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This information is current as of October 24, 2011

J Immunol; Prepublished online 24 October 2011;
doi:10.4049/jimmunol.1003507
<http://www.jimmunol.org/content/early/2011/10/23/jimmunol.1003507>

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



Integrin $\alpha\text{v}\beta 5$ -Mediated TGF- β Activation by Airway Smooth Muscle Cells in Asthma

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Severe asthma is associated with airway remodeling, characterized by structural changes including increased smooth muscle mass and matrix deposition in the airway, leading to deteriorating lung function. TGF- β is a pleiotropic cytokine leading to increased synthesis of matrix molecules by human airway smooth muscle (HASM) cells and is implicated in asthmatic airway remodeling. TGF- β is synthesized as a latent complex, sequestered in the extracellular matrix, and requires activation for functionality. Activation of latent TGF- β is the rate-limiting step in its bioavailability. This study investigated the effect of the contraction agonists LPA and methacholine on TGF- β activation by HASM cells and its role in the development of asthmatic airway remodeling. The data presented show that LPA and methacholine induced TGF- β activation by HASM cells via the integrin $\alpha\text{v}\beta 5$. Our findings highlight the importance of the $\beta 5$ cytoplasmic domain because a polymorphism in the $\beta 5$ subunit rendered the integrin unable to activate TGF- β . To our knowledge, this is the first description of a biologically relevant integrin that is unable to activate TGF- β . These data demonstrate that murine airway smooth muscle cells express $\alpha\text{v}\beta 5$ integrins and activate TGF- β . Finally, these data show that inhibition, or genetic loss, of $\alpha\text{v}\beta 5$ reduces allergen-induced increases in airway smooth muscle thickness in two models of asthma. These data highlight a mechanism of TGF- β activation in asthma and support the hypothesis that bronchoconstriction promotes airway remodeling via integrin mediated TGF- β activation. *The Journal of Immunology*, 2011, 187: 000–000.

Airway hyperresponsiveness, a key feature of asthma, is the enhanced contraction of the airway smooth muscle (ASM) layer in response to inhaled stimuli, which leads to variable airway obstruction. This phenomenon is responsible for the acute exacerbations associated with asthma. Recurrent exacerbations are a feature of severe asthma. Similarly, the structural changes of airway remodeling are also commonly associated with cases of severe asthma (1) and deteriorating lung function over time (2, 3). It has recently been shown that bronchoconstriction can induce features of airway remodeling in patients with mild asthma, including collagen deposition and goblet cell hyperplasia (4); however, the responsible mechanism is unknown.

TGF- β has been implicated in the pathogenesis of airway remodeling in asthma (5). TGF- β promotes airway remodeling, in part because of its effects on ASM cell proliferation, epithelial cell apoptosis, and potent profibrotic actions, including increasing

synthesis of collagen and fibronectin (6–8). TGF- β promotes extracellular matrix deposition, ASM proliferation, and mucous production in an animal model of allergic asthma without affecting existing airway inflammation (9). Overexpression of Smad2, a TGF- β signaling protein, causes thickening of the ASM layer and deposition of collagen following allergen challenge (10). Moreover, the importance of TGF- β signaling in asthma pathogenesis is supported by a genome-wide association study demonstrating a link between a single nucleotide polymorphism in the SMAD3 gene and asthma (11).

TGF- β is secreted from cells in noncovalent association with its propeptide, the latency-associated peptide, which renders it inactive. Activation of latent TGF- β is the rate-limiting step in its bioavailability (12), and mechanisms of TGF- β activation are fundamental to disease. Several mechanisms of activation have been described in vitro, including proteolytic activation by plasmin, matrix metalloproteases and tryptase, physical activation by extremes of heat and oxidation, and activation by thrombospondin-1 (13–17). Several studies have described increased TGF- β activity in asthma (18–20). Activation of TGF- β in asthma can occur by several mechanisms. Epithelial cells can activate TGF- β in response to damage to the epithelial layer. Mast cells, which are present in large numbers in the asthmatic bronchial mucosa, can activate TGF- β proteolytically through the release of proteases from their granules (15, 21–23). In vivo integrins appear to have a major role in TGF- β activation, at least in development (24, 25), and recently fibroblast-specific deletion of the $\alpha\text{v}\beta 8$ integrin has been shown to reduce airway remodeling by reducing TGF- β -induced CCL2 and CCL20 dependent dendritic cell migration (26). However, it is known whether smooth muscle cell TGF- β activation can directly contribute to airway remodeling.

Integrins are heterodimeric cell surface molecules involved in cell–cell interactions and cell–matrix interactions. Six of the 24

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Received for publication October 25, 2010. Accepted for publication September 28, 2011.

This work was supported by the Wellcome Trust (Project Grant 085350, Project Grant 083122, and Senior Clinical Fellowship 082265), Asthma U.K. (Project Grant 07/026), and the Nottingham Respiratory Biomedical Research Unit.

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Abbreviations used in this article: ASM, airway smooth muscle; BALF, bronchoalveolar lavage fluid; HASM, human ASM; HPRT, hypoxanthine-guanine phosphoribosyltransferase; LPA, lysophosphatidic acid; PAI1, plasminogen activator inhibitor-1; RLU, relative luciferase unit; SMA, smooth muscle actin; TMLC, transformed mink lung epithelial cell.

