ORIGINAL RESEARCH

RGS4 Overexpression in Lung Attenuates Airway Hyperresponsiveness in Mice

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Abstract

A cardinal feature of asthma is airway hyperresponsiveness (AHR) to spasmogens, many of which activate G protein–coupled receptors (GPCRs) on airway smooth muscle (ASM) cells. Asthma subtypes associated with allergy are characterized by eosinophilic inflammation in the lung due to the type 2 immune response to allergens and proinflammatory mediators that promote AHR. The degree to which intrinsic abnormalities of ASM contribute to this phenotype remains unknown. The regulators of G protein signaling (RGS) proteins are a large group of intracellular proteins that inhibit GPCR signaling pathways. RGS2- and RGS5-deficient mice develop AHR spontaneously. Although RGS4 is upregulated in ASM from patients with severe asthma, the effects of increased RGS4 expression on AHR *in vivo* are unknown. Here, we examined the impact of forced RGS4 overexpression in lung on AHR using transgenic (Tg) mice. Tg RGS4 was expressed in bronchial epithelium and ASM *in vivo*, and protein expression in lung was increased at least 4-fold in Tg mice compared with wild-type (WT) mice. Lung slices from Tg mice contracted less in response to the m3 muscarinic receptor agonist methacholine compared with the WT, although airway resistance in live, unchallenged mice of both strains was similar. Tg mice were partially protected against AHR induced by fungal allergen challenge due to weakened contraction signaling in ASM and reduced type 2 cytokine (IL-5 and IL-13) levels in Tg mice compared with the WT. These results provide support for the hypothesis that increasing RGS4 expression and/or function could be a viable therapeutic strategy for asthma.

Keywords: asthma; bronchial smooth muscle; G protein–coupled receptors; RGS proteins; signal transduction

Clinical Relevance

Genetic overexpression of RGS4 protected mice from allergen-induced airway hyperresponsiveness. Increasing RGS4 expression and/or function could be explored as a viable therapeutic strategy for asthma.

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Asthma is a chronic relapsing/remitting obstructive lung disease that reduces airflow, manifesting as wheezing, dyspnea, and cough. Although the asthmatic phenotype is somewhat heterogeneous, it is characterized universally by airway hyperresponsiveness (AHR), or increased sensitivity to inhaled bronchoconstrictor compounds such as the acetylcholine (ACh) analogs methacholine (MCh) and carbachol (CCh) (1). AHR results from inflammation of the airways, bronchial smooth muscle spasm, and increased mucus production, typically in the setting of chronic allergen or pollutant exposure (2). Although the immune response to allergens and the inflammation that is characteristic of asthma has been studied extensively, the contribution of contractile cells (e.g., airway smooth muscle [ASM] cells) to AHR is less clear (3). It is unknown whether ASM contraction signaling pathways are inherently perturbed in asthma. G protein–coupled receptor (GPCR) activation induces ASM contraction induced by some (but not all) proinflammatory mediators present in the lung, including ACh, histamine, thrombin, bradykinin, leukotrienes, and serotonin. Excitation-contraction signaling in ASM occurs principally through activation of GPCR-heterotrimeric G protein complexes containing Gαq. Ligand stimulation of the receptor induces a conformational change that results in Gαq activation by causing the exchange of the guanosine diphosphate for GTP to give the GTP-bound form of the G protein. The activated G protein then induces phospholipase C activation and the generation of inositol 1,4,5-trisphosphate (IP3). Ultimately, IP3-evoked Ca^{2+} flux into the cytosol promotes myosin light chain (MLC) phosphorylation, actin polymerization, and the generation of actin filaments aligned axially to facilitate the interaction with myosin, which culminates in cell shortening (4). A recent study of the contractile properties of ASM showed that ASM cells isolated from patients with asthma were hyperresponsive to histamine and MCh than ASM cells from healthy controls (5).

RGS proteins comprise a large family with more than 30 members in mammalian cells. These proteins negatively regulate GPCR functions through a conserved RGS domain that mediates binding to Gαi and Gαq (6). RGS proteins bind the GTP-bound activated Gα subunit and accelerate GTP hydrolysis by both Gαi and Gαq (GTPase-accelerating, or GAP, activity). This hastens the return of the Gα to the inactive GDP-bound form, thus terminating G protein signaling. Genetic knockout of individual RGS proteins in mice and human cells *ex vivo* has partially elucidated their physiological roles in the regulation of leukocyte trafficking (7), vascular smooth muscle tone and blood pressure (8), and neurological functions (9). By contrast, the physiological functions of RGS proteins in the lung have not been well studied. We have focused on the role of the R4 subfamily of RGS proteins in asthma. The R4 subfamily (RGS1–5, RGS8, RGS13, RGS18, and RGS21) contains the smallest and simplest members of the RGS family in terms of protein sequence. They are comprised of little more than the RGS box, the domain that mediates the GAP activity that is characteristic of all RGS family members. In previous studies, we detected expression of RGS2–5 in ASM cells and lung (10). RGS5 expression was increased in cultured ASM cells from asthmatic subjects, and this overexpression was associated with attenuated bronchoconstrictor-induced Ca^{2+} signaling (11). Chronic isoproterenol exposure reduced RGS5 expression in ASM, and small interfering RNA–mediated knockdown of RGS5 enhanced procontractile signaling, suggesting a mechanism underlying the paradoxical risk of increased bronchoconstriction in some patients who frequently use inhaled β-agonists (10). Subsequently, other investigators reported that a single nucleotide polymorphism in *Rgs5* was linked to severe asthma in children who responded poorly to bronchodilators (12). Corticosteroid and long-acting β-agonist (dexamethasone/salmeterol, referred to as CCS/LABA) treatment was associated with upregulation of RGS2, a selective regulator of Gαq, in human bronchial epithelial cells (BEAS-2B), which attenuated the activation (Ca^{2+} mobilization) of Gq-linked receptors by several ligands, including MCh and histamine (13). Ectopic RGS2 overexpression in BEAS-2B recapitulated this phenotype, whereas RNA interference–mediated silencing of RGS2 prevented the effects of CCS/LABA. A separate study revealed that CCS/LABA exerted similar RGS2-dependent effects on Gq-mediated signaling in human ASM cells (14). Tracheal ring contraction and plethysmography studies revealed that *Rgs2*$^{-/-}$ mice had increased bronchoconstriction in response to MCh compared with wild-type (WT) controls, providing strong evidence that RGS2 is a physiologically relevant regulator of bronchial tone. Two additional studies indicated that RGS2-deficient mice have increased AHR after allergen challenge, in part due to enhanced airway contraction after allergen challenge, and partly due to effects on airway inflammation and remodeling (15, 16). Although these published works suggest that context-dependent upregulation of expression of RGS2 protects the airways against bronchospasm, no studies have formally examined the effects of forced overexpression of RGS proteins in the lung on AHR.

In a previous study, we used immunohistochemistry to demonstrate increased quantities of RGS4 in the bronchial smooth muscle of airways from patients with severe asthma compared with airways from patients with mild/moderate asthma and controls (17). We hypothesized that increased RGS4 expression in ASM may act as a feedback mechanism to mitigate bronchoconstriction and AHR in chronic asthma. To test this hypothesis, we evaluated allergen-induced AHR, inflammation, and ASM contraction signaling in mice with forced RGS4 overexpression. We studied bacterial artificial chromosome (BAC) transgenic (Tg) mice, which express both RGS4 and enhanced green fluorescent protein (eGFP). In the transgene, an internal ribosomal entry site (IRES)-eGFP construct is inserted into the 3′ untranslated region (UTR) of RGS4. The transgene is expressed under the endogenous *Rgs4* promoter, and overexpression of this Tg *Rgs4* has not been reported to have any effect on endogenous *Rgs4* expression (18, 19). We found that RGS4 was overexpressed in the lungs of Tg mice, and that the mice were partially protected from AHR due to both reduced airway contraction and an altered inflammatory milieu after allergen sensitization and respiratory mucosal challenge.

