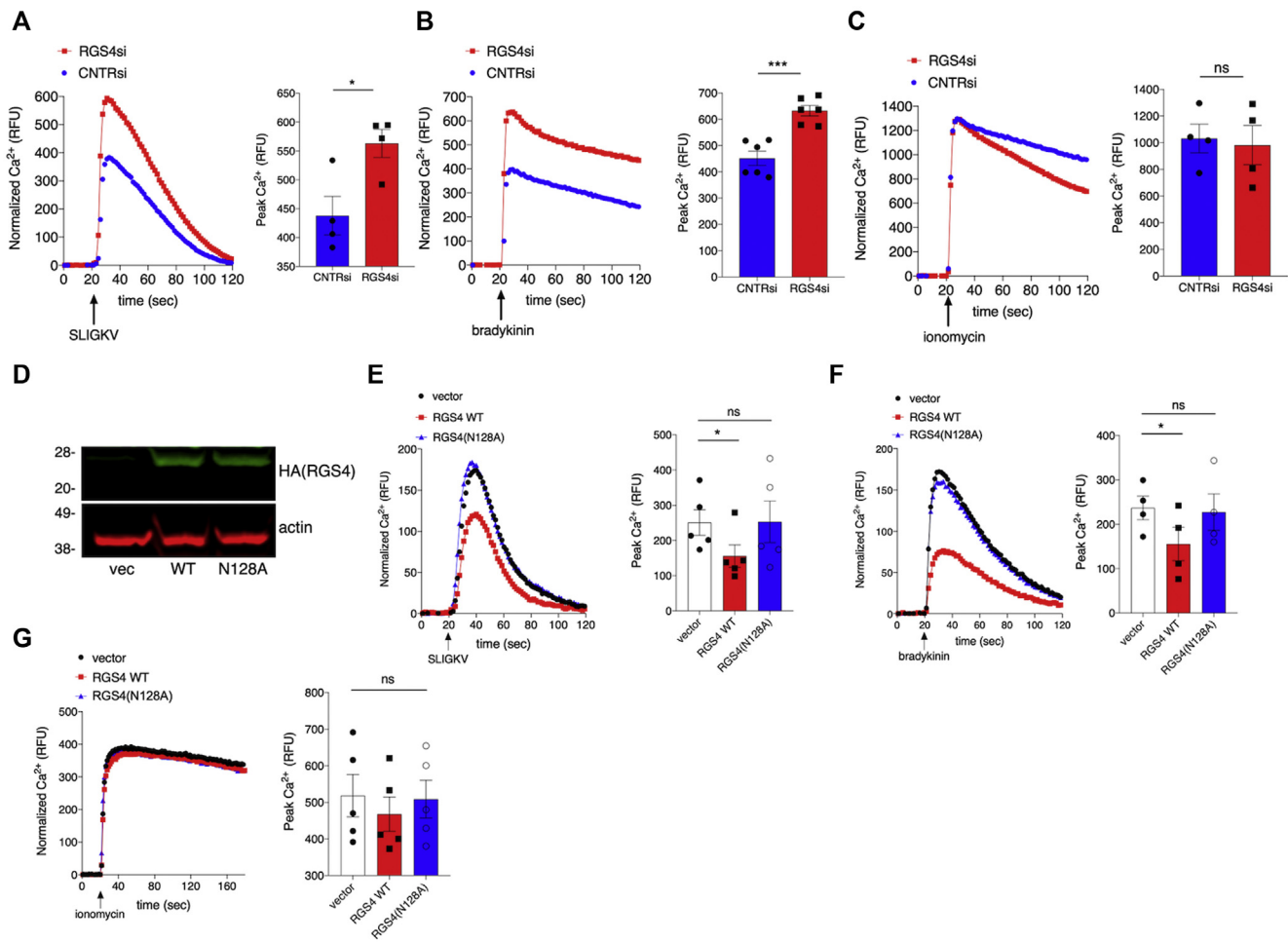


FIG 6. RGS4 inhibits GPCR-mediated Ca^{2+} signaling in AECs. Intracellular Ca^{2+} in AECs transfected with the indicated siRNAs (A-C) or BEAS2B cells transfected with the indicated plasmids (E-G) and stimulated with SLIGKV (300 μM), bradykinin (1 μM), or ionomycin (1 μM); arrows denote time of agonist addition. Bar graphs show peak Ca^{2+} response (mean $\pm$ SEM of 4-6 biological replicates evaluated in 2-3 separate experiments; $*P < .03$, $***P = .0003$, 1-way ANOVA, Tukey multiple comparisons. RFU = relative fluorescence units. D, Expression of HA(RGS4) in BEAS2B cells evaluated by immunoblotting (vec = empty control vector). ns , Not significant; $siRNA$, small-interfering RNA.

recruitment and mucus but possibly related to decreased type 2 cytokine levels.

RGS4 deficiency enhances Ca^{2+} signaling but not airway contraction *ex vivo*

RGS4 homologues (RGS2 or RGS5) inhibit AHR in mice by impairing procontractile Ca^{2+} signaling in ASM.²⁰⁻²² To evaluate how RGS4 regulates ASM contraction-related signaling pathways, we measured Ca^{2+} mobilization in tracheal ASM. Cells from *Rgs4*^{-/-} mice had increased peak Ca^{2+} responses and prolonged recovery after treatment with acetylcholine (Fig 4, A) or thrombin (Fig 4, B), but not bradykinin (Fig 4, C), compared with cells from WT mice. These results indicate that RGS4 regulates specific excitation-contraction signaling in ASM. To assess airway contraction directly, we studied precision-cut lung slices (PCLSs) from WT and RGS4-deficient mice treated with the bronchoconstrictor carbachol (CCh). Surprisingly, airway contraction in PCLSs from PBS- or *A fumigatus*-challenged

Rgs4^{Cre/fl} mice was comparable to that of *Rgs4*^{fl/fl} controls (Fig 4, D and E).

RGS4 impairs secretion of PGE2 in the airways of allergen-challenged mice

Given these findings, we hypothesized that mediators present in the airways of *Rgs4*^{-/-} mice might reduce susceptibility to AHR by mitigating contraction signaling pathways in ASM. Among many candidate bronchorelaxant factors, we focused on PGE2, a lipid mediator generated through metabolism of arachidonic acid by cyclooxygenases (cyclooxygenase 1-2) and PGE2 synthases.²³ As shown in Fig 5, A and B, PGE2 was detected at significantly higher levels in BALF from *A fumigatus*-challenged mice compared with PBS controls, and likewise at significantly higher levels in airways of *A fumigatus*-challenged *Rgs4*^{-/-} mice and ASM-specific *Rgs4* knockout mice compared with respective controls. To determine the importance of PGE2 for the attenuated AHR observed in *Rgs4*^{-/-} mice, we performed *A fumigatus* challenge together with indomethacin, a nonselective cyclooxygenase