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currently described integrins recognize and bind arginine-glycine-aspartate motifs in the latency-associated peptide of both TGF- β 1 and TGF- β 3. Four of these have been reported to activate TGF- β in vitro, including α v β 6 (27), α v β 8 (28), α v β 3 (29), and α v β 5 (30). Integrin-mediated TGF- β activation has been best characterized for the α v β 6 and the α v β 8 integrins. Activation of TGF- β by the α v β 8 integrin involves MMP14 and proteolytic cleavage of the latent TGF- β molecule, whereas α v β 3, α v β 5, and α v β 6 integrins activate TGF- β by a mechanism requiring an intact cytoskeleton and cell contraction (27, 30–32). Activation of TGF- β by α v β 6 integrins is spatially restricted to epithelial cells, whereas α v β 5, and to a lesser extent α v β 3, are present on mesenchymal cells and are able to activate mesenchymal TGF- β (30, 33, 34). This raises the possibility that cellular contraction during bronchoconstriction promotes TGF- β activation via cell surface integrins.

The aims of this study were to investigate whether contraction agonists could promote TGF- β activation in human ASM (HASM) cells, and determine whether this process was dysregulated in asthma. We found that lysophosphatidic acid (LPA) induced TGF- β activation by HASM cells via an integrin α v β 5-mediated mechanism that involved reorganization of the cytoskeleton. Furthermore, methacholine also induced TGF- β activation by HASM cells. HASM cells isolated from asthmatic patients activated more TGF- β in response to both contraction agonists than cells from nonasthmatic individuals. Furthermore, a polymorphism in the cytoplasmic domain of the integrin β 5 (*itgb5*) gene resulted in a β 5 subunit that was unable to activate TGF- β . Using the OVA model of airway remodeling we demonstrate coassociation of α v β 5 integrins and phospho-Smad2 staining in the ASM layer of remodeled airways, as well as global increases in TGF- β activation. Finally, using two distinct murine models of asthma, we show that both inhibition and genetic loss of the α v β 5 integrin results in significantly less ASM surrounding the airways despite enhanced inflammation. This finding suggests that the α v β 5 integrin has an important role in mediating airway remodeling, as well as supporting the notion that inflammation and remodeling can be dissociated in asthma. These data suggest a potential novel mechanism through which contraction of the ASM layer during asthma attacks could promote airway remodeling in patients with poorly controlled disease.

Materials and Methods

Cell culture

Tracheal and bronchial HASM cells were used. Tracheal HASM cells were obtained from postmortem tracheal specimens from donors with no history of respiratory disease or evidence of airway abnormalities as previously described (35). Bronchial HASM cells were derived from bronchial biopsy specimens and were supplied by Chris Brightling (University of Leicester, Leicester, U.K.). Asthmatic subjects recruited at Glenfield Hospital (University of Leicester) were carefully characterized and presented with an appropriate history, objective evidence of variable airflow obstruction, and airway hyperresponsiveness as described previously (36). The subjects were free from exacerbations requiring systemic corticosteroids or antibiotics for 6 wk prior to the bronchoscopy. Cells were cultured in DMEM containing 10% FCS, penicillin G (100 U/ml), streptomycin (100 μ g/ml) and L-glutamine (4 mM). All cells were used at passage 6 and were growth arrested in serum-free medium for 24 h prior to experiments. CS-1 cells, a nonadherent hamster melanoma cell line, were a gift from Dean Sheppard (University of California, San Francisco). They were cultured in RPMI 1640 plus 10% FCS, L-glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin B (2.5 μ g/ml). Following transfection with integrin β 5 constructs, the cells became adherent to plastic; they were then cultured as before with the addition of the antibiotic G418 at 500 μ g/ml, because this was the concentration that killed 100% of nontransfected CS-1 cells after 1 wk of culture (data not shown).

Transformed mink lung epithelial cell coculture assay

Transformed mink lung epithelial cells (TMLCs) that stably express a portion of the plasminogen activator inhibitor-1 (PAI1) promoter driving a luciferase gene were used as reporter cells to detect TGF- β activity (32). HASM cells were plated at 2.5×10^5 cells/ml and growth arrested for 24 h. TMLCs were plated directly on top of HASM cells at 5×10^5 cells/ml. The cells were then stimulated as required for each experiment. After 16 h, the cells were lysed using a luciferase reporter assay kit (Promega), and the luminescence was measured as relative luciferase units (RLU).

Quantitative RT-PCR

Following stimulation total cell RNA was extracted using NucleoSpin RNA II kit (Macharey Nagel) and reverse transcribed into complementary DNA (cDNA) using Moloney murine leukemia virus reverse transcriptase. cDNA was subjected to quantitative RT-PCR analysis to assess expression of the TGF- β -inducible gene PAI1 using β 2-microglobulin (β 2-M) as a housekeeping gene. Distinct primer sets were used to amplify murine DNA and hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as a housekeeping gene in these cases. Primer sequences were as follows:

PAI1 sense: 5'-TCTGCAGACCTGGTCCCCAC-3',
 PAI1 antisense: 5'-AGCCCCGTAGTTCATCCTG-3',
 β 2-M sense: 5'-AATCCAAATGCGGCATCT-3',
 β 2-M antisense: 5'-GAGTATGCCTGCCGTGTG-3',
 Fibronectin sense: 5'-CAGGATCAACTTACGGAG-3',
 Fibronectin antisense: 5'-GCCAGTGACAGCATACA-3',
 Collagen sense: 5'-CCAAATCTGTCTCCCCAGAA-3',
 Collagen antisense: 5'-TCAAAAACGAAGGGGAGATG-3',
 murine PAI1 sense: 5'-GGCCAAAGGAAAAAGCACTGT-3',
 murine PAI1 antisense: 5'-GGGCCACCAATTGATCTGTCT-3',
 murine HPRT sense: 5'-TGAAAGACTTGCTCGAGATGTCA-3', and
 murine HPRT antisense: 5'-CCAGCAGGTCAGCAAAGA-3'.