Methods

Additional details regarding the materials and methods used in this work can be found in the data supplement.

RGS4 Tg Mice

RGS4-eGFP Tg mice were generated as described previously and obtained from The Jackson Laboratory as described at jax/org https://www.jax.org/strain/007894 (18, 19). "A C57BL/6J-derived bacterial artificial chromosome (BAC; RPCI-23-82i24) was modified by inserting an IRES2-eGFP construct (and an *frt*-flanked neomycin resistance cassette) into the 3' UTR of the *Rgs4* locus. The neomycin cassette was subsequently removed by FLP-mediated recombination. BAC DNA (~225 kb) from correctly transformed and targeted bacteria was then injected into C57BL/6J x DBA F1 embryos. Tg offspring (founder line 4 (R4BAC4) were established on this B6: DBA mixed genetic background. These mice were subsequently backcrossed to C57BL/6J inbred mice for more than 12 generations. Under the control of the *Rgs4* promoter/enhancer elements, transgene expression reports dynamic developmental, regional, and cellular specific expression in developing and mature cerebral cortex neurons across all cortical domains, as well as developing and mature subcortical regions (telencephalon, diencephalon, and brainstem) (18, 19)."

Acute Allergen Challenge Model

Mice (6–12 weeks old) were sensitized intraperitoneally with *Aspergillus fumigatus* (*Af*) crude extract (20 μg; Hollister-Stier) mixed with Imject Alum (20 mg; Pierce) on days 0 and 14. The mice were challenged intranasally with PBS (naive) or *Af* extract (25 μg) on days 25, 26, and 27. They were killed 24 hours after the last challenge after studies of lung function, followed by organ harvest. All studies were performed 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).

Airway Resistance Measurements in Live Mice

Mice were anesthetized by intraperitoneal injection of a ketamine and xylazine cocktail (100 mg/kg and 10 mg/kg, respectively), followed by transtracheal intubation with a 20 gauge SURFLO Teflon intravenous catheter (Santa Cruz Animal Health). The mice were mechanically ventilated at 160 breaths/min, during which time PBS and increasing doses of MCh (0, 6.25, 12.5, 25, 50, and 100 mg/ml) were administered by nebulization. Airway resistance was measured directly using the Elan RC FinePointe system (Buxco Research Systems). Mean values (±SEM) are presented in cm H_2O/ml/s.

Results

Lung RGS4 Expression in Tg Mice

To evaluate the effects of RGS4 overexpression on respiratory function in mice, we established several founder Tg lines containing the *Rgs4-egfp* BAC. We assessed the *egfp* copy number using real-time quantitative PCR (qPCR) based on standard curves derived from *egfp* sequence-containing plasmids of known concentrations. We generated several lines of mice containing on average 10–15 copies of the *egfp* transgene/1,000 copies of GAPDH for further functional studies (Figure 1A). These values were highly correlated with the copy number per cell based on a previously established transgene quantification method (data not shown) (20). To determine whether an increased *egfp* copy number correlated with increased *Rgs4* expression, we quantified total (endogenous and Tg) *Rgs4* abundance in cDNA derived from whole lungs of founder mice by qPCR. We used founder mice for this purpose because the BAC construct includes native elements flanking the *Rgs4* coding sequences that do not permit ready differentiation of endogenous transcripts from Tg *Rgs4* transcripts. Total *Rgs4* mRNA expression was ~8- to 10-fold higher in the lungs of Tg mice than in the WT mice (Figure 1B). Our previous studies and published work have demonstrated that RGS4 is expressed in both the respiratory epithelium and bronchial smooth muscle of human lungs (17). To determine the anatomical site of RGS4 protein expression in the lungs of Tg mice, we examined GFP expression in frozen sections of lungs from WT and Tg mice by immunostaining with anti-GFP antibody. We identified ASM using an anti–smooth muscle α actin (SMAA) antibody. Whereas no GFP was detected in lungs from WT mice, we observed high GFP expression in bronchial epithelium and smooth muscle surrounding the airways of Tg mice, as evidenced by colocalization with SMAA (Figure 1C). We also detected increased total *Rgs4* mRNA expression in cultured tracheal smooth muscle cells (Figure 1D) (identified by SMAA expression; Figure E1 in the data supplement) by qPCR. We were not able to detect native or Tg RGS4 protein expression by immunoblotting. However, to enhance steady-state levels in both WT and Tg mice, we exploited the known regulation of RGS4 degradation by the proteasome (21, 22). Using the proteasome inhibitor MG132, we detected endogenous RGS4 in both WT and Tg ASM, and levels in Tg cells were increased ~4-fold compared with the WT (Figure 1E).

Increased RGS4 Expression Attenuates Airway Contraction

In patients with allergic asthma, inflammatory mediators associated with the T helper type 2 (Th2) response to allergens promotes AHR through multiple mechanisms, including bronchoconstriction, mucus hypersecretion, and airway remodeling (2). To determine the effects of increased RGS4 levels on AHR in the setting of allergic inflammation, we sensitized and challenged mice with extracts of *Af*, which contains fungal allergens linked to severe, uncontrolled forms of asthma in humans (23). After three respiratory challenges on consecutive days with *Af* or PBS as a control, we measured lung resistance in live, anesthetized mice by plethysmography after exposure to various amounts of nebulized MCh. Compared with mice challenged with PBS alone, mice treated with *Af* had increased airway resistance after inhalation of MCh (Figure 2A). Although the responses of PBS-challenged WT and Tg mice were similar, *Af*-treated Tg mice overexpressing RGS4 had significantly reduced lung resistance in response to MCh compared with *Af*-treated WT mice. These results suggested that increased RGS4 abundance in lung protects against allergen-induced AHR. To further examine the contribution of bronchoconstriction to this phenotype, we assessed airway contraction in precision-cut lung slices (PCLSs) in response to CCh by microscopy (Figure 2B). PCLSs from Tg mice sensitized with *Af* but challenged with PBS alone had decreased contractile responses to CCh (maximal effect for agonist [E_{max}] 75.9 ± 4 versus 59.62 ± 4.5%; $P = 0.01$, extra sum-of-squares F test; Figure 2C). Airways from WT mice that had been sensitized and challenged with *Af* contracted significantly

