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Mfge8 suppresses airway hyperresponsiveness in asthma by regulating smooth muscle contraction

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Airway obstruction is a hallmark of allergic asthma and is caused primarily by airway smooth muscle (ASM) hypercontractility. Airway inflammation leads to the release of cytokines that enhance ASM contraction by increasing ras homolog gene family, member A (RhoA) activity. The protective mechanisms that prevent or attenuate the increase in RhoA activity have not been well studied. Here, we report that mice lacking the gene that encodes the protein Milk Fat Globule-EGF factor 8 (*Mfge8*$^{-/-}$) develop exaggerated airway hyperresponsiveness in experimental models of asthma. *Mfge8*$^{-/-}$ ASM had enhanced contraction after treatment with IL-13, IL-17A, or TNF-α. Recombinant Mfge8 reduced contraction in murine and human ASM treated with IL-13. Mfge8 inhibited IL-13–induced NF-κB activation and induction of RhoA. Mfge8 also inhibited rapid activation of RhoA, an effect that was eliminated by an inactivating point mutation in the RGD integrin-binding site in recombinant Mfge8. Human subjects with asthma had decreased Mfge8 expression in airway biopsies compared with healthy controls. These data indicate that Mfge8 binding to integrin receptors on ASM opposes the effect of allergic inflammation on RhoA activity and identify a pathway for specific inhibition of ASM hypercontractility in asthma.

calcium sensitivity | lactadherin

Airway obstruction is a hallmark of asthma (1) and is primarily caused by an exaggerated response of airway smooth muscle (ASM) to contractile agonists. Severe bronchospasm in asthma is associated with significant morbidity and economic cost. New insights into the pathogenesis of bronchoconstriction, especially as they relate to the ASM, are of significant clinical interest. The cytokines IL-13, IL-17A, and TNF-α, all of which are elevated in the airways of patients with asthma, regulate airway hyperresponsiveness (AHR) by increasing ASM calcium sensitivity (2–5). The release of contractile agonists during airway inflammation causes excessive bronchoconstriction in sensitized ASM. The protective mechanisms that prevent or attenuate the increase in calcium sensitivity of ASM exposed to cytokines have not been well studied.

The force of ASM contraction is regulated by the phosphorylation status of myosin light chain (MLC) (6). Cytokines enhance ASM calcium sensitivity and contraction through a signaling pathway involving NF-κB, ras homolog gene family, member A (RhoA), and ROCK2 (2–4). Activation of this pathway leads to phosphorylation and inactivation of MLC phosphatase (MLCP) by ROCK2 (4, 7, 8).

Mfge8 is a multifunctional integrin-binding glycoprotein that is expressed in ASM. Mfge8 modulates inflammation by binding and targeting apoptotic cells for clearance through an integrin-dependent pathway (9, 10). We hypothesized that Mfge8 would be important in the resolution of lung inflammation in asthma. We report here that Mfge8-deficient mice (*Mfge8*$^{-/-}$ mice) developed exaggerated AHR in ovalbumin (Ova)-induced experimental models of asthma. However, enhanced AHR was not a result of increased airway inflammation. Instead, we report a unique role for Mfge8 in opposing the enhanced calcium sensitivity induced in ASM by cytokines released in allergic airway inflammation. Mfge8 reduced calcium sensitivity by integrin-dependent inhibition of RhoA activity and by inhibiting RhoA and ROCK2 protein induction by repressing activation and nuclear translocation of NF-κB.

Results

***Mfge8*$^{-/-}$ Mice Develop Exaggerated AHR After Ova Sensitization.** To examine the role of Mfge8 in allergic airway disease, we evaluated the response of *Mfge8*$^{-/-}$ mice in two models (36-d and 70-d Ova protocols) of allergen-induced asthma (11, 12). In both models, Ova-treated *Mfge8*$^{-/-}$ mice developed significantly exaggerated increases in pulmonary resistance, a measure of AHR to acetylcholine (Fig. 1 *A* and *B*).

Airway inflammation is central to the pathogenesis of asthma (13). To determine whether Mfge8 deficiency induced exaggerated airway inflammation, we examined bronchoalveolar lavage (BAL) samples in mice treated with Ova. In the 36-d Ova protocol, we observed a small increase in total BAL cell and lymphocyte counts but no difference in eosinophil counts (Fig. S1*A*). In the 70-d Ova protocol, the inflammatory response was more robust in both groups and there were no differences in BAL cell counts or in the number of BAL neutrophils, lymphocytes, eosinophils, or macrophages (Fig. S1*B*).

