

CD151, a laminin receptor showing increased expression in asthmatic patients, contributes to airway hyperresponsiveness through calcium signaling

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Background: Airway smooth muscle (ASM) contraction underpins airway constriction; however, underlying mechanisms for airway hyperresponsiveness (AHR) remain incompletely defined. CD151, a 4-transmembrane glycoprotein that associates with laminin-binding integrins, is highly expressed in the human lung. The role of CD151 in ASM function and its relationship to asthma have yet to be elucidated. **Objective:** We sought to ascertain whether CD151 expression is clinically relevant to asthma and whether CD151 expression affects AHR.

Methods: Using immunohistochemical analysis, we determined the expression of CD151 in human bronchial biopsy specimens from patients with varying asthma severities and studied the mechanism of action of CD151 in the regulation of ASM contraction and bronchial caliber *in vitro*, *ex vivo*, and *in vivo*. **Results:** The number of CD151⁺ ASM cells is significantly greater in patients with moderate asthma compared with those in healthy nonasthmatic subjects. From loss- and gain-of-function studies, we reveal that CD151 is required for and enhances G protein-coupled receptor (GPCR)-induced peak intracellular calcium release, the primary determinant of excitation-contraction coupling. We show that the localization of

CD151 can also be perinuclear/cytoplasmic and offer an explanation for a novel functional role for CD151 in supporting protein kinase C (PKC) translocation to the cell membrane in GPCR-mediated ASM contraction at this site. Importantly, CD151^{-/-} mice are refractory to airway hyperreactivity in response to allergen challenge.

Conclusions: We identify a role for CD151 in human ASM contraction. We implicate CD151 as a determinant of AHR *in vivo*, likely through regulation of GPCR-induced calcium and PKC signaling. These observations have significant implications in understanding the mechanism for AHR and the efficacy of new and emerging therapeutics. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Airway hyperresponsiveness, airway smooth muscle, asthma, contraction, calcium, CD151, G protein-coupled receptor, integrins, laminin, PKC, tetraspanin

Asthma is associated with airway hyperresponsiveness (AHR), which manifests from excessive airway obstruction in response to low concentrations of bronchospasmogens, such as methacholine. Airway smooth muscle (ASM) contraction underpins airway narrowing; however, the underlying mechanisms of AHR remain incompletely defined. We and others have shown that the accumulation of excess extracellular matrix proteins, including several laminins, drives survival signals to promote a contractile smooth muscle phenotype, ASM hyperplasia, and airway hyperreactivity.¹ Thus targeting the laminin-signaling pathway might offer new avenues for the development of therapies to reduce the increase in mass of contractile phenotype ASM cells that contributes to AHR in asthmatic patients. We also show that laminin and laminin-binding integrin expression, in particular $\alpha 7\beta 1$ integrin, is concomitantly induced in ASM cells under conditions that promote acquisition of a contractile phenotype.^{2,3} Here we provide evidence of an additional new mechanism of enhancement of ASM contraction by the laminin-associated protein CD151.

CD151 belongs to the superfamily of 4-transmembrane glycoproteins called tetraspanins. Tetraspanins are linked to and can integrate many physiologic and pathologic events (eg, cell proliferation and growth, apoptosis, and migration) through integrins and kinases, such as protein kinase C (PKC).⁴ CD151 associates selectively with laminin-binding integrins,⁵ which are expressed in abundance by mature contractile ASM cells.^{2,3} In ASM PKC mediates Ca²⁺-dependent contraction, actin

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Abbreviations used

AHR:	Airway hyperresponsiveness
ASM:	Airway smooth muscle
DMEM:	Dulbecco modified Eagle medium
GFP:	Green fluorescent protein
GPCR:	G protein-coupled receptor
MESCA:	Melbourne Epidemiological Study of Childhood Asthma
OVA:	Ovalbumin
PKC:	Protein kinase C
PMA:	Phorbol 12-myristate 13-acetate
siRNA:	Small interfering RNA

cytoskeletal dynamics, and transcription of smooth muscle-selective genes.^{6,7} To date, a role for CD151 in regulating the contractile function of ASM cells has not been examined. Notably, CD151 is highly expressed in the mature lung,⁵ but before the present study, the expression and functional role of CD151 in human ASM cells and their contribution to asthma pathophysiology had not been established.

Our data uncover a previously unknown mechanism for modulating human ASM contraction. We implicate CD151 as a determinant of AHR *in vivo*, likely through regulation of G protein-coupled receptor (GPCR)-induced calcium mobilization and PKC signaling. These observations have significant implications in understanding the mechanism for AHR and the efficacy of new and emerging therapeutics.

METHODS**Reagents**

All compounds were purchased from Sigma (St Louis, Mo), unless stated otherwise.

Human ASM cell culture

Primary cultured human ASM cells were prepared, as previously described^{2,8} and as discussed in the [Methods](#) section in this article's Online Repository at www.jacionline.org. At least 3 different human ASM cell lines between passages 3 and 14 were used in all studies. All procedures were approved by the National Healthcare Group Domain Specific Review Board, Singapore.

Subjects

Subjects were either nonasthmatic or asthmatic volunteers with varying asthma severities (classified by using Global Initiative for Asthma guidelines) recruited from the Melbourne Epidemiological Study of Childhood Asthma (MESCA) cohort (all aged 42 years at the time of biopsy), as previously described.^{9,10} Further demographic data are outlined in [Table I](#).

Western blotting

Protein lysates were size fractionated by using SDS-PAGE with loading buffer in the presence or absence of β -mercaptoethanol (β -actin and CD151, respectively), as previously described.²

Immunocytochemistry of CD151 and phospho-PKC

Primary antibodies included anti-CD151 (1:100; Serotec, Oxford, United Kingdom), anti-phospho PKC α/β (Thr 638/641; 1:50; Cell Signaling, Danvers, Mass), and secondary antibody Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (1:200; Invitrogen, Carlsbad, Calif; see the [Methods](#) section in this article's Online Repository at

TABLE I. Demographic data for subjects from the MESCA cohort from whom biopsy specimens were obtained for immunohistochemistry

	Nonasthmatic subjects	Patients with mild asthma	Patients with moderate asthma
Subjects (no.)	10	13	8
Sex (M/F)	4/6	7/6	6/2
Atopic (%)	40%	85%	100%
FEV ₁ (mean $\pm$ SEM)	110 $\pm$ 4	100 $\pm$ 3	62 $\pm$ 3*
Current smokers	70%	54%	38%
Current treatment			
β_2 -Adrenoceptor agonists	0%	62%	100%
Inhaled steroids	0%	31%	100%
Oral steroids	0%	15%	25%

* $P < .05$ compared with nonasthmatic subjects.

www.jacionline.org). Nuclear counterstaining was performed with Hoechst 33342 (10 μ g/mL; DAKO, Glostrup, Denmark).

Immunohistochemistry of CD151 in human tissue

Human bronchial specimens obtained from lung resections were quick-frozen in OCT compound, and 7- μ m cross-sections were cut. Antibodies included CD151 (11B1) and fluorescein isothiocyanate- and Texas Red-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, Pa). Fluorescence micrographs were obtained with an Olympus LX70 microscope equipped with a charge-coupled camera controlled by UltraView Software (Olympus, Center Valley, Pa).

For the MESCA biopsy cohort samples, paraffin-embedded sections (5 μ m) were stained for CD151 (11G5a, 1:750 dilution; Serotec) and smooth muscle (sm)- α -actin (1:500 dilution; DAKO). The number of CD151⁺ cells was counted with cellSens dimension software (Olympus) at $\times 20$ magnification and expressed per square millimeter of sm- α -actin-positive biopsy area.¹¹ Staining, images, and measurements were taken by a single observer in a blinded fashion.

CD151 small interfering RNA preparation and study design

The siRNA Generation Kit (Gene Therapy Systems, San Diego, Calif) was used to prepare small interfering RNA (siRNA) from human ASM cDNA with primers that amplified CD151 cDNA (537 base pairs; National Center for Biotechnology Information accession no. NM004357; forward: 5' GAC AAC ATG TGG CAC CG 3' and reverse: 5' GTC TTG CAG CAG CTG TC 3').² PCR primers were designed to amplify unique regions of CD151 that did not share sequence homology with any other tetraspanins (see the [Methods](#) section in this article's Online Repository). siRNA experiments were terminated after 6 days of serum deprivation based on preliminary studies, which showed effective silencing of CD151 mRNA expression with no evidence of cell toxicity or changes in cell shape.

CD151 overexpression studies

pEGFP-N3 and pEGFP-CD151 (2 μ g each)¹² were mixed separately with jetPRIME (Polyplus-transfection, New York, NY) in 200 μ L of transfection buffer, according to the supplier's instructions (see the [Methods](#) section in this article's Online Repository). Stable green fluorescent protein (GFP)-expressed and CD151-overexpressed cells were obtained after 2 weeks of culture with G418 selection.

Intracellular calcium assay

Human ASM cells (negative control, GFP-expressed, and CD151 overexpressed cells) were seeded on 6-well plates (see the [Methods](#) section in this article's Online Repository). The RF-5301PC intracellular ion

measurement system (Shimadzu, Kyodo, Japan) was used to monitor intracellular calcium levels.

