

Loss of regulator of G protein signaling 5 promotes airway hyperresponsiveness in the absence of allergic inflammation

Nariman A. Balenga, PhD,^a William Jester, MS,^b Meiqi Jiang, MD, PhD,^b Reynold A. Panettieri, Jr, MD,^b and Kirk M. Druey, MD^a *Bethesda, Md, and Philadelphia, Pa*

Background: Although eosinophilic inflammation typifies allergic asthma, it is not a prerequisite for airway hyperresponsiveness (AHR), suggesting that underlying abnormalities in structural cells, such as airway smooth muscle (ASM), contribute to the asthmatic diathesis. Dysregulation of procontractile G protein-coupled receptor (GPCR) signaling in ASM could mediate enhanced contractility.

Objective: We explored the role of a regulator of procontractile GPCR signaling, regulator of G protein signaling 5 (RGS5), in unprovoked and allergen-induced AHR.

Methods: We evaluated GPCR-evoked Ca^{2+} signaling, precision-cut lung slice (PCLS) contraction, and lung inflammation in naive and *Aspergillus fumigatus*-challenged wild-type and *Rgs5*^{-/-} mice. We analyzed lung resistance and dynamic compliance in live anesthetized mice using invasive plethysmography.

Results: Loss of RGS5 promoted constitutive AHR because of enhanced GPCR-induced Ca^{2+} mobilization in ASM. PCLSs from naive *Rgs5*^{-/-} mice contracted maximally at baseline independently of allergen challenge. RGS5 deficiency had little effect on the parameters of allergic inflammation, including cell counts in bronchoalveolar lavage fluid, mucin production, ASM mass, and subepithelial collagen deposition. Unexpectedly, induced IL-13 and IL-33 levels were much lower in challenged lungs from *Rgs5*^{-/-} mice relative to those seen in wild-type mice.

Conclusion: Loss of RGS5 confers spontaneous AHR in mice in the absence of allergic inflammation. Because it is selectively expressed in ASM within the lung and does not promote inflammation, RGS5 might be a therapeutic target for asthma. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Asthma, airway hyperresponsiveness, G proteins, regulator of G protein signaling proteins, *Aspergillus fumigatus*

Abbreviations used

AHR: Airway hyperresponsiveness
 ASM: Airway smooth muscle
 BALF: Bronchoalveolar lavage fluid
 DAPI: 4'-6-Diamidino-2-phenylindole dihydrochloride
 ERK: Extracellular signal-regulated kinase
 GPCR: G protein-coupled receptor
 hASM: Human airway smooth muscle
 MLC: Myosin light chain
 mtASM: Mouse tracheal airway smooth muscle
 PCLS: Precision-cut lung slice
 PI3K: Phosphoinositide 3-kinase
 PLCβ: Phospholipase Cβ
 RGS: Regulator of G protein signaling
 R_L: Lung resistance
 TLR: Toll-like receptor
 WT: Wild-type

Asthma is characterized by airway hyperresponsiveness (AHR) and infiltration of inflammatory leukocytes in the lung, which are typically associated with allergen exposure.¹ Airway smooth muscle (ASM) contraction is triggered primarily by spasmogens activating Gα_q-linked G protein-coupled receptors (GPCRs), including the M3 muscarinic, cysteinyl leukotriene, thrombin, histamine H1, and bradykinin receptors,² and levels of ligands for many of these GPCRs are increased in the lungs of allergic asthmatic patients. Exaggerated bronchoconstriction to the muscarinic receptor 3 agonist methacholine characterizes asthma.³ GPCR activation induces guanosine triphosphate binding to Gα_q, which activates phospholipase Cβ (PLCβ), evoking inositol trisphosphate formation, release of Ca^{2+} into the cytosol, cross-bridging of the actomyosin complex, and ASM shortening.

Recent work suggests that ASM-intrinsic mechanisms also contribute to AHR independently of inflammation. Mice deficient in the integrin α9β1 had baseline AHR, and tracheal smooth muscle from these mice exhibited increased force generation *ex vivo*.⁴ Mice lacking a receptor tyrosine phosphatase, CD148, had reduced baseline pulmonary resistance and were protected from allergen-induced AHR.⁵ Underlying dysregulation of ASM procontractile signaling pathways could partially explain the poor efficacy of specific antagonists of individual contraction-inducing receptors in asthmatic patients.⁶

GPCR function is controlled in part by the regulator of G protein signaling (RGS) family, which accelerate the intrinsic GTPase activity of Gα subunits to attenuate the downstream signaling pathway.⁷ RGS2 was downregulated in PBMCs and lungs of asthmatic patients and in lungs of allergen-challenged mice,⁸ and *Rgs2*^{-/-} mice had spontaneous AHR. However, because RGS2 is widely expressed in many lung constituent cells,

From ^athe Molecular Signal Transduction Section, Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases/National Institutes of Health, Bethesda, and ^bthe Pulmonary, Allergy and Critical Care Division, Airways Biology Initiative, University of Pennsylvania, Philadelphia.

Supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases/National Institutes of Health (NIH; project no. AI001746, to K.M.D.) and NIH grants R01 HL097796 and P30 ES013508 (to R.A.P.).

Disclosure of potential conflict of interest: R. A. Panettieri's institution has received funds from the National Institutes of Health, AstraZeneca, Merck, and Roche, and he has received consulting fees from J&J and Merck, as well as fees from Teva, for participating in review activities. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication May 10, 2013; revised January 24, 2014; accepted for publication January 27, 2014.

Corresponding author: Kirk M. Druey, MD, 10 Center Dr, Rm 11N242, Bethesda, MD 20892. E-mail: kdruey@niaid.nih.gov.

0091-6749

<http://dx.doi.org/10.1016/j.jaci.2014.01.019>

including epithelial and ASM cells, the attractiveness of an RGS2-specific therapeutic target for asthma is uncertain.

We found previously that expression of a closely related isoform, RGS5, is restricted to a subset of smooth muscle cells in both human subjects and mice.⁹ Exposure of cultured human airway smooth muscle (hASM) to β -adrenergic agonists, a standard bronchodilator therapy used for asthma, reduced RGS5 expression and intensified excitation-contraction responses to GPCR agonists.¹⁰ In a recent study a single nucleotide polymorphism in *RGS5* correlated with clinical response to β -agonists in asthmatic children.¹¹ Here we investigated the effects of RGS5 deficiency on both inflammation and AHR *in vivo* by using *Rgs5*^{-/-} mice. These mice had both spontaneous and inflammation-associated AHR independent of the degree of inflammation or changes in ASM mass. AHR was principally due to increased ASM excitation-contraction responses to GPCR ligands. These results warrant further investigation into the suitability of RGS5 as a drug target for AHR.

METHODS

For complete description of the methods used in this study, see the [Methods](#) section in this article's Online Repository at www.jacionline.org.

RESULTS

RGS5 inhibits GPCR-induced excitation-contraction signaling in mouse ASM

RGS5 overexpression reduced carbachol-elicited bronchoconstriction of human precision-cut lung slices (PCLSs) *ex vivo*,⁹ whereas PCLSs from *Rgs5*^{-/-} C57Bl/6 mice bronchoconstricted more to carbachol.¹⁰ To determine whether augmented excitation-contraction signaling in ASM from RGS5-deficient mice contributed to their increased responsiveness, we examined GPCR-evoked signaling in mouse tracheal airway smooth muscle (mtASM) cultures from wild-type (WT) and *Rgs5*^{-/-} mice. These cells had similar morphology, growth, and smooth muscle α -actin content (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org and data not shown). Expression of several procontractile GPCRs (see [Fig E1, B](#)) and downstream signaling components, including PLC β , G α_q , G $\alpha_{i1/2}$, G α_{i3} , myosin light chain (MLC), smooth muscle α -actin, and β -arrestin 1/2 (see [Fig E1, C](#)), was similar in WT and RGS5-deficient mtASM. Comparison of *Rgs* expression in mtASM from WT and *Rgs5*^{-/-} mice revealed that *Rgs1* and *Rgs3* were not present, and there was little difference in *Rgs2* expression (see [Fig E2, A and B](#), in this article's Online Repository at www.jacionline.org). Although *Rgs4* mRNA expression was increased 3- to 4-fold in mtASM and whole lungs of naive *Rgs5*^{-/-} mice (see [Fig E2, A and B](#)), it was decreased in lungs of allergen-challenged RGS5-deficient mice compared with expression seen in lungs of challenged WT mice (see [Fig E2, C](#)). Published studies have noted marked dissociation between RGS4 mRNA and protein levels because of post-transcriptional regulation.^{12,13} Accordingly, RGS4 protein amounts were nearly identical in mtASM cells from WT and *Rgs5*^{-/-} mice (see [Fig E2, D](#)). These results indicate that transcriptional upregulation of *Rgs4* in mtASM and lungs of naive mice is unlikely to have an effect on AHR in allergen-challenged *Rgs5*^{-/-} mice.

To evaluate excitation-contraction signaling pathways in RGS5-deficient ASM, we treated mtASM cells with various

procontractile agonists and measured cytosolic Ca²⁺ concentrations by using fluorimetry. Acetylcholine ([Fig 1, A](#)) and bradykinin ([Fig 1, B](#)) elicited significantly more Ca²⁺ flux in mtASM from *Rgs5* knockout mice than from WT mice, particularly at the highest agonist concentrations. In contrast, exposure of WT or RGS5-deficient mtASM to serotonin ([Fig 1, C](#)), thrombin ([Fig 1, D](#)), thapsigargin, or ionomycin ([Fig 1, E](#)) induced comparable Ca²⁺ responses. These experiments suggested that RGS5 inhibits Ca²⁺ signaling induced by some but not all procontractile GPCRs in mtASM and that such differences cannot be attributed to alterations in cellular Ca²⁺ stores or Ca²⁺ channel activity.

We next examined events downstream of Ca²⁺ that promote contraction. Because we could not detect robust agonist-induced MLC phosphorylation in these cells, we measured extracellular signal-regulated kinase (Erk) activation as a surrogate for engagement of the contractile apparatus because Erk1/2 regulates MLC kinase activity and induces Ca²⁺-independent MLC phosphorylation.^{14,15} Surprisingly, we observed similar basal, acetylcholine-induced, or bradykinin-induced Erk phosphorylation in mtASM cells pooled from WT or *Rgs5*^{-/-} mice by means of immunoblotting (see [Fig E3](#) in this article's Online Repository at www.jacionline.org). Because RGS5 is expressed in a subpopulation of ASM cells in both mouse and human lungs,⁹ we hypothesized that variable RGS5 expression in WT cells precluded accurate determination of its role in this signaling pathway using this method. Instead, we evaluated RGS5 expression and Erk1/2 phosphorylation in individual cells using quantitative immunofluorescence.¹⁶ RGS5 was expressed in the cytoplasm of some but not all ASM cells ([Fig 2, A](#), and see [Fig E4](#) in this article's Online Repository at www.jacionline.org), and amounts varied 3-fold ([Fig 2, C](#)). Acetylcholine-induced Erk1/2 activation (phosphorylated Erk [pErk] staining) was significantly more intense in cells from *Rgs5*^{-/-} mice than in WT cells expressing RGS5 ([Fig 2, A and B](#)). Acetylcholine-evoked pErk and RGS5 amounts correlated inversely ([Fig 2, C](#)). Thus RGS5 negatively regulates procontractile signaling pathways in ASM.