Amplification was performed using an MXPro3000 (Stratagene) with SYBR Premier Ex Taq (Takara Bio) on the following program: initial denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 15 s. Amplification of a single DNA product was confirmed by melting curve analysis. Data were expressed as relative expression using the $\Delta\Delta$ Ct equation as described previously (37).

Isolation of nuclear and cytoplasmic protein fractions

Nuclear and cytoplasmic protein fractions were isolated from LPA-stimulated HASM cells as described previously (38, 39) using the NEXtract CellLytic nuclear extraction kit (Sigma) according to the manufacturer's protocol.

Western blot

Levels of Smad2 and Smad3 in cytoplasmic and nuclear fractions were determined by Western blotting. Protein samples (30 μ g per lane) were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and electroblotted to a polyvinylidene fluoride membrane. After blocking for 1 h (5% nonfat milk in TBS plus 0.1% Tween 20), the membrane was incubated overnight at 4°C with monoclonal anti-Smad2/3 Ab (1:500 in blocking buffer; Cell Signaling Technologies). After washing, the membrane (PBS pH 7.4 plus 0.3% Tween-20) was incubated with HRP-conjugated secondary Ab (1:2000 in blocking buffer) for 1 h at room temperature. The membrane was incubated with ECL Western blotting detection reagent and visualized by exposure to Hyperfilm-ECL.

Cell titer-glow assay

The Cell Titer-Glo Luminescent Cell Viability Assay (Promega) was used to determine cell number in coculture experiments comparing nonasthmatic and asthmatic HASM cells. Additional wells of cells were prepared and treated in the same manner as the experimental wells. After the incubation period of 16 h, 100 μ l Cell Titer-Glo reagent was added to each well, and the plate was placed on a rocker for 10 min at room temperature. The luminescence was measured and data were converted into actual cell numbers using a standard curve of known cell numbers.

Stable transfections

DNA plasmid constructs corresponding to the common full-length integrin β 5 subunit and a polymorphic integrin β 5 subunit in the vector pcDNA3.1 were provided by Prof. E. Kawahara (Kanazawa University, Kanazawa,

Japan). The transfection reagent Transfast (Promega) was used to stably transfect CS-1 hamster melanoma cells according to the manufacturer's protocol.

Flow cytometry for integrin $\alpha\beta 5$

Cell surface expression of the integrin $\alpha\beta 5$ was assessed by flow cytometry as described previously (32). Cells were counted, and 0.5×10^6 cells were first blocked in goat serum for 20 min and then stained with an anti- $\alpha\beta 5$ Ab (Ab clone name ALULA raised in mouse; a gift from Prof. Dean Sheppard) at 25 $\mu\text{g}/\text{ml}$ for 20 min. After washing the cells in PBS, the cells were stained with a PE-labeled anti-mouse secondary Ab for 20 min. Negative control cells were blocked with goat serum but only stained with the secondary PE-labeled Ab. Cells were analyzed using a BD FACSCanto flow cytometer.

Coimmunoprecipitation of integrin $\beta 5$ with talin

The interaction of integrin $\beta 5$ subunit with the cytoskeletal protein talin was investigated using a Universal Magnetic coimmunoprecipitation kit (Active Motif) according to the manufacturer's instructions. A rabbit polyclonal Ab directed against integrin $\beta 5$ and a mouse monoclonal directed against talin 1 and 2 (clone 8D4) were used (both from Abcam). Protein (500 μg) was immunoprecipitated with 5 μg Ab and protein G magnetic beads. After separation of proteins by SDS-PAGE, the membrane was probed with second Ab.

OVA murine model of asthma

All animal care and procedures were approved by the University of Nottingham Ethical Review Committee and were performed under Home Office Project and Personal License authority within the Animal (Scientific Procedures) Act 1986. Female BALB/c 6-wk-old mice were purchased from Charles River (Margate, Kent, U.K.) and housed in the Biomedical Services Unit, University of Nottingham for at least 7 d after delivery. Animals had free access to food (Tekland Global 18% protein rodent diet; Bicester, Oxen, U.K.) and water. On day 0 of the study, mice were sensitized by i.p. injection of 10 μg OVA (Sigma) diluted 1:1 with the adjuvant Alum (Sigma), followed by a subsequent sensitization on day 12. At day 19, the mice were challenged daily by oropharyngeal administration of either 400 $\mu\text{g}/\text{ml}$ OVA in 50 μl saline or 50 μl saline alone for 6 d, followed by additional challenges on days 26, 28, 30, and 33. The mice were sacrificed on day 34. During the study of the inhibition of $\alpha\beta 5$, mice were sensitized as described and treated every 2 d from day 18 with 4 mg/kg anti- $\alpha\beta 5$ (clone ALULA) by i.p. injection until the end of the study. Bronchoalveolar lavage was performed using 1 ml PBS. Both frozen and formalin-fixed tissues were collected for immunohistochemistry. For formalin-fixed tissue, the left lobe was inflated with formalin and fixed in formalin overnight and then embedded in paraffin wax. For frozen tissue, the left lobe was inflated with 1 ml OCT and then frozen in precooled isopentane and stored at -80°C . The remaining four lobes were separated and snap frozen in liquid nitrogen until needed.