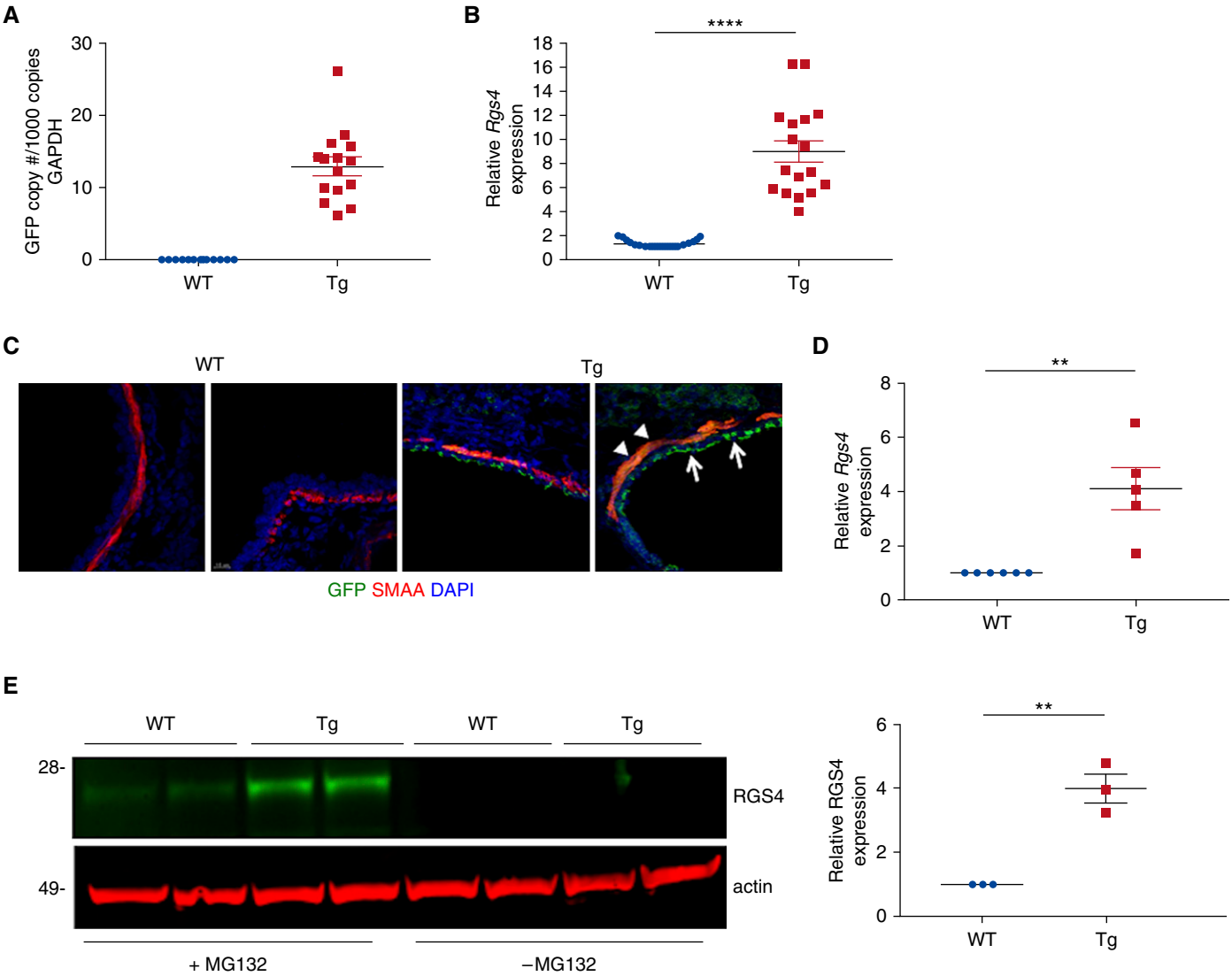

Figure 1. Transgenic (Tg) overexpression of RGS4 in mouse lungs. (*A*) Plasmids containing sequences for *egfp* and *gapdh* were used to generate standard curves. Genomic DNA was extracted from earsnips, and copies of the *egfp*-containing transgene/1,000 copies of GAPDH were assessed in each of the indicated strains by quantitative PCR (qPCR). (*B*) *Rgs4* expression in cDNA derived from whole lungs was determined by qPCR (mean ± SEM; wild-type [WT]: 1.3 ± 0.07; Tg: 9.01 ± 0.91; ****$P < 0.0001$, unpaired *t* test). (*C*) GFP and smooth muscle α actin (SMAA) were detected in frozen lung sections by immunofluorescence and confocal microscopy. Arrowheads indicate bronchial smooth muscle layer; epithelium is indicated by arrows. (*D*) Tracheal smooth muscle cells were extracted from three to four mice of each genotype and cultured. *Rgs4* expression was determined by qPCR. Each symbol represents expression relative to one WT sample, set as 1 (mean ± SEM); WT: 1.01 ± 0.005; Tg: 4.1 ± 0.79: **$P = 0.001$, unpaired *t* test). (*E*) RGS4 expression in tracheal airway smooth muscle cells was determined by immunoblotting lysates from cells left untreated or treated with the proteasome inhibitor MG132. The image represents a single experiment run in duplicate. The bar graph represents the mean ± SEM of RGS4 expression normalized to β-actin. Cells were derived from six to eight mice of each genotype, assessed in two independent experiments; **$P = 0.003$, unpaired *t* test.

more than those from PBS-challenged mice (maximal contraction ∼80% versus ∼60%, respectively) in response to CCh, as expected (Figure 2D). CCh-induced bronchoconstriction was significantly reduced in airways from *Af*-challenged Tg mice compared with those from comparably treated WT mice (E_{max} 86.7 ± 4.2 versus 73.3 ± 4.6%, $P = 0.04$). From these results, we conclude that RGS4 overexpression reduces PCLS contraction in response to CCh.

Allergen-Induced Leukocyte Recruitment Is Not Affected by RGS4 Overexpression

Mediators associated with allergic inflammation (e.g., IL-13 and TNF-α) may act on ASM cells directly to augment their contractile and synthetic functions (24). In addition to bronchi, PCLSs contain several cell types—epithelial cells and mast cells are two examples—that could affect ASM contraction indirectly through inflammatory mediator secretion. To determine whether reduced lung inflammation contributed to the attenuated AHR, we compared parameters of lung inflammation induced by *Af* sensitization and challenge in WT and Tg mice.