RGS5 regulates lung slice contraction

Lung slices from C57Bl/6 *Rgs5*^{-/-} mice exhibited constitutively increased contraction in response to muscarinic receptor agonists.¹⁰ To explore these findings in allergen-induced inflammation and to exclude strain-dependent effects, we backcrossed *Rgs5*^{-/-} mice to a BALB/c background for 10 generations and compared and evaluated PCLS contraction. Although the baseline diameter of the airways was similar in slices from either strain ([Fig 3, A](#)), airways from *Rgs5*^{-/-} mice bronchoconstricted more robustly to carbachol ([Fig 3, A and B](#)) or acetylcholine ([Fig 3, C](#)) than those from WT mice. RGS5 deficiency primarily affected the efficacy of these muscarinic receptor ligands: the median effective concentration for carbachol was similar for each strain, whereas the E_{max} differed significantly (WT mice = 37.66 $\pm$ 8.665; *Rgs5*^{-/-} mice = 63.84 $\pm$ 7.25, *P* = .0001). To determine whether the enhanced contraction of PCLSs from *Rgs5*^{-/-} mice resulted from intrinsically abnormal signaling responses to these agonists, we measured Erk1/2 phosphorylation. Although baseline pErk levels were similar, acetylcholine-induced pErk levels were higher in PCLSs from *Rgs5*^{-/-} mice than in those from WT mice ([Fig 3, D](#)).

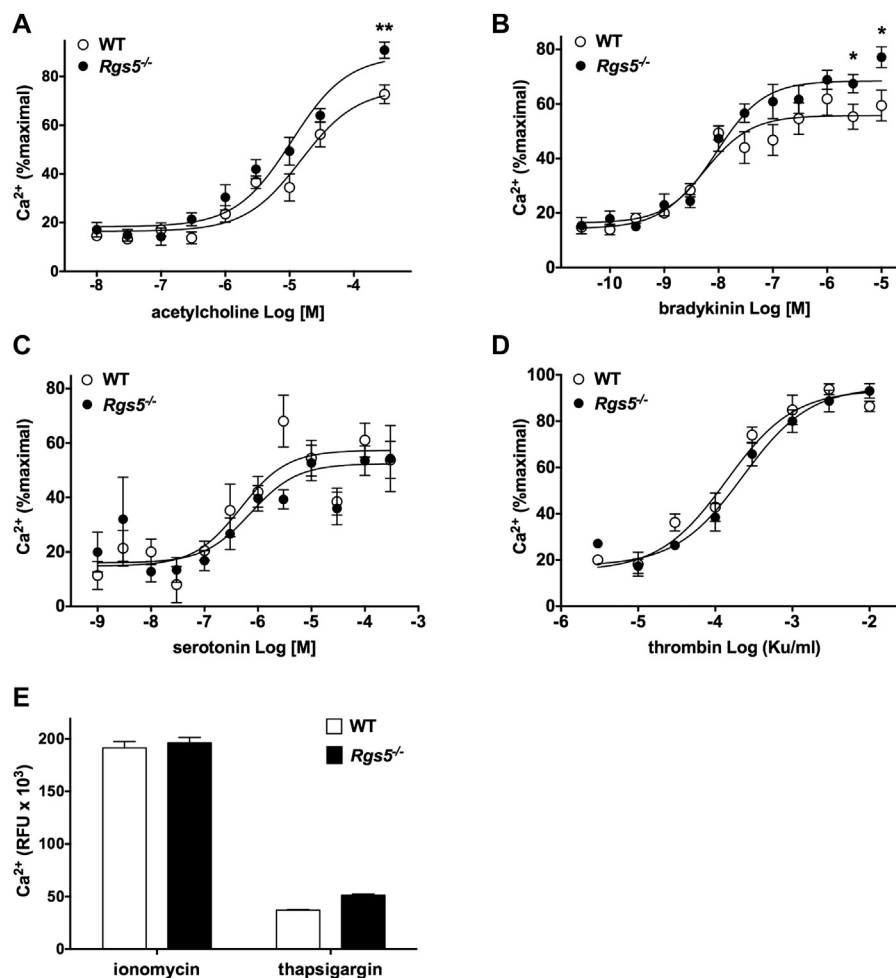


FIG 1. RGS5 regulates GPCR-mediated intracellular calcium release. Cytosolic calcium release in mtASM from WT and *Rgs5*^{-/-} mice after stimulation with acetylcholine (A), bradykinin (B), serotonin (C), thrombin (D), ionomycin (1 μ mol/L), or thapsigargin (1 μ mol/L; E) assessed by means of fluorimetry is shown. Relative fluorescence unit (RFU) values were normalized to the maximal response in Fig 1, A to D. Values are means $\pm$ SEMs from 3 to 6 independent experiments (in mtASM derived from 10 mice per group) assayed in triplicate. **P* < .04 and ***P* = .004, unpaired *t* test.

To investigate mechanisms of RGS5-mediated bronchial contraction in allergic inflammation, we sensitized and challenged mice with extracts of *Aspergillus fumigatus* and measured PCLS contraction. As expected, airways of allergen-challenged WT mice contracted significantly more to carbachol than those from naive mice ($E_{\max}$ in PCLSs from challenged mice = 75.24 ± 4.73). In contrast, constriction of PCLSs from challenged *Rgs5*^{-/-} mice was not only similar to that of naive *Rgs5*^{-/-} mice but also nearly equivalent to the responses of PCLSs from challenged WT mice. These results suggest that airways from mice lacking RGS5 respond maximally to muscarinic receptor stimulation at baseline. In other words, these mice have constitutive AHR that is dissociated from allergen-induced inflammation.

RGS5 modulates the lung cytokine environment of allergen-challenged mice

An alternate mechanism for the equivalent contraction of PCLSs from allergen-challenged WT and RGS5-deficient mice

could be reduced inflammation in *Rgs5*^{-/-} mice. However, parameters of lung inflammation after sensitization and challenge with *A. fumigatus*, including total leukocyte numbers (see Fig E5, A, in this article's Online Repository at www.jacionline.org) and composition (see Fig E5, B) in bronchoalveolar lavage fluid (BALF), peribronchial and perivascular inflammation (see Fig E5, C), ASM mass (see Fig E5, D), goblet cell metaplasia (see Fig E6, A, in this article's Online Repository at www.jacionline.org), and collagen deposition (see Fig E6, B) were similar in naive and *A. fumigatus*-challenged WT and *Rgs5*^{-/-} mice. Levels of allergen-associated inflammatory cytokines, including IL-4 and IL-5, were similar in BALF from WT and *Rgs5*^{-/-} mice in the presence or absence of *A. fumigatus* challenge (Fig 4, A and B). Surprisingly, however, IL-13 levels were nearly 3-fold lower in BALF from allergen-challenged *Rgs5*^{-/-} mice compared with those seen in WT mice (Fig 4, C). Although *Il13* mRNA expression increased 10.5-fold in WT lungs after *A. fumigatus* challenge relative to that seen in naive mice, *Il13* induction was reduced by more than 40% in *Rgs5*^{-/-} mice (*P* = .0017; Fig 4, D).

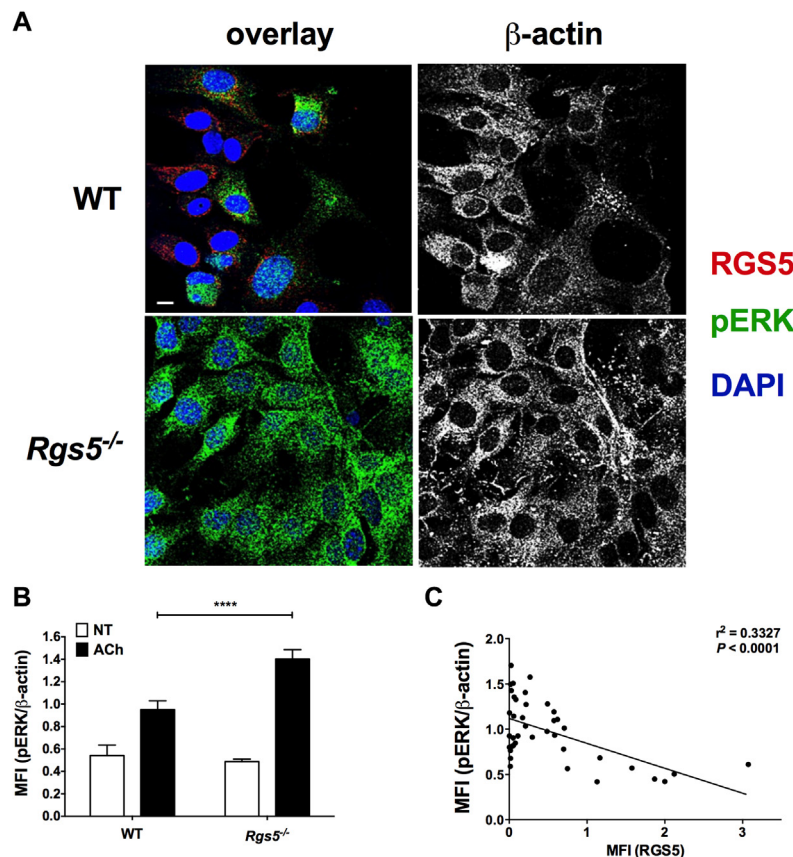


FIG 2. GPCR-induced Erk activation correlates inversely with RGS5 expression in mtASM. **A**, Cells were serum starved for 48 hours and stimulated with acetylcholine (ACh; 10 μ mol/L) for 30 minutes. Cells were immunostained, and indicated nuclei were stained with DAPI (original magnification $\times 63$; scale bar = 10 μ m). **B**, Mean fluorescence intensity (MFI) of pErk/ β -actin (RGS5⁺ cells only; mean $\pm$ SEM of 30–40 cells per group). **** $P < .0001$. **C**, MFI of pErk/ β -actin staining in a range of WT mtASM cells was plotted against the MFI of RGS5 (43 cells).

IL-33 is a major inducer of *Il13* expression in murine models of asthma.^{17,18} Both human and mouse ASM produce IL-33,¹⁹ and *Il13* expression is increased in ASM from patients with severe asthma.²⁰ We detected a 2.6-fold increase in *Il13* expression in whole lungs of WT *A fumigatus*-challenged mice compared with that seen in PBS-treated mice (Fig 4, E), whereas upregulation of *Il13* was reduced by 33% in similarly treated *Rgs5*^{-/-} mice ($P < .0001$). Recent work indicates that *Aspergillus* species components induce proinflammatory cytokine expression through Toll-like receptor (TLR) stimulation.^{21,22}

Accordingly, LPS (a TLR4 agonist) induced robust *Il13* expression in human or mouse ASM (Fig 4, F and H). TLR-evoked cytokine expression is mediated in part by phosphoinositide 3-kinase (PI3K) signaling,²³ and RGS proteins can both positively and negatively regulate PI3K through their interaction with its p85 regulatory subunit.^{24–26} Consistent with the hypothesis that TLR-evoked *Il13* expression in ASM is PI3K dependent, LPS elicited Akt phosphorylation in human or mouse ASM (Fig 4, G, and data not shown), and the PI3K inhibitor LY294002 strongly reduced LPS-evoked *Il13* expression (Fig 4, F). LPS induced significantly less *Il13* expression in mtASM from *Rgs5*^{-/-} mice than from WT mice (Fig 4, F). Surprisingly, overexpression of RGS5 in hASM cells by transduction with tat-RGS5-GFP (see Fig E7 in this article's Online Repository at www.jacionline.org) also inhibited

LPS-induced *Il13* expression compared with that seen in untreated cells or those transduced with tat-GFP alone (Fig 4, H). IL-33 induction in epithelial cells was also decreased in lungs of *Rgs5*^{-/-} mice relative to WT mice after *A fumigatus* challenge (see Fig E8 in this article's Online Repository at www.jacionline.org). Taken together, these results indicate that RGS5 levels markedly influence lung IL-33 expression in the context of allergic inflammation.