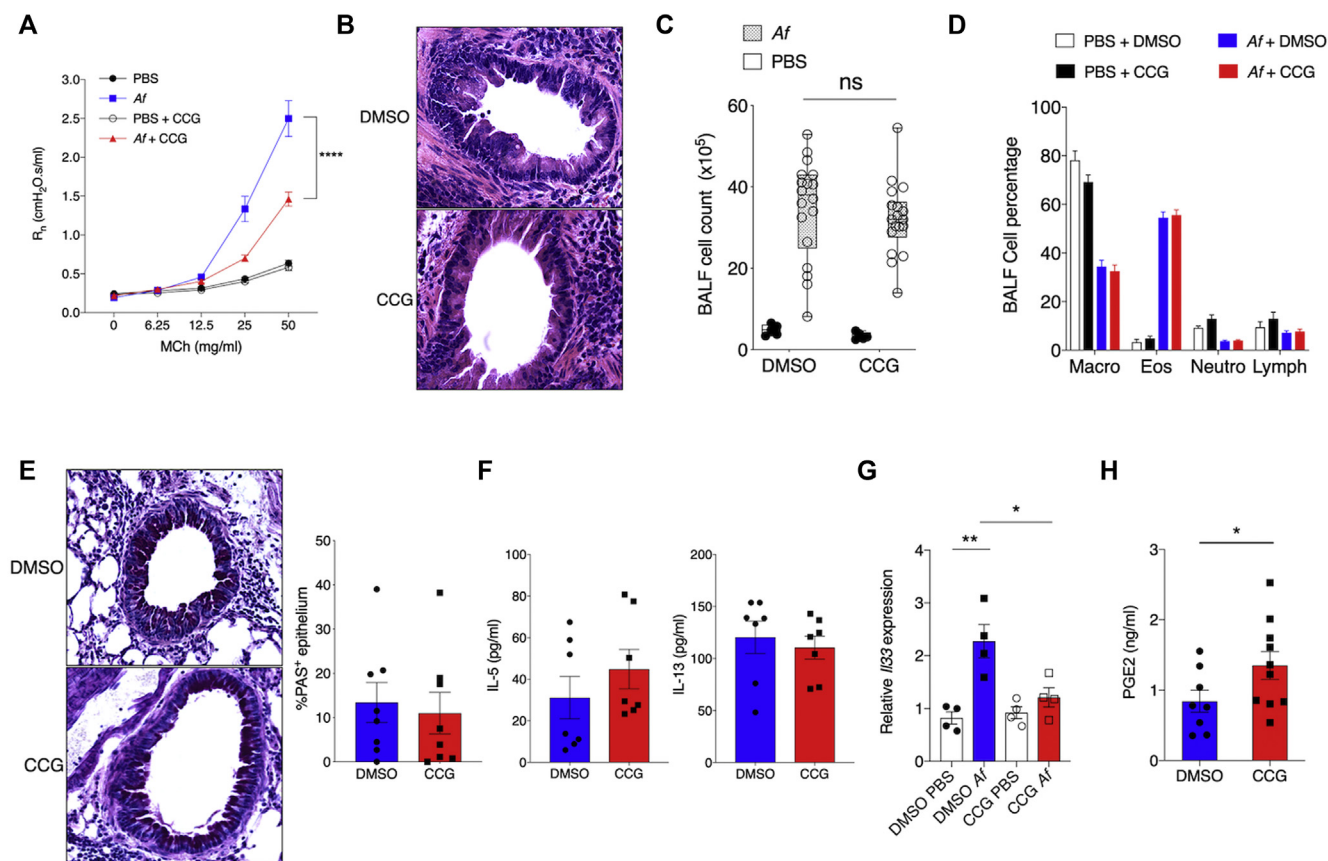


FIG 7. A–G, Pharmacological RGS4 inhibition reduces the development of allergen-induced bronchospasm in mice. Airway resistance (Fig 7, A) (**** $P < .0001$, 2-way ANOVA); airway inflammation (hematoxylin and eosin staining) (Fig 7, B); total leukocyte counts (Fig 7, C), and leukocyte composition (Fig 7, D) in BALF; airway mucus (PAS staining) (Fig 7, E); IL-5 and IL-13 in BALF (Fig 7, F); lung *Il13* expression (* $P = .01$; ** $P = .001$, 1-way ANOVA, Tukey multiple comparisons) (Fig 7, G) in Balb/c mice challenged with *A fumigatus* together with RGS4 inhibitor CCG203769 (CCG) or diluent (DMSO) alone. Images are representative of 18 mice/group analyzed in 4 independent experiments. **G,** PGE2 levels in BALF of mice challenged with *A fumigatus* together with diluent (DMSO) or RGS4 inhibitor (CCG) (mean $\pm$ SEM of 8–10 mice/group analyzed in 2 independent experiments, * $P = .04$, Mann-Whitney). DMSO, Dimethyl sulfoxide; Eos, eosinophils; Lymph, lymphocytes; Macro, macrophages; Neutro, neutrophils; ns, not significant; PAS, periodic acid-Schiff.

1-2 inhibitor (Fig 5, C). Indomethacin pretreatment of *Rgs4*^{−/−} mice significantly increased airway resistance after *A fumigatus* challenge compared with diluent alone, comparable to levels seen in *A fumigatus*-challenged WT mice (Fig 5, D). Collectively, these findings suggest that *Rgs4*^{−/−} mice do not develop AHR after allergen challenge because of an associated increase in PGE2 in the airways.

RGS4 inhibits an epithelial response to allergens

The increased airway PGE2 levels in *Rgs4*^{−/−} mice led us to explore pathways leading to PGE2 secretion that might be targeted by RGS4. *A fumigatus* and other allergenic fungi, for example, *Penicillium species* and *Alternaria alternata* (*Aa*), linked to severe asthma²⁴ secrete proteases that can elicit release of proinflammatory mediators including IL-6, IL-8, and TNF- α .^{25–27} We hypothesized that activation of proteinase-activated receptors (PARs) on AECs by allergens may be a critical step in the generation of PGE2. Allergen extracts of *Aa*, a purified serine

protease derived from *A fumigatus* that we have previously linked to AHR (alkaline protease 1)^{28,29} or the proteinase-activated receptor 2 (PAR2) agonist peptide (SLIGKV) elicited a significant increase in PGE2 secretion in AECs compared with cells treated with medium alone (Fig 5, E). Preincubation with a PAR2-specific antagonist (ENMD-1068) significantly reduced *Aa*- and SLIGKV-induced PGE2 secretion but had no effect on alkaline protease 1-mediated responses. Finally, RGS4 knockdown by small-interfering RNA enhanced *Aa*- or SLIGKV-induced PGE2 secretion by AECs compared with a control small-interfering RNA (Fig 5, F). These findings indicate that fungal allergens induce PGE2 secretion by AECs, a response that is negatively regulated by RGS4.

RGS4 inhibits prostaglandin-dependent airway relaxation

PGE2 has important anti-inflammatory actions on mast cell, lymphocyte, and ILC2 activities.³⁰ To explore the mechanisms by