Aspergillus fumigatus murine model of asthma

The University of California–San Francisco Committee on Animal Research (IACUC) approved the use of all mice for all reported experiments. The animals were allowed free access to food (Tekland Rodent Laboratory Chow) and water. $\beta 5$ knockout mice (*itgb5*^{-/-}) were generated as described previously (40) and bred onto a 129 SvJae background. The murine model of *Aspergillus fumigatus* allergic lung disease was used with methods that have been described (41). Isoflurane-anesthetized mice were given 10 μg (in 40 μl saline) of *A. fumigatus* (Hollister-Stier Laboratories) or 40 μl saline to the nostrils using a micropipette with the mouse held in the supine position. After three treatments per week for a total of nine doses, mice were euthanized 48 h after the last intranasal challenge. Bronchoalveolar lavage was performed using three separate washes with 1 ml PBS. The separate washes were combined and centrifuged at 1500 rpm for 5 min, and the resulting cell pellet was resuspended in 1 ml PBS. Total inflammatory cells were counted using a hemocytometer. Formalin-fixed tissue was collected for immunohistochemistry. The left lobe was inflated with formalin, fixed in formalin overnight, and embedded in paraffin wax.

$\alpha\text{SMA}/\alpha\beta 5$ dual staining

Sections of frozen murine lung tissue (3 μm thick) were used. After fixation in Zamboni's fixative for 20 min, the sections were boiled in 2 mM citrate buffer for 3 min and then blocked with avidin and biotin blocking solutions (Vector Laboratories) each for 15 min in PBS. The tissue was then blocked in 5% goat serum plus 0.1% BSA in PBS for 30 min and incubated with an $\alpha\beta 5$ Ab (Abcam 15459, 1:200) overnight. After washing in PBS, the

slides were incubated with a Dylight649 conjugated secondary Ab. The cells were then stained with a 1:100 dilution of an α -smooth muscle actin (SMA) Ab (clone 1A4) using a mouse-on-mouse Ab detection kit (Vector Laboratories) according to the manufacturer's instructions. Staining was visualized using a laser-scanning confocal microscope and Nikon NIS Elements image analysis software. Images were acquired at a single plane through the tissue.

$\alpha\text{SMA}/\text{P-Smad2}$ dual staining

Sections of paraffin wax-embedded tissue (5 μm thick) were used. After dewaxing and rehydration, Ag retrieval was performed by boiling the sections for 20 min in citrate buffer. The sections were blocked in 5% normal goat serum plus 0.1% BSA. The sections were incubated with anti-Phospho-Smad2 overnight (1:200) and then a DyLight594 conjugated secondary Ab. The sections were then counterstained with an anti-mouse anti- αSMA Ab using a mouse-on-mouse fluorescein kit (Vector Laboratories) according to the manufacturer's instructions. Staining was visualized using a laser-scanning confocal microscope and Nikon NIS Elements image analysis software. Images were acquired at a single plane through the tissue.

αSMA immunohistochemistry

αSMA immunohistochemistry was performed on 5- μm thick sections of paraffin wax-embedded tissue. These sections were dewaxed in xylene, rehydrated in increasing concentrations of ethanol, and then washed in PBS. Ag retrieval was performed by boiling the sections for 20 min in citrate buffer. The sections were blocked to inhibit nonspecific binding of the primary Ab in 5% normal goat serum plus 0.1% BSA in PBS for 30 min. The sections were incubated with primary Ab overnight (1:1000). Sections were then incubated with a DyLight488-conjugated secondary Ab for 30 min, and the sections were counterstained with a DAPI nuclear stain. Negative control slides that were not stained with the primary Ab prior to incubation with the secondary Ab were performed for each experiment. αSMA quantification around smaller airways was performed using NIS Elements software (Nikon) measuring area in micrometers of αSMA staining. The definition of a small airway in mice is currently ambiguous; however, a previous study has defined a small airway as one with a diameter of 100–200 μm (42). This study used this definition. Only cross-sectional airways with a radius of less than 100 μm were quantified.

Murine PAII sandwich ELISA

Snap-frozen lung lobes were defrosted in ice-cold lysis buffer (20 mM Tris-HCl, 1% Triton-X 100, 137 mM NaCl, 2 mM EDTA, 25 mM β -glycerophosphate, 10% glycerol, 1 mM Na₃VO₄, 1 mM phenylmethanesulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM DTT, and 0.1 U/ml protease inhibitor mixture). The lungs were homogenized using a hand-held homogenizer. Samples were centrifuged at 13,000 rpm for 10 min, and the supernatant was collected. The concentration of murine PAII in lung homogenates from OVA-sensitized, saline, and OVA-challenged mice was determined using a murine PAII total Ag sandwich ELISA (Patricell) according to the manufacturer's instructions.

Statistics

Statistical analysis was performed using GraphPad Prism 4 software. When data measured differences between groups, the mean values for pooled data were compared. When mechanistic pathways in normal cells were being assessed, the experiments were replicated at least three times, representative examples were shown, and experimental replicates were assessed statistically. Comparison of two data sets was performed by two-tailed unpaired *t* test. Comparison of more than two data sets was performed by two-way ANOVA; *p* < 0.05 was accepted as significant.

Results

Contraction agonist-induced TGF- β activation in HASM cells

To determine whether contraction agonists activated TGF- β in HASM cells, these cells were stimulated with LPA and methacholine, and TGF- β activity was measured using three independent assays. Both LPA and methacholine induced concentration-dependent increases in active TGF- β as measured by coculture assay (Fig. 1A, 1B). There was no effect of either agonist on the reporter cells alone (data not shown). LPA also induced a time-dependent increase in PAII mRNA expression, which was maximal at 8 h (Fig. 1C). This increase was abrogated by a pan-TGF- β neutralizing Ab confirming that LPA induced PAII gene expres-

sion was mediated by TGF- β . Methacholine was also able to induce PAI1 mRNA in a TGF- β dependent manner (Fig. 1D). Finally, both LPA and methacholine stimulation induced the translocation of Smad2 and smad3 to the nucleus (Fig. 1E, 1F). These data confirm that the contraction agonists LPA and methacholine activated TGF- β in HASM cells.