Mfge8 can regulate the differentiation of naive T cells into regulatory T cells (Tregs) and Th1 and Th17 cells (14–16). Each of these cell types has defined roles in asthma (17–20). To determine whether Ova treatment of *Mfge8*$^{-/-}$ mice affected T-cell differentiation, we isolated lymphocytes from the lung and measured cytokine production (or expression of FoxP3 for Treg cells). *Mfge8*$^{-/-}$ and WT mice had similar numbers and proportions of IFN-γ (Th1), IL-13 (Th2), IL-17A (Th17), and FoxP3 (Treg)-expressing cells, as well as total and CD4^{+} lymphocytes (Fig. S1 *C–F*). Serum Ova-specific IgE levels were also no different (Fig. S1 *G* and *H*), providing further evidence that the systemic immune response to allergen was unaffected by Mfge8 deficiency. Taken together, these data indicate that differences in the

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The authors declare no conflict of interest.

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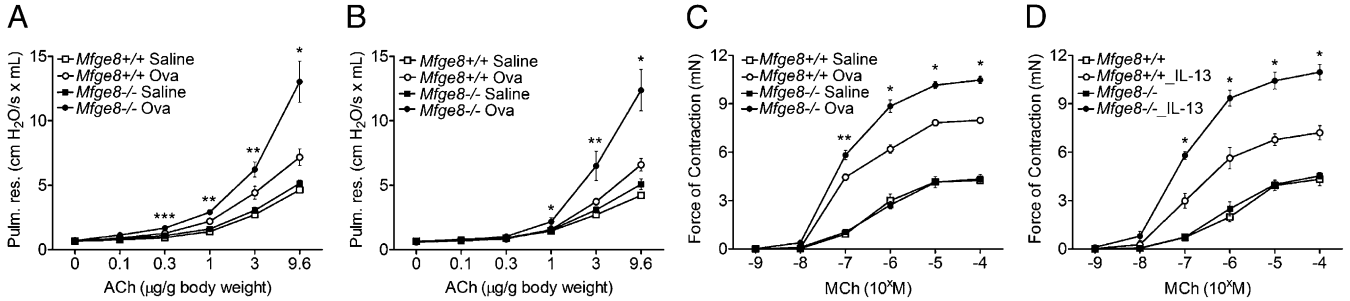

Fig. 1. *Mfge8*$^{-/-}$ mice develop exaggerated AHR after Ova sensitization and challenge. (*A*) Mice were treated with the 36-d Ova protocol as described in *Materials and Methods*. *Mfge8*$^{-/-}$ mice developed significantly greater pulmonary resistance to acetylcholine 24 h after the last Ova challenge (n = 9–10 in each experimental group; *P < 0.001, **P < 0.01, ***P < 0.05, Ova-treated *Mfge8*$^{-/-}$ vs. WT mice). (*B*) Mice were sensitized with the 70-d Ova protocol as described in *Materials and Methods*. *Mfge8*$^{-/-}$ mice developed significantly greater pulmonary resistance to acetylcholine 24 h after the last Ova challenge (n = 18–20 for Ova-treated group and n = 14–20 for saline-treated group; *P < 0.001, **P < 0.01, Ova-treated *Mfge8*$^{-/-}$ vs. WT mice). (*C*) Tracheal rings were taken from mice completing the 36-d Ova protocol, and contractile force was measured. *Mfge8*$^{-/-}$ tracheal rings generated significantly greater contraction to MCh (n = 8 for Ova-treated and n = 4 for saline solution-treated tracheal rings; *P < 0.001, **P < 0.01, Ova-treated *Mfge8*$^{-/-}$ vs. WT rings). (*D*) Tracheal rings were treated with IL-13 (100 ng/mL) for 12 h before measuring contraction. *Mfge8*$^{-/-}$ tracheal rings generated significantly greater contraction to MCh (n = 12; *P < 0.05, **P < 0.001, Ova-treated *Mfge8*$^{-/-}$ vs. WT rings).

immune response to allergen are not responsible for the effect of Mfge8 on AHR.

Mfge8 Expression in Proximal Airways Localizes to ASM. To localize Mfge8 expression, we stained tissues from saline solution- and allergen-treated WT mice for Mfge8. Mfge8 was expressed in the smooth muscle of bronchial (Fig. S2*A*) and tracheal ASM (Fig. S2*B*), but not in the epithelium. As with murine samples, Mfge8 was expressed in the ASM compartment of the human airway (Fig. S2*D*). To determine the cellular source of the Mfge8 in the ASM, we stained *Mfge8*$^{-/-}$ tissue sections for β-gal expression. β-Gal is part of the fusion protein created by the gene trapping vector used to generate *Mfge8*$^{-/-}$ mice used in these studies, and expression can be used to identify the cellular source of protein production (9). We found robust β-gal expression in ASM with no expression in the airway epithelium (Fig. S2 *E* and *F*). These data indicate that ASM produces and secretes Mfge8, which accumulates in the ASM compartment.