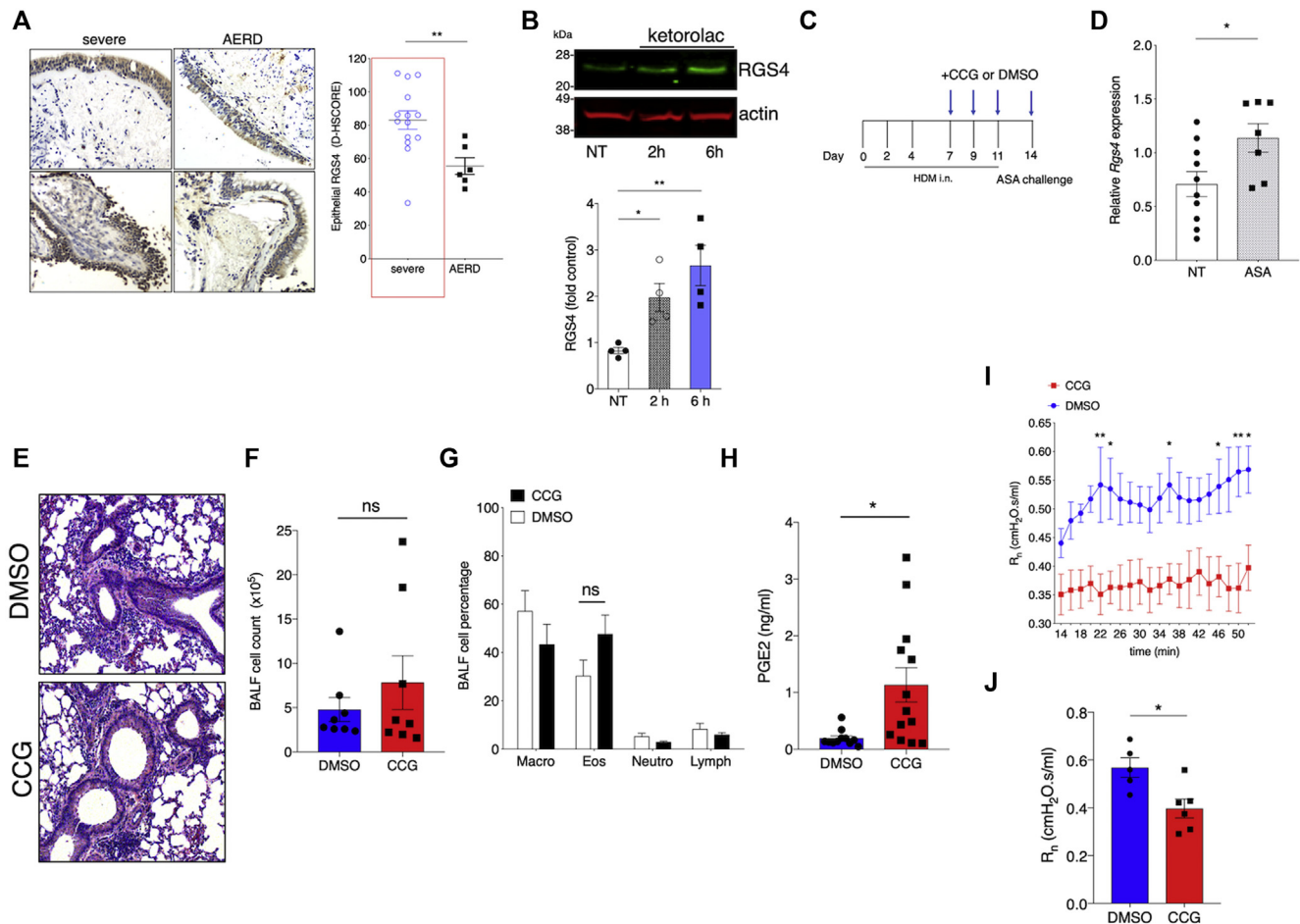


FIG 8. Potential role of RGS4 in aspirin-associated bronchospasm. **A**, Immunohistochemistry of RGS4 in lung epithelium in subjects with severe asthma (boxed data also presented in Fig 1, B) with or without a history of NSAID-associated bronchospasm; ** $P = .004$, Mann-Whitney. **B**, RGS4 expression in AECs left untreated (NT, not treated) or treated with ketorolac (200 μ g/mL) for the indicated times (mean $\pm$ SEM of 4 independent experiments using cells from 2 different individual donors, * $P = .03$, ** $P = .005$, 1-way ANOVA, Holm-Sidak multiple comparisons). **C**, *ptges1*^{-/-} mice were challenged with HDM together with diluent (DMSO) or RGS4 inhibitor (CCG) followed by a single intranasal challenge with diluent or lys-ASA. **D**, *Rgs4* transcript expression in HDM-challenged *ptges1*^{-/-} mice (mean $\pm$ SEM, relative to a calibrator value, set as "1"; * $P = .02$, unpaired *t* test). **E–J**, Analysis of HDM-challenged *ptges1*^{-/-} mice as follows: hematoxylin and eosin staining (Fig 8, E); BALF leukocyte counts (Fig 8, F); BALF leukocyte composition (Fig 8, G); and BALF PGE2 levels (Fig 8, H) (mean $\pm$ SEM of 5–13 mice/group analyzed in 2–3 independent experiments, * $P = .01$, Mann-Whitney); airway resistance after challenge with lys-ASA starting at $t = \sim 8$ minutes postchallenge (Fig 8, I); and end point resistance at 50 minutes postchallenge (mean $\pm$ SEM of 4–5 mice/group analyzed in 2 independent experiments; * $P < .04$, ** $P < .005$, 2-way ANOVA, Sidak multiple comparisons) (Fig 8, J). HDM, House-dust mite; DMSO, dimethyl sulfoxide; ns, not significant.

which PGE2 reduces AHR in *Rgs4*^{-/-} mice, we first assessed mast cell activation by measuring leukotriene E4, a stable mast cell-derived cysteinyl leukotriene metabolite, in BALF.³¹ Leukotriene E4 levels in BALF from allergen-challenged WT and *Rgs4*^{-/-} mice were indistinguishable from one another (Fig 5, G). This result and the observed increase in ILC2-derived type 2 cytokines in *Rgs4*^{-/-} mice (Fig 2, K) suggest that PGE2 decreases AHR through mechanisms that are in part independent of allergic inflammation.

To examine the effects of RGS4 on PGE2-mediated bronchial tone via direct means, we used a recently developed small molecule (CCG203769, hereafter referred to as CCG) that inhibits RGS4-G α interactions through covalent modification of Cys residues in the RGS domain and has high selectivity for

RGS4 over close homologues such as RGS16 or RGS19 (IC₅₀ = 140 nM for RGS4 vs 6 μ M for RGS16/19).³² We applied CCG or diluent alone (dimethyl sulfoxide) to PCLSs of naive mice, followed by sequential measurements of CCh-induced bronchoconstriction and responses to the mouse PAR2 agonist SLIGRL, a hexapeptide that induces prostaglandin-dependent bronchorelaxation *ex vivo*.^{33–36} Similar to the results of our studies featuring RGS4-deficient mice (Fig 5, E), CCG had no effect on Balb/c WT PCLS contraction in response to CCh (see Fig E7 in this article's Online Repository at www.jacionline.org). Administration of SLIGRL resulted in significant dilation of precontracted airways (12.2 $\pm$ 1.3%; $P < .0001$); relaxation of airways in CCG-pretreated PCLSs was nearly double that of controls (20.1% $\pm$ 3.3%; $P = .01$; Fig 5, H). These results support the conclusion

that PAR2 mediates bronchodilation through an RGS4-regulated pathway in mice.

RGS4 modulates GPCR signaling in epithelial cells

To understand the mechanism(s) by which RGS4 modulates PGE2 secretion, we evaluated PAR2-mediated signaling in AECs. PAR2 couples to Gαq, which induces intracellular Ca²⁺ mobilization, Erk phosphorylation, and generation of arachidonic acid through the actions of cytosolic phospholipase A2.³⁷ Because RGS4 is a GAP for Gαq,³⁸ we tested its effects on this pathway by measuring intracellular Ca²⁺ flux in AECs transfected with RGS4 small-interfering RNA or treated with CCG. Either manipulation enhanced the peak Ca²⁺ response to SLIGKV or to the GPCR agonist, bradykinin (Fig 6, A and B; see Fig E8, A, in this article's Online Repository at www.jacionline.org). In contrast, neither treatment had an effect on Ca²⁺ flux induced by a GPCR-independent stimulus (Ca²⁺ ionophore) (Fig 6, C, and Fig E8, B). In a separate set of experiments, overexpression of WT RGS4, but not a mutant lacking GAP activity (N128A)³⁹ in AECs (BEAS2B) (Fig 6, D) inhibited SLIGKV- and bradykinin-mediated Ca²⁺ flux but had no effect on ionomycin-induced responses (Fig 6, E-G). Taken together, these results indicate that RGS4 inhibits PGE2 secretion primarily by limiting GPCR-induced G protein activation, upstream of PGE2 biosynthesis.

Pharmacological RGS4 inhibition mimics genetic RGS4 deficiency

The lower predisposition of *Rgs4*^{-/-} mice to develop a severe asthma-like phenotype prompted us to investigate pharmacological inhibition of RGS4 activity in this disease model. We sensitized mice with *A fumigatus* as previously and then challenged intranasally with *A fumigatus* together with CCG or diluent (dimethyl sulfoxide) alone (schematic shown in Fig E9, A, in this article's Online Repository at www.jacionline.org). Although CCG had virtually no effect on airway resistance in PBS-challenged WT mice, allergen-challenged mice treated with CCG had significantly reduced MCh-induced bronchospasm compared with mice treated with diluent alone (Fig 7, A). CCG had no effect on AHR in *Rgs4*^{-/-} mice regardless of allergen exposure (Fig E9, B), confirming its specificity. CCG had no impact on lung inflammation, airway mucus, or type 2 cytokines (Fig 7, B-F). However, CCG reduced lung *Il33* expression (Fig 7, G) and significantly increased airway PGE2 levels (Fig 7, H) in *A fumigatus*-challenged mice compared with controls. These findings suggest that allergen-induced AHR is modulated by the actions of RGS4 on a PGE2-dependent pathway.

Potential role of RGS4 in aspirin-associated bronchospasm

Given our findings on the relationship between RGS4, PGE2, and allergen-induced AHR, we speculated that RGS4 regulates bronchospasm related to aspirin (ASA) intake. RGS4 expression in bronchial epithelium of biopsies from patients with severe asthma and a history of ASA-induced bronchospasm was lower than that detected in subjects with asthma of comparable severity but lacking responses to ASA (Fig 8, A). However, RGS4 was transiently upregulated in AECs after acute treatment with the

water-soluble nonsteroidal anti-inflammatory drug ketorolac (Fig 8, B), suggesting potential relevance to ASA-induced respiratory reactions. To investigate this relationship further, we used a mouse model of aspirin-exacerbated respiratory disease (AERD) that features mice lacking the gene encoding microsomal PGE2 synthase 1 (*ptges1*^{-/-} mice). *ptges1*^{-/-} mice have reduced (but detectable) PGE2 levels in lungs at homeostasis⁴⁰ and develop bronchoconstriction after ASA challenge in the setting of existing allergic inflammation (house-dust mite allergen challenge).⁴¹

To determine whether RGS4 inhibition mitigates the AERD-like phenotype of *ptges1*^{-/-} mice, we applied house-dust mite repeatedly to the lower airway mucosa in the presence or absence of CCG, followed by an additional treatment with diluent alone (dimethyl sulfoxide) or CCG and intranasal inhalation of lysine-ASA 2 hours later (Fig 8, C). Paralleling the human studies, we detected increased *Rgs4* expression in lungs from house-dust mite-challenged *ptges1*^{-/-} mice after ASA challenge compared with diluent-treated mice (Fig 8, D). CCG did not significantly affect airway inflammation including lung histology (Fig 8, E), inflammatory cell counts, or airway eosinophils (Fig 8, F and G). In contrast, although levels of PGE2 in BALF from both groups were lower overall than in allergen-challenged WT mice, they were significantly increased in CCG-treated mice compared with controls (Fig 8, H). Consistent with published studies, these mice developed increased lung resistance over a period of 50 minutes after ASA inhalation (basal: 0.46 ± 0.03 vs end point: 0.57 ± 0.04; *P* = .03, paired *t* test) (Fig 8, I). Pretreatment with CCG significantly reduced bronchospasm induced by ASA challenge (Fig 8, I and J). Collectively, our findings suggest that acute upregulation of RGS4 may contribute to ASA-induced bronchospasm by exacerbating PGE2 deficiency in the airways.

