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IL-17A Recruits Rab35 to IL-17R to Mediate PKCα-Dependent Stress Fiber Formation and Airway Smooth Muscle Contractility

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IL-17A is a critical proinflammatory cytokine for the pathogenesis of asthma including neutrophilic pulmonary inflammation and airway hyperresponsiveness. In this study, by cell type–specific deletion of IL-17R and adaptor Act1, we demonstrated that IL-17R/Act1 exerts a direct impact on the contraction of airway smooth muscle cells (ASMCs). Mechanistically, IL-17A induced the recruitment of Rab35 (a small monomeric GTPase) and DennD1C (guanine nucleotide exchange factor [GEF]) to the IL-17R/Act1 complex in ASMCs, resulting in activation of Rab35. Rab35 knockdown showed that IL-17A–induced Rab35 activation was essential for protein kinase Cα (PKCα) activation and phosphorylation of fascin at Ser39 in ASMCs, allowing F-actin to interact with myosin to form stress fibers and enhance the contraction induced by methacholine. PKCα inhibitor or Rab35 knockdown indeed substantially reduced IL-17A–induced stress fiber formation in ASMCs and attenuated IL-17A–enhanced, methacholine-induced contraction of airway smooth muscle. Taken together, these data indicate that IL-17A promotes airway smooth muscle contraction via direct recruitment of Rab35 to IL-17R, followed by PKCα activation and stress fiber formation. ***The Journal of Immunology*****, 2019, 202: 1540–1548.**

It is estimated that, worldwide, more than 300 million people have asthma, and 8% of them suffer from the severe type of this disease (1). These patients are typically unresponsive or poorly responsive to currently available asthma drugs and frequently require high doses of systemic steroids. Several studies suggest a central role for IL-17 (also called IL-17A) in severe asthma (2–4). High levels of IL-17A are found in induced sputum, bronchial biopsies, and serum obtained from patients with severe asthma (5–7). IL-17A is a major proinflammatory cytokine that coordinates local tissue inflammation via the upregulation of proinflammatory and neutrophil-mobilizing cytokines and chemokines. Deficiency of IL-17A signaling components leads to diminished neutrophilic pulmonary inflammation and airway hyperresponsiveness (AHR) in both allergic and nonallergic asthma mouse models (8–10).

IL-17A, the prototypic IL-17 family member, functions either as a homodimer or as a heterodimer with IL-17F. Upon IL-17A stimulation, Act1 is recruited to IL-17R through a SEFIR-dependent interaction (11–14). Act1, in turn, interacts with multiple TRAFs for various downstream pathways, including NF-κB activation (15–18). Emerging evidences implicate cell type–specific activation of IL-17A–induced, Act1-mediated signaling, orchestrating the complex pathogenic processes. Although IL-17A signaling in airway epithelial cells plays a critical role for neutrophilic pulmonary inflammation (10), IL-17A has been implicated in AHR by increasing the contractility of airway smooth muscle (ASM) (19, 20). However, whether and how IL-17A signaling directly impacts on contractility of ASM cells (ASMCs) remains unclear.

We now deleted IL-17RC subunit of IL-17R complex and Act1 in ASMCs by breeding IL-17RC– and Act1-floxed mice with smooth muscle actin (SMA)–rtTA-Cre transgenic mice. IL-17A enhanced methacholine (MCh)–induced contraction, which was abolished in the ASMC-specific or IL-17RC– or Act1-deficient

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Abbreviations used in this article: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; ASMC, ASM cell; GEF, guanine nucleotide exchange factor; I.N., intranasal(ly); KO, knockout; Mass Spec, mass spectrometry; MCh, methacholine; PKCα, protein kinase Cα; RT, room temperature; shRNA, short hairpin RNA; SMA, smooth muscle actin; SM-MHC, smooth muscle–myosin H chain; WT, wild-type.

tracheal rings. To our knowledge, these results, for the first time, provided genetic evidence that IL-17A signaling in ASMCs exerts a direct impact on trachea contractility.

Although IL-17A was previously shown to increase the levels of RhoA and its downstream effector, ROCK2, in ASMCs (19), in this study, we report a novel IL-17A–signaling axis that plays a direct role in ASMC contractility. By mass spectrometry (Mass Spec) analysis, we identified Rab35 (a small monomeric GTPase) (21) as an interacting protein of IL-17R. We found that IL-17A induced the recruitment of Rab35 (22) and its activator DennD1C (guanine nucleotide exchange factor [GEF]) (22, 23) to the IL-17R/Act1 complex in ASMCs, resulting in activation of Rab35. Furthermore, we demonstrated that IL-17A–induced Rab35 activation was essential for protein kinase Cα (PKCα) activation and phosphorylation of fascin at Ser39 in ASMCs, allowing F-actin to interact with myosin to form stress fibers and generate contraction force. Consistently, PKCα inhibitor or Rab35 knockdown attenuated IL-17A–induced actin/myosin interaction (stress fiber formation) in ASMCs and reduced IL-17A–enhanced, MCh-induced contraction of ASM. Taken together, these data indicate that Rab35/PKC/fascin cascade is a novel mechanism for IL-17A–mediated ASM contraction.

Materials and Methods

Mice

IL-17RC–deficient mice were obtained from Dr. W. Ouyang (18) (Genentech) and α-SMA promoter (α-sm-rTTA) and (tetO)7-cre mice were obtained from Dr. D. Sheppard (University of California, San Francisco). Both strains were described previously (24). Act1-floxed mice were generated in Dr. X. Li (13) laboratory and described previously. Rosa-LSL-TdTomato mice were purchased from The Jackson Laboratory.

IL-17RC–floxed mice were generated for Dr. Li by Cyagen Biosciences using gene-targeting technology (Supplemental Fig. 1). A targeting vector containing a 5′homology arm, a 3′homology arm, and a conditional region was generated by PCR. The targeting construct also contained loxP sequences flanking the conditional knockout (KO) region and the Neo expression cassette (for positive selection of embryonic stem cells), flanked by FRT sequences (for subsequent removal of the Neo cassette). The final targeting construct is shown in Supplemental Fig. 1A. Successfully targeted embryonic stem cells were injected into blastocysts and implanted into pseudopregnant females. Chimeric male offspring were mated to wild-type (WT) C57BL/6 female, and germline transmission of the mutant IL-17RC allele was confirmed by Southern blot (data not shown) and PCR analyses (Supplemental Fig. 1B). The following primers were used: Clevelandclinic009_F1: 5′-CCTAGTTTATGTCACAGAGCAGCCATG-3′.