Mfge8 Regulates ASM Contraction After Ova Sensitization. The lack of an exaggerated immune response in *Mfge8*$^{-/-}$ mice after allergen challenge and the localization of Mfge8 around ASM suggested a role for Mfge8 in regulating ASM contraction. To test this hypothesis, we removed tracheal rings from *Mfge8*$^{-/-}$ and WT mice that had been sensitized and challenged with Ova or saline solution in vivo and measured the force of contraction in response to methacholine (MCh) and KCl. Tracheal rings from saline solution-treated mice generated similar contractile responses. However, tracheal rings from *Mfge8*$^{-/-}$ mice treated with Ova had significantly enhanced contraction to MCh and KCl (Fig. 1*C* and Fig. S3, respectively). These data indicate that Mfge8 regulates the force of ASM contraction in the setting of allergic inflammation.

In human asthma and mouse models of asthma, repeat exposure to allergen can induce an increase in ASM mass (21, 22). We therefore wanted to determine whether the enhanced AHR in *Mfge8*$^{-/-}$ mice could be the result of an increase in ASM mass. To evaluate ASM mass in vivo, we measured the expression of α-smooth muscle actin, a marker of ASM, in total lung homogenates taken from *Mfge8*$^{-/-}$ and WT mice treated with Ova. Although Ova induced an increase in total α-smooth muscle actin expression, we did not detect differences between experimental groups (Fig. S4*A*). We also did not find a difference in the thickness of the smooth muscle layer after Ova treatment (Fig. S4*B*), indicating that enhanced AHR is not a result of an increase in smooth muscle mass.

Mfge8 Prevents Cytokine-Enhanced ASM Contraction. IL-13 is a central regulator of asthma (23) and promotes airway obstruction by increasing the sensitivity of smooth muscle to calcium (2, 3). As Mfge8 deficiency did not result in an increase in the total number or proportion of IL-13–producing Th2 cells in the lung, we determined whether Mfge8 altered the ASM response to IL-13. We harvested tracheal rings from untreated mice and incubated them with IL-13 for 12 h before measuring contraction in response to MCh and KCl. IL-13-treated, but not vehicle control-treated, *Mfge8*$^{-/-}$ tracheal rings had significantly increased contraction (Fig. 1*D* and Fig. S5*A*) to MCh and KCl, indicating that Mfge8 modulated the ASM response to IL-13. To determine whether this effect of Mfge8 was specific for IL-13, we evaluated the effects of incubating tracheal rings with TNF-α and IL-17A, cytokines that have key roles in AHR and also increase calcium sensitivity. *Mfge8*$^{-/-}$ tracheal rings had enhanced contraction after treatment with each of these cytokines (Fig. S5 *B* and *C*). These data indicate a general role for Mfge8 in suppressing cytokine-induced enhancement of ASM contraction. To determine whether Mfge8 suppressed cytokine-enhanced smooth muscle contraction in other organ systems, we examined small intestinal smooth muscle. Smooth muscle strips from the jejunum of *Mfge8*$^{-/-}$ mice treated with IL-13 had enhanced contraction, which was completely rescued by incubation with recombinant Mfge8 (rMfge8; Fig. S5*D*).

As airway narrowing in asthma occurs in the bronchial airways, we wanted to confirm that enhanced contraction in *Mfge8*$^{-/-}$ tracheal rings accurately reflected airway narrowing mediated by bronchial smooth muscle. We therefore studied bronchial airway narrowing in an in vitro lung slice model (24). We incubated lung slices for 12 h with IL-13. We then measured airway narrowing by quantifying airway luminal diameter before and after treatment with MCh. Lung slices from *Mfge8*$^{-/-}$ mice incubated with IL-13 had significantly increased airway narrowing (Fig. S5 *D* and *E*), reflecting enhanced bronchial smooth muscle contraction.

Effect of Mfge8 on Tracheal ASM Contraction Is Independent of Airway Epithelium. The accumulation of Mfge8 around smooth muscle and the lack of Mfge8 in the airway epithelium suggested that the effect of Mfge8 on ASM was independent of interactions between ASM and the adjacent epithelium. However, IL-13 affects the airway epithelium (25) and ASM (26), and airway epithelium has an inhibitory effect on ASM contraction (27, 28). To investigate whether smooth muscle–epithelial interactions were important for the function of Mfge8 in ASM contraction, we repeated the tracheal ring contraction assay after denuding the epithelium layer from each ring (4). The removal of the epithelium resulted in

greater contractile responses in all experimental groups. However, IL-13–treated *Mfge8*$^{-/-}$ tracheal rings lacking epithelium still had significantly greater contraction then their WT counterparts (Fig. S6), indicating that the effect of Mfge8 on ASM is not dependent on smooth muscle–epithelial interactions.