DISCUSSION

Here, we have identified a lung epithelial response to allergens that is in part mediated by activation of PAR2; the response includes production of PGE2 and is inhibited by RGS4, a protein upregulated in the respiratory epithelium in subjects with asthma. In patients without asthma, RGS4 is expressed at lower levels, facilitating PGE2 secretion after allergen exposure and its resultant protective effects on allergic inflammation and bronchial tone. As such, RGS4 may be a critical target in specific asthma endotypes such as AERD or fungal asthma.

The beneficial effects of RGS4 loss of function were quite unexpected given the results of previous studies of RGS family members in asthma. *Rgs2*^{-/-} or *Rgs5*^{-/-} mice exhibit spontaneous AHR even in the absence of allergen challenge and manifest allergic inflammation essentially equivalent to respective controls.^{21,42} Our previous work on RGS4 demonstrated that pretreatment of PCLSs with platelet-derived growth factor increased RGS4 expression and diminished CCh-induced contraction.¹³ In a separate study, transgenic mice expressing extra copies of human *RGS4* under the control of its native promoter were less susceptible to allergen-induced AHR primarily due to reduced ASM contraction and with equivalent inflammatory responses.⁴³ Here, we analyzed loss of RGS4 expression and function and its impact on the airways in experiments performed with *Rgs4*^{-/-} mice or with an RGS4 inhibitor, respectively. As such, it is not possible to compare the results of our current work with the findings presented previously.

Because it principally targets heterotrimeric G proteins, RGS4 may exert pleiotropic effects on inflammation and AHR by simultaneously regulating several GPCR pathways. In support of this hypothesis are findings that document that the loss of RGS4 expression (1) enhances PAR2-mediated PGE2 secretion in AECs (Fig 6, E); (2) increases expression of *Il33*, which encodes an epithelial-derived proinflammatory cytokine (Fig 3, H); and (3) promotes excitation-contraction signaling in ASM cells (Fig 5, A and B). Increased *Il33* expression detected in *A fumigatus*-challenged *Rgs4*^{-/-} mice suggests that in addition to its effects on ASM cells, RGS4 regulates epithelial activation, which in turn may affect cytokine production by activated ILC2s.⁴⁴ Because previous work has demonstrated that PGE2 inhibits human ILC2 functions including production of IL-5 and IL-13,⁴⁵ it is critical to consider the possibility that the anti-inflammatory actions of PGE2 might counteract the actions of allergic cytokines. Specifically, in contrast to germline *Rgs4*^{-/-} mice, we detected less *Il33* expression in both ASM-specific RGS4 knockout mice and RGS4 inhibitor-treated mice than in respective controls, suggesting that increased PGE2 in these mice may inhibit ILC2-mediated cytokine production. PGE2 is also known to inhibit eosinophil migration in response to eotaxin and other chemoattractants,⁴⁶ which may account for the discrepancy between IL-5 levels and the degree of eosinophil infiltration observed.

Our studies suggest that RGS4 promotes AHR by affecting PGE2-dependent bronchorelaxation. PGE2 is a potent bronchodilator of both human and mouse airways *ex vivo*.^{47,48} However, although PGE2 inhalation protects against the development of immediate bronchoconstriction in patients with AERD after ingestion of nonsteroidal anti-inflammatory drugs³⁰ and in subjects with asthma after allergen challenge,⁴⁹ its role in regulating airway tone in chronic allergic asthma remains uncertain.⁵⁰ In addition to PGE2 deficiency, additional mechanisms may contribute to bronchospasm in AERD, including reduced expression of and signaling through the EP2 receptor expressed on ASM.^{51,52} PGE2 also inhibits 5-lipoxygenase, an enzyme required for synthesis of bronchoconstrictive cysteinyl leukotrienes, and PGE2 deficiency in AERD thereby leads to increased lung cysteinyl leukotriene levels.³⁰ We did not detect significantly altered levels of mast cell–secreted leukotriene E4 in lungs of PBS- or *A fumigatus*-challenged *Rgs4*^{-/-} mice compared with WT mice (Fig 6, G), nor did we observe changes in the expression of *cyclooxygenase 1-2*, *ptges1*, or *ptger1-4* (encoding PGE2 receptors EP1-4) by RNA sequencing (data not shown). Thus, RGS4 may promote AHR principally by inhibition of PGE2 biosynthesis by activated epithelial cells upstream of enzymatic steps and independent of transcription. Overall, our findings are consistent with those identified in related mouse models, including those in mice lacking 15-hydroxyprostaglandin dehydrogenase, the major catabolic enzyme of PGE2. *Hpgd*^{-/-} mice have elevated airway PGE2 levels at homeostasis and reduced AHR.⁵³

RGS4 expression in bronchial epithelium of biopsies from patients with severe asthma and a history of ASA-induced bronchospasm was surprisingly lower than that detected in subjects with asthma of comparable severity but lacking responses to ASA (Fig 8, A), and administration of NSAIDs induced upregulation of RSG4 in AECs (Fig 8, B) and in the *ptges1*^{-/-} mouse AERD model (Fig 8, D). Although these results suggest the possibility of dysregulated RGS4 expression in AERD, more extensive studies of RGS4 expression in cells and tissue

from patients with AERD will be required to clarify the role of RGS4 in disease pathogenesis. Most important, pharmacological RGS4 inhibition sustainably reduced bronchospasm and increased PGE2 levels in airways in models of severe and ASA-associated asthma, suggesting a tractable therapeutic approach meriting further investigation.

We thank Helene F. Rosenberg (National Institute of Allergy and Infectious Diseases/National Institutes of Health) for critically reviewing the manuscript.

Key messages

- RGS4 expression is increased in respiratory epithelium in severe asthma, a finding that correlates with functional lung impairment.
- RGS4 promotes AHR in a mouse model of allergic lung inflammation and inhibits PGE2 synthesis in the airways.
- A small molecule RGS4 antagonist inhibits allergen- and nonsteroidal anti-inflammatory drug-induced AHR in mice, suggesting a new therapeutic approach.

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METHODS

Mouse strains

Balb/c, C57/BL6, *Rgs4^{LacZ/LacZ}*, and *Rgs4^{fl/fl}* mice were purchased from the Jackson Laboratories (Bar Harbor, Me). Smooth muscle α actin-Cre and *ptges1^{-/-}* mice were kindly provided by Dr Fred Finkelman (University of Cincinnati School of Medicine, Cincinnati, Ohio) and Dr Garret FitzGerald (University of Pennsylvania School of Medicine, Pennsylvania, Pa), respectively. All mice were bred and maintained under pathogen-free conditions at an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility at the National Institute of Allergy and Infectious Diseases and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under an animal study proposal (LAD3e) approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Reagents, cells, and antibodies

Normal human bronchial epithelial cells and BEAS2B cells were obtained from the American Type Tissue Collection and cultured in epithelial growth medium recommended by the manufacturer. PAR2 peptides SLIGKV and SLIGRL were from Abcam (Cambridge, United Kingdom). Carbachol, acetylcholine, methacholine, bradykinin, thrombin, ketorolac tris salt, aspirin, lysine-aspirin, indomethacin, forskolin, isoproterenol, MG132, CCG203769, and ENMD-1068 were purchased from Sigma-Aldrich. Antibodies used in this study were purchased from the following sources: rabbit anti-RGS4 antibody (1:500) and mouse anti- β -actin (1:2500) used for immunoblotting were from Sigma (St Louis, Mo); RGS4 antibody used for immunohistochemistry (1:400) was from Proteintech (Rosemont, Ill)^{E1}; anti-MUC5AC was from Abcam; and anti-HA (1:1000) was from Cell Signaling Technology (Danvers, Mass). For detection of endogenous RGS4 by immunoblotting, proteasomal inhibitor MG132 (10 μ M) was added to cells for 1 hour before cell lysis.