RGS5 deficiency evokes allergen-independent AHR

To examine how the loss of RGS5 affects pulmonary function *in vivo*, we measured total lung resistance (R_L) on naive anesthetized mice and after methacholine inhalation. Although R_L and dynamic compliance were similar in untreated WT and *Rgs5*^{-/-} mice before methacholine treatment, *Rgs5* gene deletion resulted in substantially increased resistance (Fig 5, A) and reduced dynamic compliance (Fig 5, B) in response to a range of methacholine concentrations. We also examined the effects of RGS5 deficiency on *A fumigatus*-induced AHR and found little difference between responses to *A fumigatus* in WT and *Rgs5*^{-/-} mice (data not shown). These results indicate that the loss of RGS5 evoked spontaneous AHR in untreated mice.

To evaluate the effect of reduced lung IL-13 concentrations on AHR in allergen-challenged *Rgs5*^{-/-} mice, we measured R_L after

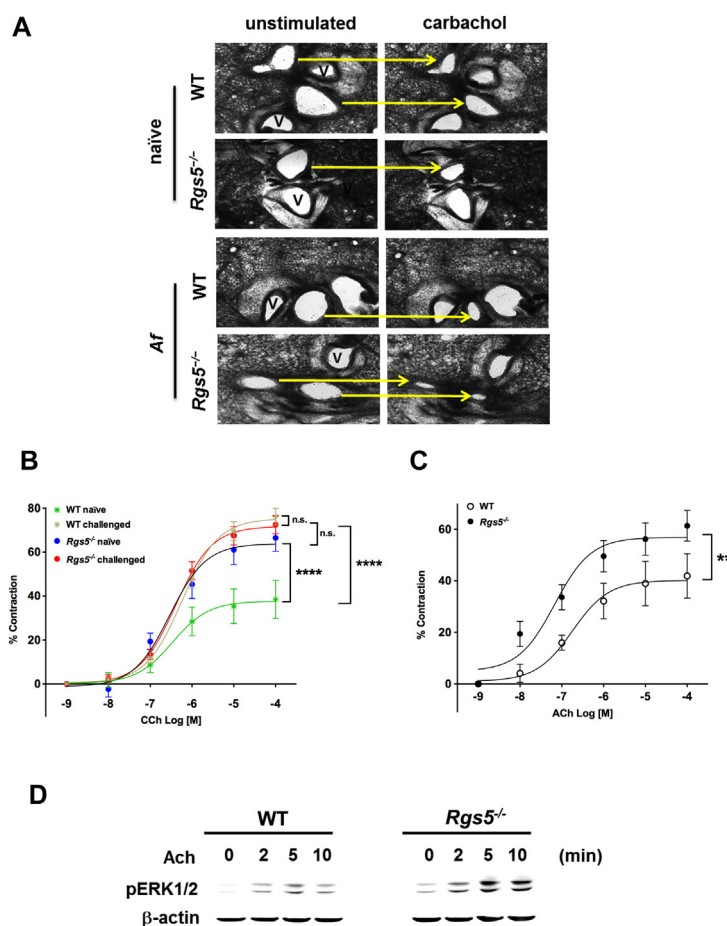


FIG 3. RGS5 regulates PCLS contraction independently of allergen challenge. **A-C**, WT and *Rgs5*^{-/-} mice were sensitized and challenged with PBS or *A fumigatus*, followed by measurement of PCLS contraction induced by carbachol (CCh; Fig 3, A and B) or acetylcholine (ACh; Fig 3, C). Arrows show contracting airways. V, Vessel. Graphs show percentage contraction (mean $\pm$ SEM from 4 mice [25–50 airways in total] per group). ***P* = .004 and *****P* < .0001. n.s., Not significant. **D**, Mouse PCLSs prepared from WT or *Rgs5*^{-/-} mice were serum starved for 48 hours and left untreated (0 minutes) or stimulated with acetylcholine (ACh; 10 μ M/L) for the indicated times, followed by immunoblotting of lysates with the indicated antibodies.

intratracheal IL-13 instillation. IL-13 did not induce significant changes in inflammatory cell composition in BALF (see Fig E9 in this article's Online Repository at www.jacionline.org) but did enhance methacholine-evoked R_L compared with saline alone, particularly at the highest methacholine concentration (R_L saline vs IL-13 in WT mice = 5.16 ± 1.3 cm H₂O $\cdot$ s/mL vs 11.62 ± 3.5 [*P* < .0001]; R_L saline vs IL-13 in *Rgs5*^{-/-} mice = 7.3 ± 1.5 vs 15.29 ± 3.9 [*P* < .0001 for either strain]). However, there was no significant difference between IL-13-treated WT and *Rgs5*^{-/-} mice in response to methacholine (Fig 5, C). These results indicate that lung structural cells respond to IL-13, as expected in the absence of RGS5.

DISCUSSION

This study supports a role for RGS5 in the pathophysiology of asthma. Depletion of RGS5 in mice augments GPCR-triggered Ca²⁺ signaling, promoting spontaneously increased R_L and exaggerated airway contraction after methacholine challenge. Some parameters of allergen-induced inflammation (eg, *Il13* and *Il33* expression) were actually decreased in *Rgs5*^{-/-} mice relative to

WT, indicating that the loss of RGS5 enhances AHR specifically by increasing ASM contractility.

Although asthma is considered an inflammatory disease typified by eosinophilia and T_H2 immune responses^{27,28} and although inflammation promotes AHR,²⁹ the diagnosis of asthma can be made in the absence of inflammation. A subset of asthmatic patients with prominent neutrophilic airway inflammation respond poorly to inhaled corticosteroids,³⁰ suggesting that intrinsic abnormalities in lung structural cells, including ASM, underlie disease pathogenesis in some cases. Although increased expression of proteins of the contractile apparatus has been previously reported in ASM from asthmatic patients,^{31–34} we did not detect differential expression of PLC β , G α_q , G $\alpha_{i1/2}$, G α_{i3} , and MLC in mtASM cells from WT and *Rgs5*^{-/-} mice, suggesting that AHR resulted from increased activity of excitation-contraction signaling pathways. Our study suggests that dysregulated bronchoconstrictor signaling caused by abnormal RGS protein expression can contribute to AHR in asthma independently of the inflammatory response. Similar uncoupling of airway inflammation and AHR caused by ozone has also been reported.³⁵

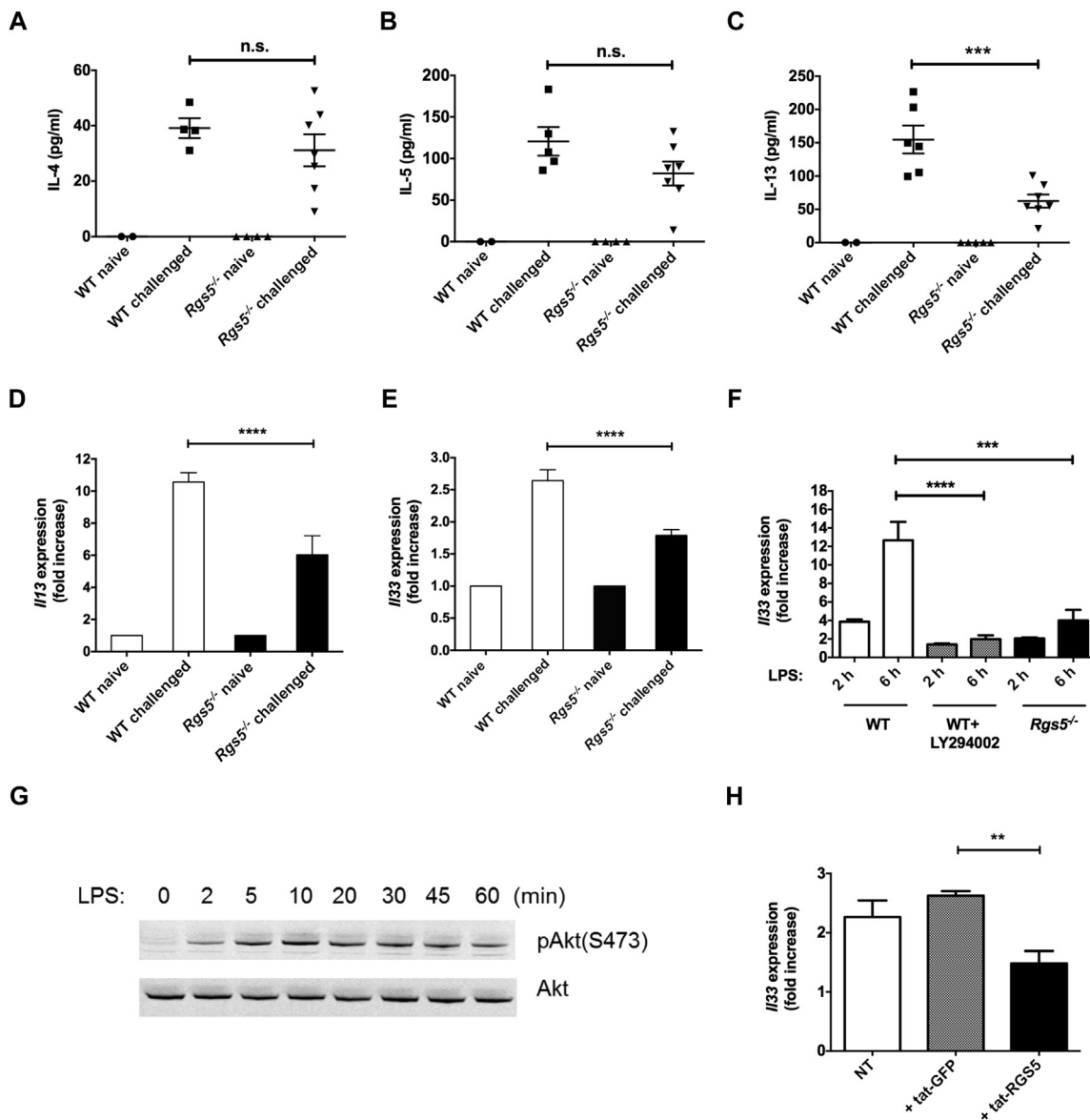


FIG 4. RGS5 regulates IL-13 production after allergen challenge through IL-33. **A-C**, BALF cytokine concentrations (mean $\pm$ SEM of 3-8 mice per group). *** P < .001. n.s., Not significant. **D** and **E**, *Il13* (Fig 4, **D**) or *Il33* (Fig 4, **E**) expression in lungs from naive and *A fumigatus*-challenged WT and *Rgs5*^{-/-} mice determined by means of quantitative PCR. **F**, LPS-induced *Il33* expression in mtASM cells. **G**, LPS-induced Akt phosphorylation in hASM cells (representative of 3 independent experiments). **H**, LPS-evoked *Il33* expression in hASM cells left untreated (NT) or transduced with tat-GFP or tat-RGS5-GFP. Results in Fig 4, **D** through **F** and **H**, are represented as the fold increase relative to that seen in naive or untreated control animals and set as 1 (mean $\pm$ SEM of 3-8 mice per group [Fig 4, **D-E**]). Results are from 2 to 3 experiments using cells pooled from 7 mice of each genotype (Fig 4, **F**) or 2 experiments assayed in triplicate (Fig 4, **H**). ** P < .004, *** P = .0004, and **** P < .0001, 1-way ANOVA.