Sensitization and allergen challenge protocol

Female mice aged 6 to 8 weeks were purchased from the Animal Resource Centre (Canning Vale, Australia; wild-type, C57Bl/6) or from an existing breeding colony (CD151^{-/-} mice). All procedures were approved by the Institutional Guidelines for Animal Care and Use Committee of the National University of Singapore. Mice were sensitized by means of intraperitoneal injection of 20 µg of ovalbumin (OVA) and 4 mg of aluminum hydroxide constituted in 0.1 mL of saline on days 0 and 14. On days 22, 23, and 24, mice were challenged with 1% OVA aerosol for 30 minutes (see the [Methods](#) section in this article's Online Repository). Control mice received saline aerosols through an identical protocol. Twenty-four hours after the last challenge, mouse airway reactivity was determined,¹ and lungs were collected for protein and immunohistochemical analysis thereafter (see the [Methods](#) section in this article's Online Repository). Immunohistochemical staining of sm- α -actin was done (1:500; DakoCytomation), and the area of sm- α -actin-positive cells was measured by a single observer in a blinded fashion.^{1,11} Digital photographs of lung sections were analyzed at $\times 20$ magnification. The cellSens dimension software (Olympus) was used for analysis. In a separate group of mice, bronchoalveolar lavage fluid was collected for total cell counts and cytokine measurement by means of ELISA, as described previously.¹³

Isometric contraction studies

In a separate group of experiments, following the above OVA sensitization and challenge protocol, mouse trachea were dissected and cut into 3 segments, each with 3 cartilage rings (see the [Methods](#) section in this article's Online Repository), and mounted onto an organ bath (model 610M; DMT, Aarhus, Denmark), which was filled with 5 mL of Krebs solution (95% O₂ and 5% CO₂ at 37°C) with a resting tension of 0.3 mN. Indomethacin (3 µmol/L) was administered to inhibit the effect of epithelium. The response of the tracheal segments to cumulative concentrations of methacholine was monitored by using LabChart software and determined based on the maximum response after each dose administration.

Statistical analysis

Where appropriate, comparisons were made with repeated-measures 1-way ANOVA, followed by the Bonferroni *post hoc t* test. Two-way repeated-measures ANOVA was used to analyze the effect of different doses of methacholine on changes in lung resistance and dynamic compliance in the 2 groups of mice (wild-type and CD151^{-/-} mice), as well as to analyze the effect of OVA challenge on increases in the area of sm- α -actin-positive staining in wild-type versus CD151^{-/-} mice. The Kruskal-Wallis test was used for human clinical data. A *P* value of less than .05 was considered significant. Error bars indicate SEMs.

RESULTS

ASM CD151 expression

To identify whether CD151 plays a role in ASM function, we first ascertained whether the expression of CD151 in ASM is clinically relevant. We used immunohistochemistry to assess the presence of CD151 protein in human bronchial biopsy specimens from patients with varying asthma severity. Histologic sections of biopsy samples were obtained from an existing collection of biopsy samples from the MESCA cohort. Earlier findings from this cohort showed that ASM volume was increased with increasing asthma severity.^{10,14} We show that the frequency of CD151 expression in ASM is significantly increased in patients with moderate asthma compared with nonasthmatic subjects

(*P* < .05, Kruskal-Wallis test; [Fig 1](#)). We note that the localization of CD151 appears to be heterogeneous, with some ASM cells showing plasma membrane staining of CD151, whereas others are perinuclear.

CD151 is a marker of but not required for ASM acquisition of a contractile phenotype

A number of studies suggest that ASM from asthmatic patients can be hypercontractile¹⁵⁻²⁰ or can display altered contractile properties.^{21,22} However, full agreement is yet to be reached,^{23,24} and the mechanism for such a change in function remains to be confirmed. Mechanisms that underpin phenotype change have been studied by using cultured human ASM cells, which can be induced to a supercontractile state through prolonged maintenance in serum-free conditions.⁶ Here we show that the abundance of CD151 protein increases in human ASM cells because they are induced from a synthetic/proliferative phenotype to a contractile phenotype by maintaining cells in serum-free media for up to 7 days ([Fig 2, A](#)). Moreover, using fluorescence microscopy and immunoprecipitation assays, we show that CD151 co-localizes and coassociates with integrin $\alpha 7$, which we previously reported also accumulates during prolonged serum-free culture ([Fig 2, B and C](#)).² The CD151 integrin $\alpha 7$ association is retained with increasing detergent stringency ([Fig 2, C](#), and see [Fig E1](#) in this article's Online Repository at www.jacionline.org).

We next assessed whether the accumulation of CD151 is required for human ASM cells to acquire a contractile (mature) phenotype. Using siRNA, we selectively reduced CD151 protein abundance after 6 days, a time point selected from preliminary experiments that showed abrogation of CD151 mRNA without any evidence of cell toxicity ([Fig 2, D and E](#)). Also, as a negative control in all experiments, we used siRNA targeting GFP. CD151 knockdown with siRNA did not affect serum withdrawal-induced accumulation of the intermediate filament protein desmin, a marker of contractile phenotype ASM cells ([Fig 2, E and F](#)).² These results reveal that although CD151 accumulates and is a marker for ASM switching from a synthetic phenotype, the protein is not necessary for contractile phenotype acquisition *per se*.

CD151 associates with and recruits PKC to the cell membrane

ASM contraction induced by GPCR agonists is mediated by phospholipase C-catalyzed production of diacylglycerol and inositol triphosphate. Diacylglycerol can activate PKC, a process that includes PKC translocation to the plasma membrane.²⁵ In ASM PKC mediates Ca²⁺-dependent contraction, actin cytoskeletal dynamics, and transcription of smooth muscle-selective genes.⁶ Of note, in epithelial cells PKC directly associates with CD151 to promote cell-cell contact, forming a multimolecular complex that recruits integrin $\alpha 3 \beta 1$.^{4,26} Given that CD151 abundance is markedly greater in contractile human ASM cells, we tested whether it has a role in PKC signaling mechanisms that regulate contraction.

Using isolated cell membrane fractions, we show that CD151 can immunoprecipitate with phospho-PKC after stimulation of human ASM cells with a direct activator of PKC, phorbol 12-myristate 13-acetate (PMA; [Fig 3, A](#)). In ASM cells a role for CD151 in PKC translocation and signaling has not been demonstrated previously. Furthermore, not only do we show

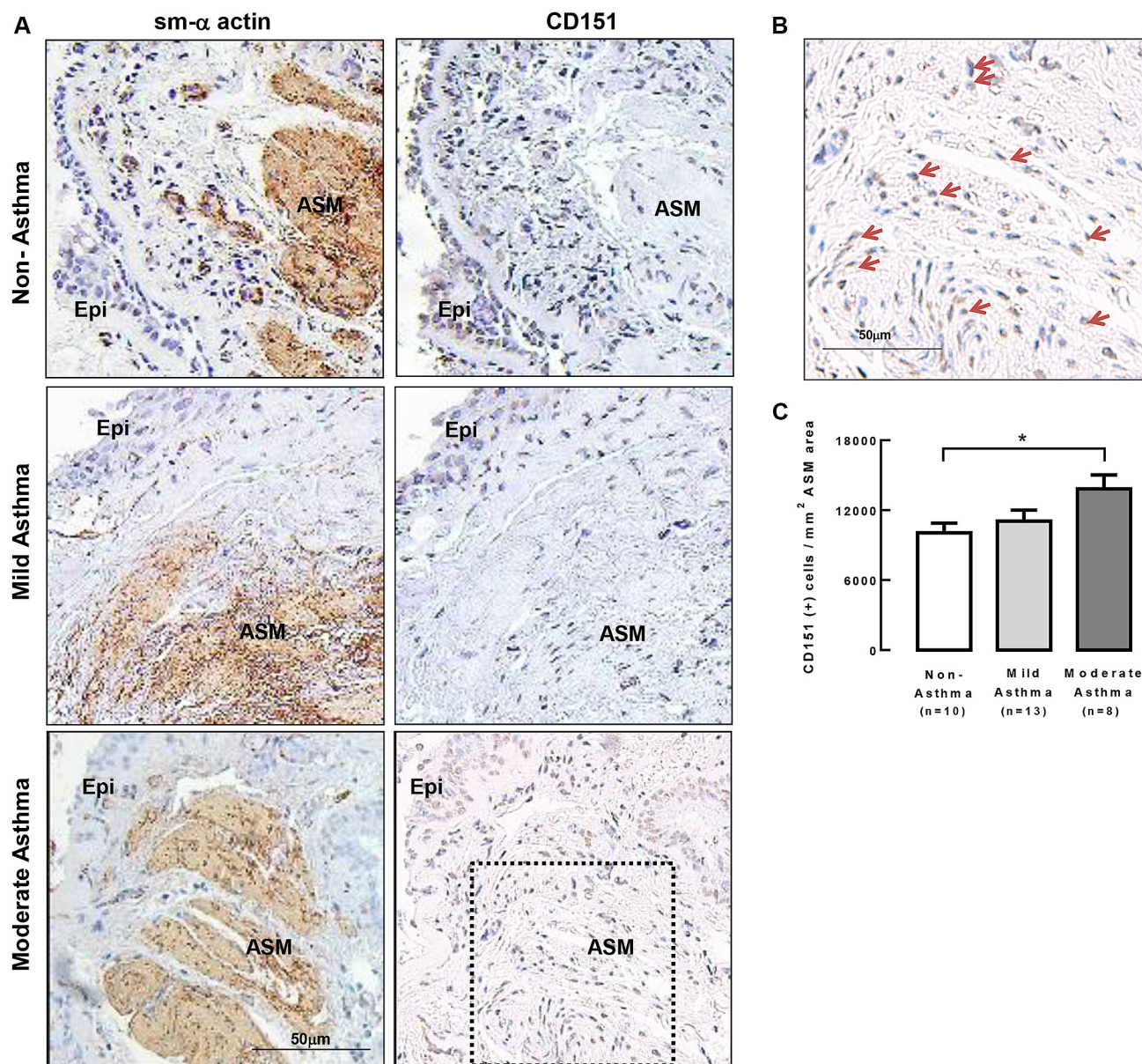


FIG 1. CD151 is highly expressed in cultured human ASM cells and bronchial ASM bundles from donors with moderate asthma. **A**, Representative matched immunohistochemical images of human lung samples stained for sm-α-actin and CD151. Epi, Epithelium. **B**, Enlarged image showing perinuclear staining (examples indicated by brown arrows) of CD151 in a moderate asthma sample. Blue represents nucleus staining. **C**, Quantitation of CD151⁺ cell numbers in biopsy specimens per square millimeter of quantifiable sm-α-actin-positive biopsy area. *P < .05 compared with nonasthmatic subjects.