Recombinant Mfge8 Reduces *Mfge8*$^{-/-}$ and WT ASM Contraction. We were next interested in defining the time at which Mfge8 was required for its effect on ASM contraction. To address this question, we incubated tracheal rings that had been treated with IL-13 for 12 h with rMfge8 for 1 h before measuring contraction. Incubation with rMfge8 for 1 h completely rescued enhanced contraction in *Mfge8*$^{-/-}$ tracheal rings (Fig. 2*A*). These data indicate that the effect of Mfge8 on ASM does not require new protein synthesis and is unlikely to be the result of chronic structural changes in *Mfge8*$^{-/-}$ ASM. We next examined whether longer incubation with rMfge8 would have a greater effect on reducing ASM contraction. A 12-h incubation of IL-13–treated *Mfge8*$^{-/-}$ tracheal rings with recombinant protein markedly reduced ASM contraction to untreated *Mfge8*$^{-/-}$ levels whereas it had no effect on the force of untreated tracheal ring contraction (Fig. 2*A*). We also examined whether rMfge8 would decrease WT tracheal ring contraction. A 1-h incubation with rMfge8 significantly reduced, and 12 h incubation completely abrogated, the IL-13–induced increase in contractility in WT tracheal rings treated with IL-13 (Fig. 2*B*).

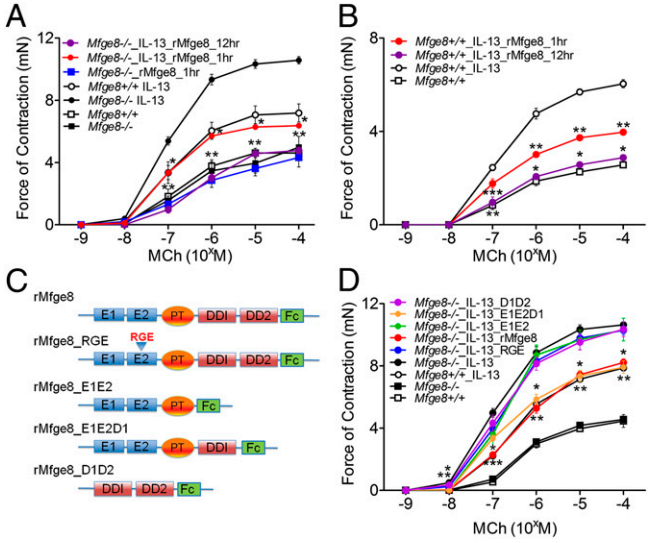

Fig. 2. Mfge8 reduces ASM contraction through its integrin-binding and discoidin domains. (*A*) IL-13–treated tracheal rings were incubated with rMfge8 for 1 or 12 h before challenge with MCh (n = 5–14; *P < 0.001, IL-13–treated *Mfge8*$^{-/-}$ rings treated with rMfge8 vs. IL-13–treated rings receiving no additional treatment for 1 h; **P < 0.001, IL-13–treated *Mfge8*$^{-/-}$ rings treated with rMfge8 vs. IL-13–treated rings receiving no additional treatment for 12 h). There was no significant difference between vehicle-treated *Mfge8*$^{-/-}$ rings treated with rMfge8 and vehicle-treated rings receiving no additional treatment. (*B*) IL-13–treated WT tracheal rings generate significantly less contraction when incubated with rMfge8 (n = 6–12; *P < 0.001, **P < 0.01, and ***P < 0.05, IL-13–treated WT rings treated with rMfge8 vs. IL-13–treated rings receiving no additional treatment). (*C*) Mfge8 mutated protein constructs fused to a human Fc domain. Mfge8 contains two EGF domains (E1 and E2), two discoidin domains (D1 and D2), and a proline-threonine rich mucin-like domain (PT). (*D*) The full-length construct and a construct lacking the second discoidin domain (E1E2D1) significantly reduced contraction (n = 5–29; *P < 0.001, *Mfge8*$^{-/-}$_IL-13_rMfge8 vs. *Mfge8*$^{-/-}$_IL-13 treatment; **P < 0.001, ***P < 0.001, *Mfge8*$^{-/-}$_IL-13_E1E2D1 construct vs. *Mfge8*$^{-/-}$_IL-13 treatment). A one-way ANOVA followed by a Bonferroni t test for subsequent pairwise comparison was used for all statistical analysis. All data are expressed as mean ± SEM.