RGS5 did not affect bronchoconstrictor-induced signaling universally; in fact, RGS5 deficiency augmented Ca²⁺ responses to acetylcholine and bradykinin but had little effect on those to thrombin and serotonin in mtASM. PCLSs from *Rgs5*^{-/-} mice contracted more than those from WT mice after application of the muscarinic receptor ligands acetylcholine and carbachol; however, the poor contractile response of mouse PCLSs to agonists other than acetylcholine/carbachol, including bradykinin,⁹ precluded a more thorough assessment of the regulation of pro-contractile receptors by RGS5 *ex vivo*. Receptor- or cell type–

specific features of RGS proteins could account for this phenotype. A published report demonstrated that RGS5 diminished angiotensin II type 1A but not carbachol-mediated mitogen-activated protein kinase activation in rat aortic smooth muscle cells.³⁶ Second, thrombin receptor (PAR1) and some receptors for serotonin might induce Ca²⁺ flux through activation of both G α_q and G $\alpha_{12/13}$.^{37,38} Because RGS5 regulates G α_q but not G $\alpha_{12/13}$, the overall responses to these agonists might be minimally affected by the loss of RGS5. Third, RGS5 expression is unequally distributed among ASM cells in mice and human

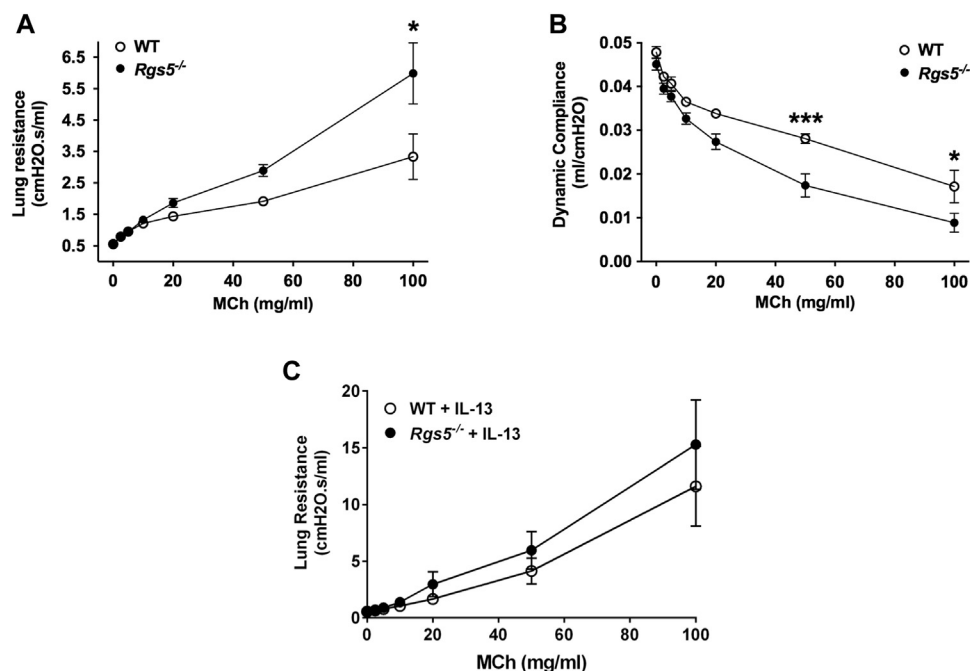


FIG 5. *Rgs5*^{-/-} mice have spontaneous AHR. **A** and **B**, R_L (Fig 5, **A**) and dynamic compliance (Fig 5, **B**) in response to increasing doses of aerosolized methacholine (MCh) in naive BALB/c WT and *Rgs5*^{-/-} mice (mean $\pm$ SEM of 4 mice per group). * $P < .05$ and *** $P < .001$. **C**, R_L in naive mice after intratracheal instillation of recombinant IL-13 (n = 4 mice per genotype).

subjects,⁹ which was confirmed in this study. Future work might allow more precise comparison of responses to other procontractile agonists in WT and RGS5-deficient cells through single-cell analysis.

Surprisingly, we did not detect increased contraction of lung slices from *A fumigatus*-challenged *Rgs5*^{-/-} mice compared with those from WT mice. Although PCLSs from *Rgs5*^{-/-} mice might be maximally responsive to carbachol at baseline, our results suggest that RGS5 also regulates production of IL-13, which augments ASM contractility downstream of GPCRs by upregulating expression of RhoA and RhoA kinase.^{39,40} The equivalent responses of WT and *Rgs5*^{-/-} mice to inhaled IL-13 also indicate that RGS5 principally controls GPCR-mediated airway constriction. Moreover, reduced IL-13 quantities in lungs of *A fumigatus*-challenged *Rgs5*^{-/-} mice might mitigate the AHR induced by allergen exposure.

Innate lymphoid type 2 cells appear to be the main source of lung IL-13, and epithelium- and ASM-derived IL-33 is a prominent inducer of IL-13 expression in innate lymphoid type 2 cells.^{17,19} *Il13* expression in *A fumigatus*-challenged lungs from *Rgs5*^{-/-} mice was significantly less than that seen in control animals, suggesting that RGS5 regulates *Il13* expression indirectly through IL-33. Because RGS5 overexpression also inhibited LPS-induced *Il13* expression in ASM, however, it might control this response in a complex manner (Fig 4, *F-H*). Published studies have indicated that PI3K both positively²³ and negatively⁴¹ modulates LPS-induced proinflammatory cytokine expression, and that RGS proteins might enhance or inhibit PI3K activation in various contexts.²⁴⁻²⁶ In addition, recent work has demonstrated that TLR-induced cytokine production is inversely related to G α i2 activity,^{42,43} which is profoundly affected by RGS5 levels.

Furthermore, apoptosis and necrosis promote processing, secretion, and activation of IL-33 protein.⁴⁴ In a study of mouse ovarian cancer cells *in vivo*, RGS5 levels correlated with increased survival and increased tumor cell necrosis.⁴⁵ Here we found that ASM cells devoid of RGS5 have enhanced basal viability and increased resistance to necrosis-inducing agents (H₂O₂) than their WT counterparts (data not shown), suggesting an additional posttranscriptional mechanism of IL-33 regulation by RGS5. The cause of reduced IL-33 expression in lung epithelial cells of *Rgs5*^{-/-} mice relative to WT mice (see Fig E8) is less clear because these cells do not express RGS5. We speculate that reduced secretion of IL-33-inducing cytokines (eg, TNF- α) by RGS5-deficient ASM could indirectly attenuate epithelial IL-33 expression.

In contrast to RGS5, RGS2 and RGS4 are highly expressed in epithelial cells.^{8,26} In addition to spontaneous AHR, *Rgs2*^{-/-} mice had increased baseline ASM mass and mucin production. In separate studies RGS2 inhibited MUC5AC secretion in airway epithelial cells directly,⁴⁶ whereas RGS5 did not regulate ATP-induced *Muc5ac* expression in NCI-H292 epithelial cells.⁴⁷ We found that RGS5 deficiency did not have a substantial effect on allergen-induced airway remodeling, including mucus production, ASM mass, and subepithelial fibrosis, in mice, all of which are believed to contribute to AHR in chronic asthma in human subjects.^{48,49} The selective modulation of ASM contractility by RGS5 in the absence of inflammation suggests that it is a drug candidate for asthma. However, future strategies, including genetic targeting or overexpression of RGS5 specifically in ASM in mice and studies of RGS5 expression in subpopulations of asthmatic patients with varying disease characteristics, are needed to fully elucidate its role in the pathophysiology of this disease and its feasibility as a therapeutic target.

Key messages

- RGS5 is selectively expressed in ASM within the lung and negatively regulates procontractile GPCR signaling.
- Loss of RGS5 promotes baseline AHR in the absence of allergen exposure.
- Dysregulated GPCR signaling in ASM can underlie asthmatic endotypes that do not feature prominent inflammation.