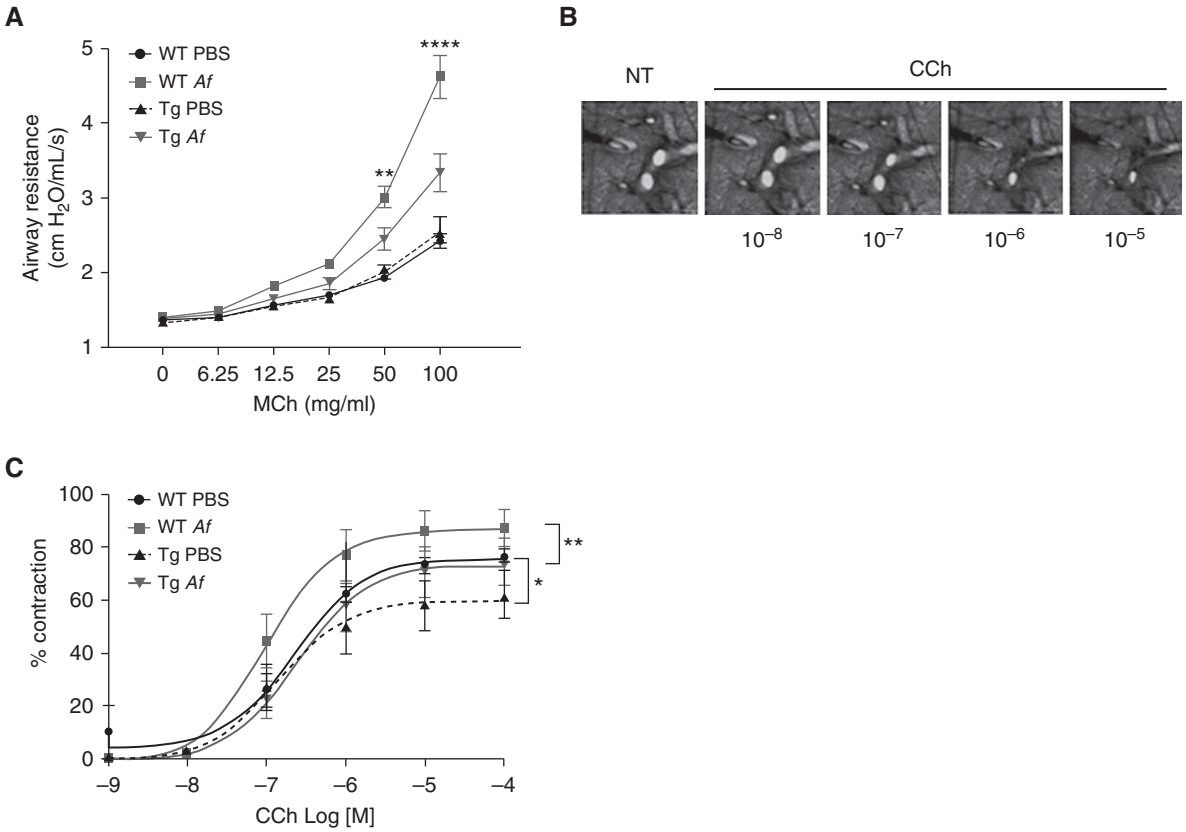

Figure 2. RGS4 overexpression attenuates acute allergen-induced airway hyperresponsiveness. (*A*) Airway resistance (cm H_2O ml^{-1}/s) after administration of the indicated concentrations of nebulized methacholine (MCh) was determined by plethysmography. Graphs represent the mean ± SEM from 10–13 mice/group, measured in four independent experiments (***P* = 0.001;*****P* < 0.0001, two-way ANOVA, Tukey multiple comparisons of WT versus Tg *Aspergillus fumigatus* [*Af*]). (*B*) Representative images of airway contraction in precision-cut lung slices in response to the indicated doses of carbachol (CCh). (*C*) Airway contraction in precision-cut lung slices obtained from naive or *Af*-sensitized and challenged mice treated with increasing doses of CCh was assessed by measuring the airway diameter by microscopy. Graphs are mean ± SEM of 9–13 mice/group evaluated in four independent experiments (**P* = 0.03; ***P* = 0.009, two-way ANOVA, Bonferroni multiple comparisons). NT = not treated.

We specifically assessed leukocyte infiltration, mucus cell hypertrophy, and proinflammatory cytokines in BAL fluid (BALF). We observed a nearly 20-fold increase in the total number of inflammatory cells in BALF from mice sensitized and challenged with *Af* compared with mice challenged with PBS alone (Figure 3A). As anticipated, a substantial increase in the percentage of eosinophils accounted for the difference (Figure 3B). Neither the quantity nor the composition of inflammatory cells in the BALF of Tg mice differed substantially from what was observed in the WT. Histological examination of lung sections from WT and Tg PBS-challenged mice revealed no structural differences (Figure 3C). In contrast to the lungs from naive mice, the lungs from allergen-sensitized and -challenged mice had prominent peribronchial and perivascular leukocyte infiltrates and mucus cell hypertrophy, as expected, but there was little difference between the lungs of WT and Tg mice. These findings suggest that RGS4 overexpression does not reduce AHR by inhibiting recruitment of inflammatory cells to the lung in response to allergen challenge.

Alterations in the Lung Inflammatory Microenvironment in RGS4 Tg Mice

To determine whether the proinflammatory microenvironment is altered by the overexpression of RGS4 in lung, we assessed BALF cytokines in PBS- or allergen-challenged mice by multiplexed ELISA. Compared with PBS treatment alone, sensitization and challenge with *Af* led to significantly increased levels of the Th2-associated cytokines IL-4, IL-5, and IL-13 in the BALF of WT mice, but not Tg mice (Figures 4A–4C). The levels of IL-5 and IL-13 were significantly reduced in Tg mice compared with the WT, and IL-4 showed a similar trend that did not reach statistical significance. The Th1 cytokine IL-12 was significantly upregulated in BALF from Tg, but not WT, *Af*-challenged mice compared with PBS-treated mice, but the levels were similar in WT and Tg mice (Figure 4D). We did not detect upregulation of other cytokines tested, including IL-1β, IL-9, IL-10, IL-17, eotaxin, IFN-γ, and TNF-α, in the *Af*-treated WT or Tg mice compared with controls (data not shown). Because RGS4 is prominently expressed in epithelial cells (25), we investigated whether RGS4 overexpression affects the production of epithelial-derived cytokines that are capable of driving allergic inflammation, such as IL-25 and IL-33. We did not find increased levels of IL-25 in BALF from allergen-challenged mice compared with naive mice (Figure 5A), but there was a trend toward