Clevelandclinic009_R1: 5′-CCCAGTTCTAAAGCACGTATCTCCTACA-3′.

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Reagents

We purchased Abs from following companies: FLAG M2 tag and hemagglutinin tag (Sigma-Aldrich and Cell Signaling); V5 tag (Invitrogen); myc tag (Cell Signaling); smooth muscle–myosin H chain (SM-MHC) and fascin (Abcam); SMA (Sigma-Aldrich); Rab35 (Cell Signaling); activated Rab35-GTP (NewEast Biosciences); p-IκB, IκB, p-p65, PKCα, p-PKCα, and p-myosin (Cell Signaling); p-fascin (ECM Biosciences); Act1 (custom generated); GAPDH (Ambion); and β actin (Cytoskeleton).

Rhodamine phalloidin for F-actin staining was purchased from Life Technologies.

PKC inhibitor Gö6976 was purchased from Cell Signaling.

Mouse ASMC isolation

Mouse smooth muscle cells were isolated as described previously (25, 26). Shortly, the excised, longitudinally cut tracheas were digested in 0.15% Pronase solution (Roche) at 4˚C overnight. Next day, the tracheas were brushed with a cotton swab to remove remaining epithelial cells, cut into small pieces (~30 per trachea), and transferred to a 100-cm^2 tissue culture dish for attachment and outgrowth of ASMCs.

Intranasal instillation of rIL-17A

Mice were exposed to inhaled anesthesia with isoflurane. Recombinant murine IL-17A, carrier-free (R&D Systems) and resuspended according to manufacturer's instruction, was instilled into a nasal opening in a 20-μl aliquot per mouse (1 μg/mouse).

Tracheal ring contraction

The mice were injected with IL-17A (1 μg in 20 μl) or PBS via intranasal (I.N.) route. Twelve hours later, the tracheas were excised and cut into 3-mm rings and placed in DMEM12 with HEPES and L-glutamine. For some experiments, the rings were incubated with or without IL-17A or PKC inhibitor for 12 h in a 37˚C incubator with 5% CO_2.

Rings were suspended in a 15-ml organ bath filled with oxygenated modified Krebs–Henseleit solution and connected to a force-displacement transducer (FT03; Grass Instrument, West Warwick, RI), and a resting tension of 0.5 g was applied. After a 15-min equilibration period, concentration/response curves to MCh were constructed.

Lentiviral I.N. delivery

Replication-incompetent, vesicular stomatitis virus G protein–enveloped lentivirus (pLKO.1-puro) containing either control or Rab35 short hairpin RNA (shRNA) (10^7–10^{10} PFU) in 30 μl was administered via I.N. route to mice anesthetized with injectable agents on day 0. Seventy-two hours after inoculation, the mice were treated I.N. with 1 μg of IL-17A for 12 h.

Rab35 shRNA (TRCN0000100532) Sigma-Aldrich, Clone ID: NM_198163.1-232s1c1.

Sequence: 5′-CCGGGCTGTTACGATTCGCAGACAACTCGAGTTGTCTGCGAATCGTAACAGCTTTTTG-3′.

Control shRNA (pLKO.1 nontargeting, SH002 Sigma-Aldrich).

Proximity ligation assays (Duolink)

IL-17RC-M2 or Act1-M2 and Rab35^{WT}-V5 were overexpressed in HeLa cells grown on glass coverslips. Twenty-four hours posttransfection, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature (RT) and blocked with 10% normal goat serum for 1 h at RT. The cells were next permeabilized with 0.1% Triton X-100 in goat serum for 15 min at RT and incubated with primary Abs (dilution 1:1000) against epitope tags overnight: rabbit anti-FLAG M2 (Cell Signaling) or mouse anti-V5 (Invitrogen). Duolink, based on in situ proximity ligation assay, was performed according manufacturer instructions (Sigma-Aldrich).

Immunohistochemistry

Cells grown on the glass coverslips were fixed with 4% paraformaldehyde for 10 min at RT and blocked with 10% normal goat serum for 1 h at RT. The cells were next permeabilized with 0.1% Triton X-100 in goat serum for 15 min at RT and incubated with primary Abs (dilution 1:1000) against epitope tags overnight or with phalloidin/rhodamine (dilution 1:1000). Next day, the appropriate secondary fluorescent Ab was applied (1:500). The cells were costained with DAPI.

Western blotting

Cells stimulated as indicated were harvested, washed once with cold PBS, and lysed for 30 min at 4˚C in 1% Triton X-100, 20 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, and protease and phosphatase inhibitors (Roche). Cellular debris were removed by centrifugation at 16,000 relative centrifugal force for 10 min. For immunoblotting, cell extracts were fractionated by SDS-PAGE and transferred to Immobilon-P transfer membranes (MilliporeSigma), using a wet transfer apparatus (Bio-Rad Laboratories). Immunoblot analysis was performed, and the bands were visualized with HRP-coupled goat anti-rabbit, goat anti-mouse, or donkey anti-goat Ig as appropriate (Rockland Immunochemicals), using the ECL Western blotting detection system (GE Healthcare). Protein levels were equilibrated with the Protein Assay Reagent (Bio-Rad Laboratories).

Coimmunoprecipitation

For coimmunoprecipitations, cells were harvested, washed once with cold PBS, and lysed in a Triton X-100–containing buffer (0.5% Triton X-100, 20 mM HEPES [pH 7.4], 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, and protease and phosphatase inhibitors [Roche]). Cell extracts were incubated with 1 μg of indicated Ab for 2 h, followed by incubation for 12 h with 30 μl of Protein G Sepharose beads (prewashed and resuspended in lysis buffer at a 1:1 ratio). After incubations, the beads were washed four times with lysis buffer, separated by SDS-PAGE, and analyzed by immunoblotting.