Contraction agonist-induced TGF- β activation was mediated via $\alpha\text{v}\beta 5$ integrin

To identify the mechanism of TGF- β activation in HASM cells, an $\alpha\text{v}\beta 5$ blocking Ab was used. LPA- and methacholine-induced TGF- β activation was assessed by measuring PAI1 mRNA levels, and both were abrogated by the $\alpha\text{v}\beta 5$ blocking Ab (Fig. 2A, 2B).

Because cytoskeletal proteins are central to contraction-induced TGF- β activation (27, 30), the pharmacologic inhibitor of actin reorganization (cytochalasin D) was used. LPA-induced TGF- β activation was inhibited by cytochalasin D (Fig. 2C). To determine whether bronchodilation inhibited LPA-induced TGF- β activation, the $\beta 2$ -adrenoreceptor ($\beta 2$) agonist formoterol was used. The addition of formoterol completely inhibited LPA-induced increases in PAI1 gene expression (Fig. 2D).

A polymorphism in the $\beta 5$ subunit cytoplasmic tail abrogated $\alpha\text{v}\beta 5$ -mediated TGF- β activation

To establish whether cytoskeletal interactions with the $\beta 5$ cytoplasmic domain were involved in mediating TGF- β activation,

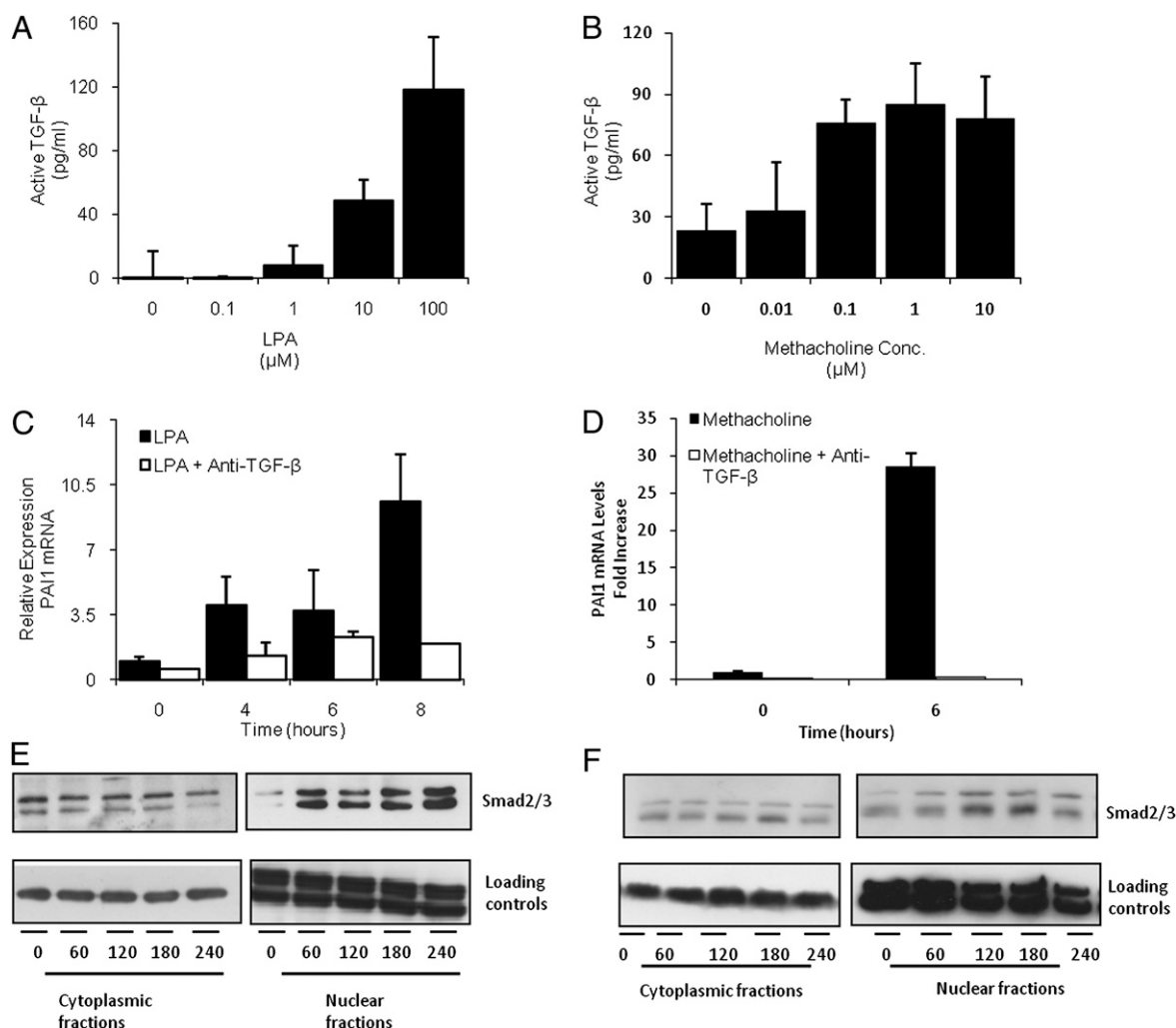


FIGURE 1. Contraction agonist-induced TGF- β activation by HASM cells. **A**, HASM cells in coculture with TMLCs were stimulated with 0, 0.1, 1, 10, and 100 μM LPA. Data are expressed as mean TGF- β activity $\pm$ SEM; $n = 3$ independent primary HASM cell lines derived from nonasthmatic donors; $p < 0.05$ for 10 μM LPA, $p < 0.05$ for 100 μM LPA. **B**, HASM cells in coculture with TMLCs were stimulated with 0, 0.01, 0.1, 1, and 10 μM methacholine. Data are expressed as mean TGF- β activity $\pm$ SEM; $n = 2$ independent experiments using nonasthmatic HASM cells; $p < 0.05$ for 1 μM methacholine. **C**, HASM cells were stimulated with 20 μM LPA in the presence (white bars) or absence (black bars) of 10 $\mu\text{g}/\text{ml}$ pan-TGF- β neutralizing Ab, and relative expression of PAI1 mRNA levels was measured. LPA increased PAI1 expression ($p < 0.05$), which was abrogated by pan-TGF- β neutralizing Ab ($p < 0.05$). Data were normalized to housekeeping gene $\beta 2$ -M and expressed as mean fold increase in PAI1 mRNA $\pm$ SEM. Data were replicated three times and a representative example is shown. **D**, HASM cells were stimulated with 1 μM methacholine in the presence (white bars) or absence (black bars) of 10 $\mu\text{g}/\text{ml}$ pan-TGF- β neutralizing Ab, and relative expression of PAI1 mRNA levels was measured. Methacholine