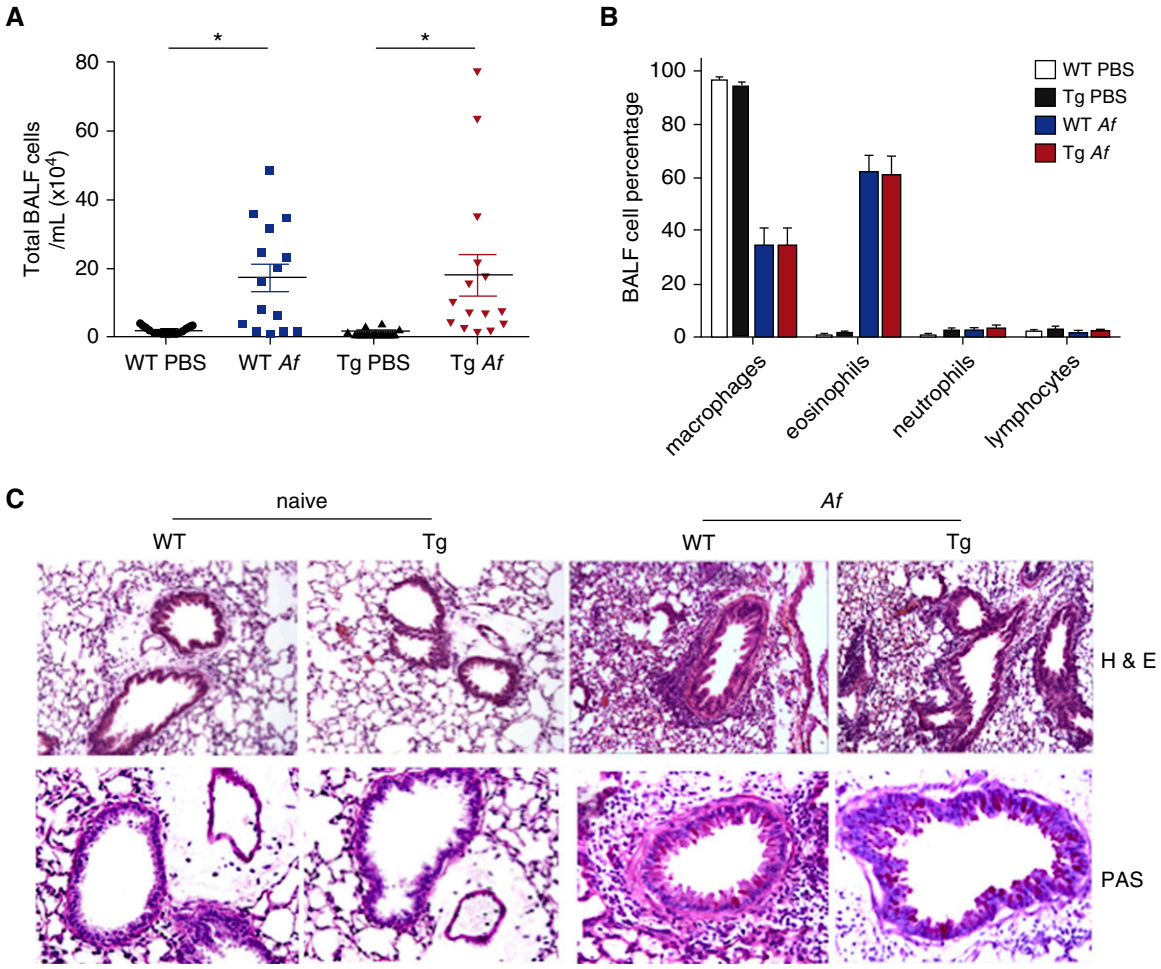

Figure 3. RGS4 overexpression does not reduce allergen-induced leukocyte recruitment to lung. (*A* and *B*) Total BAL fluid (BALF) leukocyte counts (*A*) and percentages of BALF inflammatory cell types (*B*) from naive and *Af*-challenged mice (mean ± SEM of 10–13 mice/group in four independent experiments; $^*P = 0.02$, one-way ANOVA, Tukey *post hoc* test). (*C*) Representative lung sections stained with hematoxylin and eosin (H&E) (top panel) or periodic acid–Schiff (PAS, bottom panel).

increased IL-33 levels in BALF and statistically significant increases in *Il33* mRNA expression in whole lungs of allergen-challenged mice compared with PBS-treated mice of either genotype (Figures 5B and 5C). There were no significant differences in IL-33 expression in WT versus Tg mice. We also examined expression of the epithelial-related mucin gene *Muc5ac*, which promotes airway obstruction through increased mucus production. *Muc5ac* was upregulated in lungs from *Af*-challenged mice relative to naive animals, but RGS4 overexpression did not significantly affect *Muc5ac* expression (Figure 5D) (26). Collectively, these findings suggest that RGS4 overexpression dampens the Th2 cytokine response to *Af* through as yet unknown mechanisms, but reduces AHR through various mucus-independent pathways, including reduced airway contraction.

Increased RGS4 Expression Inhibits Contraction Signaling in ASM

To determine whether RGS4 overexpression attenuates AHR by inhibiting contraction-inducing signaling pathways in bronchial smooth muscle, we examined intracellular Ca^{2+} mobilization induced by GPCR agonists in individual tracheal ASM cells by confocal microscopy. ACh or thrombin, another potent bronchoconstrictor, induced prominent and transient spikes in intracellular Ca^{2+} levels over baseline. ACh- or thrombin-evoked Ca^{2+} fluxes were significantly blunted in Tg cells overexpressing RGS4 compared with WT cells (Figures 6A and 6B). Consistent with this reduced upstream signal, downstream effector activation (MLC phosphorylation) induced by thrombin was also decreased in RGS4 Tg cells compared with WT cells (Figure 6C). Thus, increased RGS4 expression diminishes excitation-contraction signaling in ASM to mitigate bronchoconstriction.

Discussion

Here, we investigated the effects of overexpression of the GPCR-related regulatory protein RGS4 on acute allergic inflammation in mice. We used Tg mice with extra copies of *Rgs4* coding sequences downstream of its endogenous promoter. We showed that increased levels of RGS4 in the lung reduced allergen-evoked airway contraction *in vivo* and *ex vivo* after

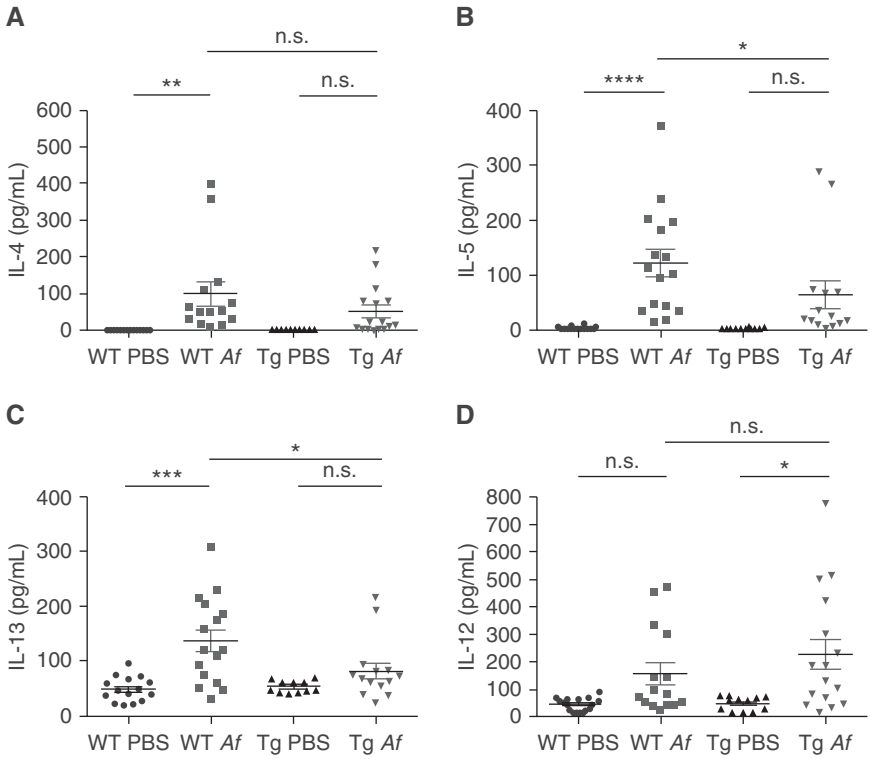

Figure 4. Lung inflammatory cytokines in RGS4 Tg mice. (*A–D*) Cytokine levels in BALF from naive or *Af*-challenged WT or Tg mice as assessed by multiplexed ELISA. Each symbol represents an individual mouse; bars are mean ± SEM; $^{*}P < 0.05$, $^{**}P = 0.002$, $^{***}P = 0.001$, $^{****}P < 0.0001$; n.s. = not significant; one-way ANOVA; Tukey or Newman–Keuls multiple comparisons.

challenge with allergen. Both intrinsically altered contraction signaling in ASM and changes in the lung inflammatory microenvironment appear to contribute to this phenotype.

Multiple lines of evidence indicate that variations in expression of RGS4 and several other R4 RGS proteins in the lung modify AHR in both mice and humans owing to their ability to inhibit Gq-mediated ASM contraction and proliferation. *Rgs2* knockout mice have spontaneous AHR due to increased ASM Ca^{2+} signaling and contraction in response to spasmogens. The airways of these mice also have increased peribronchial smooth muscle mass in the absence of allergen challenge caused by faster growth of ASM cells through Erk- and phosphatidylinositol 3-kinase (PI3K)-dependent mechanisms (15, 16). RGS2 expression is decreased in lungs from patients with asthma compared with controls, and two related single nucleotide polymorphisms in the *RGS2* promoter linked to decreased RGS2 expression are more prevalent in patients with asthma versus controls (65 versus 44%) (27). In previous work, we demonstrated increased RGS4 expression in bronchial smooth muscle bundles in lungs from patients with asthma by immunohistochemistry (17), and the amount of immunoreactive RGS4 correlated with clinical asthma severity. Concurrently, we showed that *RGS4* knockdown in PCLS enhanced airway contraction to histamine and ACh, whereas platelet-derived growth factor (PDGF) reduced PCLS contraction by upregulating RGS4 expression. However, the role of RGS4 in AHR *in vivo* had not been previously evaluated, and no studies have formally tested the impact of increased expression of any RGS protein in the lung on bronchial smooth muscle tone and AHR in a whole animal model. Here, we extended our previous observations by demonstrating that RGS4 overexpression *in vivo* decreases airway contraction, due in part to blunted GPCR-mediated Ca^{2+} signaling and MLC phosphorylation in ASM cells.