Results

IL-17A signaling in ASMCs promotes contractility

IL-17A stimulation substantially enhanced MCh-induced WT tracheal ring contraction, which was completely abolished in IL-17RC–deficient tracheal rings (data not shown). Because IL-17A–driven contractility was retained in tracheal rings denuded of epithelium (19), we hypothesized that IL-17A may directly act on smooth muscle cells to enhance contraction. To test this hypothesis, we specifically deleted IL-17RC in ASMC cells by breeding IL-17RC–floxed mice with SMA-rtTA-Cre transgenic mice [expressing the reverse tetracycline transactivator under the control of the α-SMA promoter (α-sm-rTTA) and (tetO)7-cre] (24). When SMA-rtTA-Cre mice were crossed onto Rosa26-LSL-tdTomato background, we detected the colocalization of tdTomato with SMA (Fig. 1A) in ASMC of trachea. The enhanced MCh-induced contraction in response to IL-17A stimulation was abolished in the ASMC-specific, IL-17RC–deficient tracheal rings (Fig. 1B). We also examined the impact of ASMC-specific Act1 (adaptor of IL-17R) deficiency on tracheal contractility. IL-17A–induced enhancement of MCh-induced contraction was also abolished in ASMC-specific, Act1-deficient tracheal rings (Fig. 1C). These results suggest that IL-17A signaling in ASMCs directly enhances tracheal contractility.

Rab35 is recruited to the IL-17R/Act1 complex

We next studied how IL-17A signaling impacts ASMC function. Through the Mass Spec search for IL-17RC versus IL-17RB novel interacting proteins, we identified several proteins that were specifically associated with IL-17RC. Among these IL-17RC–specific interacting proteins, we became interested in Rab35 (Supplemental Fig. 2) because Rab35 was shown to play a critical role in actin dynamics and in bundling of actin (21, 22, 27, 28), an important component for ASMC contractility (21, 22). We confirmed the interaction of Rab35 with IL-17RC by proximal ligation assay (Duolink) and coimmunoprecipitation (Fig. 2A, 2B). Interestingly, we also found that IL-17A–induced Rab35 and IL-17RC interaction was Act1 dependent, suggesting that Act1 is required for the recruitment of Rab35 to IL-17RC in response to IL-17A stimulation (Fig. 2B).

Although $Rab35^{S22N}$ is the GDP-bound inactive form of Rab35, $Rab35^{Q67L}$ is the GTP-bound active form of Rab35 (27, 29–32). Compared with the WT Rab35, the active form of Rab35 ($Rab35^{Q67L}$) lost binding to IL-17RC. Interestingly, the inactive form of Rab35 ($Rab35^{S22N}$) binds well to the IL-17RC (Fig. 2C). These data suggest that IL-17RC may preferentially binds to the inactive form of Rab35, and activation of Rab35 may lead to its dissociation from IL-17RC. Furthermore, we found that SEFIR domain of IL-17RC is required for its interaction with Rab35 (Fig. 2D).

In contrast, coimmunoprecipitation experiments showed that Act1 preferentially interacted with GTP-bound active form of Rab35 ($Rab35^{Q67L}$ mutant) (Fig. 3A). Rab family proteins need to be activated via GEFs, which mediate the exchange of GDP to GTP (23). IL-17A stimulation indeed was able to induce Rab35 activation, which was abolished in Act1-deficient ASMCs (Fig. 3B). It has been shown previously that DennD1C is specific Rab35 GEF, which uniquely

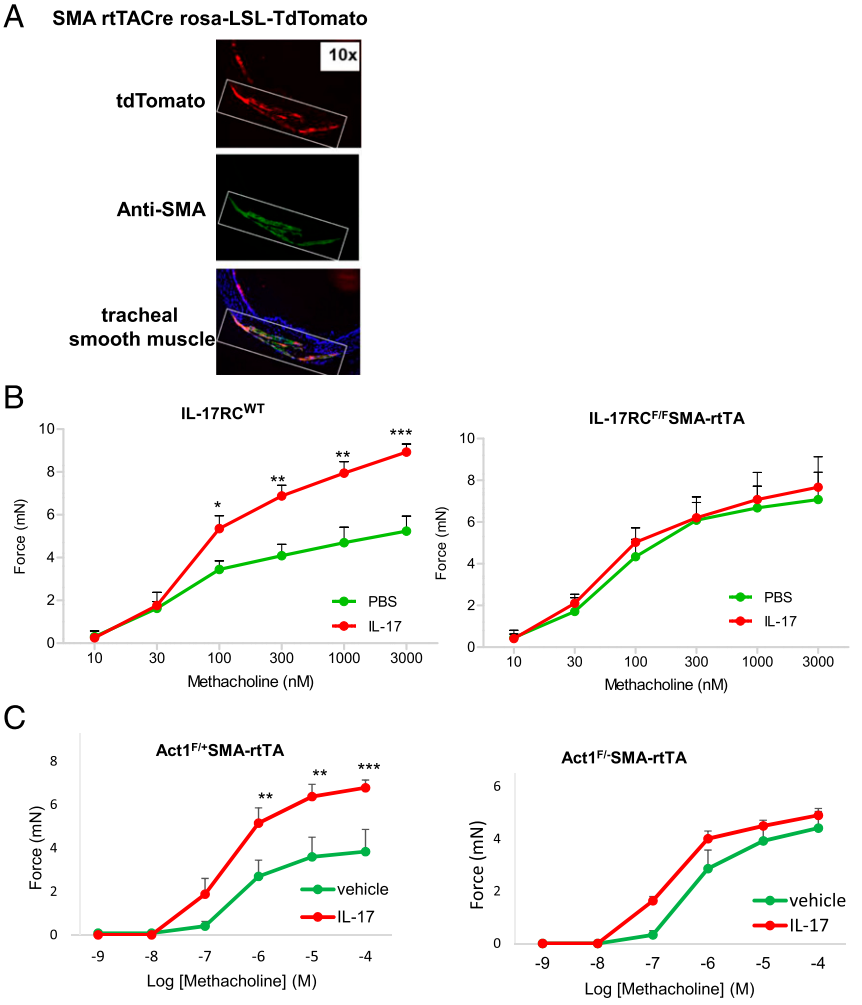

FIGURE 1. IL-17RC is required for IL-17A–induced AHR. (**A**) Immunohistochemistry staining of tracheas isolated from SMA-rtTA-Cre mice that were bred onto Rosa26-LSL-tdTomato background. The sections were counterstained with α-SMA Ab. TdTomato (red) is expressed in ASMC, and it colocalizes with SMA (green). (**B**) Contractile force measurements from tracheal rings stimulated with MCh after I.N. treatment with PBS or IL-17A of IL-17RC WT and smooth muscle–specific IL-17RC KO ($IL\text{-}17RC^{F/F}$SMArtTA) mice. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Data are means ± SEM of at least five tracheal rings per group. (**C**) Contractile force measurements from tracheal rings stimulated with MCh after in vitro treatment with PBS or IL-17A of WT control ($Act1^{F/+}$SMArtTA) and smooth muscle–specific Act1 KO ($Act1^{F/-}$SMArtTA) mice. $**p < 0.01$, $***p < 0.001$. Two-way ANOVA with repeated measures of variance, followed by Tukey *t* test for subsequent pairwise analysis was used to calculate statistical significance. Data are means ± SEM of at least five tracheal rings per group.