REFERENCES

- Chung F, Adcock I. Asthma: application of cell and molecular biology techniques to unravel causes and pathophysiological mechanisms. *Methods Mol Med* 2000; 44:1-29.
- Penn RB. Embracing emerging paradigms of G protein-coupled receptor agonism and signaling to address airway smooth muscle pathobiology in asthma. *Naunyn Schmiedeberg Arch Pharmacol* 2008;378:149-69.
- Kang SH, Kim HY, Seo JH, Kwon JW, Jung YH, Song YH, et al. Bronchial hyper-responsiveness to methacholine and AMP in children with atopic asthma. *Allergy Asthma Immunol Res* 2012;4:341-5.
- Chen C, Kudo M, Rutaganira F, Takano H, Lee C, Atakilit A, et al. Integrin $\alpha 9 \beta 1$ in airway smooth muscle suppresses exaggerated airway narrowing. *J Clin Invest* 2012;122:2916-27.
- Katsumoto TR, Kudo M, Chen C, Sundaram A, Callahan EC, Zhu JW, et al. The phosphatase CD148 promotes airway hyperresponsiveness through SRC family kinases. *J Clin Invest* 2013;123:2037-48.
- Bartho L, Benko R. Should antihistamines be re-considered as antiasthmatic drugs as adjuvants to anti-leukotrienes? *Eur J Pharmacol* 2013;701:181-4.
- Bansal G, Druey KM, Xie Z. R4 RGS proteins: regulation of G-protein signaling and beyond. *Pharmacol Ther* 2007;116:473-95.
- Xie Y, Jiang H, Nguyen H, Jia S, Berro A, Panettieri RA Jr, et al. Regulator of G protein signaling 2 is a key modulator of airway hyperresponsiveness. *J Allergy Clin Immunol* 2012;130:968-76.e3.
- Yang Z, Balenga N, Cooper PR, Damera G, Edwards R, Brightling CE, et al. Regulator of G-protein signaling-5 inhibits bronchial smooth muscle contraction in severe asthma. *Am J Respir Cell Mol Biol* 2012;46:823-32.
- Yang Z, Cooper PR, Damera G, Mukhopadhyay I, Cho H, Kehrl JH, et al. Beta-agonist-associated reduction in RGS5 expression promotes airway smooth muscle hyper-responsiveness. *J Biol Chem* 2011;286:11444-55.
- Labuda M, Laberge S, Briere J, Berube D, Krajcinovic M. RGS5 gene and therapeutic response to short acting bronchodilators in paediatric asthma patients. *Pediatr Pulmonol* 2013;48:970-5.
- Bodenstein J, Sunahara RK, Neubig RR. N-terminal residues control proteasomal degradation of RGS2, RGS4, and RGS5 in human embryonic kidney 293 cells. *Mol Pharmacol* 2007;71:1040-50.
- Xie Y, Wolff DW, Wei T, Wang B, Deng C, Kirui JK, et al. Breast cancer migration and invasion depend on proteasome degradation of regulator of G-protein signaling 4. *Cancer Res* 2009;69:5743-51.
- Mansfield PJ, Shayman JA, Boxer LA. Regulation of polymorphonuclear leukocyte phagocytosis by myosin light chain kinase after activation of mitogen-activated protein kinase. *Blood* 2000;95:2407-12.
- Zhu HQ, Cheng XW, Xiao LL, Jiang ZK, Zhou Q, Gui SY, et al. Melatonin prevents oxidized low-density lipoprotein-induced increase of myosin light chain kinase activation and expression in HUVEC through ERK/MAPK signal transduction. *J Pineal Res* 2008;45:328-34.
- Albeck JG, Mills GB, Brugge JS. Frequency-modulated pulses of ERK activity transmit quantitative proliferative signals. *Mol Cell* 2013;49:249-61.
- Wills-Karp M, Finkelman FD. Innate lymphoid cells wield a double-edged sword. *Nat Immunol* 2011;12:1025-7.
- Kamijo S, Takeda H, Tokura T, Suzuki M, Inui K, Hara M, et al. IL-33-mediated innate response and adaptive immune cells contribute to maximum responses of protease allergen-induced allergic airway inflammation. *J Immunol* 2013;190:4489-99.
- Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;23:479-90.
- Prefontaine D, Lajoie-Kadoch S, Foley S, Audusseau S, Olivenstein R, Halayko AJ, et al. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol* 2009;183:5094-103.
- Millien VO, Lu W, Shaw J, Yuan X, Mak G, Roberts L, et al. Cleavage of fibrinogen by proteinases elicits allergic responses through Toll-like receptor 4. *Science* 2013;341:792-6.
- Shaw JL, Fakhri S, Citardi MJ, Porter PC, Corry DB, Kheradmand F, et al. IL-33-responsive innate lymphoid cells are an important source of IL-13 in chronic rhinosinusitis with nasal polyps. *Am J Respir Crit Care Med* 2013;188:432-9.
- Li X, Tupper JC, Bannerman DD, Winn RK, Rhodes CJ, Harlan JM. Phosphoinositide 3 kinase mediates Toll-like receptor 4-induced activation of NF-kappa B in endothelial cells. *Infect Immun* 2003;71:4414-20.
- Bansal G, Xie Z, Rao S, Nocka KH, Druey KM. Suppression of immunoglobulin E-mediated allergic responses by regulator of G protein signaling 13. *Nat Immunol* 2008;9:73-80.
- Liang G, Bansal G, Xie Z, Druey KM. RGS16 inhibits breast cancer cell growth by mitigating phosphatidylinositol 3-kinase signaling. *J Biol Chem* 2009;284:21719-27.
- Damera G, Druey KM, Cooper PR, Krymskaya VP, Soberman RJ, Amrani Y, et al. An RGS4-mediated phenotypic switch of bronchial smooth muscle cells promotes fixed airway obstruction in asthma. *PLoS One* 2012;7:e28504.
- Leckie MJ, ten Brinke A, Khan J, Diamant Z, O'Connor BJ, Walls CM, et al. Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000; 356:2144-8.
- Borish LC, Nelson HS, Lanz MJ, Claussen L, Whitmore JB, Agosti JM, et al. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. *Am J Respir Crit Care Med* 1999;160:1816-23.
- Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, Karp CL, et al. Interleukin-13: central mediator of allergic asthma. *Science* 1998;282:2258-61.
- Green RH, Brightling CE, Woltmann G, Parker D, Wardlaw AJ, Pavord ID. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 2002;57:875-9.
- Sakai H, Chiba Y, Hirano T, Misawa M. Possible involvement of CPI-17 in augmented bronchial smooth muscle contraction in antigen-induced airway hyper-responsive rats. *Mol Pharmacol* 2005;68:145-51.
- Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, et al. IL-17A produced by α beta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. *Nat Med* 2012; 18:547-54.
- McGraw DW, Elwing JM, Fogel KM, Wang WC, Glinka CB, Mihlbachler KA, et al. Crosstalk between Gi and Gq/Gs pathways in airway smooth muscle regulates bronchial contractility and relaxation. *J Clin Invest* 2007;117:1391-8.
- Penn RB, Benovic JL. Regulation of heterotrimeric G protein signaling in airway smooth muscle. *Proc Am Thorac Soc* 2008;5:47-57.
- Cooper PR, Mesaros AC, Zhang J, Christmas P, Stark CM, Douaidy K, et al. 20-HETE mediates ozone-induced, neutrophil-independent airway hyper-responsiveness in mice. *PLoS One* 2010;5:e10235.
- Wang Q, Liu M, Mullah B, Siderovski DP, Neubig RR. Receptor-selective effects of endogenous RGS3 and RGS5 to regulate mitogen-activated protein kinase activation in rat vascular smooth muscle cells. *J Biol Chem* 2002;277: 24949-58.
- Hannon J, Hoyer D. Molecular biology of 5-HT receptors. *Behav Brain Res* 2008; 195:198-213.
- Price RD, Weiner DM, Chang MS, Sanders-Bush E. RNA editing of the human serotonin 5-HT_{2C} receptor alters receptor-mediated activation of G13 protein. *J Biol Chem* 2001;276:44663-8.
- Chiba Y, Nakazawa S, Todoroki M, Shinozaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. *Am J Respir Cell Mol Biol* 2009;40:159-67.
- Goto K, Chiba Y, Matsusue K, Hattori Y, Maitani Y, Sakai H, et al. The proximal STAT6 and NF-kappaB sites are responsible for IL-13- and TNF-alpha-induced RhoA transcriptions in human bronchial smooth muscle cells. *Pharmacol Res* 2010;61:466-72.
- Luyendyk JP, Schabbauer GA, Tencati M, Holscher T, Pawlinski R, Mackman N. Genetic analysis of the role of the PI3K-Akt pathway in lipopolysaccharide-induced cytokine and tissue factor gene expression in monocytes/macrophages. *J Immunol* 2008;180:4218-26.
- Fan H, Li P, Zingarelli B, Borg K, Halushka PV, Birnbaumer L, et al. Heterotrimeric G α (i) proteins are regulated by lipopolysaccharide and are

- anti-inflammatory in endotoxemia and polymicrobial sepsis. *Biochim Biophys Acta* 2011;1813:466-72.
43. Li P, Neubig RR, Zingarelli B, Borg K, Halushka PV, Cook JA, et al. Toll-like receptor-induced inflammatory cytokines are suppressed by gain of function or overexpression of Galpha(i2) protein. *Inflammation* 2012;35:1611-7.
 44. Miller AM. Role of IL-33 in inflammation and disease. *J Inflamm (Lond)* 2011; 8:22.
 45. Altman MK, Nguyen DT, Patel SB, Fambrough JM, Beedle AM, Hardman WJ, et al. Regulator of G-protein signaling 5 reduces HeyA8 ovarian cancer cell proliferation and extends survival in a murine tumor model. *Biochem Res Int* 2012;2012: 518437.
 46. Liu C, Li Q, Zhou X, Kolosov VP, Perelman JM. Regulator of G-protein signaling 2 inhibits acid-induced mucin5AC hypersecretion in human airway epithelial cells. *Respir Physiol Neurobiol* 2013;185:265-71.
 47. Song KS, Kim HJ, Kim K, Lee JG, Yoon JH. Regulator of G-protein signaling 4 suppresses LPS-induced MUC5AC overproduction in the airway. *Am J Respir Cell Mol Biol* 2009;41:40-9.
 48. Holgate ST. The sentinel role of the airway epithelium in asthma pathogenesis. *Immunol Rev* 2011;242:205-19.
 49. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167:1360-8.

METHODS

Mouse strains and asthma model

C57BL/6 *Rgs5*^{-/-} mice were generated, as described previously,^{E1} and backcrossed onto a BALB/c background for 10 generations. *Rgs5*^{-/-} mice and their WT littermates were used for all experiments. Acute allergen-induced airway inflammation was established by sensitizing 8- to 10-week-old female mice intraperitoneally with 20 μ g of *A fumigatus* extract (Hollister-Stier, Spokane, Wash) mixed with 20 mg of Alum (Imject Alum; Pierce, Rockford, Ill) on days 0 and 14. Mice were challenged intranasally with PBS (naive) or *A fumigatus* extract (25 μ g, challenged) on days 25, 26, and 27. Twenty-four hours after the last challenge, mice were killed and used for experiments. Intratracheal instillation of IL-13 (3 μ g in 30 μ L of saline) was performed after ketamine/xylazine (100/20 mg/kg) anesthesia. All studies were run in accordance with institutional guidelines provided by the National Institute of Allergy and Infectious Diseases Animal Use and Care Committee under an approved study (ASP LAD3e).

Reagents and antibodies

Antibodies used in this study were purchased from the following sources: mouse anti-ERK1/2, rabbit anti-pERK1/2, rabbit anti-Akt, mouse anti-phosphoAkt (Ser473), rabbit anti-MLC, rabbit anti- β -arrestin 1/2 were all from Cell Signaling Technology (Danvers, Mass); mouse anti- β -actin, goat anti-smooth muscle α -actin (for immunoblotting), and mouse anti-smooth muscle α -actin (for immunofluorescence) were from Sigma (St Louis, Mo); mouse anti-glyceraldehyde-3-phosphate dehydrogenase and rabbit anti-B2R were from Abcam (Cambridge, United Kingdom); mouse anti-PLC β was from BD Biosciences (San Jose, Calif); mouse anti-PAR1 was from R&D Systems (Minneapolis, Minn); goat anti-AChR was from Santa Cruz Biotechnology (Santa Cruz, Calif); rabbit anti-5HT2A was from antibodiesonline.com; rabbit anti-G $\alpha_{q/11}$, rabbit anti-G $\alpha_{i/2}$, and rabbit anti-G α_{i3} were from EMD Biosciences (San Diego, Calif); rabbit anti-RGS4 was from Millipore (Temecula, Calif); and chicken anti-RGS5 was from ProSci (Loveland, Colo). Carbachol, acetylcholine, bradykinin, serotonin, thrombin, ionomycin, thapsigargin, BSA, LPS, and Triton X-100 were purchased from Sigma. LY294002 was purchased from EMD Biosciences.

Invasive measurement of lung function in live anesthetized mice

R_L , dynamic compliance, elastance, tissue damping, tissue elastance, and airway resistance were recorded.^{E2} Briefly, mice were anesthetized with an intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (20 mg/kg) mixture. After anesthesia, a 0.5-cm incision was performed from the rostral to caudal direction. The flap of the skin was retracted, connective tissue was dissected away, and the trachea was exposed. The trachea was then cannulated between the second and third cartilage rings with a blunt-end stub adapter and secured with suture. The mouse was connected to the FlexiVent system (SCIREQ, Montreal, Quebec, Canada), and spontaneous respirations were terminated with an intramuscular injection of pancuronium bromide (3 mg/kg). Parameters of mechanical ventilation included a rate of 150 breaths/min and a 0.25-mL tidal volume. The respiratory mechanics were measured, as previously described. Airway responsiveness was measured after inhalation of nebulized saline and increasing concentrations of nebulized methacholine (0, 2.5, 5, 10, 20, 50, and 100 mg/mL).