Discoidin and Integrin-Binding Domains of Mfge8 Are Necessary for the Effect of Mfge8 on ASM Contraction. The domain structure of Mfge8 consists of two EGF domains followed by two discoidin domains (29). The second EGF domain has an RGD sequence that binds integrins (30). To determine which domains were necessary for ASM contraction, we tested the ability of a series of mutated Mfge8 recombinant protein constructs (Fig. 2*C*) to rescue enhanced contraction in *Mfge8*$^{-/-}$ tracheal rings. Although recombinant full-length Mfge8 rescued enhanced contraction, constructs with a point mutation changing the RGD sequence to RGE (rMfge8_RGE), lacking both EGF domains (rMfge8_D1D2), or lacking both discoidin domains (rMfge8_E1E2) failed to rescue enhanced ASM contraction (Fig. 3*D*). A construct lacking the second but retaining the first discoidin and both EGF domains (rMfge8_E1E2D1) completely rescued enhanced contraction (Fig. 2*D*). These data indicate that the effect of Mfge8 on ASM contraction is dependent on the integrin-binding and discoidin domains. Of the RGD binding integrins, Mfge8 binds αvβ3 and αvβ5 (30, 31), but not α5β1, αvβ6, or αvβ8 (29). Whether Mfge8 binds α8β1 or αvβ1 is unknown. αvβ5, αvβ3, and α8β1 are all expressed in ASM (32–35). Therefore, the potential integrin receptors for Mfge8 in ASM are αvβ3, αvβ5, αvβ1, or α8β1. The discoidin domains of Mfge8 have been shown to bind phosphatidylserine (PS) on apoptotic cells (30). To determine whether Mfge8 modulated ASM contraction by binding PS through its discoidin domains, we examined the effects of annexin V, a competitive binder of PS, on rMfge8 rescue of *Mfge8*$^{-/-}$ tracheal ring contraction. The addition of annexin V did not inhibit rMfge8 from rescuing enhanced contraction (Fig. S7).

Mfge8 Regulates ASM Calcium Sensitivity by Regulating RhoA Induction and Activity. Force generation by ASM is dependent on actin–myosin cross-bridge formation. When cytosolic calcium levels are increased, the combination of calcium and calmodulin activate MLC kinase, which phosphorylates MLC, leading to enhanced contraction (6, 36). Muscarinic agonists such as MCh increase cytosolic calcium concentration through release of intracellular stores of calcium from the sarcoplasmic reticulum. KCl increases cytosolic calcium primarily by inducing depolarization-dependent opening of voltage-gated calcium channels and allowing for calcium entry from the extracellular compartment. *Mfge8*$^{-/-}$ tracheal rings treated with IL-13 had increased contraction in response to KCl and MCh. These data suggest that the effect of Mfge8 on ASM contraction is mediated through modulation of calcium sensitivity rather than through increasing cytosolic calcium concentrations. To confirm that the response of *Mfge8*$^{-/-}$ ASM to KCl was independent of muscarinic receptors, we repeated the tracheal ring assay in the presence of atropine. Whereas atropine completely blocked MCh-induced contraction, it did not change the enhanced contraction induced by KCl in IL-13–treated *Mfge8*$^{-/-}$ tracheal rings (Fig. S8 *A* and *B*). To directly examine whether Mfge8 modulated the changes in cytosolic calcium in response to MCh, we measured calcium oscillations (37) in IL-13–treated lung slices from *Mfge8*$^{-/-}$ and WT mice. We did not find differences between experimental groups (Fig. S8 *C* and *D*).

Because IL-13 enhances ASM calcium sensitivity and Mfge8 opposed the effect of IL-13, we determined whether Mfge8 reduced calcium sensitivity by evaluating the phosphorylation status of MLC and the myosin-binding subunit of MLCP (MYPT1). Western blots of *Mfge8*$^{-/-}$ tracheal rings treated with IL-13 for 12 h had increased levels of phosphorylated MLC and MLCP (Fig. 3*A*), indicating that Mfge8 regulates ASM calcium sensitivity. There was no difference in baseline phosphorylation levels of *Mfge8*$^{-/-}$ and control samples, consistent with the lack of an observed difference in unstimulated ASM contraction. Increased ASM calcium sensitivity induced by IL-13 and IL-17A occurs through NF-κB activation, with subsequent induction of RhoA