Previously published work and the current study suggest that increased RGS4 expression in ASM can mitigate AHR in asthma through RGS4's capacity to inhibit bronchoconstriction. However, its impact on asthmatic diathesis may be multifaceted. A previous study reported that forced RGS4 expression in mouse heart driven by the α-myosin heavy chain promoter was maladaptive: hearts exposed to chronic pressure overload became dilated and hypocontractile, followed by rapid clinical decompensation (28). Similarly, in severe, uncontrolled asthma, airways may be less contractile due to ASM hyperplasia and stiffness, leading to "fixed" airway obstruction that is unresponsive to bronchodilators (29). Mitogens such as PDGF stimulate the growth of human ASM in part by activating PI3K-dependent pathways. RGS4 and other R4 family members could affect ASM growth through their known physical interactions with the p85 regulatory subunit of Class IA PI3Ks, which occur independently of GAP activity (17, 30, 31). In a previous study, we demonstrated that small interfering RNA–mediated knockdown of RGS4 expression in human ASM cells reduced PDGF-evoked proliferation and PI3K activity (17). Here, we did not detect significant differences in the growth of WT and RGS4 Tg ASM cells in culture, nor did we detect any spontaneous increases in peribronchial smooth muscle mass in Tg mice (data not shown). Because the short-term allergen sensitization and challenge model used here does not feature prominent increases in ASM mass around airways, further studies of *Rgs4* gene-deleted strains chronically exposed to allergen may be needed to fully evaluate its role in airway remodeling. Finally, recent work has also shown that PI3Kδ induces bronchoconstriction in PCLS through RhoA activation, suggesting that RGS4's interaction with PI3K could affect ASM contraction through its regulation of both G protein and PI3K pathways (32).

RGS4 overexpression reduced some (but not all) parameters of airway inflammation in allergen-challenged Tg mice through unclear mechanisms. Mice deficient in Gq were previously shown to have impaired eosinophil recruitment to lungs after sensitization and challenge with ovalbumin (33), whereas mice deficient in RGS2, a negative regulator of Gαq, displayed increased eosinophil accumulation in lungs after repeated intranasal inoculations with house dust mite allergen (15). These findings suggest

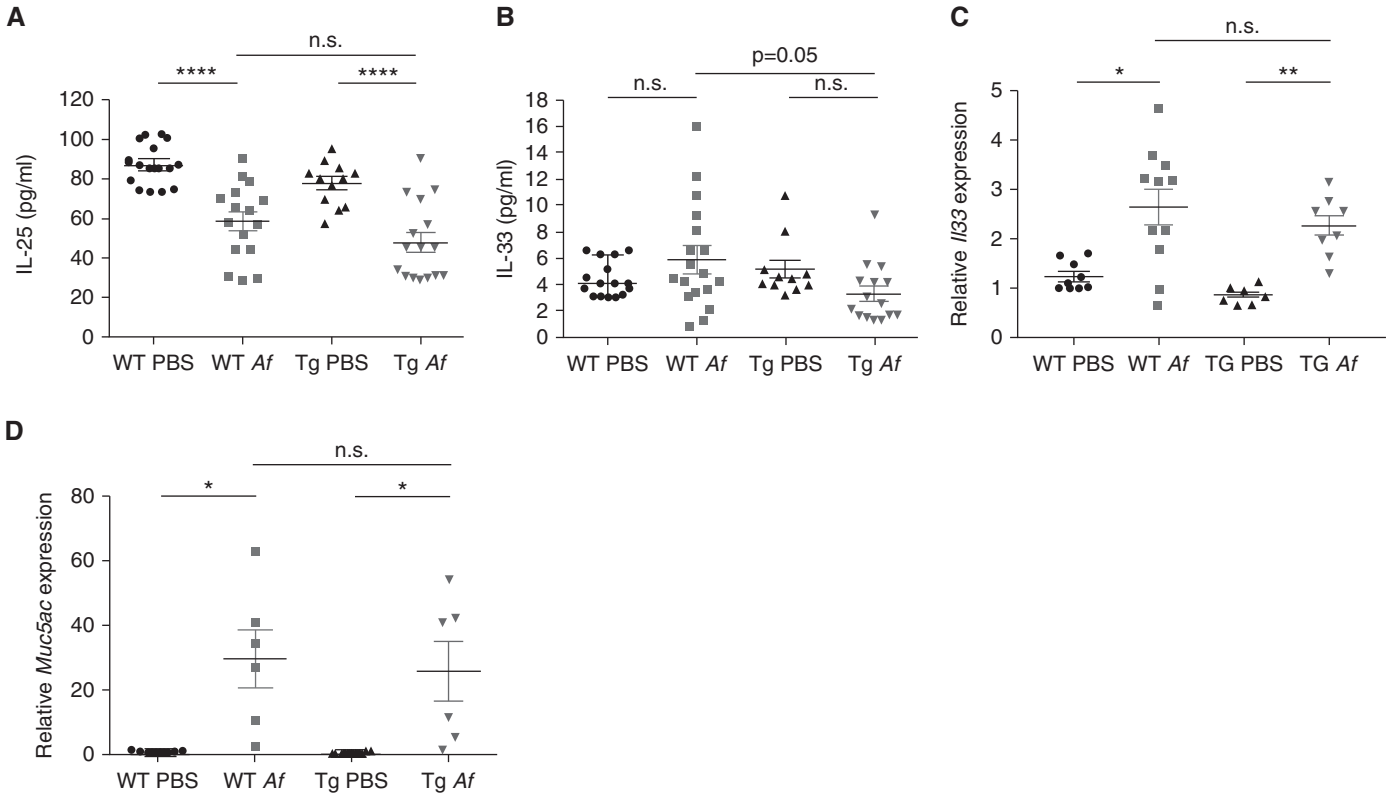

Figure 5. Epithelial-associated cytokines and gene expression in RGS4 Tg mice. (*A* and *B*) Cytokine levels in BALF were determined as in Figure 4. ****$P < 0.001$, one way ANOVA. (*C* and *D*) *Il33* expression in cDNA derived from whole lungs was determined by qPCR using gene-specific primers. Each symbol represents an individual mouse; bars are mean ± SEM; *$P = 0.02$, **$P = 0.002$, Kruskal–Wallis ANOVA, Benjamini–Hochberg corrected *P* values for nonnormally distributed data. (*D*) *Muc5ac* expression in lungs was determined by qPCR; *$P < 0.04$, Kruskal–Wallis, Dunn's multiple comparisons.

the existence of as yet unidentified Gq signaling pathways in leukocytes that promote inflammation. Such pathways could be targets for regulation by RGS4 because it interacts with Gαq. However, our results differ from the above findings in that we observed intact eosinophil recruitment to lungs after allergen exposure in Tg mice, but reduced type 2 cytokine levels in the airways compared with the WT. Variations in the allergen challenge models used in the respective studies could account for these differences. For instance, recent work has demonstrated that fungal challenge of mice leads to a more severe asthma phenotype than that induced by house dust mite, which is associated with higher levels of IL-33 and greater leukocyte numbers in lung tissue (34). Finally, because IL-13 directly enhances ASM responses to spasmogens, the reduced levels of IL-13 in the lungs of Tg mice are sufficient to explain the reduced AHR we observed in these mice after allergen challenge (35).