PCLS preparation and measurement of airway contraction

Mice were killed by means of isoflurane inhalation and gentle cervical dislocation. After exposure of the trachea, silk sutures (Havel's, Cincinnati, Ohio) were threaded underneath the trachea, and the upper part of the trachea was nicked with a scalpel. Lungs were inflated with 1 mL of SeaPlaque low-melting temperature agarose (2% wt/vol in PBS without Ca²⁺ and Mg²⁺; Lonza, Basel, Switzerland) by using a blunt-ended needle inserted into the tracheal nick. Agarose was then flushed into the alveolar spaces by using 200 μ L room air. Lungs were cooled on ice for 10 minutes to solidify agarose, followed by excision of the left lobe and embedding in mold-plunger assembly

of the Tissue Embedding Unit (Alabama Research and Development) containing 2% agarose for 15 minutes. The hardened samples were transferred to a Krumdieck Tissue Slicer MD4000 (Alabama Research and Development) with a plunger, and 200- μ m-thick slices were prepared. Slices containing intact bronchi (diameter, 250–450 μ m) were washed several times and incubated overnight in serum-free Ham F-12 medium at 37°C with a 5% CO₂ incubator. Slices were weighed down with platinum wire-containing nylon threads (Harvard Apparatus, Holliston, Mass). Images were obtained with a Leica DMI400 inverted microscope ($\times 5$ objective; Leica, Wetzlar, Germany) equipped with Retiga2000R camera (QImaging, Surrey, British Columbia, Canada). The baseline airway area was measured with Image-Pro Plus software (Media Cybernetics, Rockville, Md). Slices were stimulated with increasing concentrations of agonists (10⁻⁹ to 10⁻⁴ mol/L), and the airway luminal area was assessed after stimulation with each agonist concentration for 4 minutes. Contraction was calculated as a percentage of the baseline luminal area.

Mouse tracheal smooth muscle cell isolation and immunostaining

Naive WT and *Rgs5*^{-/-} mice (n = 5 in each group) were killed, followed by excision of tracheas using aseptic conditions. The trachea was separated from adjacent epithelium and connective tissues, and single-cell suspensions were prepared with a papain dissociation kit (Worthington Biochemical, Lakewood, NJ) supplemented with Collagenase D, Dispase II, and DNase I. Cells were cultured in Ham F-12 medium supplemented with 10% FBS, penicillin, streptomycin, and amphotericin. The purity of mtASM cells was evaluated by means of immunofluorescence staining with anti-smooth muscle α -actin antibody. Cells were seeded on 4-well chamber slides (Lab-Tek, Nunc), washed with PBS, and fixed in 3.7% paraformaldehyde for 10 minutes. Cells were permeabilized with 0.3% Triton X-100 for 5 minutes and washed once with PBS. The Mouse on Mouse basic kit (MOM; Vector Laboratories, Burlingame, Calif) was used to block Fc receptor binding, followed by incubation with mouse anti-smooth muscle α -actin, biotinylated anti-mouse IgG, and Texas red-conjugated streptavidin. 4'-6-Diamidino-2-phenylindole dihydrochloride (DAPI)-counterstained images were obtained with a Leica SP5 confocal microscope.

hASM preparation and protein transduction

hASM cells were extracted from lungs of healthy donors obtained postmortem from the National Disease Research Interchange (NDRI; Philadelphia, Pa), as previously described.^{E3} Plasmids encoding tat-GFP and tat-RGS5-GFP were constructed by means of PCR with pEGFP and pEGFP-RGS5 as templates. tat fusion proteins were prepared essentially as described previously,^{E4} and cells were incubated with tat proteins at a final concentration of 250 nmol/L for 1 to 2 hours before further cell manipulations.

Measurement of intracellular Ca²⁺ mobilization

mtASM cells from WT and *Rgs5*^{-/-} mice were seeded (2 $\times$ 10⁴ cells/well) in black clear-bottom 96-well plates (Corning, Corning, NY), allowed to attach for 48 hours, and serum starved in Ham F-12 medium for 24 hours before measurement of intracellular Ca²⁺ concentration. Ca²⁺ indicator dye (FLIPR Calcium 3 Assay; Molecular Devices, Sunnyvale, Calif) was added to each well, followed by incubation for 1 hour at 37°C with 5% CO₂. Thapsigargin, ionomycin, or serial dilutions of GPCR agonists (acetylcholine, bradykinin, serotonin, or thrombin) were prepared in a separate plate at 5 $\times$ concentrations for robotic addition to cells with the FLEXStation II fluorimeter (Molecular Devices). Relative fluorescence units were measured every 1.5 seconds for a period of 160 seconds after agonist addition. Results were plotted as the maximum-minimum signal over this time period, with the minimum signal defined as the stable baseline value before agonist addition. Data were normalized to the maximal response and presented as a percentage of maximal Ca²⁺ release.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from mtASM and mouse lungs with the RNeasy Mini Kit (Qiagen, Hilden, Germany) per the manufacturer's instructions. Total

RNA (1 μ g) was reverse transcribed into cDNA with SuperScript III First Strand Synthesis Kit (Invitrogen, Carlsbad, Calif). Quantitative real-time PCR was performed with TaqMan gene expression assay probes (Life Technologies). The 20- μ L reaction contained 1 $\times$ TaqMan Universal Master Mix, 1 μ L specific gene or β -actin gene expression assay probe, and 1 μ L of cDNA. The reactions were run for 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Data were analyzed by using the $2^{-\Delta\Delta C_t}$ method.^{E5,E6} Data represent tissue derived from 4 to 8 mice or from 4 separate batches of mtASM, each derived from pooled tracheas of 5 to 7 WT or 5 *Rgs5*^{-/-} mice and assayed in 2 separate experiments in triplicate.

Immunoblotting

mtASM cells were serum starved for 48 hours, followed by stimulation with bradykinin, acetylcholine, or LPS 1 μ g/mL for indicated times. Lysates of mtASM or hASM cells were prepared by using radioimmunoprecipitation lysis buffer supplemented with protease inhibitor and phosphatase inhibitor cocktails (Roche, Mannheim, Germany). Lysates were centrifuged at 13,000 rpm for 10 minutes at 4°C, and supernatants were boiled in sample reducing buffer at 95°C for 10 minutes. Primary antibody staining was detected by using near-infrared conjugated secondary antibodies and quantified with the LI-COR Odyssey Imaging System (LI-COR Biosciences). PCLSs prepared from WT and *Rgs5*^{-/-} mice were cultured in serum-free Ham F-12 medium for 48 hours. Preparation of lysates from PCLSs was performed, as previously described,^{E7} with minor modifications. Five slices were stimulated with 200 μ L of acetylcholine (10 μ mol/L) in microcentrifuge tubes for the indicated times. Dry ice-cold acetone containing 20% trichloroacetic acid and 10 mmol/L dithiothreitol was added to each tube to stop the stimulation, and samples were kept at -20°C overnight. PCLSs were sonicated (3 cycles), and lysates were spun at 13,000g for 10 minutes at 4°C. Pellets were washed 2 times with cold acetone containing 10 mmol/L dithiothreitol, air-dried, and boiled in sample reducing buffer at 95°C for 10 minutes.

Immunofluorescence

mtASM cells derived from WT and *Rgs5*^{-/-} mice were seeded on 4-well chamber slides (Lab-Tek, Nunc). Cells were serum starved for 48 hours and then stimulated with acetylcholine (10 μ mol/L) for 30 minutes. Stimulation was terminated by addition of ice-cold PBS. Cells were then fixed with 3.7% paraformaldehyde for 10 minutes at room temperature and washed with PBS. Cells were permeabilized with 0.2% Triton X-100 for 10 minutes and then blocked in 5% BSA for 1 hour at room temperature. Cells were incubated with chicken anti-RGS5 (1:1000), rabbit anti-pERK (1:250), and mouse anti- β -actin (1:500) antibodies overnight at 4°C, followed by staining with Alexa Fluor 594 goat anti-chicken (1:1000), Alexa Fluor 488 goat anti-rabbit (1:500), and Alexa Fluor 680 goat anti-mouse (1:500) secondary antibodies. Nuclei were counterstained with DAPI. A minimum of 10 fields ($\times 63$ magnification) per condition were evaluated with a Leica SP5 X-WLL confocal microscope. The mean fluorescence intensities of RGS5, pERK, and β -actin were measured with ImageJ software. hASM cells transfected with tat proteins were washed with PBS, fixed in 4% paraformaldehyde, and imaged as above after mounting on glass cover slips with Vectashield plus DAPI, as above.

BALF cytokine and cell analysis

For cytokine analysis, lungs were perfused with 1.5 mL of ice-cold HBSS, and lavage fluid was centrifuged at 1500 rpm at 4°C for 10 minutes. BALF was stored at -80°C for cytokine assays. Red blood cells in the pellet were lysed with ACK lysis buffer. Total cell numbers were determined by using hemocytometry, and differentials were evaluated by means of light microscopic examination of a Diff-Quick (Siemens)-stained cytospin preparation

(700 cells in a minimum of 15 fields at $\times 40$ magnification). Cytokines were analyzed with a customized MilliPlex kit (Millipore) by using magnetic beads. Samples were run in duplicate in a Bio-Plex 200 system with HTF (Bio-Rad Laboratories, Hercules, Calif).

Lung histologic analysis

Lungs were perfused with 20 mL of ice-cold PBS injected into the left ventricle, followed by excision and fixation in 10% neutral buffered formalin. Sections (5 μ m) were prepared from paraffin-embedded lungs and stained with hematoxylin and eosin, periodic acid-Schiff, Masson trichrome, rabbit anti-smooth muscle α -actin, or anti-IL-33 antibodies. Images were obtained with a Leica DMI400 light microscope equipped with Retiga2000R camera (QImaging) and analyzed with QCapture software.

IL-33 immunohistochemistry

Mouse lungs were perfused with 20 mL of ice-cold PBS injected into the left ventricle, followed by excision and fixation in 10% neutral buffered formalin. Sections (5 μ m) were prepared from paraffin-embedded lungs. After deparaffinization and antigen retrieval, sections were blocked in BSA and incubated with goat anti-IL-33 antibody (R&D Systems) at a 1:100 dilution overnight. Sections were then incubated with biotinylated anti-goat antibody (1:500; Dako, Glostrup, Denmark) for 30 minutes, followed by horseradish peroxidase-conjugated streptavidin (1:400, Dako) for 30 minutes. Slides were developed with diaminobenzidine mounted with VectaMount (Vector Laboratories) with glass cover slips. Images were obtained with a Leica DMI400 light microscope equipped with Retiga2000R camera (QImaging). Images were analyzed with Image-Pro Plus software (Media Cybernetics). The intracellular stained pixels within each section were chosen by using the "Segmentation" tool, and the area of stained pixels in the section was measured by using the "Count and Measure Objects" tool and normalized to the total section area.

Statistical analysis

Data are presented as means $\pm$ SEMs, where applicable, and were analyzed by using 1- or 2-way ANOVA or the Student *t* test with PRISM software (GraphPad Software, La Jolla, Calif). We considered a *P* value of less than .05 to be statistically significant.

REFERENCES

- Cho H, Park C, Hwang IY, Han SB, Schimel D, Despres D, et al. *Rgs5* targeting leads to chronic low blood pressure and a lean body habitus. *Mol Cell Biol* 2008; 28:2590-7.
- Haczku A, Emami K, Fischer MC, Kadlecsek S, Ishii M, Panettieri RA, et al. Hyperpolarized ³He MRI in asthma measurements of regional ventilation following allergic sensitization and challenge in mice—preliminary results. *Acad Radiol* 2005;12:1362-70.
- Balg Z, Balenga N, Cooper PR, Damara G, Edwards R, Brightling CE, et al. Regulator of G-protein signaling-5 inhibits bronchial smooth muscle contraction in severe asthma. *Am J Respir Cell Mol Biol* 2012;46:823-32.
- Bansal G, Xie Z, Rao S, Nocka KH, Druey KM. Suppression of immunoglobulin E-mediated allergic responses by regulator of G protein signaling 13. *Nat Immunol* 2008;9:73-80.
- Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45.
- Balenga NA, Aflaki E, Kargl J, Platzer W, Schroder R, Blattermann S, et al. GPR55 regulates cannabinoid 2 receptor-mediated responses in human neutrophils. *Cell Res* 2011;21:1452-69.
- Mukherjee S, Trice J, Shinde P, Willis RE, Pressley TA, Perez-Zoghbi JF. Ca²⁺ oscillations, Ca²⁺ sensitization, and contraction activated by protein kinase C in small airway smooth muscle. *J Gen Physiol* 2013;141:165-78.