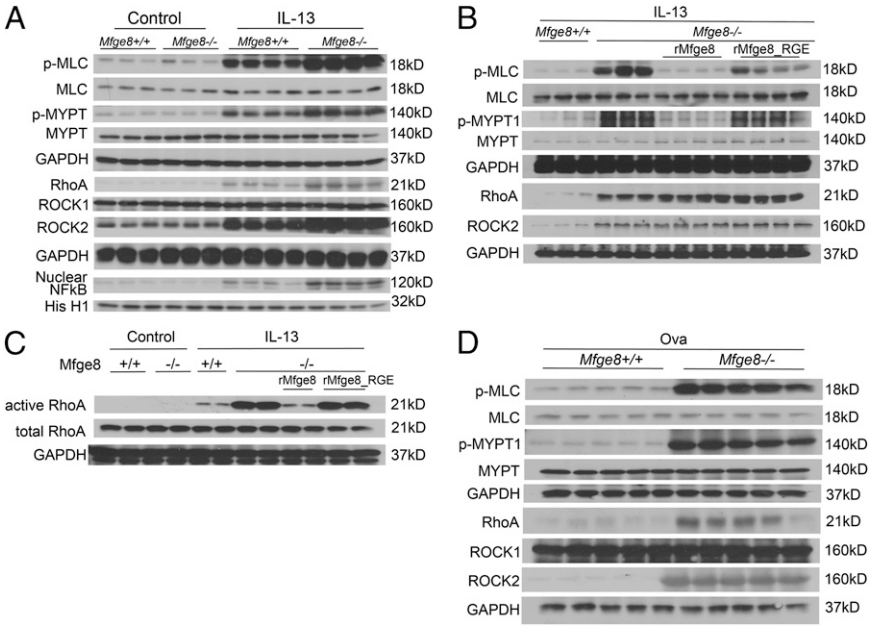

Fig. 3. Mfge8 regulates calcium sensitivity. (*A*) Tracheal ring muscle strips were incubated with IL-13 for 12 h and treated with MCh for 15 min, after which protein was extracted for Western blotting and examined with antibodies directed against MLC, pMLC, MYPT1, pMYPT1, GAPDH, RhoA, ROCK1, and ROCK2. For quantification of NF-κB, the nuclear fraction was probed with antibodies directed against NF-κB and histone H1 as a loading control. (*B*) Tracheal ring muscle strips were incubated with IL-13 for 12 h and rMfge8 (10 μg/mL) or rMfge8_RGE (10 μg/mL) for 1 h and treated with MCh for 15 min, and Western blots performed as described in *A*. (*C*) Primary ASM isolated from tracheal rings were incubated with IL-13 (100 ng/mL) and rMfge8 (10 μg/mL), rMfge8_RGE (10 μg/mL), or vehicle control for 1 h and stimulated with 5-hydroxytryptamine for 15 min, after which active and total RhoA was evaluated by using a GST pull-down assay and Western blot. (*D*) Tracheal rings from mice completing the 36-d Ova protocol were incubated with MCh for 15 min and then processed for Western blot as described in *A*.

protein. After 12 h incubation with IL-13, *Mfge8*$^{-/-}$ tracheal rings had increased nuclear NF-κB translocation and increased total RhoA expression. Expression of ROCK2, the downstream kinase targeted by RhoA in smooth muscle that is responsible for phosphorylation of MLCP, was also increased (Fig. 3*A*). As incubation with rMfge8 for 1 h rescued enhanced tracheal ring contraction, we assessed the effect of recombinant protein on calcium sensitivity. After a 12-h incubation with IL-13, 1 h treatment with rMfge8, but not the rMfge8_RGE mutant, reduced MLC and MLCP phosphorylation to control levels (Fig. 3*B*) while having no effect on RhoA and ROCK2 protein levels.

As 1 h incubation with rMfge8 did not decrease RhoA and ROCK2 protein, we examined whether Mfge8 affected RhoA activity in addition to RhoA expression. To test this hypothesis, we incubated primary ASM cells for 1 h with IL-13 and then treated them with 5-hydroxytryptamine for 15 min. We then measured RhoA activation by a Rho binding domain GST pull-down assay. IL-13 induced an increase in RhoA activation in WT cells and a marked increase in RhoA activation in *Mfge8*$^{-/-}$ cells (Fig. 3*C*). The addition of rMfge8 completely reversed the increase in RhoA activation whereas the rMfge8_RGE mutant had no effect.

To verify that differences in calcium sensitivity after IL-13 treatment reflected the effects of Ova treatment in vivo, we evaluated calcium sensitivity from tracheal ring samples after in vivo sensitization with Ova. Tracheal rings were harvested 24 h after the last intranasal allergen challenge of the 36-d Ova protocol, stimulated with MCh in vitro for 15 min, and prepared for Western blot. As with the IL-13–treated samples, *Mfge8*$^{-/-}$ tracheal rings treated with Ova had a marked increase in MLC and MLCP phosphorylation and RhoA and ROCK2 protein induction (Fig. 3*D*). Taken together, these data suggest a model by which Mfge8 binding to integrin receptors on ASM opposes the increase in ASM calcium sensitivity induced by airway inflammation by negatively regulating the expression and activity of RhoA (Fig. S9).

Mfge8 Is Expressed in Human ASM and Inhibits Human ASM Contraction Induced by IL-13. We were next interested in determining whether the pathway we identified was active in human ASM. To determine the functional role of Mfge8, we treated bronchial rings obtained from human lungs with IL-13 and recombinant lactadherin (the human orthologue of Mfge8). Incubation with two different doses of lactadherin significantly reduced bronchial ring contraction with the higher dose decreasing contraction to near baseline levels (Fig. 4*A*). These data indicate that lactadherin opposes enhanced bronchial smooth muscle contraction induced by IL-13.