Analysis of beta galactosidase activity

β -Gal activity in formalin fixed, paraffin-embedded was determined using the β -gal staining kit (Imgenex, San Diego, Calif) as per the manufacturer's instructions.

Immunohistochemistry

Human and mouse lung sections were deparaffinized and antigens retrieved at 70°C for 40 minutes using citrate buffer (pH 6.0) before incubation with RGS4 (1:400) or MUC5AC (1:500) antibodies followed by 3,3'-diaminobenzidine (DAB) staining and hematoxylin counterstaining. Immunoreactivity was quantified using a method previously described for clinical biopsies.^{E2} Briefly, the global scale of the image analysis was set as 2950 pixels = 1 mm, with a pixel ratio of 1. RGS4 or MUC5AC⁺ pixels within a region of interest within the epithelium were chosen using the DAB Color Deconvolution plugin. Mean DAB intensity ranged from 0 (black) to 255 (total white). DAB intensity (digital HSCORE, D-HSCORE) was calculated using the formula $f = 255 - i$, where f is the final DAB intensity and i is the mean DAB intensity of the selected area from ImageJ. When multiple pictures were taken from the same slide, the mean D-HSCORE was calculated for each section.

Flow cytometry

Single-cell suspensions were prepared from lungs as described previously.^{E3} Cells were then restimulated with phorbol 12-myristate 13-acetate (100 ng/mL) plus ionomycin (1 μ M) at 37°C for 4 hours. Cell viability was assessed using Zombie Live/Dead Aqua (ThermoFisher, Waltham, Mass) and cells were fixed, permeabilized using Fix/Perm buffer (BD Biosciences, San Jose, Calif), and stained with the antibodies listed in Table E1.

Model of AERD and measurement of PGE2

Responses to aspirin in *ptges1^{-/-}* mice were evaluated essentially as described previously. Mice were challenged intranasally with house-dust mite allergen extract (*Dermatophagoides farinae*, Hollister-Stier Allergy, 3 μ g) every other day for a period of 2 weeks. For the final 3 challenges, dimethyl sulfoxide or CCG203769 (6 ng/kg total dose) was administered intranasally with allergen. Twenty-four hours after the last allergen challenge, a final dose of CCG was administered intranasally. Two hours later, mice were given a single dose of lys-ASA (1.2 μ g) intranasally. They were then anesthetized and mechanically ventilated, and airway resistance recorded approximately 8 minutes after ASA inhalation for a period of 50 minutes. PGE2 levels in BALF and cell supernatants were determined by ELISA as per the manufacturer's instructions (Enzo Life Sciences, Farmingdale, NY).

Measurement of airway mechanics in PCLSs

Mice were killed and the trachea cannulated with a blunt-end stub adapter secured with a suture. Lungs were inflated with 2% SeaPlaque low melting point temperature agarose in PBS without Ca^{2+} and Mg^{2+} using a syringe. Lungs were placed on ice for 1 hour to solidify agarose. The left lobe was excised and embedded in a mold using low melting point agarose. Lung slices (210 μ m) were prepared using the Krumdiek Tissue Slicer MD4000 (Alabama Research and Development, Munford, Ala). They were washed 2 times with serum-free Ham's F-12 medium and were incubated overnight at 37°C in a tissue culture incubator (5% CO_2). After the slices had been weighted down with platinum wire-containing nylon threads, they were stimulated with increasing concentration of carbachol (10^{-9} to 10^{-4} mol). Images of the airway were taken before and at $t = 5$ minutes poststimulation. Images were analyzed using ImageJ, and contraction of the airway was calculated by measuring the luminal area after stimulation as a percentage of the baseline luminal area. For analysis of airway relaxation, slices were contracted with carbachol (1 μ M) for 10 minutes before addition of SLIGRL (100 μ M). Images were obtained at $t = 5$ minutes after peptide addition. Airways not exhibiting contraction or relaxation were excluded from the final analysis.

Tracheal smooth muscle cell isolation

Mice were euthanized by isoflurane inhalation and tracheas excised. Luminal epithelium and surrounding muscular tissues were removed. We used a papain dissociation kit (Worthington Biochemical, Lakewood, NJ) supplemented with collagenase D, dispase II, and DNase I (Sigma) to prepare single-cell suspensions. Cells were cultured in Ham's F-12 medium, 10% FBS, penicillin, streptomycin, and amphotericin (ThermoFisher). Cells were passaged up to 4 times.

Measurement of intracellular Ca^{2+}

ASM cells were seeded (1.3×10^4 cells/well) in u-slide 8-well, ibitreat plates (Ibidi Scientific, Fitchburg, Wis) in Ham's F-12 medium supplemented with 10% FBS. Cells were serum starved in Ham's F-12 medium supplemented with insulin, transferrin, and selenium (ThermoFisher) for 7 days. Fluo-8 calcium indicator dye (ThermoFisher) was added to each well, followed by incubation in the dark for 30 minutes at 37°C and an additional 30 minutes at room temperature. Using a Leica SP5 confocal microscope, we obtained images every 20 seconds before and after the addition of agonist over a period of 180 seconds. Mean gray scale measurements of a cytoplasmic region of interest for each responding cell in each time frame were recorded using Imaris software (Zurich, Switzerland). All mean grayscale values were normalized to the values obtained in frame 1. Normal human bronchial epithelial cells were plated in 96-well plates at a density of 2.5×10^3 cells/well. Ca^{2+} Fluo-6 indicator (Molecular Devices, San Jose, Calif) was added to each well for 2 hours. Agonists were added robotically to wells using the Flex-Station III instrument (Molecular Devices), and fluorescence was measured every 1.5 seconds for 180 seconds. Each reading was subtracted from the initial value to obtain the normalized Ca^{2+} value.

RNA isolation and quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (mouse ASM and lung) or RNeasy Lipid Tissue Mini Kit (brain) (Qiagen, Germantown, Md) and reverse transcribed into cDNA using the SuperScript III First-Strand Synthesis SuperMix (ThermoFisher). Gene expression was evaluated using the 1× TaqMan Gene Expression Master Mix and specific TaqMan assay probes (ThermoFisher).

Immunoblotting and transfections

Cells or tissues were lysed in radioimmunoprecipitation buffer supplemented with protease inhibitor and phosphatase inhibitor cocktails (Roche). Clarified lysates were electrophoresed on NuPAGE Bis-Tris gels (ThermoFisher). Primary antibody binding was detected using near-infrared conjugated secondary antibodies and quantified using the Odyssey Imaging System (Li-Cor Biosciences, Lincoln, Neb). Normal human bronchial epithelial cells were transfected with a pool of small-interfering RNAs (nontargeting control [D-001810-10] or RGS4 [LQ-009900-00-0005]) using Dharmafect reagent 1 per the manufacturer's protocol (Dharmacon, Lafayette, Colo) for 48 to 72 hours before analysis of gene expression. BEAS2B cells were transfected with

plasmids pcDNA3 (vector), pcDNA3-HA-RGS4 WT, or pcDNA3-RGS4 (N128A)^{E4} using Lipofectamine 2000 (ThermoFisher).

Statistical analysis

Unless otherwise noted, data are presented as mean ± SEM and analyzed using *t* test or ANOVA by PRISM (GraphPad, San Diego, Calif). Nonparametric Spearman correlation coefficients were calculated in [Figures 1 and 2](#). *P* values less than .05 were considered statistically significant.

REFERENCES

- E1. Xue X, Wang L, Meng X, Jiao J, Dang N. Regulator of G protein signaling 4 inhibits human melanoma cells proliferation and invasion through the PI3K/AKT signaling pathway. *Oncotarget* 2017;8:78530-44.
- E2. Fuhrich DG, Lessey BA, Savaris RF. Comparison of HSCORE assessment of endometrial beta3 integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ). *Anal Quant Cytopathol Histopathol* 2013;35:210-6.
- E3. Garvy BA, Qureshi MH. Delayed inflammatory response to *Pneumocystis carinii* infection in neonatal mice is due to an inadequate lung environment. *J Immunol* 2000;165:6480-6.
- E4. Druey KM, Kehrl JH. Inhibition of regulator of G protein signaling function by two mutant RGS4 proteins. *Proc Natl Acad Sci U S A* 1997;94:12851-6.