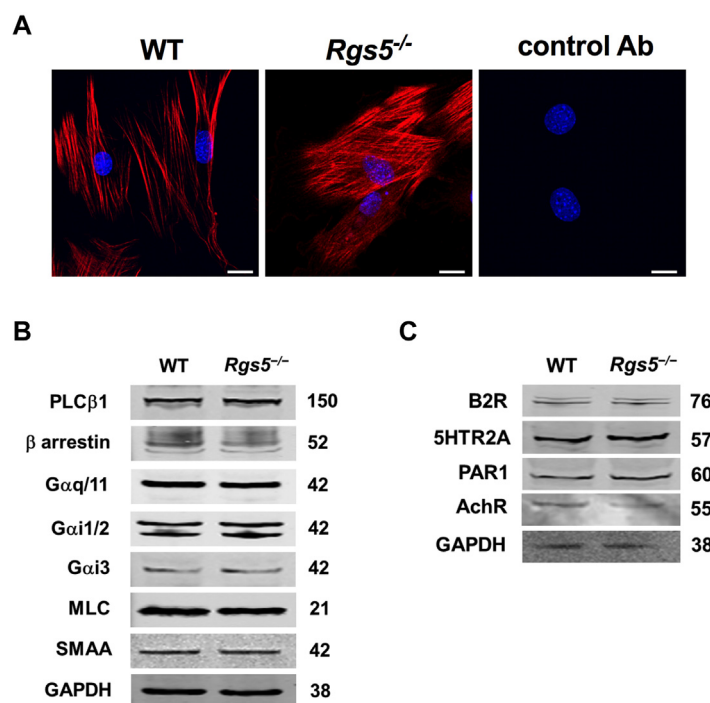


FIG E1. Phenotype of mtASM from WT and *Rgs5*^{-/-} mice. **A**, mtASM cells were stained with smooth muscle α-actin antibody (red) and DAPI (nuclei, blue; original magnification ×63). Scale bar = 10 μm. **B** and **C**, Whole-cell lysates of mtASM from WT and *Rgs5*^{-/-} mice were immunoblotted with indicated antibodies. Ab, Antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

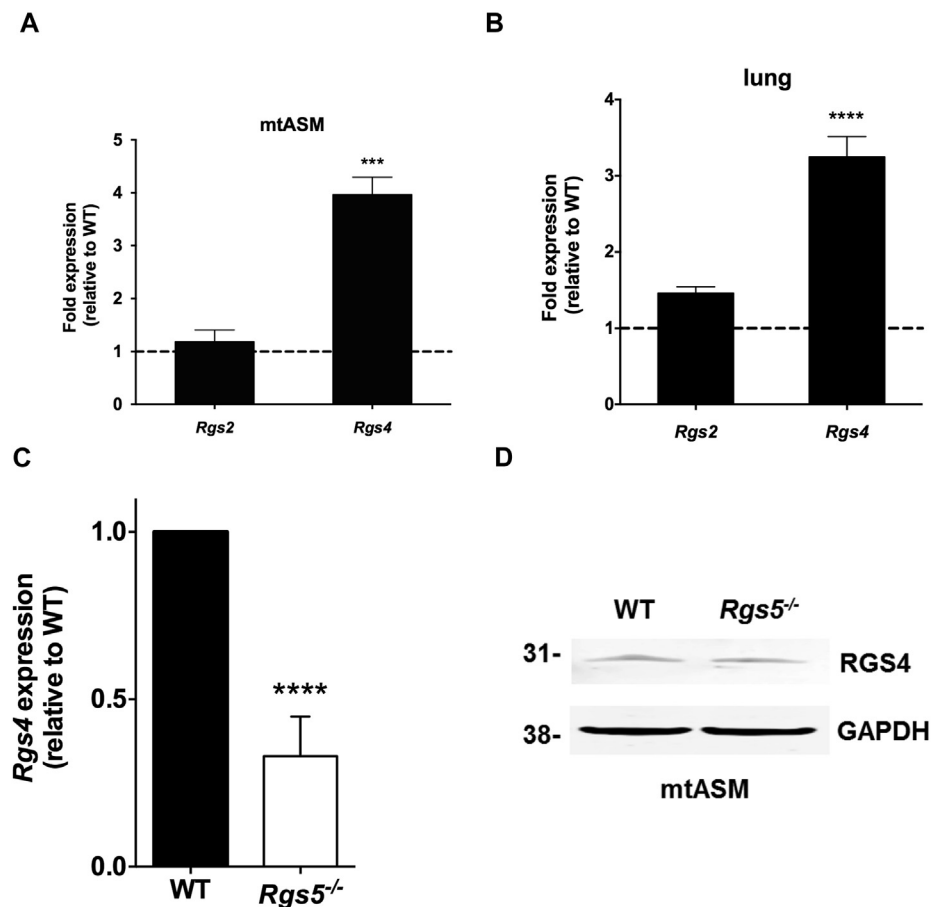


FIG E2. Expression of RGS family members in lungs and ASM devoid of RGS5. **A** and **B**, Expression of *Rgs2* and *Rgs4* in mtASM (Fig E2, A) or whole lungs (Fig E2, B) from WT and *Rgs5*^{-/-} mice was determined by means of quantitative real-time PCR. Data are presented as expression in *Rgs5*^{-/-} mice relative to WT set as 1 (mean $\pm$ SEM of 2 independent experiments with 5 mice per genotype [mtASM] or 4 mice per genotype). *** P < .0005 and **** P < .0001, paired t test. **C**, *Rgs4* expression in allergen-challenged mice. Mice were sensitized and challenged with *A. fumigatus*, as per the [Methods](#) section. RNA was extracted from whole lungs 24 hours after the final allergen challenge, and *Rgs4* expression was analyzed by using quantitative real-time PCR. **** P = .00001, t test. **D**, RGS4 amounts in ASM from WT and *Rgs5*^{-/-} mice were determined by means of immunoblotting. The blot is representative of 3 similar experiments using cells derived from 5 to 7 mice of each genotype. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

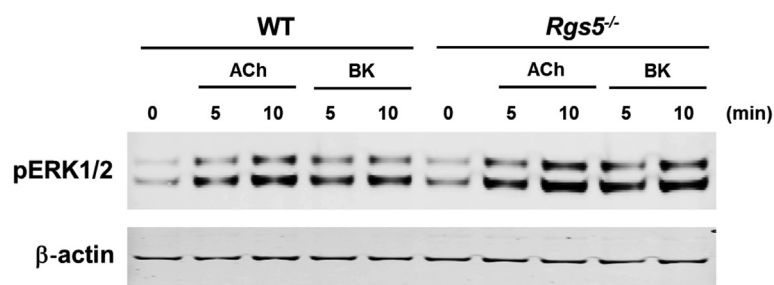


FIG E3. GPCR-induced Erk phosphorylation in pooled mtASM cells from WT and *Rgs5*^{-/-} mice. **A**, mtASM were serum starved for 48 hours and left untreated (0 minutes) or stimulated with acetylcholine (*ACh*; 10 μ mol/L) and bradykinin (*BK*; 10 nmol/L) for 5 and 10 minutes. Whole-cell lysates were immunoblotted with the indicated antibodies.

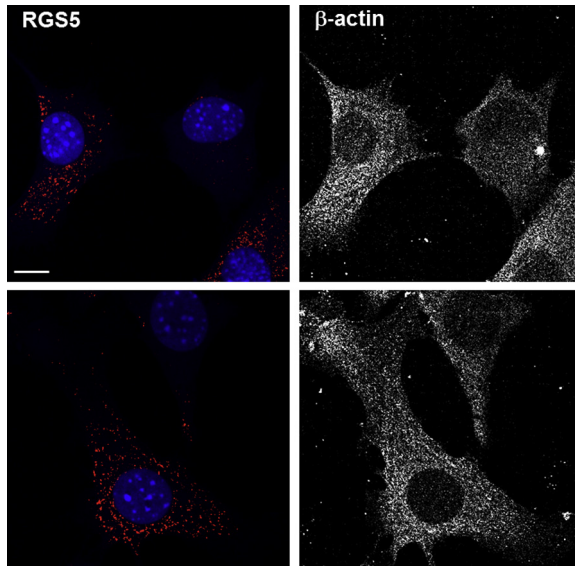


FIG E4. Variable cytosolic RGS5 expression in WT mtASM cells. mtASM cells from WT mice were fixed and stained with indicated antibodies, as outlined in the [Methods](#) section (*red* = RGS5; *white* = β -actin; *blue* = DAPI; original magnification $\times 63$). Scale bar = 20 μ m.