Mfge8 Expression Is Decreased in Airways of Patients with Asthma. As Mfge8/lactadherin modulated the effect of IL-13 on bronchial smooth muscle contraction in vitro, we were interested in seeing whether there were differences in Mfge8 expression in the airways of patients with asthma. We examined Mfge8 and α-smooth muscle actin (Fig. 4*B*) expression in endobronchial biopsy samples obtained from subjects with mild to moderate asthma and from normal subjects. Asthmatic subjects and healthy control subjects had short-term exposure to ozone (one of the asthmatic subjects was exposed to particular matter). Interestingly, Mfge8 expression was decreased in all five samples, compared with three healthy control samples. To quantify expression, we normalized by densitometry Mfge8 expression to the loading control, histone H3, and found a significant decrease in Mfge8 expression in asthmatic patients (Fig. 4*C*). We also normalized α-smooth muscle actin expression to loading control expression. As expected, α-smooth muscle actin expression was significantly greater in patients with asthma compared with healthy control subjects (Fig. 4*D*). We also examined the ratio of normalized Mfge8 expression to normalized α-smooth muscle actin expression (Fig. 4*E*) and found a marked decrease in patients with asthma. Taken together, these data indicate that in asthmatic subjects exposed to environmental oxidative stress, there is a decrease in the absolute amount of Mfge8 expression in the airways as well as a decrease in the amount of Mfge8 expression relative to smooth muscle mass.

Discussion

In these studies, we identify a pathway that regulates bronchoconstriction in allergic airway disease by suppressing the effect of cytokines on ASM contraction. *Mfge8*$^{-/-}$ mice develop exaggerated AHR with minimal changes in airway inflammation. *Mfge8*$^{-/-}$ ASM has enhanced contractile responses after in vivo treatment of mice with Ova or in vitro treatment with IL-13, TNF-α, and IL-17A. Mfge8 rescues enhanced ASM contraction, and this effect is dependent on its integrin-binding and discoidin domains. Mechanistically, Mfge8 binding to integrin receptors

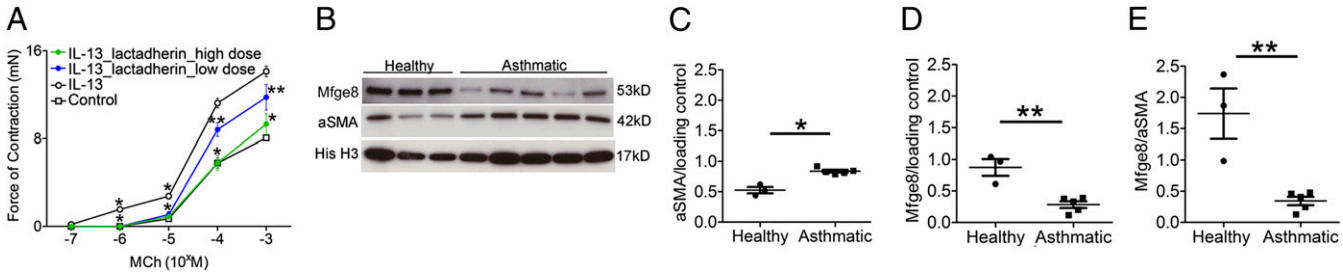

Fig. 4. Mfge8 regulates contraction in human ASM, and expression is decreased in human asthmatic patients. (*A*) Human bronchial rings were treated with IL-13 and lactadherin (10 and 20 μg/mL) for 12 h, and force of contraction was measured ($n = 5–10$; $*P < 0.001$, $**P < 0.05$, IL-13–treated rings vs. lactadherin, one-way ANOVA followed by Bonferroni test for subsequent pairwise comparison). (*B*) Homogenates from endobronchial biopsies of healthy and asthmatic human subjects were separated by SDS/PAGE and probed for Mfge8, α-smooth muscle actin, and histone H3 as a loading control. (*C*, *D*, and *E*) Blots were subsequently analyzed by densitometry and normalized to loading control pixel density values. Biopsies from asthmatic patients expressed higher levels of α-smooth muscle actin (*C*) but lower levels of Mfge8 (*D*; $**P < 0.01$, $*P < 0.001$). (*E*) Expression of Mfge8 was normalized to α-smooth muscle actin. Asthmatic patients expressed less Mfge8 relative to α-smooth muscle actin than healthy controls.

leads to reduced calcium sensitivity through inhibition of cytokine-induced RhoA expression and activation. Mfge8 is expressed in human ASM, and rMfge8 completely abrogates the effect of IL-13 in enhancing human bronchial ASM contraction. In human subjects exposed to environmental oxidative stress, airway Mfge8 expression is markedly reduced in asthmatic patients compared with healthy subjects.

IL-13, TNF-α, and IL-17A each enhance calcium sensitivity through a signaling pathway consisting of NF-κB activation, increased RhoA and ROCK2 expression, and ROCK2 phosphorylation of MLCP (2–4, 38). We show here that Mfge8 inhibits ASM contraction in part by suppressing this pathway. *Mfge8*$^{-/-}$ ASM had increased nuclear translocation of NF-κB, increased RhoA and ROCK2 expression, and increased phosphorylation of MLCP and MLC after 12 h treatment with IL-13. We further show that Mfge8 inhibits rapid RhoA activation induced by IL-13 in primary ASM cells, establishing dual roles for Mfge8 in inhibiting RhoA expression and activity. These dual effects provide an explanation for why 12 h incubation with rMfge8—compared with 1 h—leads to a greater reduction in *Mfge8*$^{-/-}$ and WT ASM contraction. The effects of 1 h treatment with rMfge8 are likely a result of prevention of RhoA activation whereas those of 12 h treatment are a result of inhibition of RhoA expression and activation.