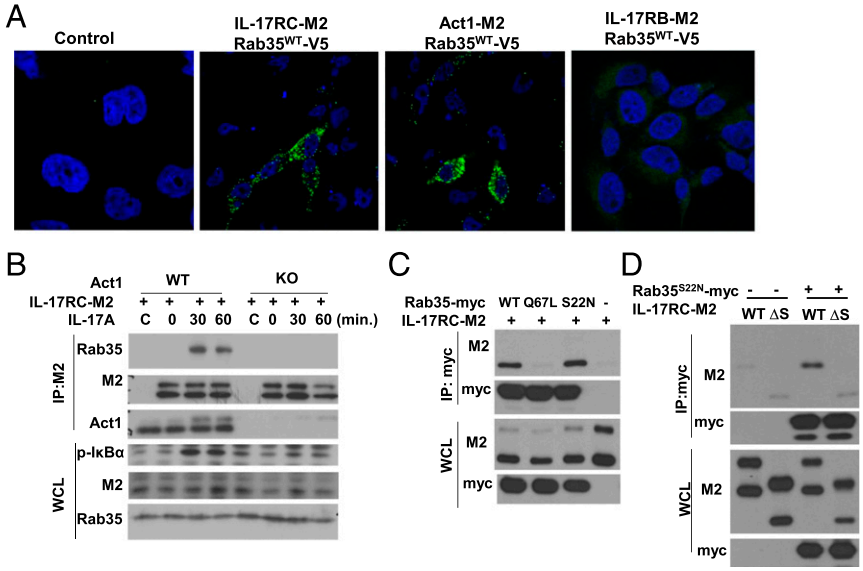

FIGURE 2. Rab35 forms interaction complex with IL-17RC. (**A**, **C**, and **D**) HeLa cells were transiently cotransfected with plasmids encoding (A) M2-tagged Act1, IL-17RC, or IL-17RB with V5-tagged Rab35^{WT} (C), M2-tagged IL-17RC WT with myc-tagged Rab$^{WT/S22N/Q67L}$ (D), M2-tagged IL-17RC WT, or δ SEFIR (ΔS) with myc-tagged Rab^{S22N}. (A) Cells were fixed, and proximity ligation assay was performed according to manufacturer instruction. Original magnification ×63. (C and D) Lysates of transfected cells were subjected to immunoprecipitation (IP) with indicated Ab, after which they were analyzed by Western blotting (WB) whole-cell lysate (WCL). All experiments were performed three times, with representative blots shown. (**B**) WT (Act1-WT) and Act1-deficient (Act1-KO) ASMCs infected with retroviral M2-tagged IL-17R and left untreated (0) or treated for 30 or 60 min with IL-17A (50 ng/ml); lysates were subjected to IP with anti-flag M2 Ab; after which, they were analyzed by WB. Data are representative of three independent experiments.

activates Rab35 for its role in actin regulation (30, 33–35). We confirmed that DennD1C was expressed in ASMC (Fig. 3C), and we found that DennD1C was also detected in IL-17RC/Act1 complex in a SEFIR domain–dependent manner (Fig. 3D), suggesting that Act1 might promote Rab35 activation via GEF recruitment.

Rab35 is required for IL-17A–induced ASM contractility

To determine the function of Rab35 in IL-17A signaling, we knocked down Rab35 by lentiviral shRNA in ASMCs. Notably, shRNA-mediated Rab35 knockdown did not have any impact on IL-17A–mediated NF-κB activation (Fig. 4A) or gene expression (Fig. 4B). Although Rab35 was implicated in regulation of actin bundling (22), we found that IL-17A stimulation induced formation of stress fibers in ASMCs, including actin polymerization and actin/myosin bundling (Fig. 4C). Importantly, both Act1 deficiency and Rab35 knockdown substantially attenuated IL-17A–induced stress fiber formation (Fig. 4C, 4D).