increased PAI1 expression ($p < 0.005$), which was abrogated by pan-TGF- β neutralizing Ab ($p < 0.005$). Data were normalized to housekeeping gene $\beta 2$ -M and expressed as mean fold increase in PAI1 mRNA $\pm$ SEM. Data were replicated three times and a representative example is shown. **E**, HASM cells were stimulated with 20 μM LPA for 0, 60, 120, 180, and 240 min. Cytoplasmic (lanes 1–6) and nuclear (lanes 7–12) proteins were separated by SDS-PAGE. Loading controls of GAPDH for cytoplasmic proteins and lamin A/C for nuclear proteins are shown. Data were replicated three times and a representative example is shown. **F**, HASM cells were stimulated with 1 μM methacholine for 0, 60, 120, 180, and 240 min. Cytoplasmic (lanes 1–6) and nuclear (lanes 7–12) proteins were separated by SDS-PAGE. Loading controls of GAPDH for cytoplasmic proteins and lamin A/C for nuclear proteins are shown. Data were replicated three times and a representative example is shown.

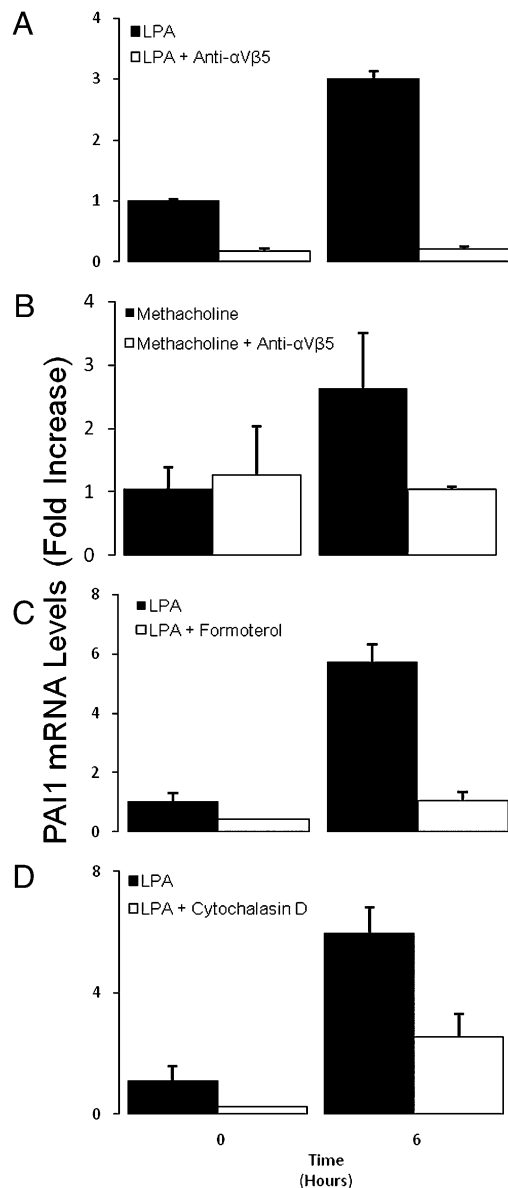


FIGURE 2. LPA-induced TGF- β activation is mediated via α v β 5 integrin and involves the cytoskeleton. **A**, HASMs were stimulated in duplicate with 20 μ M LPA with or without an α v β 5 neutralizing Ab (10 μ g/ml), and PAI1 mRNA levels were measured. The addition of α v β 5 neutralizing Ab inhibited LPA-induced PAI1 ($p < 0.01$). Data are expressed as mean fold increase in PAI1 mRNA $\pm$ SEM. Data were replicated three times and a representative example is shown. **B**, HASM cells were stimulated with 1 μ M methacholine with or without an α v β 5 neutralizing Ab (10 μ g/ml), and PAI1 mRNA levels were measured. The addition of an α v β 5 neutralizing Ab inhibited methacholine-induced TGF- β activation. Data are expressed as mean fold increase in PAI1 mRNA $\pm$ SEM. Data were replicated three times and a representative experiment is shown. **C**, HASM cells were stimulated with 20 μ M LPA with or without formoterol (1 μ M), and PAI1 mRNA levels were measured. Formoterol inhibited LPA-induced PAI1 expression ($p < 0.05$). Data are expressed as mean fold increase in PAI1 mRNA $\pm$ SEM. Data were replicated three times and a representative example is shown. **D**, HASM cells were stimulated with 20 μ M LPA with or without cytochalasin D (1 μ M), and PAI1 mRNA levels were measured. Cytochalasin D inhibited LPA-induced PAI1 expression ($p < 0.01$). Data are expressed as mean fold increase in PAI1 mRNA $\pm$ SEM. Data were replicated three times and a representative example is shown.