that CD151 associates with PKC but also that it is required for translocation of the active form of PKC to the cell membrane because CD151 protein expression by siRNA treatment prevents PMA-induced PKC phosphorylation and its recruitment to the membrane (Fig 3, B). To confirm the involvement of CD151 in PMA-induced activation of PKC, we used immunoblotting to measure the phosphorylation of CPI-17, a direct downstream target of PKC, and found that the PMA-induced appearance of phospho-CPI-17 is ablated in human ASM subjected to CD151 knockdown by using siRNA (Fig 3, C). Furthermore, using immunofluorescence imaging, we show that after PMA treatment, only ASM cells that are elongated in shape (indicative of ASM contractile phenotype) show colocalization of phospho-PKC

and CD151 (Fig 3, D). In cells that are not of a contractile phenotype, exhibiting a round nonelongate morphology, nuclear staining of phospho-PKC and lack of costaining for CD151 were observed. Taken together, these data implicate a functional role for CD151 both in binding phospho-PKC at the cell membrane and for supporting PKC activation to regulate ASM contraction.

CD151 is required and sufficient for Ca²⁺ mobilization and contraction of ASM cells

Ca²⁺ is a central trigger for ASM contraction,²⁷ being mobilized in response to contractile agonist stimulation either through

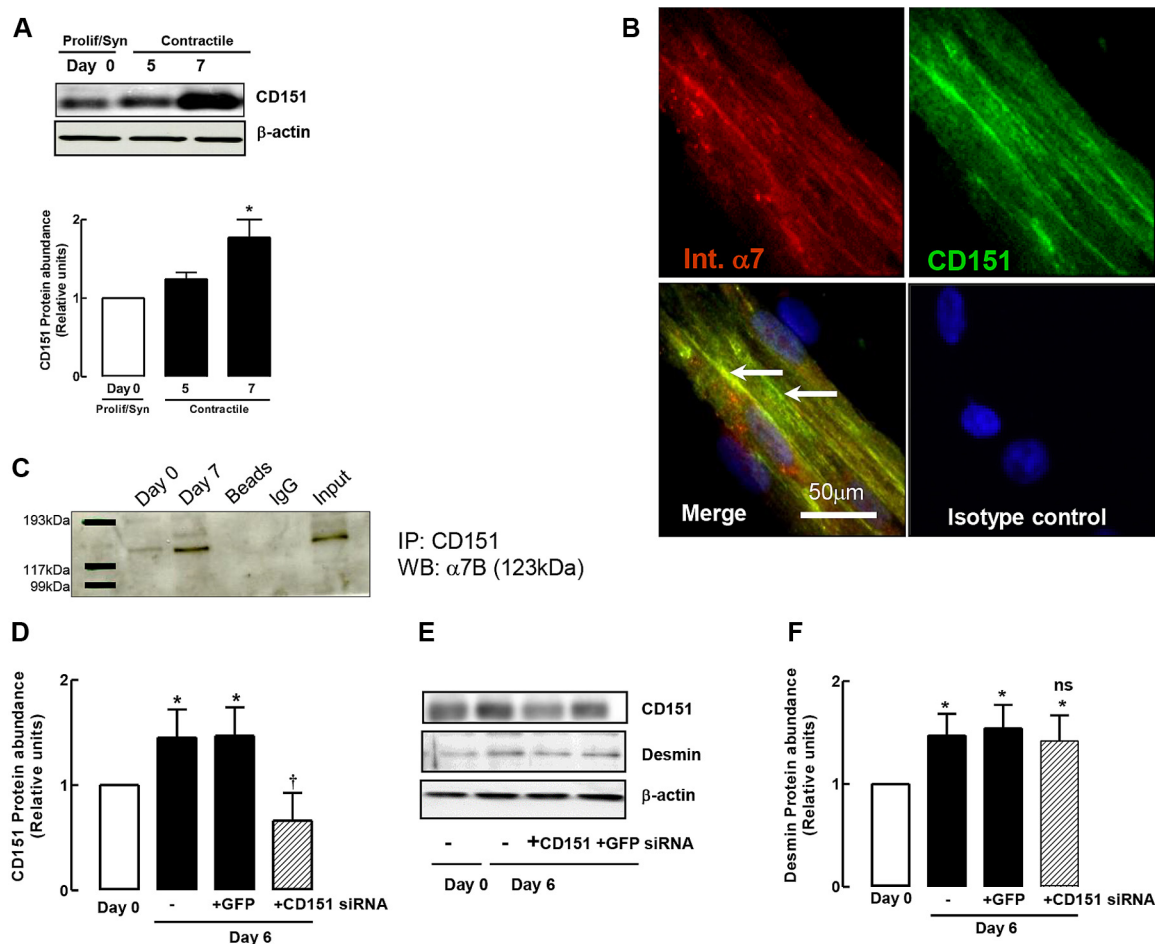


FIG 2. A, CD151 expression is enhanced after 7 days of serum deprivation. B and C, CD151 colocalizes (Fig 2, B) and coimmunoprecipitates (IP; Fig 2, C) with laminin-binding integrin $\alpha 7$ under triton detergent conditions (see the [Methods](#) section in this article's Online Repository for other detergent conditions). Day 7 denotes total protein lysate collection after 7 days of serum deprivation versus day 0. Input denotes protein lysate before IP. WB, Western blotting. D-F, Effect of CD151 siRNA on protein abundance of CD151 (Fig 2, D and E) and desmin ($n = 4$; Fig 2, E and F). * $P < .05$ compared with day 0 relative to β -actin. † $P < .05$ and ns (not significant) compared with 6-day control siRNA. The proliferative/synthetic versus contractile phenotype is determined from our earlier studies showing that the accumulation of desmin (marker of contractile phenotype) is highly expressed after more than 5 days of serum-free media compared with day 0, where the protein abundance of desmin is low.^{2,6}

influx across the plasma membrane or through release from intracellular stores. We assessed the function of CD151 in modulating Ca^{2+} mobilization and associated contractile responses induced by physiologically relevant agonists in human ASM cells by comparing responses in cells treated with siRNA for CD151 or with a nontargeting siRNA. Knockdown of CD151 protein expression with siRNA treatment (Fig 4, A) was sufficient to significantly reduce baseline Ca^{2+} levels (Fig 4, B). We stimulated ASM cells with 3 different asthma-relevant GPCR agonists (methacholine, histamine, and bradykinin) and observed that peak intracellular Ca^{2+} mobilization induced by each agonist was markedly reduced when CD151 was lacking (Fig 4, C and D). We also performed the same experimental conditions with another commercial CD151 siRNA (Dharmacon, Lafayette, Colo) and found similar observations (see Fig E2 in this article's Online Repository at www.jacionline.org). As further confirmation that the knockdown of CD151 was selective and not caused by off-target influences on intracellular calcium increases, we

also performed our experiments in the presence of ionomycin stimulation. Ionomycin bypasses signal transduction to increase calcium levels. We show that ionomycin-induced increases in calcium levels were unaffected by CD151 siRNA treatment (Fig 4, E and F).