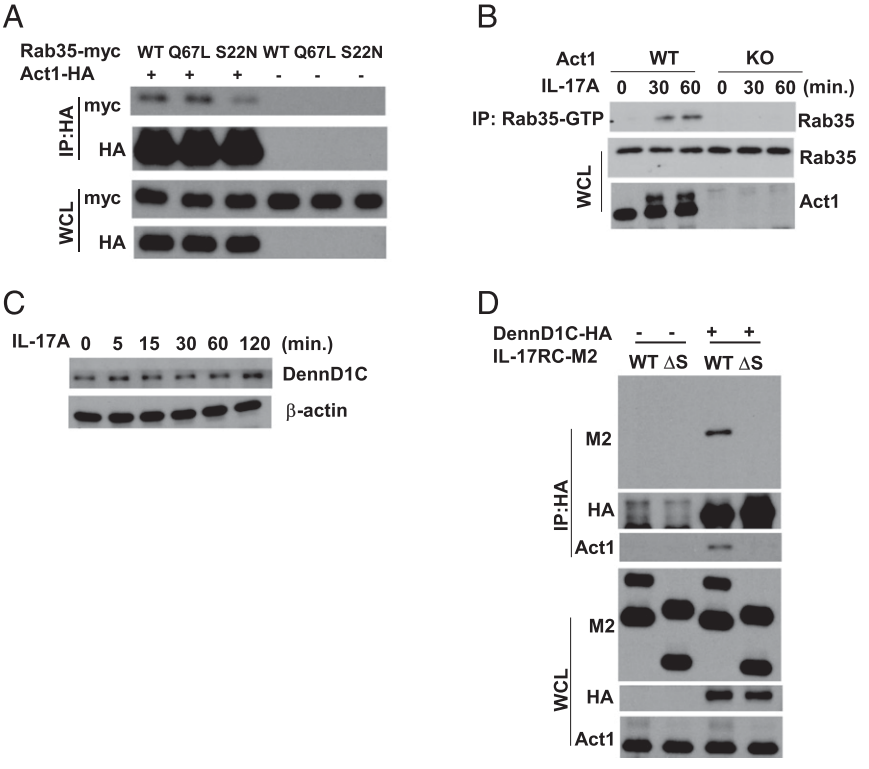

FIGURE 3. Act1 is required for Rab35 activation and IL-17RC interaction. (**A** and **D**) HeLa cells were transiently cotransfected with plasmids encoding (A) hemagglutinin (HA)–tagged Act1 with myc-tagged Rab$^{WT/S22N/Q67L}$ (D), M2-tagged IL-17RC WT, or δ SEFIR (ΔS) with HA-tagged DennD1C. Lysates of transfected cells were subjected to immunoprecipitation (IP) with indicated Ab, after which they were analyzed by Western blotting (WB) whole-cell lysate (WCL). All experiments were performed three times, with representative blots shown. (**B**) WT (Act1-WT) and Act1-deficient (Act1-KO) ASMCs left untreated (0) or treated for 30 or 60 min with IL-17A (50 ng/ml); lysates were subjected to IP with anti-Rab35^{GTP} Ab; after which, they were analyzed by WB. Data are representative of three independent experiments. (**C**) Lysates from WT ASMC were subjected to WB with indicated Ab.

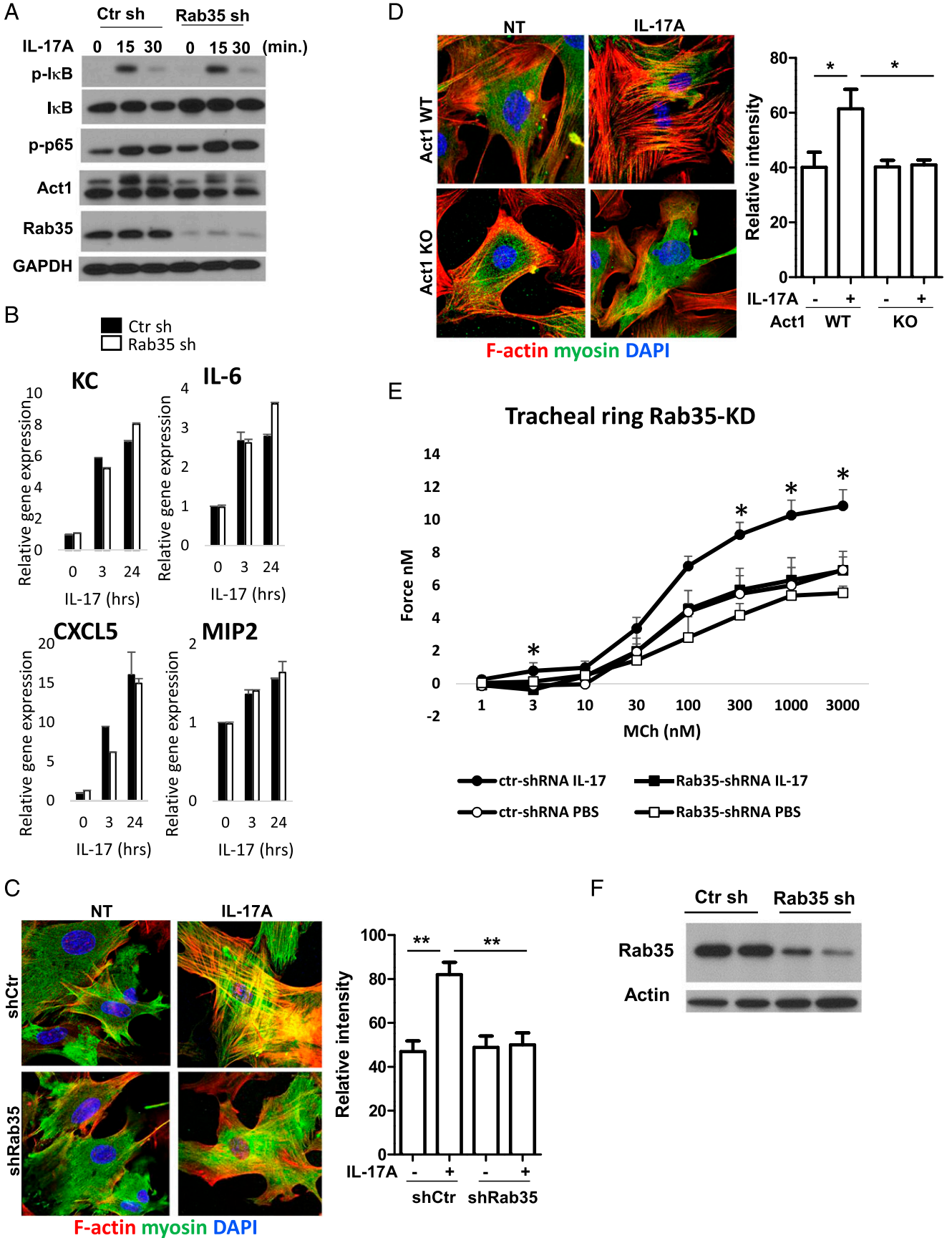

FIGURE 4. Rab35 is required for IL-17–induced smooth muscle cell contraction. (**A** and **B**) Mouse ASMCs were infected with control or Rab35 shRNA and treated with IL-17 for indicated times. Cell lysates were subjected to Western blot (WB) with indicated Abs (A) or to real-time PCR analysis for KC, IL-6, CXCL5, and MIP2 (B). (**C**) Mouse ASMCs were infected with lentivirus containing control or Rab35 shRNA and treated with IL-17A for 12 h and fixed and stained with phalloidin/rhodamine and SM-MHC (green). Relative intensity of rhodamine (F-actin) was quantified using ImageJ software. (**D**) WT (Act1-WT) and Act1-deficient (Act1-KO) ASMCs were treated with IL-17A for 12 h, fixed and stained with *(Figure legend continues)*

To examine the role of Rab35 in ASM contraction in vivo, we knocked down Rab35 in the airway by I.N. infection of lentiviral Rab35/shRNA, followed by the measurement of the MCh-induced tracheal ring contraction. Rab35 knockdown substantially reduced IL-17A–enhanced ring contraction as compared with control tracheas (Fig. 4E), indicating that Rab35 is required for IL-17A–induced contractility. Western blotting confirmed decreased expression of Rab35 in ASMCs (Fig. 4F).