the effect of a previously described β 5 polymorphism on LPA-induced TGF- β activation was investigated (43). This polymorphism involves a 9-bp deletion within the integrin β 5 cytoplasmic domain, resulting in a β 5 subunit that lacks amino acids FNK^{767–769}. Full-length DNA constructs corresponding to both the common allele of β 5 (FNKFNK) and the polymorphic β 5 (FNK; both were gifts from Prof. E. Kawahara) were stably transfected into CS-1 hamster melanoma cells, which do not express any endogenous *ITGB3* or *ITGB5* genes. After transfection, the cell surface expression of α v β 5 was assessed by flow cytometry. Both cell lines transfected with the common and polymorphic allele expressed α v β 5 integrin on the cell surface (Fig. 3A, 3B), although levels of expression were higher after transfection of the polymorphic allele. TGF- β activation was assessed in these cell lines by coculture assay. Coculture of cells expressing the common allele with TMLC reporter cells demonstrated that the common allele was able to activate TGF- β basally (Fig. 3C), as shown by a decrease in luciferase activity in the presence of a TGF- β neutralizing Ab. However, cells transfected with the polymorphic allele were unable to activate TGF- β basally (Fig. 3D).

The deletion of FNK^{767–769} in the polymorphic β 5 subunit occurs within an NxxY talin binding domain. To assess whether talin interactions with the β 5 cytoplasmic domain were altered in cells expressing the polymorphic β 5 subunit, coimmunoprecipitation studies were performed. Initially the β 5 integrin subunit was immunoprecipitated from CS-1 cell lysates expressing either full-length or polymorphic β 5 and immunoblotted for talin (Fig. 4A). To further investigate these interactions, talin was immunoprecipitated and immunoblots for β 5 integrin subunit were performed (Fig. 4B). Full-length talin (220 kDa) bound to both common and polymorphic β 5 subunits, whereas the talin rod domain (190 kDa) consistently bound only to the polymorphic allele of the β 5 subunit.

Asthmatic HASM cells activated more TGF- β than nonasthmatic HASM cells

Having demonstrated that the contraction agonists LPA and methacholine induced α v β 5 integrin-mediated TGF- β activation in HASM cells via the cytoskeleton, we wanted to determine whether there was any difference between HASM cells from patients with asthma compared with controls. When asthmatic HASM cells were stimulated with increasing concentrations of LPA, they activated more TGF- β compared with nonasthmatic cells (Fig. 5A; $p < 0.005$). LPA also induced TGF- β activation in a time-dependent manner as measured by PAI1 mRNA levels ($p < 0.05$), and asthmatic HASM cells elicited an exaggerated response to LPA stimulation at each time point tested ($p < 0.05$), which was a maximum of 6 h after stimulation (Fig. 5B). Furthermore, asthmatic HASM cells activated more TGF- β than did nonasthmatic cells in response to methacholine stimulation, as measured by both coculture assay (Fig. 5C) and by PAI1 quantitative RT-PCR (Fig. 5D). To determine whether this increased TGF- β activation resulted in increased expression of genes associated with airway remodeling we assessed expression of both fibronectin and collagen type 1A (COL1A) by quantitative RT-PCR in response to LPA stimulation. Asthmatic HASM cells expressed greater levels of fibronectin mRNA in response to LPA treatment than did nonasthmatic cells (Fig. 5E). Although there was a trend toward increased COL1A expression, no statistically significant differences between diseased and nondiseased HASM cells were detected (Fig. 5F). Finally, total TGF- β levels were measured and found to be 2-fold higher in unstimulated asthmatic HASM cells compared with nonasthmatic HASM cells ($p < 0.005$; Fig. 5G).