We also performed gain-of-function studies whereby CD151 was constitutively overexpressed in ASM cells to determine whether it is sufficient to modulate mobilization of the Ca^{2+} response to contractile agonists. Human ASM cells were transfected with a plasmid vector encoding GFP-tagged human CD151. Based on immunofluorescence assessment of the CD151-GFP tag, more than 90% of cells exhibited CD151 overexpression after 2 weeks of G418 selection (Fig 5, A). Overexpression had no effect on Ca^{2+} baseline levels (Fig 5, B), but peak Ca^{2+} mobilization induced by histamine, bradykinin, or methacholine was significantly enhanced in comparison with that seen in corresponding nontransfected or GFP tag-only-transfected ASM cells (Fig 5, C and D). These observations

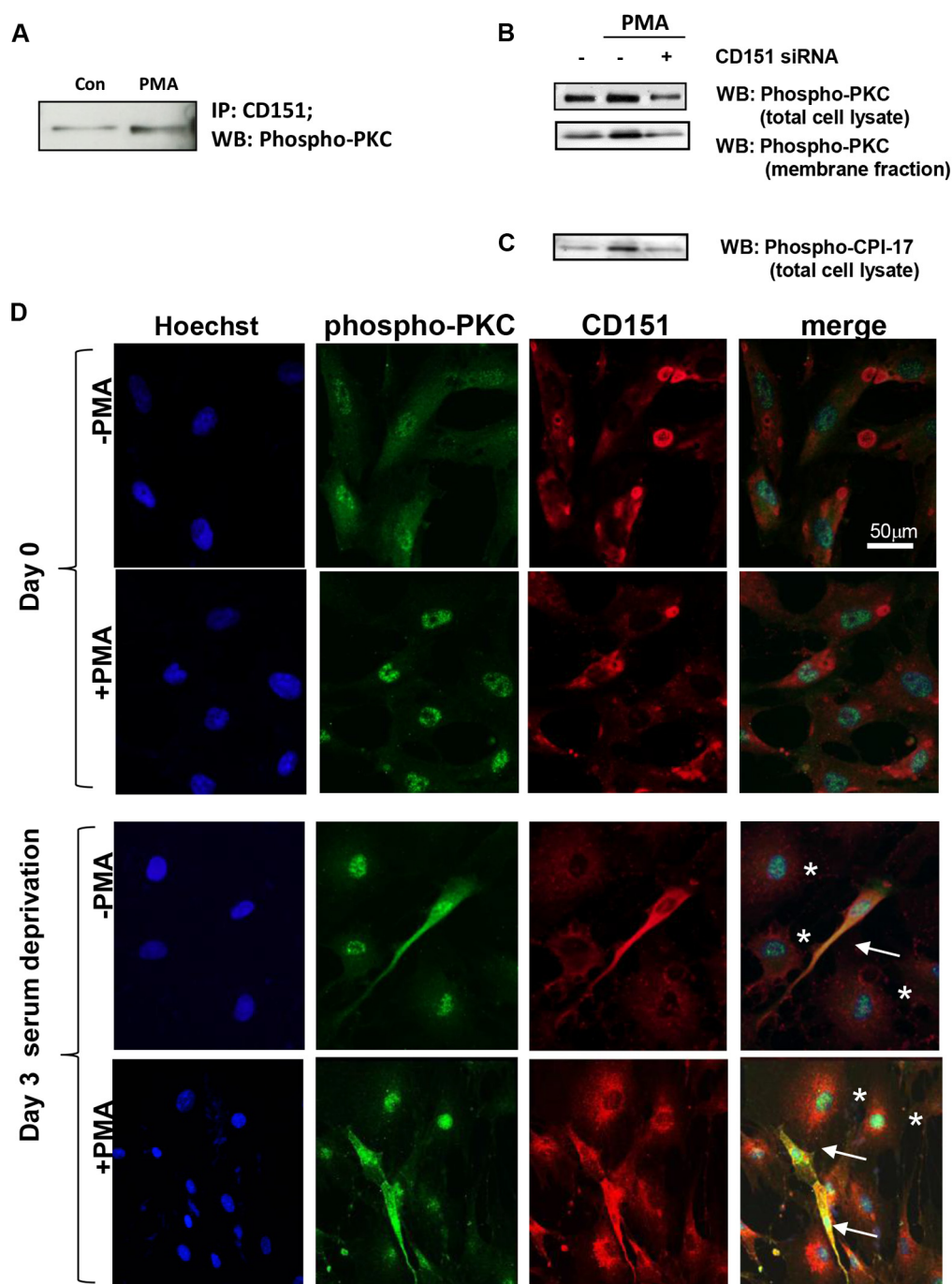


FIG 3. A-C, CD151 immunoprecipitates with phospho-PKC (Fig 3, A) and is required for recruitment of phospho-PKC up to the cell membrane (Fig 3, B; membrane fractions) and phosphorylation of CPI-17 (Fig 3, C). D, Representative images showing colocalization of phospho-PKC with CD151 after 1 hour of stimulation with PMA (1 μ mol/L). Arrows indicate ASM "contractile"-expressing phenotype cells; asterisks indicate ASM "proliferative" phenotype cells. WB, Western blotting.

demonstrate that CD151 modulates Ca^{2+} mobilization induced by physiologically relevant agonists in ASM cells.

Finally, to determine whether CD151 regulation of Ca^{2+} flux is a determinant of the functional contractile response, we performed collagen gel contraction experiments using ASM cells treated with either CD151 siRNA or nontargeting siRNA.

Whereas control siRNA (nontargeting siRNA) was without effect, CD151 silencing underpinned the significant loss of gel contraction induced by histamine (see Fig E3 in this article's Online Repository at www.jacionline.org). Taken together, this is new evidence suggesting that CD151 is required for Ca^{2+} mobilization, which is central to the regulation of ASM contraction.

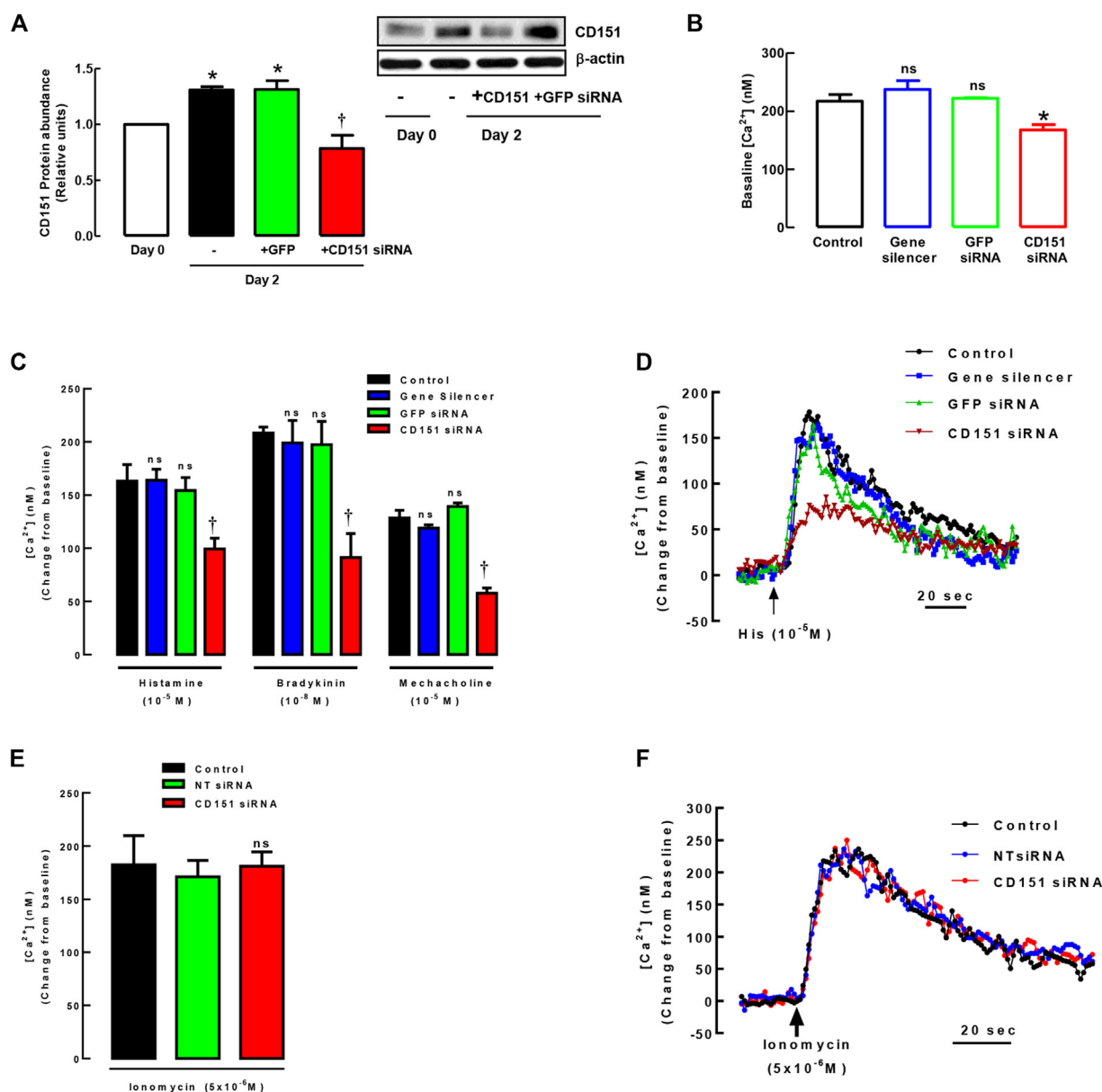


FIG 4. A, Grouped data and representative Western blot showing confirmation of knockdown of CD151 protein. B and C, Grouped data showing basal values (Fig 4, B) and the effect of the GPCR agonists histamine, bradykinin, or methacholine (Fig 4, C) on peak calcium levels in the presence and absence of CD151 siRNA. * $P < .05$ compared with basal/control; ns, not significant compared with day 0/control and † $P < .05$ compared with GFP/control siRNA values ($n = 3-5$). ns, Not significant. D, Representative calcium tracing. E and F, Grouped (Fig 4, E) and representative (Fig 4, F) calcium tracing showing the effect of ionomycin treatment in the presence and absence of CD151 siRNA treatment.