Rab35 is required for IL-17A–induced PKCα activation and actin/myosin interaction

We then investigated the molecular mechanism for IL-17A–induced, Rab35-mediated formation of stress fibers in ASMCs and ASM contraction. Rab35 was shown to regulate actin bundling via its interaction with fascin (22). IL-17A stimulation induced fascin serine 39 (Ser39) phosphorylation in ASMCs, which was abolished in Rab35 knockdown and in Act1 KO cells (Fig. 5A). Notably, PKCα induces the phosphorylation of fascin at serine 39, which releases fascin from F-actin (36), allowing F-actin/myosin interaction for generation of contraction force. In support of this, fascin depletion enhanced the formation of stress fibers rendering increased cell contractility (37, 38). In addition to fascin phosphorylation, IL-17A was also able to induce the phosphorylation of PKCα serine 657 (Ser657) in ASMCs, which was completely abolished in Rab35 knockdown as well as in Act1 KO cells (Fig. 5A).

Furthermore, whereas IL-17A induced Rab35 activation, the GTP-bound active mutant of Rab35 interacted with fascin and PKCα in response to IL-17A stimulation (Fig. 5B). Moreover, IL-17A–induced fascin/PKCα interaction was Rab35 dependent, as fascin/PKCα complex formation was abolished in Rab35-deficient ASMCs (Fig. 5C). These results suggest that IL-17A–induced Rab35 activation promotes PKCα/fascin interaction, which may allow PKCα to phosphorylate and modulate the function of fascin, allowing F-actin/myosin interaction for generation of contraction force (Fig. 5D). In support of the hypothesis, PKCα-inhibitor Gö6976 indeed blocked IL-17A–induced stress fibers (Fig. 6A). Moreover, IL-17A induced actin/myosin interaction, which was substantially blocked by PKC inhibitor Gö6976 (Fig. 6B) and Rab35 deficiency (Fig. 6C).

To further test our hypothesis, we examined the impact of IL-17A–induced PKCα activation on human bronchial rings contractility. PKCα inhibitor was used to treat the bronchial rings, followed by the measurement of the MCh-induced tracheal ring contraction. PKCα inhibitor substantially reduced IL-17A–enhanced ring contraction as compared with control bronchial rings (Fig. 6D). Because we have shown that Rab35 was required for IL-17A–induced PKCα activation, these results suggest that PKCα activation might play a critical role in Rab35-dependent, IL-17A-induced ASM contractility.

Discussion

This study demonstrated that IL-17A signaling, specifically in ASMCs, directly contributes to AHR by increasing ASM contractility. IL-17A–enhanced, MCh-induced contraction was abolished in the ASMC-specific IL-17RC– or Act1-deficient tracheal rings. Mechanistically, we found that IL-17A promotes ASM contraction via Rab35-mediated PKCα activation. IL-17A stimulation induced the recruitment of Rab35 and DennD1C to the IL-17R/Act1 complex in ASMCs, resulting in activation of Rab35. By knocking down Rab35, we showed that Rab35 was required for IL-17A–induced PKCα activation and phosphorylation of fascin in ASMCs, resulting in actin/myosin interaction to generate contractile force. IL-17A stimulation indeed induced actin/myosin interaction in ASMCs, which was attenuated by PKCα inhibitor or Rab35 knockdown. Likewise, PKCα inhibitor or Rab35 knockdown substantially reduced IL-17A–enhanced, MCh-induced contraction of ASM, indicating the functional importance of this novel IL-17R/Rab35/PKCα axis in exacerbating ASM contraction.

IL-17 receptor family encompasses five members that form specific heterodimers and respond to their cognate ligands. In this study, we specifically focused on the pathogenic mechanism of IL-17A, which has been previously shown to be elevated in patients with severe asthma. In addition, we and others have found that ASMC are highly responsive to IL-17A. To this end, IL-17RC, a receptor required for IL-17A (but not other ligands such as IL-25 signaling), is the right target for the delineation of IL-17A function. Mass Spec analysis showed that Rab35 specifically interacts with IL-17RC, but not IL-17RB receptor subunit. Rab35, an evolutionary conserved small monomeric GTPase has a variety of cellular functions (21), including actin dynamics in mammalian cells (21, 27, 28) and actin bundling during bristle formation in Drosophila (22). Notably, Rab35 controls actin bundling by recruiting fascin as an effector protein (22). Fascin is an F-actin–bundling protein, which is important for the formation of actin-based protrusions and for maintaining cytoplasmic F-actin bundles. Rabs oscillate between active GTP-bound and inactive GDP-bound states, which are regulated by GEFs. DennD1C is the relevant GEF for Rab35 facilitating the impact of Rab35 on actin function (30, 34). DennD1C was shown to interact with actin/fascin via its C-terminal actin-binding domain where it mediates the GDP-to-GTP exchange on Rab35 (34). We found that upon IL-17A stimulation, DennD1C was recruited to the IL-17R/Act1 complex. We propose that the recruited DennD1C has two functions at the receptor complex: mediating Rab35/GDP conversion to Rab35/GTP; and bringing actin/fascin bundles to IL-17R/Act1 complex via its C-terminal actin-binding motif. It is important to note in this study that another Rab35 GEF/DennD1B was implicated in asthma pathogenesis via modulation of TCR in Th2 cells (39).

The bundling activity of fascin is inhibited by phosphorylation of its serine residue Ser39. PKCα was reported to phosphorylate fascin, leading to the dissociation of fascin from the F-actin bundles and a diffuse localization of fascin (37). In this study, we showed that, upon IL-17A treatment, both PKCα and fascin were phosphorylated. The fact that IL-17A induced the complex formation among Rab35/PKCα/fascin implicates the critical role of PKCα activation in IL-17A–induced fascin phosphorylation driving the dissociation of actin from fascin/actin bundles. The released actin can then interact with myosin, resulting in stress fiber formation and smooth muscle contraction. In support of this,

phalloidin-rhodamine and SM-MHC (green). Relative intensity of rhodamine (F-actin) was quantified using ImageJ software. Student *t* test was used to calculate statistical significance. (C and D) Original magnification ×63. (**E**) Contractile force measurements from tracheal rings stimulated with MCh after I.N. treatment with PBS or L-17A from C57/B6 WT mice infected I.N. with lentivirus coding control (ctrl) or Rab35 shRNA. Data are means ± SEM of at least 5 tracheal rings per group. Two-way ANOVA with repeated measures of variance, followed by Tukey *t* test for subsequent pairwise analysis was used to calculate statistical significance. (**F**) Western blot shows deletion efficiency of Rab35 in tracheal ASMC isolated from two pairs of mice. $*p < 0.05$, $**p < 0.01$.