The ability of rMfge8 to rescue enhanced RhoA activation in primary ASM cells and enhanced contraction in tracheal rings was dependent on an intact integrin-binding domain, as a point mutation changing the RGD sequence of Mfge8 to RGE completely abrogated the effect of recombinant protein. Integrin-mediated activation of RhoA is well established in the formation of stress fibers and cell contraction (39, 40). There is also some evidence that integrin engagement can at least transiently suppress RhoA activity (41). Our findings indicate that ligation of integrin receptors by Mfge8 suppresses RhoA activation. It is unclear whether this is a result of Mfge8 directly binding integrins or competitively inhibiting the binding of an alternate integrin ligand that normally promotes RhoA activation. In addition to the requirement for integrin binding, the effect of Mfge8 on ASM contraction was dependent on the presence of at least one discoidin domain. As our data indicate that RhoA activation is secondary to integrin ligation, we speculate that the discoidin domains tether Mfge8 to the smooth muscle surface, thereby promoting binding of the RGD-containing EGF domain to integrins. Alternatively, the discoidin domain may anchor Mfge8 to the ECM surrounding smooth muscle.

Our work and the work of others have identified a role for Mfge8 in suppressing inflammation (9, 10). The role of Mfge8 in regulating lung inflammation appears to be dependent on the model of lung injury. We have previously found no differences in lung inflammation in bleomycin-treated *Mfge8*$^{-/-}$ mice (29). Other studies have shown that *Mfge8*$^{-/-}$ mice develop exaggerated lung inflammation after intratracheal LPS instillation or after reperfusion injury secondary to intestinal ischemia (42, 43). Given the important role of the immune system in asthma, we were curious to determine whether there would be differences in airway inflammation in Ova-challenged *Mfge8*$^{-/-}$ mice. We observed a small increase in airway inflammation that was limited to one of the two Ova models examined. We do not believe this difference caused enhanced AHR for the following reasons. Although we found a statistically significant increase in BAL total and lymphocyte cell counts in the 36-d Ova protocol, there were no differences in BAL cell counts in the 70-d Ova model even though there was a marked increase in AHR in this model. We also evaluated lung T-cell subsets in the 36-d Ova protocol and did not find any difference in the number or proportion of cells expressing FoxP3, IL-13, IL-17A or INF-γ, or the total number or proportion of lymphocytes or $CD4^+$ lymphocytes. Although we cannot exclude a contribution to enhanced AHR from small differences in inflammation, we believe that effect on ASM calcium sensitivity was the dominant mechanism regulating AHR.

We also found several lines of evidence to indicate a role for Mfge8 in ASM hypercontractility in human asthma. We show that Mfge8 is expressed in the ASM of human lungs. In functional assays, we show that rMfge8 markedly reduces IL-13–enhanced bronchial contraction, confirming that the Mfge8 pathway is active in human ASM. Most importantly, we show that asthmatic subjects exposed to environmental oxygen stress have a significant reduction in the expression of Mfge8 in airway biopsies compared with healthy controls. This reduction is particularly marked when we examine the expression of Mfge8 relative to α-smooth muscle expression. Our interpretation of these results is that, in patients with asthma, the expansion of the ASM compartment occurs without a compensatory expression in Mfge8. Alternatively, the effect of exposure to environmental oxidative stress may down-regulate Mfge8 expression in asthmatic subjects compared with healthy subjects. In either scenario, the exposure of ASM to the cytokine-rich environment of the asthmatic airway results in bronchospasm caused by the lack of counterregulatory factors such as Mfge8.

Although a significant body of research has focused on understanding the effects of IL-13, IL-17A, and other cytokines on coordinating the immune response in asthma (17, 23, 44–46), the importance of these mediators on ASM contractility has been more recently appreciated (2, 4). Most of the therapeutic strategies in asthma target specific steps of the inflammatory response with varying success (47). Outside of bronchodilator therapy, less effort has focused on treatments that target the ASM, despite its importance in the pathophysiology of bronchospasm and

the progression of asthma. Although bronchodilator therapy nonspecifically relaxes smooth muscle, Mfge8 specifically suppresses the molecular pathway that induces smooth muscle hypercontractility in the cytokine-rich environment of allergic airway disease. Such treatments have a potentially high impact given the central role of ASM in bronchospasm.

Materials and Methods

All methods are described in detail in *SI Materials and Methods*. All experiments using mice were approved by the University of California, San Francisco (UCSF), Institutional Animal Care and Use Committee. All experiments using human tissue were approved by the UCSF Committee on Human Research. Informed consent was obtained from all human subjects as according to the UCSF Commitee on Human Research.

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