Functional relevance of CD151 to ASM function *in vivo*

We next investigated whether the role of CD151 observed for ASM contraction *in vitro* contributes to pathophysiology, specifically AHR that develops in mice sensitized and challenged with allergen to induce an asthma-like phenotype. We first confirmed, using RT-PCR, that CD151^{-/-} mice are deficient in CD151 by determining the level of CD151 transcript (Fig 6, A) and then compared airway reactivity of allergen-naïve and OVA-sensitized and challenged wild-type and CD151^{-/-} mice. We observed that allergen exposure induction of increased airway

reactivity to methacholine in wild-type mice was absent in CD151^{-/-} mice (Fig 6, B). Furthermore, CD151^{-/-} mice exhibited increased lung compliance, suggesting that loss of airway-lung interdependence contributes to increased airway narrowing (Fig 6, C). Interestingly, in correlation with the development of airway hyperreactivity, allergen exposure increased CD151 protein expression in wild-type mouse lung homogenates (Fig 6, D). Conversely, the abundance of the smooth muscle contractile marker protein α -actin was not different between CD151^{-/-} mice that were allergen sensitized and challenged versus saline control mice (Fig 6, E and F), suggesting that the

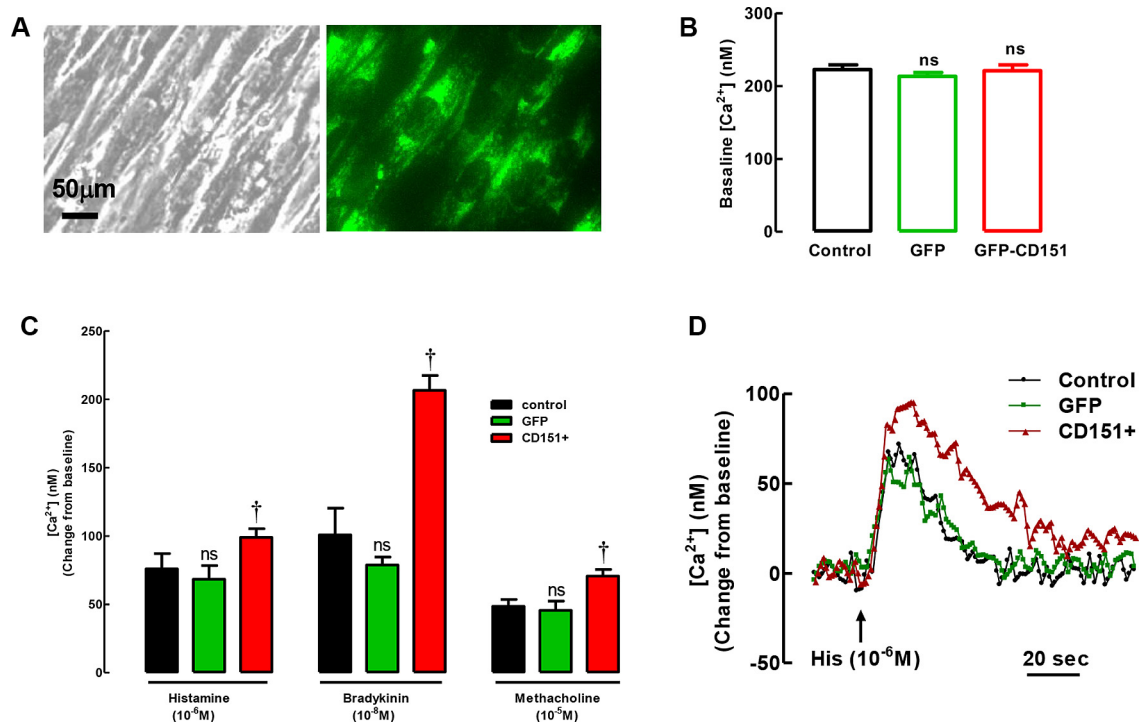


FIG 5. A, Phase contrast and immunofluorescence analysis confirming overexpression of CD151 protein. B and C, Grouped data showing basal values (Fig 5, B) and the effect of the GPCR agonists histamine, bradykinin, or methacholine (Fig 5, C) on peak calcium levels in ASM cells overexpressing GFP-tagged CD151 versus vector control. ns, Not significant compared with control; $\dagger P < .05$ compared with GFP ($n = 3-8$). ns, Not significant. D, Representative calcium tracing.

reduction in airway reactivity observed in CD151 $^{-/-}$ mice is not the direct result of a reduction in ASM abundance. Assessment of mouse bronchoalveolar lavage fluid revealed that total cell recruitment is significantly increased in OVA-sensitized and challenged wild-type mice and that this was significantly reduced in the allergen-exposed CD151 $^{-/-}$ mice (Fig 6, G). In addition, compared with allergen-exposed wild-type mice, levels of the $\text{T}_\text{H}2$ cytokine IL-5 were significantly reduced in the allergen-exposed CD151 $^{-/-}$ mice, with a concomitant increase in levels of the $\text{T}_\text{H}1$ cytokine IFN- γ (Fig 6, H and I).

We further investigated the role of CD151 in modulating GPCR-induced ASM contraction *ex vivo* by assessing isometric contraction of mouse tracheal ring preparations. To confirm that any difference in the contractile response of tracheal rings from CD151 $^{-/-}$ mice is not due to anatomic differences or a generalized loss of contractile capacity, we challenged tracheal rings with 60 mmol/L KCl to induce membrane depolarization and induce receptor-independent contraction. We observed no differences in KCl-induced force development between wild-type and CD151 $^{-/-}$ mice (Fig 7, A). Moreover, in allergen-naïve samples tracheal isometric contraction responses were similar between wild-type and CD151 $^{-/-}$ mice (Fig 7, B). However, just as we observed for airway responses *in vivo*, isometric contraction responses of tracheal preparations from CD151 $^{-/-}$ mice were blunted compared with those in wild-type tissues obtained from mice subjected to OVA sensitization and challenge (Fig 7, C). These data support a direct functional role of CD151 in enhanced excitation-contraction coupling that occurs in ASM as the result of repeated allergen exposure.

DISCUSSION

Despite advances in drug administration and use of combination therapy involving long-acting β_2 -adrenergic receptor agonists and glucocorticoids, current asthma treatment is focused on symptom relief and prevention (eg, reducing wheezing and breathlessness [Global Initiative for Asthma²⁸]). This focus relates to control of airway conductance, of which ASM contraction is a principal determinant. A number of studies suggest that ASM from asthmatic patients can be hypercontractile¹⁵⁻²⁰ or can display altered contractile properties.²¹ However, full agreement is yet to be reached,^{23,24} and the mechanism for such a change in function remains to be confirmed. Full understanding of the underlying mechanisms that contribute to asthma symptoms because of changes in ASM contractility is essential to optimize treatment efficacy. Before our current study, the role of CD151 in regulating ASM contractility had not been appreciated or assessed. Our findings describe a novel mechanism by which ASM contraction can be modulated under asthmatic conditions by physiologic agonists.

For the first time, we show that the frequency of CD151 expression in ASM is significantly increased in patients with moderate asthma compared with that in nonasthmatic/healthy control subjects. The clinical samples were obtained from an existing collection of histologic sections of biopsy samples that were originally described in the MESCA study cohort, which showed that ASM volume increased with increasing asthma severity.^{10,14} The uniqueness of this collection is that it is the longest and most comprehensive follow-up study of childhood asthma and that data have been collected every 7 years from