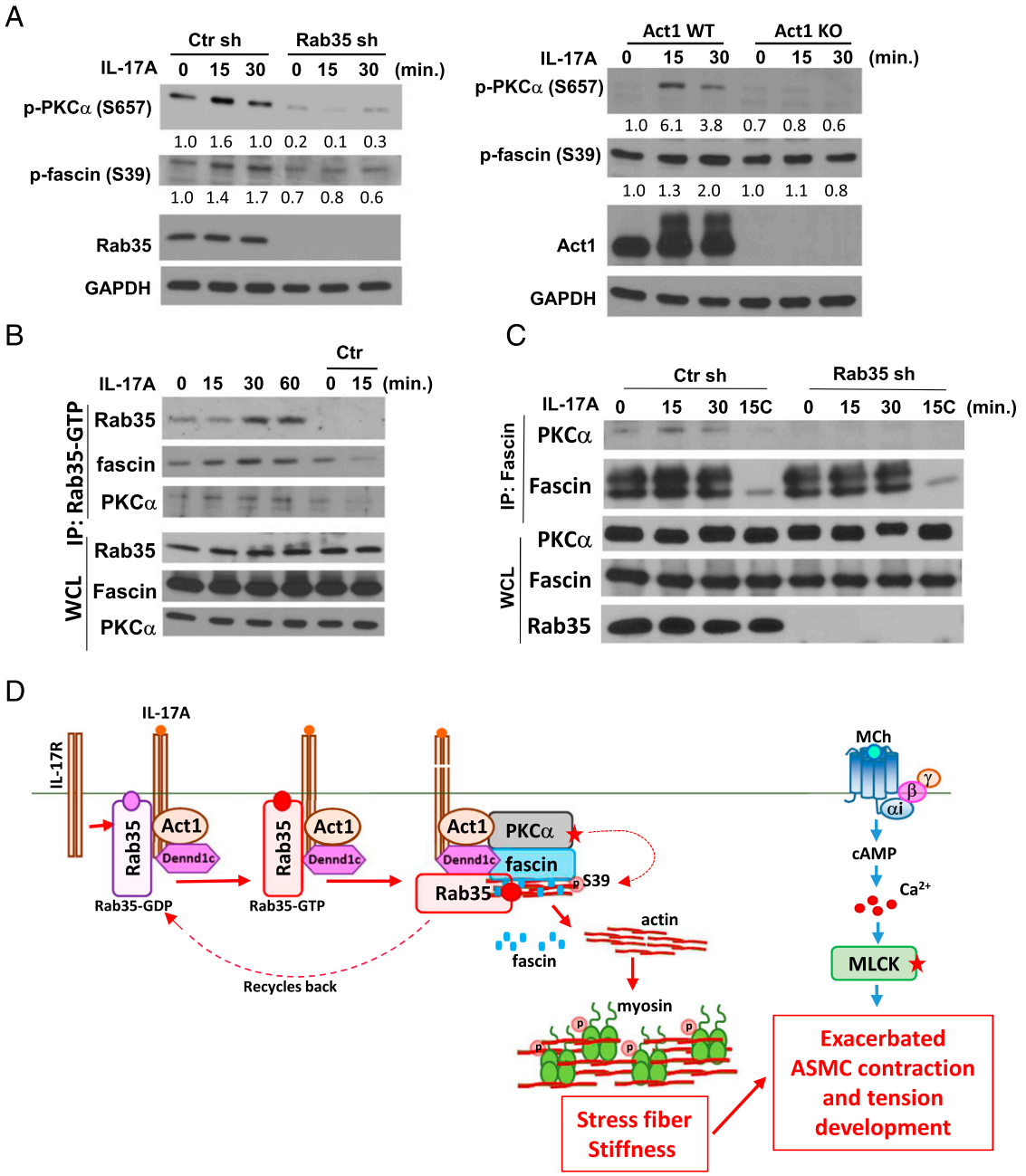

FIGURE 5. Rab35 is required for IL-17A–induced phosphorylation of PKCα and fascin in ASMCs. (**A**) Mouse WT, Act1 KO ASMC, and AMSC infected with lentivirus containing control (ctrl) or Rab35 shRNA were treated with IL-17A for 0, 15, and 30 min. Cell lysates were subjected to Western blot (WB) with indicated Abs. (**B**) Mouse ASMCs were treated with IL-17A for 0, 15, 30, and 60 min. Lysates were subjected to immunoprecipitation (IP) with anti-Rab35^{GTP} Ab, after which they were analyzed by WB with indicated Abs. (**C**) WT mouse ASMCs were infected with lentivirus containing ctrl or Rab35 shRNA and treated with IL-17A for 0, 15, and 30 min. Lysates were subjected to IP with anti-fascin Ab, after which they were analyzed by WB with indicated Abs. (**D**) Model: upon IL-17A stimulation (orange dots), Rab35/GDP is recruited to IL-17R/Act1 complex, which, in turn, recruits DennD1C, switching Rab35/GDP (purple) to Rab35/GTP (red). While the complex Act1/Rab35/GTP mediates PKCα activation (p-S657; phosphorylation is depicted as red asterisk), DennD1C brings actin/fascin bundles close to the complex. The activated PKCα then interacts with and phosphorylates fascin on Ser39, resulting in the dissociation of fascin from actin bundles. The release of actin bundles from fascin allows them to interact with myosin assembling into stress fibers, generating contraction force.

PKCα inhibitor or Rab35 knockdown substantially reduced IL-17A–induced actin/myosin interaction in ASMCs, and also attenuated IL-17A–enhanced, MCh-induced contraction of ASM. It is important to note in this study that vesicular stomatitis virus G protein–enveloped virus, which was used to knockdown Rab35 may infect other cell types in addition to smooth muscle cells. Therefore, conditional Rab35 KO mice are required for better assessment of cell type–specific contribution of Rab35-mediated trachea contraction.