There are several limitations to our study. Congenital forced overexpression of RGS4 might affect many cell types that contribute to lung development and/or function and thus confound our interpretation of lung function. For example, although we detected reduced CCh-induced contraction of PCLS from naive Tg mice compared with WT mice *ex vivo*, we were unable to discern similar differences *in vivo* in live mice using direct measurements of airway resistance. Plethysmography takes into account whole lung mechanics, and RGS4 could play roles in other cells (e.g., skeletal muscle) that affect airway resistance and dynamic compliance. PCLS is more sensitive than plethysmography and simply measures luminal narrowing. Finally, the effects of RGS4 overexpression in mice are mostly evident at higher CCh concentrations, affecting E_{max}. In naive mice, it is possible that the effective CCh concentrations that are necessary to see changes in airway resistance in the whole lung are not attainable in live animals due to systemic side effects (e.g., tachycardia, hypotension, and asphyxiation).

The mechanisms by which RGS4 overexpression reduces allergen-induced type 2 cytokines are unclear. Although the precise cellular source(s) of these mediators in various experimental models of allergic asthma has not been fully elucidated, lung structural cells produce a number of cytokines, including IL-25, IL-33, and thymic stromal lymphopoietin. Secretion of these cytokines triggers production of IL-4, IL-5, and IL-13 by various leukocytes, including Th2 and ILC2 cells (36). ASM cells, for example, produce IL-33 in response to LPS stimulation in a PI3K-dependent manner, and RGS5 overexpression inhibits LPS-induced *Il33* expression in ASM. RGS4 could regulate pattern recognition receptor–induced cytokine expression in ASM. RGS4 is also prominently expressed in epithelial cells, which secrete these mediators. However, in this study we

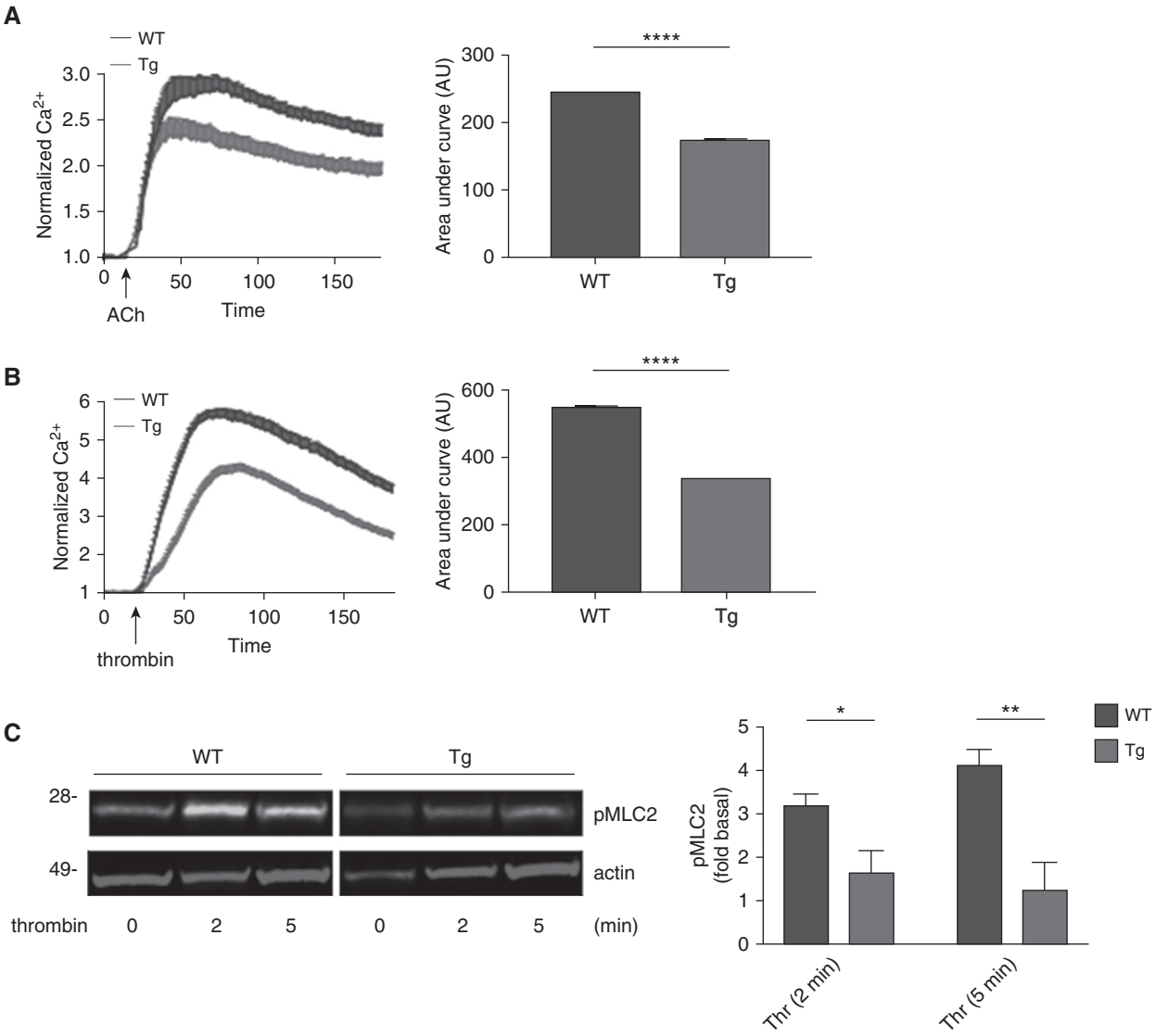

Figure 6. RGS4 is overexpressed in Tg airway smooth muscle cells and inhibits contraction signaling. (*A* and *B*) Left: intracellular Ca^{2+} over time normalized to initial values (n = 31–40 cells/genotype for acetylcholine [ACh]; 82–83 cells/genotype for thrombin). The arrow indicates time of addition of the indicated agonist. Right: bar graphs show the area under curve (mean ± SEM of four to six experiments; AU = arbitrary units); ****$P < 0.0001$, unpaired Welch's corrected t test. (*C*) Myosin light chain 2 phosphorylation (pMLC2) was assessed by immunoblotting. The image is from a single representative experiment. The bar graph is the mean ± SEM of the fold increase at each time point compared with unstimulated cells measured in four independent experiments; *P = 0.04, **P = 0.002, two-way ANOVA, Holm–Sidak multiple comparisons. Thr = thrombin.

did not detect significantly decreased IL-33 expression in allergen-challenged Tg mice compared with the WT.