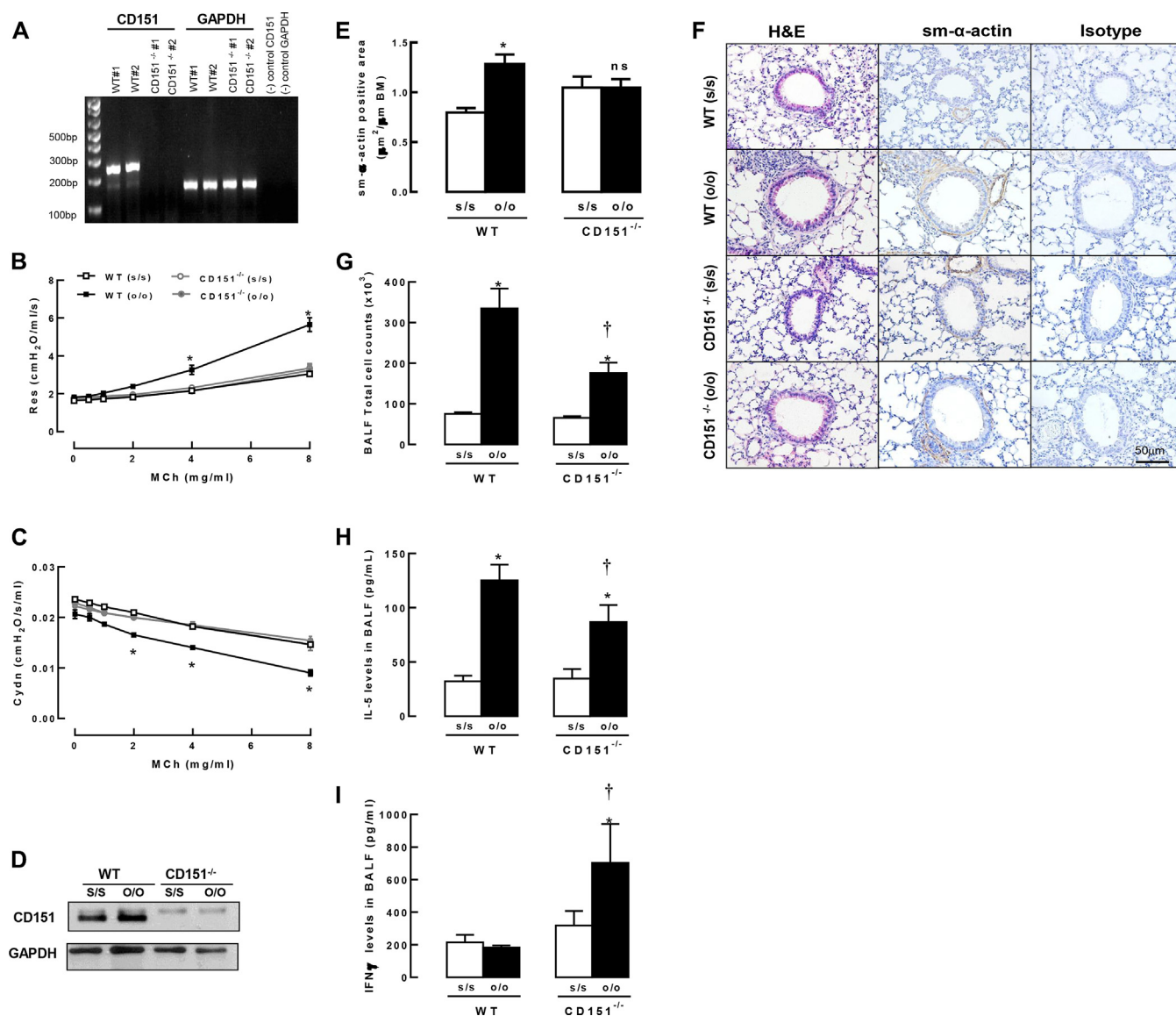


FIG 6. **A**, Confirmation of CD151 knockdown by means of PCR. **B** and **C**, Airway resistance (*Res*; Fig 6, **B**) and dynamic compliance (*Cdyn*; Fig 6, **C**) in wild-type (WT) and CD151^{-/-} mice. **P* < .05 compared with WT control mice (*n* = 4-7 per group). **D**, CD151 expression is increased in the OVA/OVA group relative to the saline/saline WT group. **E** and **F**, Grouped data (Fig 6, **E**) and representative (Fig 6, **F**) immunohistochemistry staining for sm-α-actin and hematoxylin and eosin (H&E; *n* = 4-5 per group). **G-I**, Assessment of mouse bronchoalveolar lavage fluid (BALF) for total cell numbers (Fig 6, **G**), IL-5 levels (Fig 6, **H**), and IFN-γ levels (Fig 6, **I**). **P* < .05 compared with WT (saline/saline) mice; *ns*, not significant; and †*P* < .05 compared with WT (OVA/OVA) mice (*n* = 4-8 per group). *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase.

participants since they were 7 years of age (now 59 years of age). Moreover, a control group (nonasthmatic subjects) who did not have asthma on entry into the study at age 7 years was included.

Consistent with our *in vitro* immunofluorescence data in which we show that, under basal conditions, CD151 location is unipolar/punctate (Fig 3), we show from immunohistochemical staining for CD151 in human biopsy samples that CD151 expression is also unipolar/perinuclear (Fig 1, **B**). Although early findings have classified CD151 as a “cell-surface membrane” protein that belongs to the 4-transmembrane glycoprotein family, the finding that the localization of CD151 can also be perinuclear/cytoplasmic offers an explanation for a novel functional role

(recruitment of PKC from the cytoplasm to the cell membrane) for CD151 at this site. Further studies examining determinants of CD151 localization remain to be carried out. Although there are many other pathways that could also be important (eg, phospholipase C and RhoA/RhoA kinase), we chose to look at PKC based on findings by others that CD151 associates with PKC.⁴ We extend these studies to show the functional role of the CD151-PKC association in ASM contraction and possible relevance to asthma pathogenesis. Because ASM cells are heterogeneous, we cannot exclude the possibility of PKC-independent actions of CD151 on ASM contraction. The mechanism or mechanisms by which CD151 recruits PKC to the cell membrane

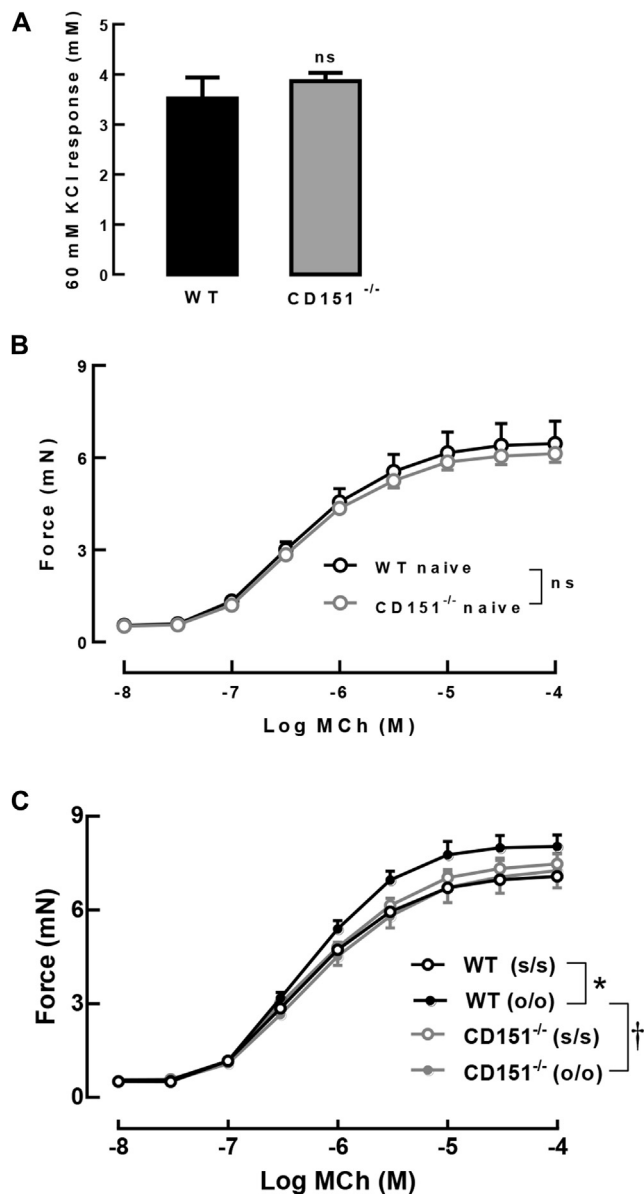


FIG 7. A, Decrease in ASM contraction in CD151^{-/-} mice is not due to loss of muscle integrity because both wild-type (WT) and CD151^{-/-} mice tracheal preparations were equally responsive to KCl. B and C, Tracheal isometric contraction studies for WT and CD151^{-/-} mice at baseline (naive; Fig 7, B) and after OVA sensitization and challenge (Fig 7, C). ns, Not significant. **P* < .05 compared with WT (saline/saline) mice and †*P* < .05 compared with WT (OVA/OVA) mice (n = 4–8 per group).

in ASM cells remains to be fully explored. Studies in epithelial cells implicate CD151 as a regulator of actin cytoskeletal reorganization through PKC- and Cdc-42-dependent mechanisms.¹² Thus CD151-mediated changes in cytoskeletal dynamics and Ca²⁺ levels could in turn contribute to PKC trafficking to the cell membrane.

Using a mouse model of passive cutaneous anaphylaxis, Abdala-Valencia et al²⁹ show upregulation of *de novo* synthesis of multiple inflammatory cytokines in isolated bone marrow-derived mast cells of CD151^{-/-} mice *ex vivo*. Thus decreasing CD151 expression could result in enhanced mediator and cytokine release, which could make asthma worse. Although we

have not examined the role of mast cells in our mouse model of AHR, we show that CD151^{-/-} mice have reduced total cell recruitment and reduced levels of the T_H2 cytokine IL-5 with corresponding upregulation of the T_H1 cytokine IFN-γ. The role of CD151 could be organ type specific (skin vs lung) and/or that our study better reflects the *in vivo* situation in which it is subjected to immune regulation. Also, whether ASM-dependent inflammation³⁰ could concomitantly be reduced under these conditions to contribute to reductions in AHR remains to be further elucidated.

Studies in mice suggest that CD151 might be important in renal function, and therefore any systemic therapy that would inhibit CD151 actions would be a potential limitation. However, it should be noted that the role of CD151 in renal function appears to be mouse strain specific: CD151^{-/-} mice on the FVB/N background exhibit renal failure,³¹ whereas CD151^{-/-} mice on the C57Bl/6 background (used in our study) do not, which is consistent with the published literature.^{32,33} Interestingly, recent studies on CD151^{-/-} mice on the FVB/N background that were generated by using 129SvEv ES cells (instead of 129/Ola and C57Bl.6 ES cells, which were used previously) are not affected by renal disease, and the authors speculate that there could be differences in key modifier genes proximal to the CD151 locus.³⁴ Thus more studies on CD151 biology in both mice and human subjects are warranted before pharmacologic intervention with CD151 antagonists can be considered for asthma therapy in human subjects.