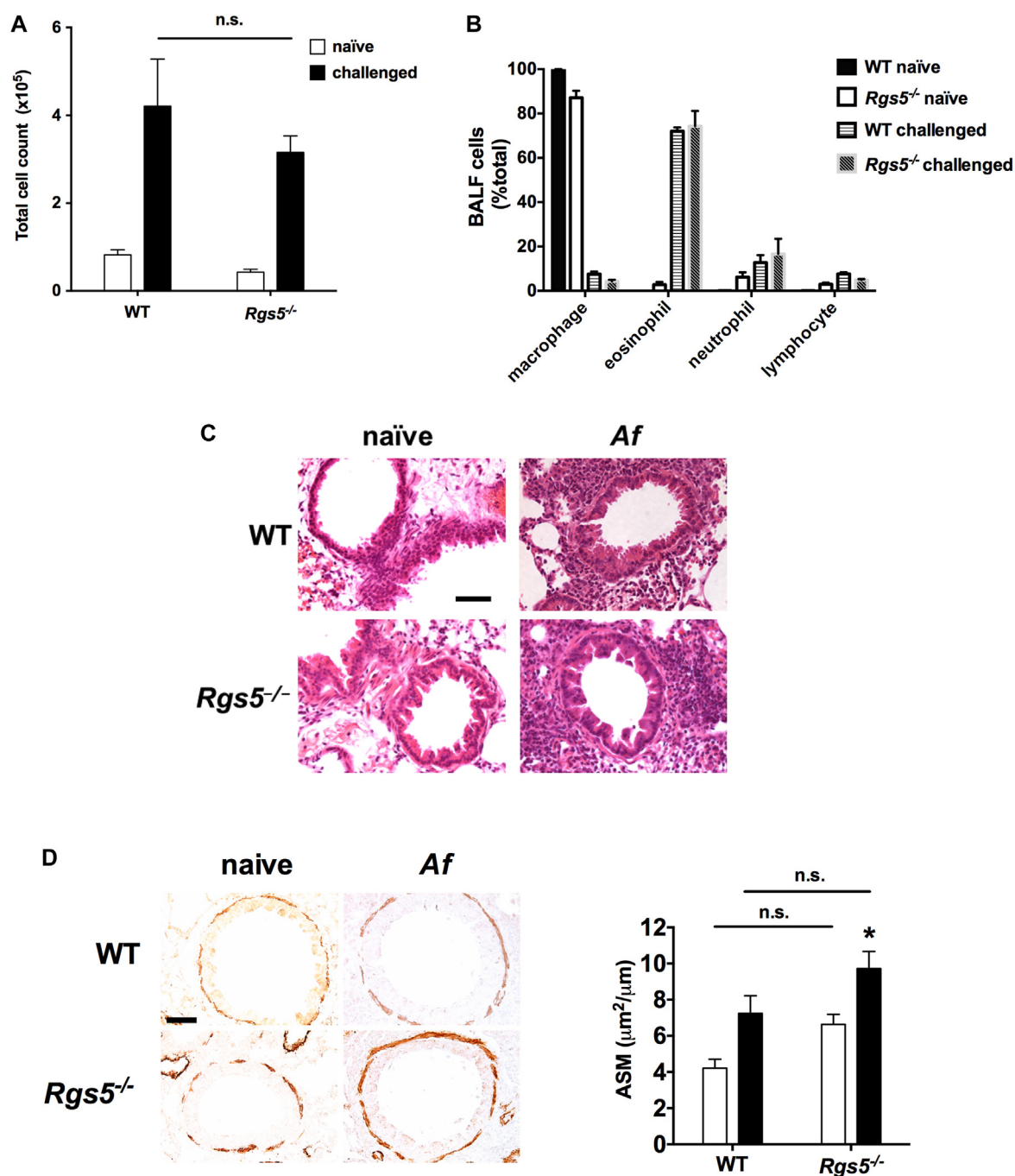


FIG E5. RGS5 does not affect lung inflammation in *A fumigatus* (*Af*)-challenged mice. **A** and **B**, Total cell counts (Fig E5, A) and differentials (Fig E5, B) were assessed in BALF cytospin preparations. Data are means $\pm$ SEMs from 3 to 9 mice per group. **C**, Histology of paraffin-embedded lungs from naïve and *A fumigatus*-challenged WT and $Rgs5^{-/-}$ mice was evaluated by using hematoxylin and eosin staining. Images are representative of 3 to 5 mice per group (original magnification $\times 40$). Scale bar = 50 μm . **D**, ASM mass was evaluated in naïve and *A fumigatus*-challenged mice by means of immunohistochemistry with smooth muscle α -actin antibody (mean $\pm$ SEM from 3-9 mice per group). n.s., Not significant. * $P < .05$, 1-way ANOVA, naïve versus challenged $Rgs5^{-/-}$ mice (original magnification $\times 40$). Scale bar = 50 μm .

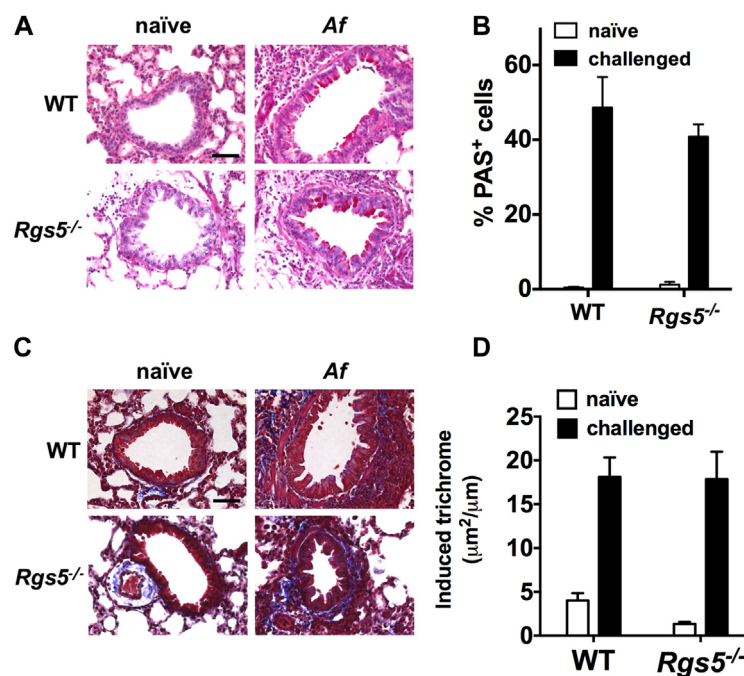


FIG E6. RGS5 does not modulate airway remodeling after acute allergen challenge. Naïve and *A fumigatus* (*Af*)-challenged WT and *Rgs5*^{-/-} mice were killed by means of isoflurane anesthesia and immediately perfused with cold PBS through the left ventricle. Paraffin-embedded lung sections were stained with periodic acid–Schiff (**A**) or Masson trichrome (**B**). Images are representative of 3 to 5 mice per group (original magnification $\times 40$). Scale bar = 50 μ m. Goblet cell metaplasia is represented as the percentage of periodic acid–Schiff–positive cells among total bronchial epithelial cells (Fig E6, *A*, right panel). Peribronchial collagen staining (induced trichrome) was quantified by using Image-Pro Plus software and normalized to the length of the basement membrane. Data are means $\pm$ SEMs of 20 to 40 airways per group.

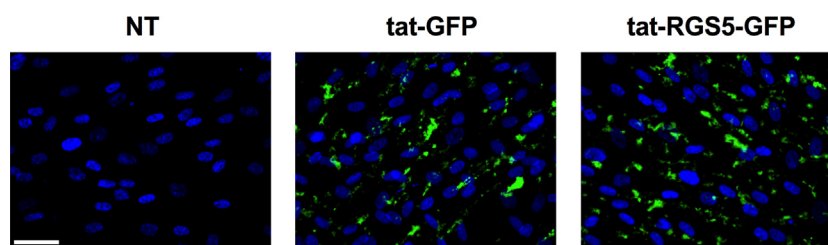


FIG E7. Transduction of hASM cells with RGS5 protein. hASM cells were either left untreated or incubated with tat proteins at a final concentration of 250 nmol/L (tat-GFP or tat-RGS5-GFP) for 2 hours. Nuclei of fixed cells were stained with DAPI. Images are representative of 2 independent experiments (original magnification $\times 40$). *Scale bar* = 50 μm .

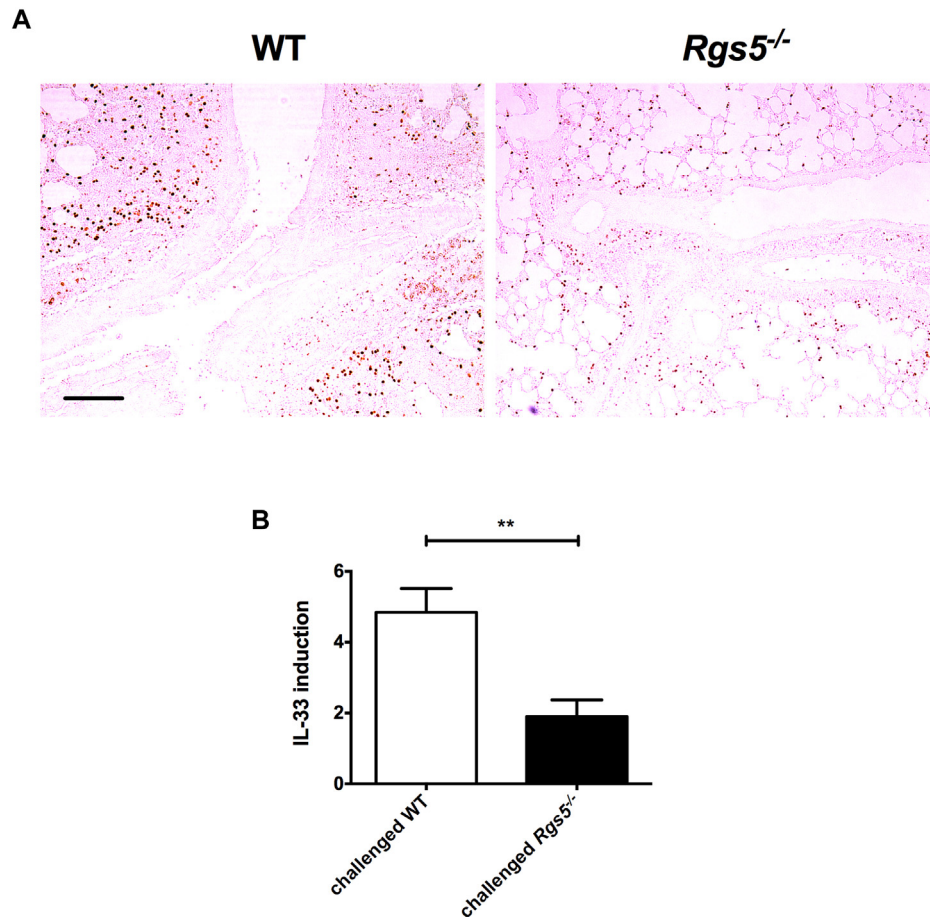


FIG E8. Lung epithelial IL-33 expression after allergen challenge is regulated by RGS5. **A,** Representative images of IL-33 immunostaining in lung sections of *A fumigatus*-challenged WT or *Rgs5*^{-/-} mice ($n = 4$ mice per genotype). **B,** Fold induction of IL-33 relative to that seen in naive mice (4 mice per genotype) was assessed by using Image-Pro Plus software (original magnification $\times 10$). $**P = .006$, t test. Scale bar = 100 μm .

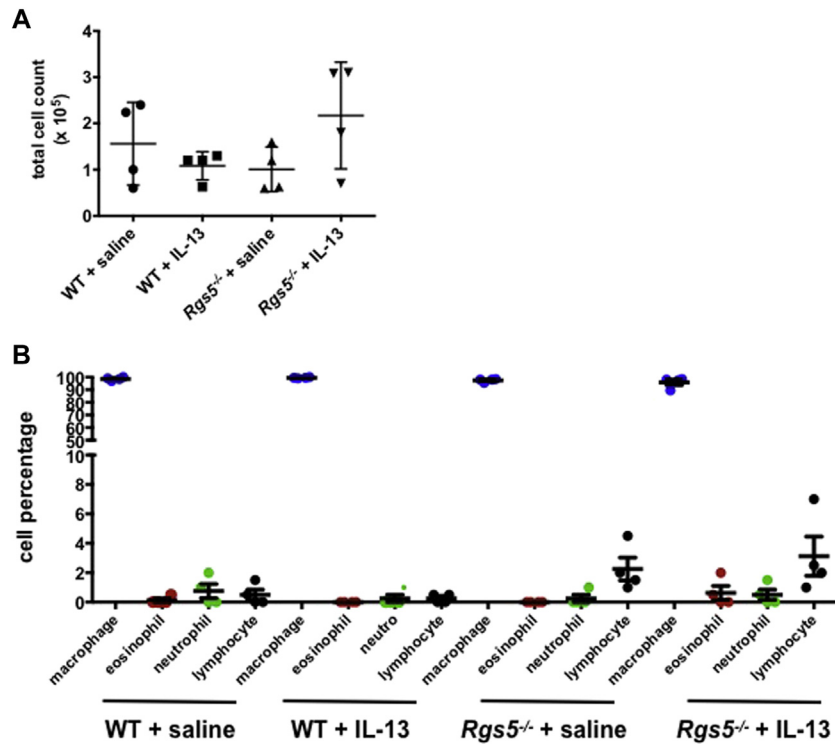


FIG E9. Inflammatory cell composition in BALF after intratracheal administration of recombinant IL-13. IL-13 (3 μ g in 30 μ L of saline) or saline alone was administered to mice, followed by bronchoalveolar lavage after 24 hours. Total cell numbers (**A**) and differential cell counts (**B**) were determined as in Fig E5.