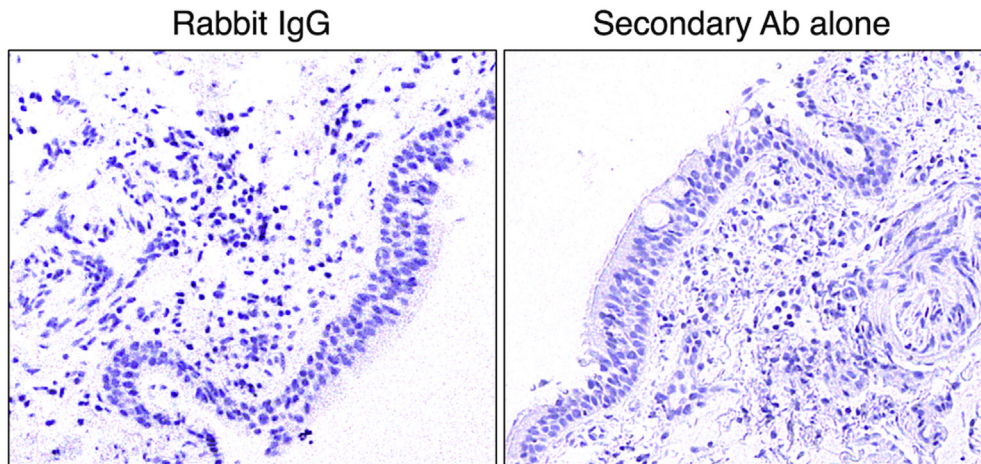


FIG E1. Antibody controls for immunohistochemistry. Human lung sections from a subject with moderate asthma were probed with rabbit IgG or antirabbit IgG secondary antibody alone as controls for rabbit anti-RGS4. *Ab*, Antibody.

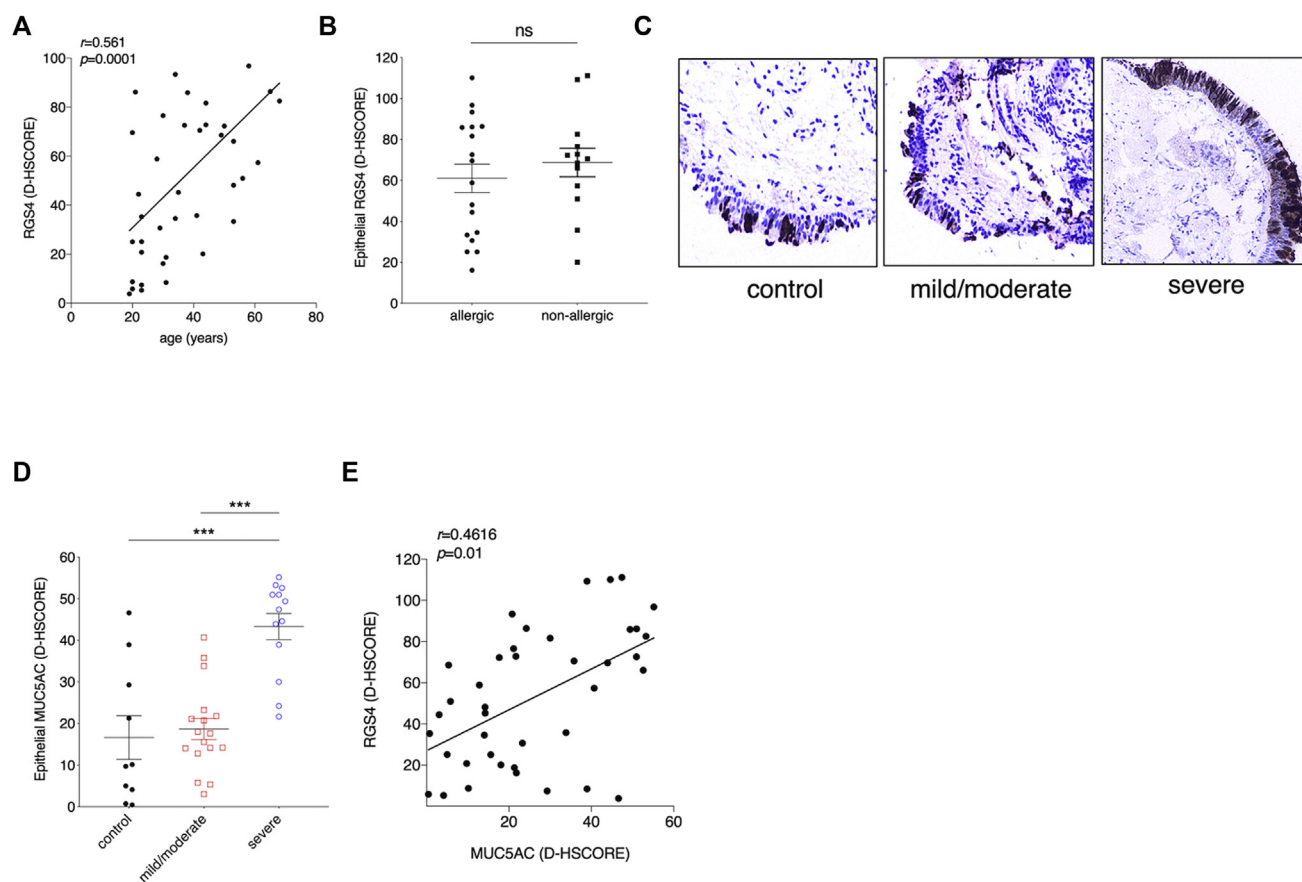


FIG E2. Characteristics associated with RGS4 expression in respiratory epithelium. **A**, Correlation between RGS4 immunoreactivity and age of subjects. **B**, RGS4 expression in epithelium in allergic and nonallergic subjects. *ns*, Not significant, Mann-Whitney. **C** and **D**, Immunohistochemistry of MUC5AC in lung epithelium; *** $P = .0003$, Kruskal Wallis, Dunn's multiple comparisons. **E**, Correlation between RGS4 and MUC5AC immunoreactivity.

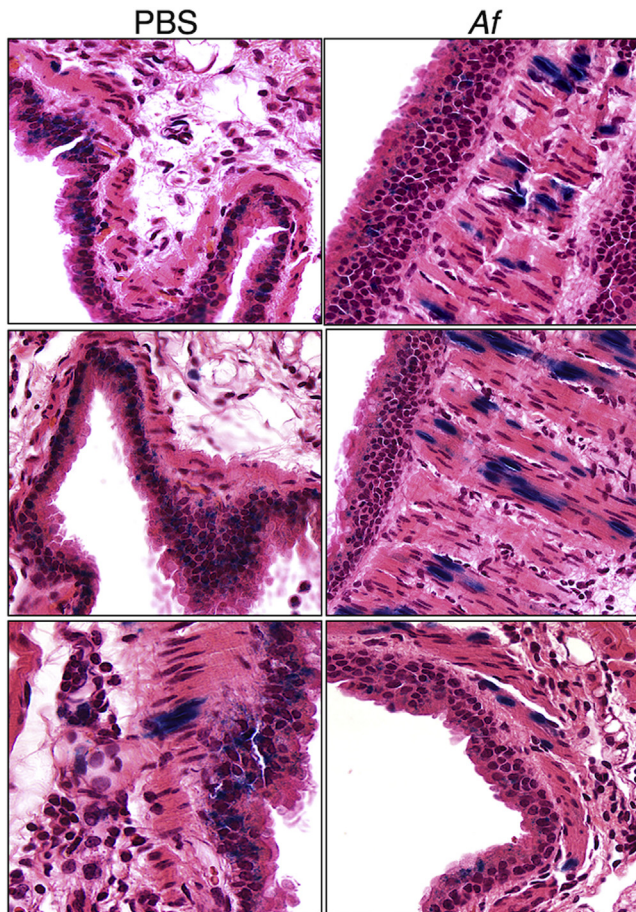


FIG E3. β -Gal activity in lungs of naive or *A fumigatus*-challenged *Rgs4*^{LacZ/LacZ} mice. Images are representative of 3 mice/group.