The finding that CD151 regulates Ca²⁺ homeostasis is significant given that Ca²⁺ plays a central role in transduction of signals leading to diverse cellular responses, such as cell migration, metabolism, protein, and/or hormone secretion and proliferation. Targeting CD151 function not only has implications for muscle function but also for many pathophysiologic signaling processes that require Ca²⁺ regulation. It has been proposed that differential Ca²⁺ handling of T_H1 and T_H2 lymphocytes might contribute to distinct cytokine production patterns.^{35,36} Our *in vivo* data show that relative to allergen-induced wild-type mice, allergen-induced CD151^{-/-} mice exhibit significantly lower levels of the T_H2 cytokine IL-5, with a corresponding higher level of the T_H1 cytokine IFN-γ. The fact that CD151 is also expressed on T lymphocytes³⁷ suggests that we cannot exclude the contribution of lymphocytes to our *in vivo* experiments, in which reduction in AHR could be due to CD151 having a direct role in skewing the T_H1/T_H2 response. Thus the mechanism by which the development of AHR is prevented in CD151^{-/-} mice can also include regulation of Ca²⁺ at the level of lymphocytes in addition to ASM. Future work would be to decipher the signaling mechanisms by which CD151 can affect Ca²⁺ regulation. Finally, given the broad and significant role that smooth muscle contraction plays in the physiology and pathophysiology of a number of human diseases, our findings will be applicable across other fields, such as the pathophysiology of the vasculature, gastrointestinal tract, and bladder.^{26,38}

Taken together, we uncover a new mechanism involving CD151 that markedly modulates ASM contraction. We demonstrate a clinically relevant role for CD151 in ASM because its expression is greatly increased in human subjects with asthma. We describe the role this protein plays in disease pathophysiology, and our results suggest CD151 could underpin the disparity in response to asthma therapies between patients and in subjects under different conditions. This also has implications in deciphering patient response to therapy, as well as for direction

in designing new therapies that target ASM contraction in patients with asthma and other chronic lung conditions.

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Key messages

- We uncover a previously unknown connection between the tetraspanin protein CD151 and GPCR-mediated calcium increases in ASM, leading to ASM contraction.
- Our *in vitro* and *in vivo* data from human specimens and murine models implicate CD151 as a determinant of ASM contraction and allergen-induced AHR.

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METHODS

Human ASM cell culture

Primary cultured human ASM cells were prepared from macroscopically healthy segments of second- to fourth-generation main bronchus obtained after lung resection surgery, according to methods previously described.^{E1,E2} At least 3 different human ASM cell lines between passages 3 and 14 were used in all studies. All procedures were approved by the National Healthcare Group Domain Specific Review Board, Singapore. Unless otherwise indicated, human ASM cells were seeded at a density of 1×10^4 cells/cm² and grown to confluence in Dulbecco modified Eagle medium (DMEM) containing 10% vol/vol FBS. Confluent cultures were switched to DMEM supplemented with 5 μ g/mL insulin, 5 μ g/mL transferrin, and 5 ng/mL selenium (ITS) for up to 7 days, as described previously, to induce a contractile phenotype in human ASM.^{E1} Serum-free medium was replaced every 3 days. With this protocol, increased expression of desmin and α -actin was consistently induced, which is indicative of phenotype maturation in primary cultured human ASM cells.

Immunocytochemistry of CD151 and phospho-PKC

Primary immortalized human ASM cells were grown on cover slips at a density of 150,000 cells. Cells were fixed in 4% paraformaldehyde for 2 hours, followed by permeabilization with 0.1% Triton X-100 for 15 minutes. Cells were blocked with Image iT-FX Signal Enhancer (Invitrogen) for 30 minutes, followed by blocking buffer containing 5% goat serum and 1% BSA for 30 minutes. Primary antibody incubation was performed overnight with the following dilutions in blocking buffer: anti-CD151 (1:100, Serotec) and anti-phospho pan-PKC α/β (Thr 638/641; 1:50; Cell Signaling). Cells were rinsed in 0.2% Tween buffer before incubation of fluorescently conjugated secondary antibody: Alexa Fluor 488 goat anti-mouse and Alexa Fluor 594 goat anti-rabbit (1:200, Invitrogen). Nuclear counterstaining was performed with Hoechst 33342 (10 μ g/mL, DAKO) for 45 seconds before mounting in FluorSave (Invitrogen). Cells were viewed with an upright Olympus fluorescence microscope.

CD151 siRNA preparation and study design

The siRNA Generation Kit (Gene Therapy Systems) was used to prepare siRNA from human ASM cDNA with primers that amplified CD151 cDNA (537 base pairs; National Center For Biotechnology Information accession no. NM004357; forward: 5' GAC AAC ATG TGG CAC CG 3' and reverse: 5' GTC TTG CAG CAG CTG TC 3'), exactly according to our previous published work.^{E1} PCR primers were designed to amplify unique regions of CD151 that did not share sequence homology with any other tetraspanins. Briefly, the day before siRNA transfection, confluent human ASM cells were reseeded into 6-well plates in DMEM/10% vol/vol FBS so that 50% to 60% confluence was achieved 16 hours later. Transfection of siRNA (1 μ g/mL) was performed with Gene Silencer reagent (Gene Therapy Systems), according to the manufacturer's instructions, and cells were maintained in serum-free DMEM/ITS thereafter for 2 days (calcium studies). For negative control studies, the transfection protocol was performed in the absence of siRNA (gene silencer reagent) or in the presence of negative control GFP siRNA.

CD151 overexpression studies

Primary human ASM cells were seeded onto 6-well plates at a density of 2×10^5 cells/well 24 hours before transfection. The next day, 2 μ g of pEGFP-N3 and 2 μ g of pEGFP-CD151^{E3} was mixed separately with 4 μ L of jetPRIME (Polyplus-transfection) in 200 μ L of transfection buffer, according to the supplier's instructions. After 10 minutes of incubation, the transfection mix was added evenly onto the cells. Four hours after transfection, the transfection mix was removed, and the cells were incubated in DMEM containing 10% FBS. G418 (1 mg/mL) was added to the culture medium 48 hours later. Stable GFP-expressed and CD151-overexpressed cells were obtained after 2 weeks of culture with G418 selection.

Calcium assay

Primary human ASM cells (negative control, GFP-expressed, and CD151-overexpressed cells) were seeded on 6-well plates. Forty-eight hours before calcium assay, cell-culture medium was replaced with serum-free medium. On the day of the experiment, serum-free medium was replaced with fresh calcium buffer (145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L HEPES, 1 mmol/L CaCl₂, 100 mmol/L glucose, and 2% BSA) containing 5 μ mol/L Fura2-AM at 37°C for 30 minutes. After washing with calcium buffer twice, the cells were trypsinized and suspended in calcium buffer to a final cell density of approximately 2.5×10^5 /mL. Two milliliters of cell suspension was transferred to a cuvette and mixed by stirring. The RF-5301PC intracellular ion measurement system (Shimadzu) was used to monitor intracellular calcium levels. Once the baseline was stable, cells were treated with 10^{-5} mol/L methacholine (Sigma, St Louis, Mo), 10^{-5} mol/L histamine, and 10^{-8} mol/L bradykinin individually. After responding time, the cells were administrated with 100 μ L of Triton and later 100 μ L of 0.5 mol/L EDTA to achieve the maximum and minimum calcium levels. The emission wavelength was set to 510 nm, and the excitation wavelength was set to 380 nm and 340 nm. The ratio of these 2 fluorescence intensities was recorded, and the concentration of intracellular calcium was calculated from the program.

Sensitization and allergen challenge protocol

Female mice aged 6 to 8 weeks were purchased from the Animal Resource Centre (wild-type, C57Bl/6) or from an existing breeding colony (CD151^{-/-} mice). All procedures were approved by the Institutional Guidelines for Animal Care and Use Committee of National University of Singapore. Mice were sensitized by means of intraperitoneal injection of 20 μ g of OVA and 4 mg of aluminum hydroxide constituted in 0.1 mL of saline on days 0 and 14. On days 22, 23, and 24, mice were challenged with 1% OVA aerosol for 30 minutes. Aerosols were generated with an ultrasonic nebulizer (OMRON ultrasonic nebulizer NE-U17, OMRON Healthcare, Kyoto, Japan) on the indicated days. Control mice received saline aerosols through an identical protocol. Twenty-four hours after the last challenge, mouse airway reactivity was performed, as previously described.^{E4} After this, mouse lungs were collected, with 2 lobes frozen in liquid nitrogen and stored at -80°C , and the rest of the lobes were fixed in 10% formalin (Sigma). A quarter of a frozen mouse lung lobe from each of the mouse lungs was homogenized in 500 μ L of lysis buffer (containing 100 mmol/L NaCl; 10 mmol/L Tris HCl, pH 7.5; 2 mmol/L EDTA, pH 7.5; 0.5% wt/vol deoxycholate; 1% vol/vol Triton X-100; 1 mmol/L phenylmethylsulfonyl fluoride; 10 mmol/L MgCl₂; 5 μ g/mL aprotinin; and 100 μ mol/L sodium orthovanadate) by using TissueLyser LT (Qiagen, Hilden, Germany) to obtain lung lysates. The homogenized lung lobes were centrifuged at 9600g for 10 minutes at 4°C. The lysates were collected and stored at -20°C until required for Western blotting.