Force generation by ASM is mediated by actin/myosin interaction, which is critically dependent on myosin phosphorylation. In this article, we propose an additional novel IL-17A–dependent mechanism via Rab35/PKC/fascin cascade contributing to ASM contraction. Based on our findings, we propose a working model (Fig. 5D): upon IL-17A stimulation, Rab35/GDP is recruited to IL-17R/Act1 complex, which, in turn, recruits DennD1C, switching Rab35/GDP to Rab35/GTP. Although the complex Act1/Rab35/GTP mediates PKCα activation (p-S657), DennD1C

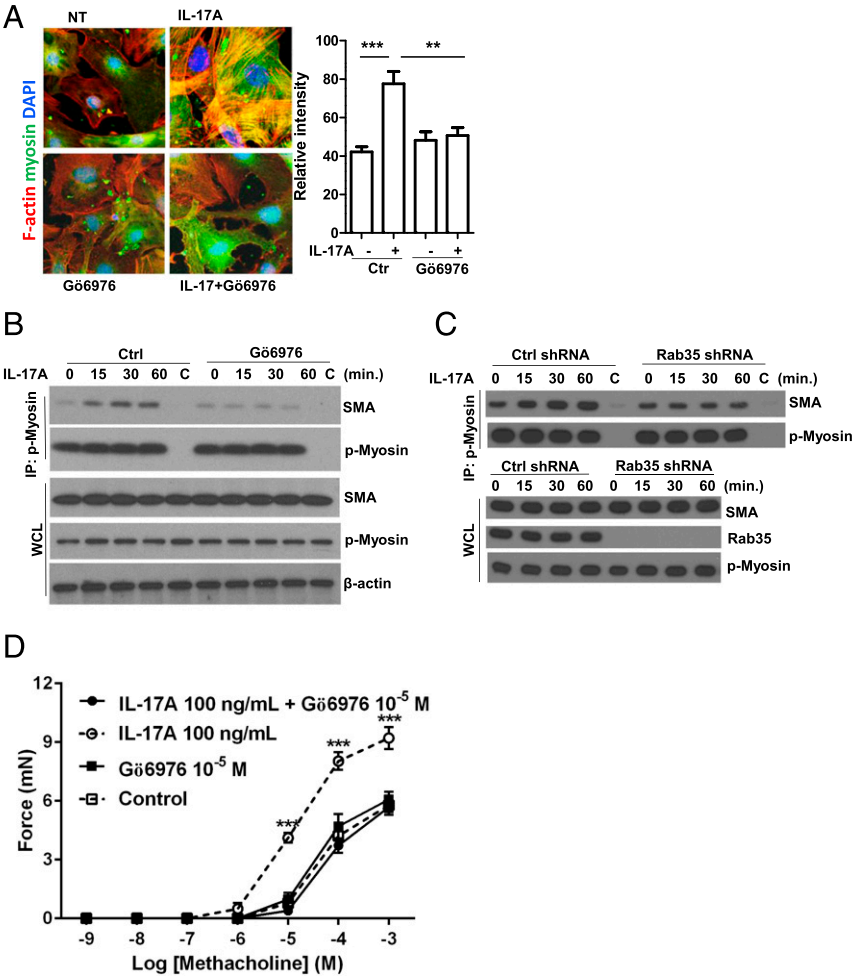

FIGURE 6. PKCα activation is required for IL-17A–induced contraction. (**A**) WT mouse ASMCs were pretreated either with DMSO or PKC inhibitor Gö6976 for 1 h, next treated with IL-17A for 12 h, and fixed and stained with phalloidin/rhodamine and SM-MHC (green). Original magnification ×63. (**B** and **C**) WT (B) and Rab35 knockdown (C) mouse ASMCs were pretreated either with DMSO or PKC inhibitor Gö6976 for 1 h following the treatment with IL-17A for 0, 15, 30, and 60 min. Lysates were subjected to immunoprecipitation (IP) with p-myosin Ab, after which they were analyzed by Western blot (WB) with indicated Abs. (**D**) Contractile force measurements from human bronchial rings stimulated with MCh after treatment with PBS, IL-17A, Gö6976, or IL-17A/Gö6976 for 1 h. The statistics used were a two-way ANOVA with repeated measures of variance, followed by Tukey *t* test for subsequent pairwise analysis. $^{**}p < 0.01$, $^{***}p < 0.001$.

brings actin/fascin bundles close to the complex. The activated PKCα then interacts with and phosphorylates fascin on Ser39, resulting in the dissociation of fascin from actin bundles. The release of actin bundles from fascin allows them to interact with myosin assembling into stress fibers, generating contraction force. As it has been shown that IL-17A has an important role in driving neutrophilic inflammation in asthmatic airway, our present findings may have important implications for the development of new therapies for treating AHR in a subset of patients with severe asthma associated with high levels of IL-17A.

Myosin phosphorylation is modulated via both calcium-dependent calmodulin and RhoA/ROCK2 pathway. IL-17A was previously shown to increase the level of RhoA and ROCK2 via NF-κB, leading to increased myosin L-chain phosphorylation (19, 20). Although we did not detect the induction of RhoA and ROCK2 expression in our primary smooth muscle cells (data not shown), we did notice that NF-κB was only weakly induced in these cells, suggesting that the Rab35/PKC/fascin cascade was the main contributor to the enhanced contractility induced by IL-17A in our system. Thus, it is possible that, in response to robust NF-κB activation, the RhoA/ROCK2 pathway may be induced to work in concert with Rab35/PKC/fascin cascade for enhanced contractility. Future studies are required to investigate how these pathways work together in the pathogenesis of asthma.

The role of IL-17 signaling as a target for asthma treatment is an emerging field of intense investigation. Elevation of IL-17A levels has been reported to associate with a severe form of asthma characterized by neutrophilic inflammation. Although previous clinical trial using brodalumab, an IL-17RA blockade Ab, did not detect robust activity in patients with moderate to severe asthma (40), a recent report found that secukinumab (IL-17A–neutralizing Ab) treatment mediated therapeutic response in patients with lower levels of serum IgE (41). This highlights the heterogeneous nature of the disease. Our study, along with other reports, demonstrates a crucial role for IL-17A signaling in the pathogenesis of AHR using mouse models of asthma. Taken together, future clinical trials are needed to further evaluate the efficacy of IL-17A neutralization for the management of severe asthma.

Disclosures

The authors have no financial conflicts of interest.

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