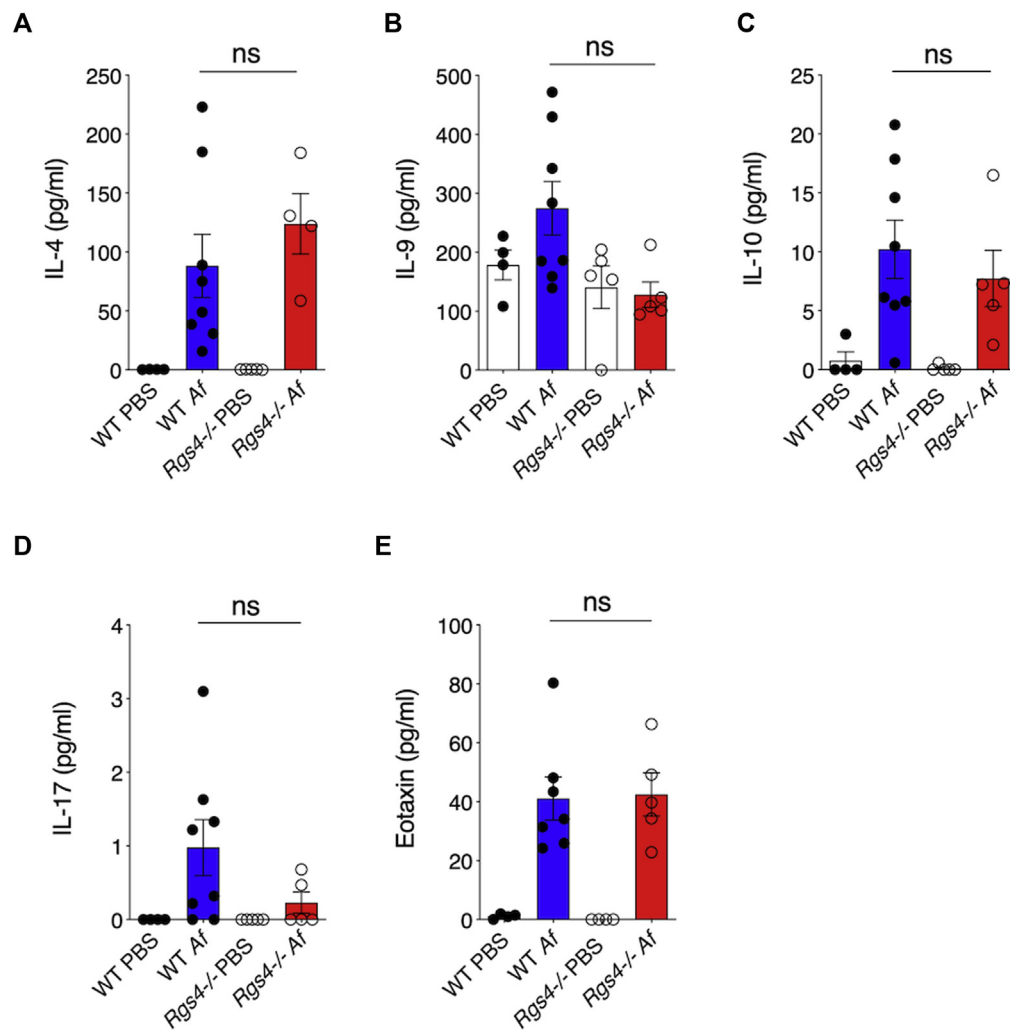


FIG E4. A-E, Cytokines in BALF of naive and allergen-challenged WT or *Rgs4*^{-/-} mice (mean $\pm$ SEM, ns, not significant, 1-way ANOVA). ns, Not significant.

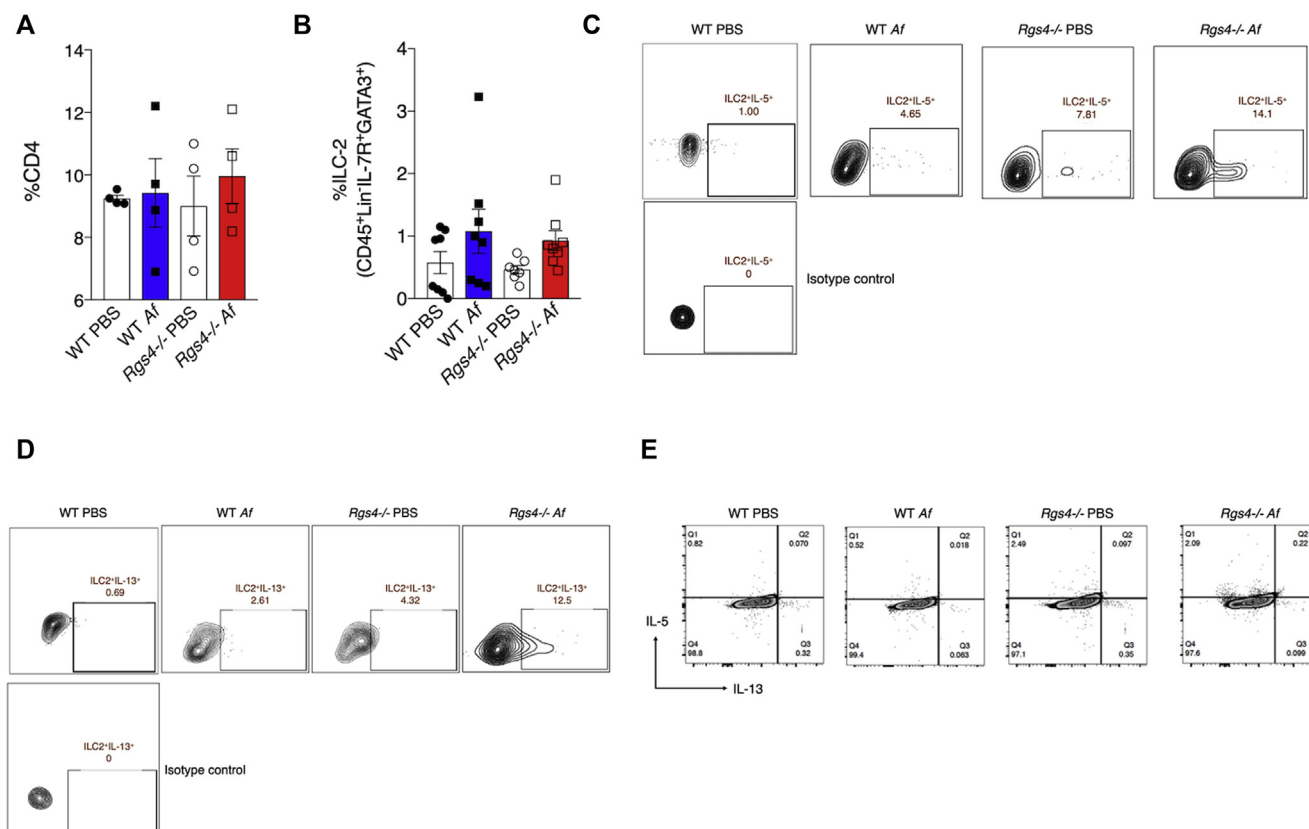


FIG E5. Lung cell immunophenotyping. Single-cell lung suspensions were prepared and analyzed by flow cytometry as per the Methods. **A** and **B**, CD4⁺ T cells (Fig E5, A) and ILC2s (CD45⁺Lin⁻IL-7R⁺GATA3⁺) (Fig E5, B) as a percentage of total live cells. **C-E**, Representative flow plots of cytokine⁺ ILC2s (Fig E5, C and D) CD4⁺ T cells (Fig E5, E).

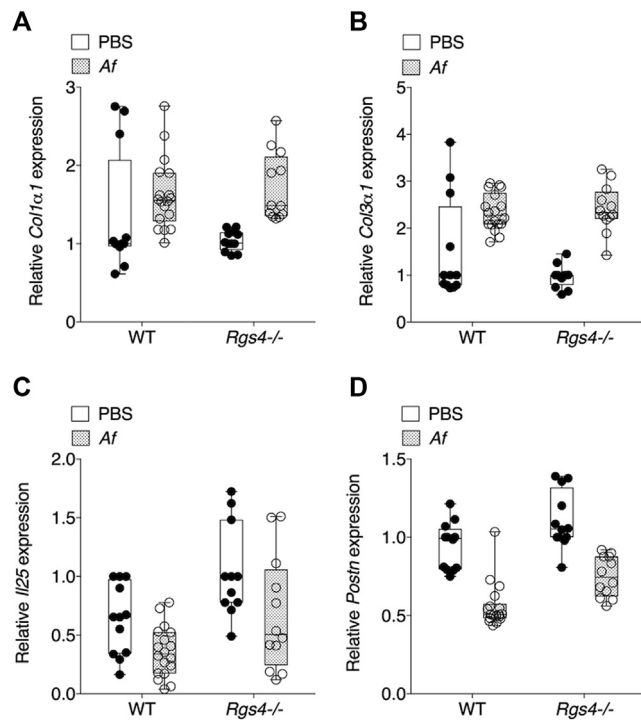


FIG E6. Expression of proinflammatory and remodeling genes in allergen-challenged mice. **A-D**, Relative expression of *Col1α1*, *Col1α3*, *Il25*, or *Pstn* in naive and allergen-challenged WT or *Rgs4*^{-/-} mice was determined by quantitative PCR (mean ± SEM, ns, not significant, 1-way ANOVA).