Our studies provide the first proof-of-concept evidence that overexpression of an RGS protein in lung provides protection against AHR induced by acute allergen challenge. Currently, no compounds that increase RGS activity and could be exploited for treatment are available. However, several cellular mechanisms that modulate RGS4 steady-state levels, such as the ubiquitin-proteasome pathway, have been described (37). Application of a proteasome inhibitor (such as bortezomib) to the respiratory mucosa might represent a viable strategy for the treatment of asthma, although such inhibitors are likely to affect the steady-state levels of many proteins that contribute to AHR. At present, mouse strains with tissue-specific deletion of RGS4 in ASM and epithelial cells are being generated to fully delineate its regulation of ASM contraction and proinflammatory pathways, respectively, in the setting of acute and chronic allergen exposure. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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References

1. Nair P, Martin JG, Cockcroft DC, Dolovich M, Lemiere C, Boulet LP, *et al*. Airway hyperresponsiveness in asthma: measurement and clinical relevance. *J Allergy Clin Immunol Pract* 2017;5:649–659.e2.
2. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015;16:45–56.
3. Prakash YS. Emerging concepts in smooth muscle contributions to airway structure and function: implications for health and disease. *Am J Physiol Lung Cell Mol Physiol* 2016;311:L1113–L1140.
4. Penn RB, Benovic JL. Regulation of heterotrimeric G protein signaling in airway smooth muscle. *Proc Am Thorac Soc* 2008;5:47–57.
5. An SS, Mitzner W, Tang WY, Ahn K, Yoon AR, Huang J, *et al*. An inflammation-independent contraction mechanophenotype of airway smooth muscle in asthma. *J Allergy Clin Immunol* 2016;138:294–297.e4.
6. Sjögren B. The evolution of regulators of G protein signalling proteins as drug targets—20 years in the making: IUPHAR Review 21. *Br J Pharmacol* 2017;174:427–437.
7. Xie Z, Chan EC, Druey KM. R4 regulator of G protein signaling (RGS) proteins in inflammation and immunity. *AAPS J* 2016;18:294–304.
8. Osei-Owusu P, Blumer KJ. Regulator of G protein signaling 2: a versatile regulator of vascular function. *Prog Mol Biol Transl Sci* 2015;133: 77–92.
9. Gerber KJ, Squires KE, Hepler JR. Roles for regulator of G protein signaling proteins in synaptic signaling and plasticity. *Mol Pharmacol* 2016;89:273–286.
10. Yang Z, Cooper PR, Damera G, Mukhopadhyay I, Cho H, Kehrl JH, *et al*. β-agonist-associated reduction in RGS5 expression promotes airway smooth muscle hyper-responsiveness. *J Biol Chem* 2011; 286:11444–11455.
11. 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;46:823–832.
12. Labuda M, Laberge S, Brière J, Bérubé D, Krajinovic M. RGS5 gene and therapeutic response to short acting bronchodilators in paediatric asthma patients. *Pediatr Pulmonol* 2013;48:970–975.
13. Holden NS, George T, Rider CF, Chandrasekhar A, Shah S, Kaur M, *et al*. Induction of regulator of G-protein signaling 2 expression by long-acting β2-adrenoceptor agonists and glucocorticoids in human airway epithelial cells. *J Pharmacol Exp Ther* 2014;348:12–24.
14. Holden NS, Bell MJ, Rider CF, King EM, Gaunt DD, Leigh R, *et al*. β2-Adrenoceptor agonist-induced RGS2 expression is a genomic mechanism of bronchoprotection that is enhanced by glucocorticoids. *Proc Natl Acad Sci USA* 2011;108:19713–19718.
15. George T, Bell M, Chakraborty M, Siderovski DP, Giembycz MA, Newton R. Protective roles for RGS2 in a mouse model of house dust mite-induced airway inflammation. *PLoS One* 2017;12:e0170269.
16. 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;130:968–976.e3.
17. 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.
18. Ebert PJ, Campbell DB, Levitt P. Bacterial artificial chromosome transgenic analysis of dynamic expression patterns of regulator of G-protein signaling 4 during development. I. Cerebral cortex. *Neuroscience* 2006;142:1145–1161.
19. Ebert PJ, Campbell DB, Levitt P. Bacterial artificial chromosome transgenic analysis of dynamic expression patterns of regulator of G-protein signaling 4 during development. II. Subcortical regions. *Neuroscience* 2006;142:1163–1181.
20. Joshi M, Keith Pittman H, Haisch C, Verbanac K. Real-time PCR to determine transgene copy number and to quantitate the biolocalization of adoptively transferred cells from EGFP-transgenic mice. *Biotechniques* 2008;45:247–258.
21. Siedlecki AM, Jin X, Thomas W, Hruska KA, Muslin AJ. RGS4, a GTPase activator, improves renal function in ischemia-reperfusion injury. *Kidney Int* 2011;80:263–271.
22. Wang Q, Traynor JR. Opioid-induced down-regulation of RGS4: role of ubiquitination and implications for receptor cross-talk. *J Biol Chem* 2011;286:7854–7864.
23. Ghosh S, Hoselton SA, Schuh JM. Allergic Inflammation in *Aspergillus fumigatus*-induced fungal asthma. *Curr Allergy Asthma Rep* 2015;15: 59.
24. Ramakrishna L, de Vries VC, Curotto de Lafaille MA. Cross-roads in the lung: immune cells and tissue interactions as determinants of allergic asthma. *Immunol Res* 2012;53:213–228.
25. 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;41:40–49.
26. Evans CM, Kim K, Tuvim MJ, Dickey BF. Mucus hypersecretion in asthma: causes and effects. *Curr Opin Pulm Med* 2009;15:4–11.
27. Jiang H, Xie Y, Abel PW, Wolff DW, Toews ML, Panettieri RA Jr, *et al*. Regulator of G-protein signaling 2 repression exacerbates airway hyper-responsiveness and remodeling in asthma. *Am J Respir Cell Mol Biol* 2015;53:42–49.
28. Rogers JH, Tamirisa P, Kovacs A, Weinheimer C, Courtois M, Blumer KJ, *et al*. RGS4 causes increased mortality and reduced cardiac hypertrophy in response to pressure overload. *J Clin Invest* 1999;104: 567–576.
29. Zhang L, He L, Gong J, Liu C. Risk factors associated with irreversible airway obstruction in asthma: a systematic review and meta-analysis. *Biomed Res Int* 2016;2016:9868704.
30. Liang G, Bansal G, Xie Z, Druey KM. RGS16 inhibits breast cancer cell growth by mitigating phosphatidylinositol 3-kinase signaling. *J Biol Chem* 2009;284:21719–21727.
31. 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.
32. Koziol-White CJ, Yoo EJ, Cao G, Zhang J, Papanikolaou E, Pushkarsky I, *et al*. Inhibition of PI3K promotes dilation of human small airways in a rho kinase-dependent manner. *Br J Pharmacol* 2016;173:2726–2738.
33. Borchers MT, Justice PJ, Ansay T, Mancino V, McGarry MP, Crosby J, *et al*. Gq signaling is required for allergen-induced pulmonary eosinophilia. *J Immunol* 2002;168:3543–3549.
34. Castanhinha S, Sherburn R, Walker S, Gupta A, Bossley CJ, Buckley J, *et al*. Pediatric severe asthma with fungal sensitization is mediated by steroid-resistant IL-33. *J Allergy Clin Immunol* 2015;136:312–322.e7.
35. Nesmith AP, Agarwal A, McCain ML, Parker KK. Human airway musculature on a chip: an in vitro model of allergic asthmatic bronchoconstriction and bronchodilation. *Lab Chip* 2014;14: 3925–3936.
36. Mitchell PD, O'Byrne PM. Epithelial-derived cytokines in asthma. *Chest* 2017;151:1338–1344.
37. Wang J, Xie Y, Wolff DW, Abel PW, Tu Y. DHHC protein-dependent palmitoylation protects regulator of G-protein signaling 4 from proteasome degradation. *FEBS Lett* 2010;584:4570–4574.