Isometric contraction studies

In a separate group of experiments following the above OVA sensitization and challenge protocol, mice were anesthetized, and whole trachea beneath the larynx was immediately removed and placed into fresh Krebs solution (118 mmol/L NaCl, 1.2 mmol/L KH₂PO₄, 1.2 mmol/L MgSO₄, 25 mmol/L NaHCO₃, 4.7 mmol/L KCl, 2.8 mmol/L CaCl₂, and 11 mmol/L glucose). The trachea was dissected free of surrounding tissue under a microscope (Zeiss Stemi 2000; Carl Zeiss, Oberkochen, Germany) and cut into 3 segments, each with 3 cartilage rings. Each segment was mounted on 2 L-shaped pin supports in an organ bath (DMT model 610M, Denmark), with the ASM strip between the pins. The organ bath was filled with 5 mL of Krebs solution, which was continuously aerated with 95% O₂ and 5% CO₂ and maintained at 37°C. After a 1-hour equilibration time, with solution change every 15 minutes, the tracheal segments were stretched to a resting tension of 0.3 mN, which we previously determined from our preliminary studies to be the maximum response to methacholine. After this, 3 treatments with 60 mmol/L KCl treatment were administrated to produce a stable response to KCl, followed by restretching the segments back to 0.3 mN after each KCl. After the final wash, 3 μ mol/L indomethacin (Sigma) was administrated to inhibit the effect of epithelium. Thirty minutes later, cumulative

concentrations (0.01–100 $\mu\text{mol/L}$) of methacholine were added to each chamber at 5-minute intervals. The response of the tracheal segments to methacholine was monitored by using the software LabChart and determined based on the maximum response after each dosage administration.

Mouse lung tissue histology

Lung tissue sections were deparaffinized with xylene and rehydrated with graded alcohol. Antigen retrieval was performed by heating the slides in the antigen-unmasking solution (Vector Laboratories, Burlingame, Calif). Immunoreactivity staining was carried out with the Vector Mouse on Mouse (M.O.M.) immunodetection kit (Vector Laboratories), according to the manufacturer's instructions. Briefly, slides were incubated with the Dako peroxidase blocking solution (DakoCytomation) and M.O.M. mouse immunoglobulin blocking reagent. Slides were then incubated with a primary antibody directed at sm- α -actin (1:500, DakoCytomation), followed by subsequent incubations with the biotinylated anti-mouse IgG reagent and the Vectastain Elite ABC reagent (Vector Laboratories). Counterstaining was performed with hematoxylin. The area of sm- α -actin-positive cells was calculated on the basis of a modification of a previously described method,^{E4,E5} and the cellSens dimension software (Olympus) was used for analysis. Digital photographs of lung sections were analyzed at $\times 20$ magnification. Sm- α -actin-positive areas were measured by a single observer in a blinded fashion.

Collagen gel contraction assay

The collagen gel contraction assay was carried out with a commercial collagen assay kit (Cell Biolabs, San Diego, Calif), according to the manufacturer's instructions. Briefly, human ASM cells were seeded onto 100-mm culture dish and harvested at 90% to 100% confluence after 7 days of growth in complete media. Approximately 5×10^5 cells were resuspended in

100 μL of FBS-free culture medium for each assay. The cells were then treated with either 12.5 μg of CD151 siRNA or a nontargeting siRNA pool as the control. This was then followed by mixing 100 μL of cell suspension with 400 μL of cold collagen gel solution prepared in advance. This cell-gel mixture was then added in a 24-well plate and incubated at 37°C in a 5% CO_2 incubator for 1 hour to polymerize. After gel polymerization, 1 mL of FBS-free culture medium was added to each well. The cultures were then incubated for 2 days, during which stress developed. Before the contraction test, the gels were released from the sides of the wells carefully by using thin pipette tips. After 5 minutes of equilibration time, 10 $\mu\text{mol/L}$ histamine (Sigma) was added to the gels, and the gel images were taken at various time points after histamine administration. The images were analyzed by using ImageJ software (National Institutes of Health, Bethesda, Md) by calculating the percentage decrease in gel area relative to control.

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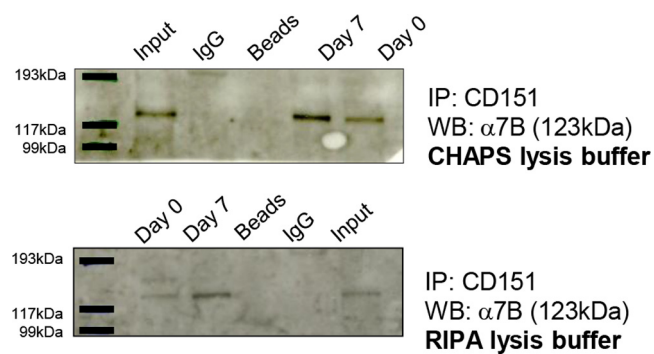


FIG E1. CD151 coimmunoprecipitates (*IP*) with the laminin-binding integrin α 7 under CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) and RIPA (Radioimmunoprecipitation assay) detergent conditions. Day 7 denotes protein lysate collection after 7 days of serum deprivation versus day 0. Input denotes total protein lysate before IP. *WB*, Western blotting.

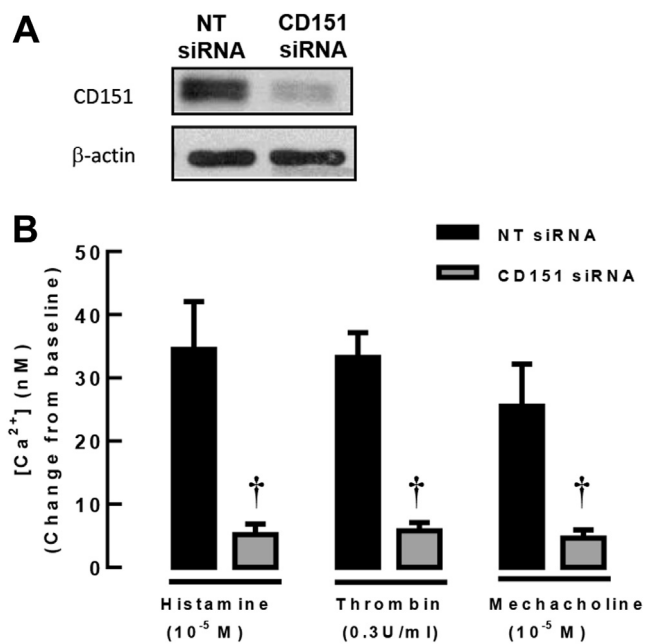


FIG E2. **A**, Representative western blot showing confirmation of knock-down of CD151 protein with a second commercial siRNA kit. **B**, Grouped data showing the effect of 3 GPCR agonists on peak calcium levels in the presence and absence of CD151 siRNA relative to nontargeting (NT) siRNA. * $P < .05$ compared with basal/control values ($n = 8$).

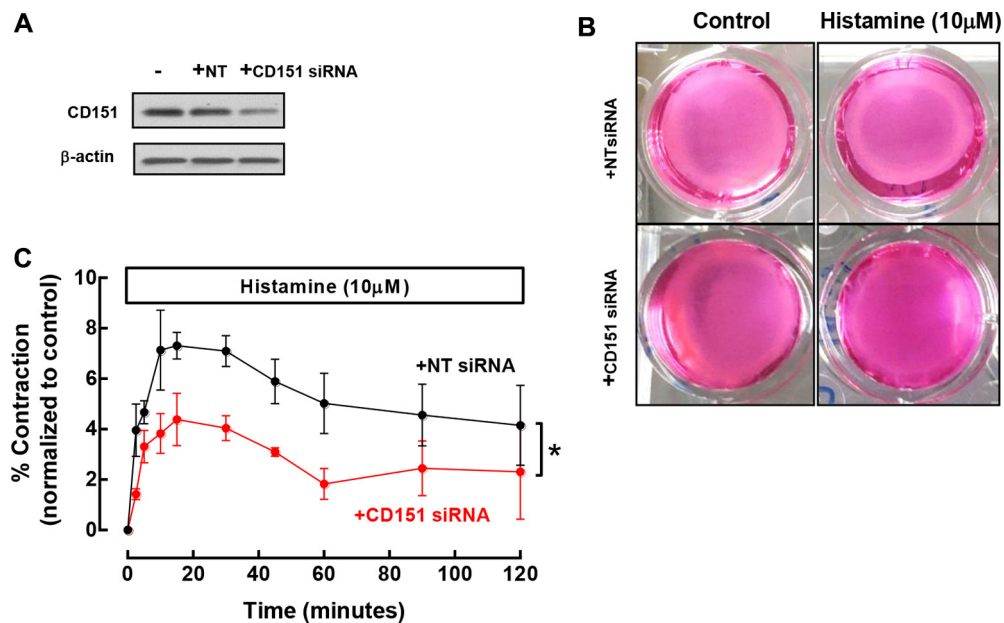


FIG E3. Silencing of CD151 protein expression by siRNA inhibits histamine-induced gel contraction of ASM cells. **A**, Confirmation of knockdown of CD151 protein abundance by CD151 siRNA compared with nontargeting (NT) control siRNA. **B** and **C**, Representative (Fig E3, *B*) and grouped (Fig E3, *C*) data of time course showing gel contraction with exogenous stimulation (histamine). Gels containing ASM cells contracted with histamine in the presence of control siRNA (NT), whereas gels containing ASM cells that were pre-treated with CD151 siRNA contracted less after histamine stimulation. Results are shown as a percentage decrease in gel area relative to control untreated gels. * $P < .05$ compared with NT-siRNA-treated cells ($n = 3$). Error bars indicate SEMs.