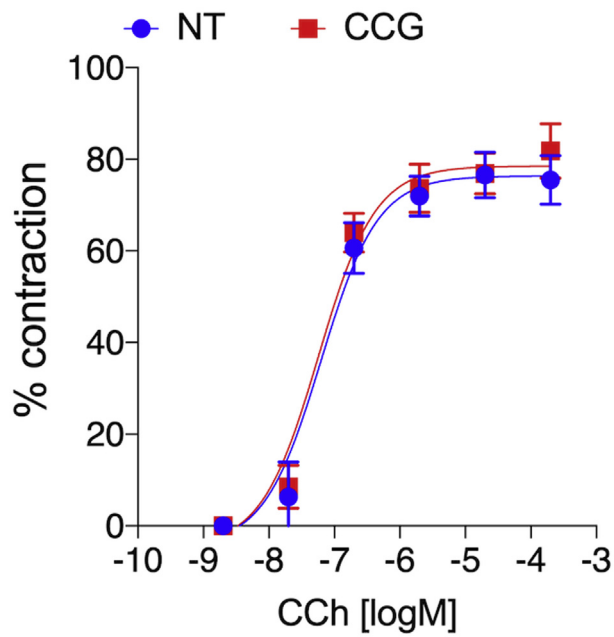


FIG E7. RGS4 inhibitor (CCG203769) does not affect airway contraction *ex vivo*. PCLSs from naive Balb/c mice were pretreated with diluent alone (DMSO) or CCG (2.5 μ M) for 1 hour before assessment of airway contraction in response to CCh (mean $\pm$ SEM of >40 airways/group analyzed in 2 independent experiments). *DMSO*, Dimethyl sulfoxide.

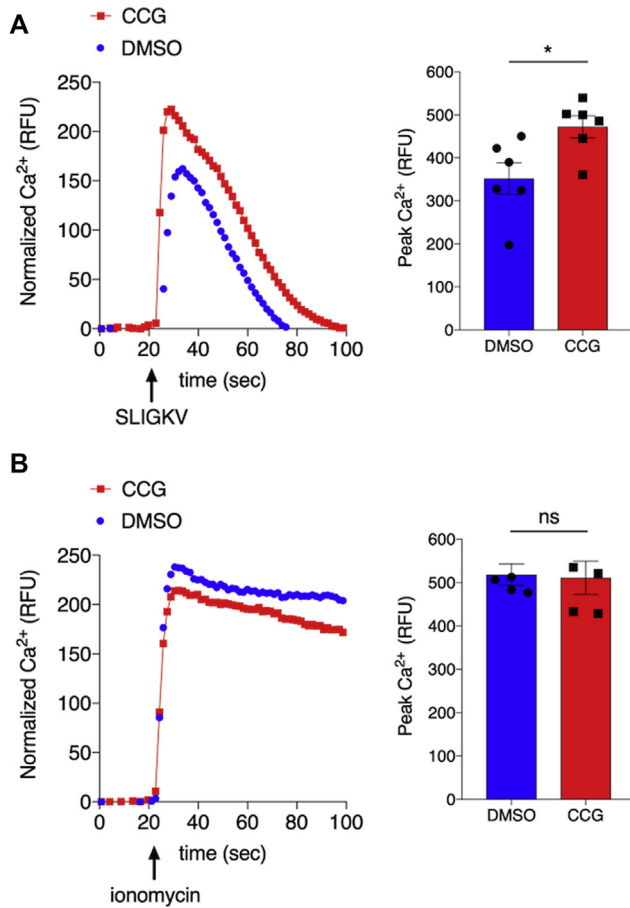


FIG E8. CCG modulates Ca^{2+} signaling in AECs. **A** and **B**, Cytosolic Ca^{2+} in NHBE cells treated with SLIGKV (Fig E8, **A**) or ionomycin (Fig E8, **B**); time of agonist addition is denoted by the arrow. Bar graph shows peak Ca^{2+} (mean $\pm$ SEM of 3 independent experiments; $*P = .02$, t test). *DMSO*, Dimethyl sulfoxide; *NHBE*, normal human bronchial epithelial cells, *ns*, not significant.

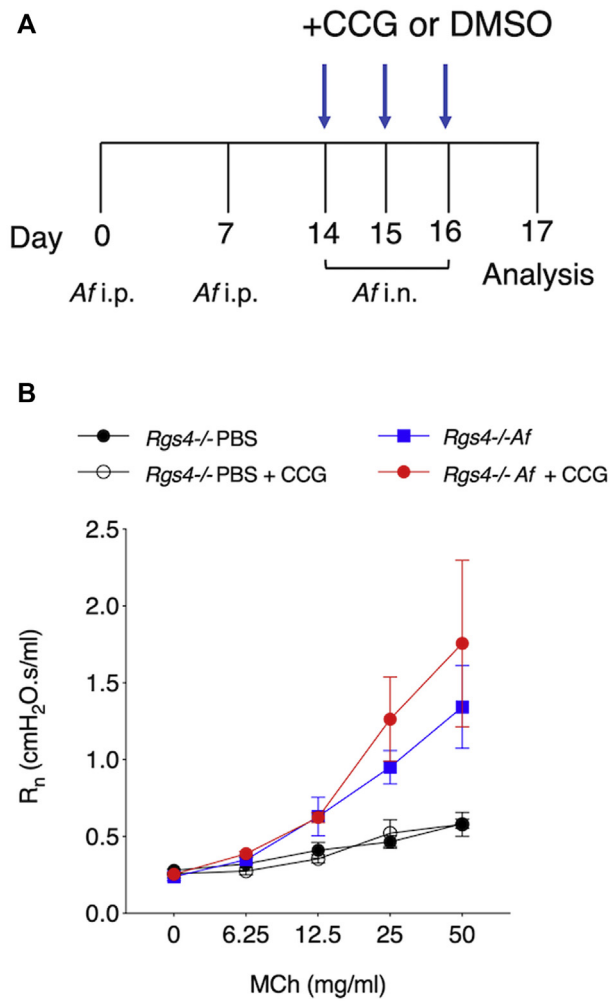


FIG E9. RGS4 inhibitor has no effect on airway resistance in *Rgs4*^{-/-} mice. **A**, Schematic of inoculation schedule for RGS4 inhibitor experiments. **B**, Airway resistance in naive or *A. fumigatus*-challenged *Rgs4*^{-/-} mice treated with diluent (DMSO) or CCG was evaluated by plethysmography (mean $\pm$ SEM of 3-4 mice/group). DMSO, Dimethyl sulfoxide.

TABLE E1. Antibodies used for flow cytometry

Marker	Fluorophore	Source	Clone	Dilution
IL-5	APC	Biolegend	TRFK5	1:100
CD49b	PE	ThermoFisher	DX5	1:100
CD11c	PE	ThermoFisher	N418	1:100
NK1.1	PE	ThermoFisher	PK136	1:100
CD19	PE	ThermoFisher	MB19-1	1:100
F4/80	PE	ThermoFisher	BM8	1:100
Ly6G/C	PE	ThermoFisher	RB6-8C5	1:100
CD3e	PE	ThermoFisher	145-2C11	1:100
CD4	BV650	Biolegend	GK1.5	1:100
CD3e	PE-Cy7	ThermoFisher	145-2C11	1:100
CD45	PerCP	Biolegend	30-F11	1:100
CD127 (IL-7R)	AF700	ThermoFisher	A7R34	1:100
GATA3	BV711	BD Biosciences	L50-823	1:100
IL-13	AF488	ThermoFisher	eBio13A	1:100

APC, Allophycocyanin; PE, phycoerythrin.

TABLE E2. Clinical characteristics of patients studied

Characteristic	Controls	Mild/moderate	Severe	AERD
Number	10	19	14	6
Age (y)	24.8 (1.5)	36.9 (2.8)	46.5 (4.1)	48.8 (8.5)
Male/female	3/7	10/9	8/6	5/6
Prebronchodilator FEV ₁ (% predicted)	101.5 (88-111)	85.8 (53-115)	68 (30-103)	74.8 (62-99)
Inhaled CCS	None	10 (500)	14 (1652)	5 (2200)
Atopy	None	10	8	4