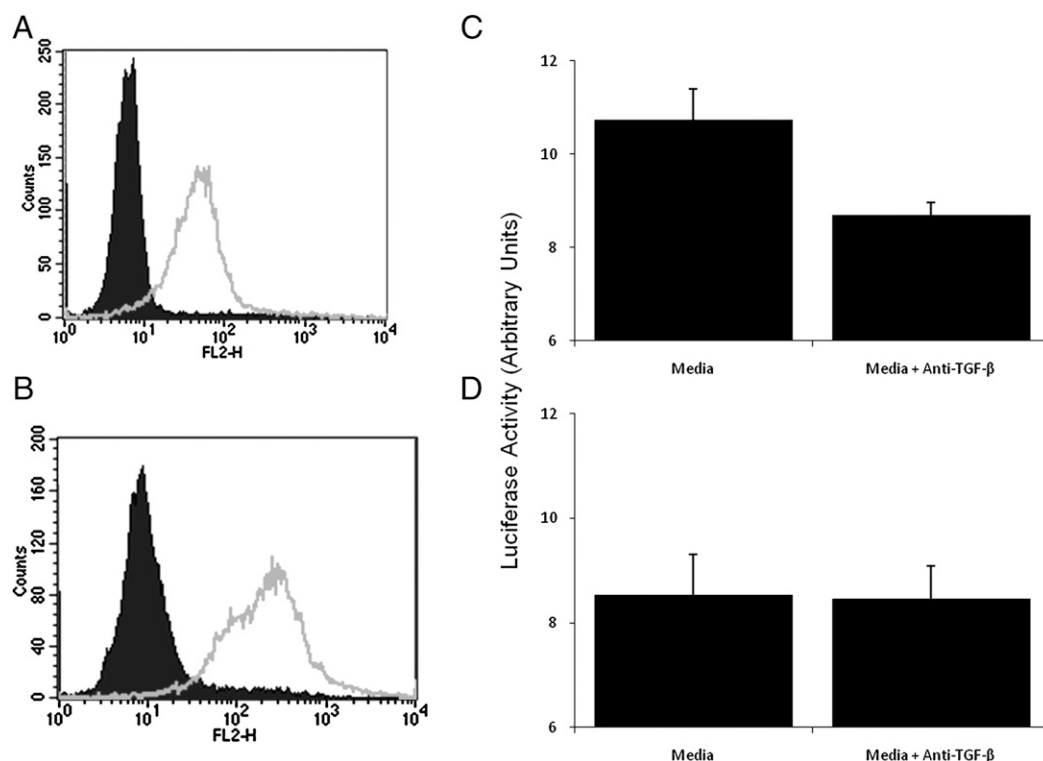


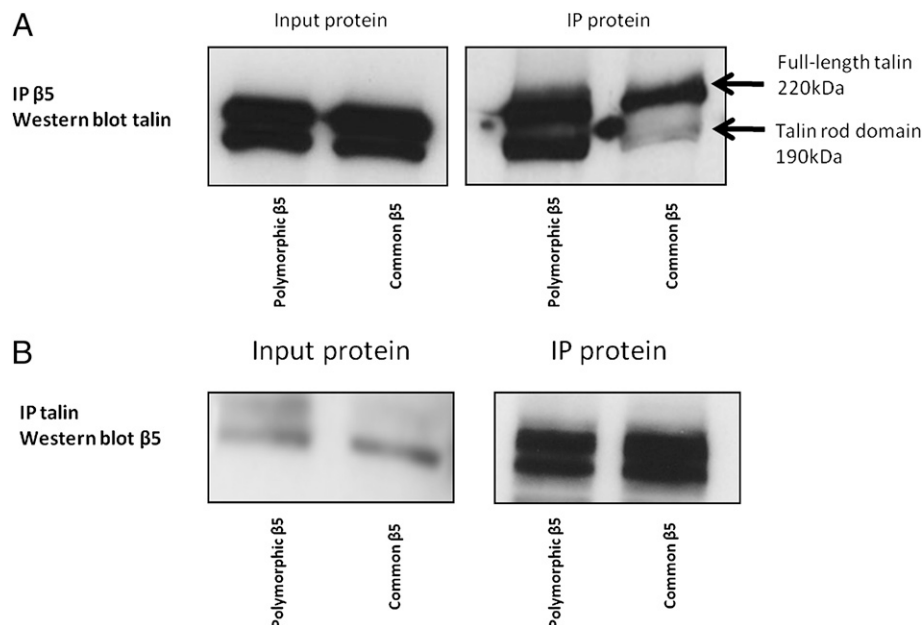
FIGURE 3. A polymorphism in the $\beta 5$ cytoplasmic tail abrogates $\alpha v\beta 5$ -mediated TGF- β activation. *A*, The cell surface expression of $\alpha v\beta 5$ by CS-1 cells transfected with full length $\beta 5$ construct (FNKFNK) was assessed by flow cytometry. The mean PE fluorescence intensity is shown. *B*, The cell surface expression of $\alpha v\beta 5$ by CS-1 cells transfected with polymorphic $\beta 5$ construct (FNK) was assessed by flow cytometry. The mean PE fluorescence intensity is shown. *C*, FNKFNK-transfected CS-1 cells were cocultured with TMLCs with or without a pan-TGF- β neutralizing Ab. Pan-TGF- β neutralizing Ab inhibited basal luciferase activity ($p < 0.05$). Data are expressed as mean RLU $\pm$ SEM. Data were replicated four times and a representative experiment is shown. *D*, FNK-transfected CS-1 cells were cocultured with TMLC with or without a pan-TGF- β neutralizing Ab. Data are expressed as mean RLU $\pm$ SEM. Data were replicated four times and a representative experiment is shown.

$\alpha v\beta 5$ integrin mediated increased TGF- β activity in asthmatic cells, but this was not due to increased cell surface $\alpha v\beta 5$ integrin expression

To determine whether the enhanced TGF- β activity observed in asthmatic HASM cells was mediated by the $\alpha v\beta 5$ integrin, we stimulated HASM cells from asthmatic patients with LPA in the

presence of a $\alpha v\beta 5$ blocking Ab (Fig. 6A, 6B). The $\alpha v\beta 5$ integrin blocking Ab completely abrogated LPA-induced increases in TGF- β activity, as assessed by coculture assay in asthmatic and nonasthmatic HASM cells (Fig. 6A, 6B). This finding indicated that the increased LPA-induced TGF- β activation observed in asthmatic HASM cells was mediated via the $\alpha v\beta 5$ integrin. To

FIGURE 4. Talin interacts with both full-length and polymorphic $\beta 5$ subunits. *A*, The interaction between talin and the $\beta 5$ subunit was assessed by coimmunoprecipitation. $\beta 5$ was immunoprecipitated from 500 μ g total cell protein, and any associated talin was detected by Western blot. Talin migrates at 220 and 190 kDa; 10 μ g input protein (prior to immunoprecipitation) was used as a loading control. Data were replicated three times and a representative example is shown. *B*, The interaction between talin and the $\beta 5$ subunit was assessed by coimmunoprecipitation. Talin was immunoprecipitated from 500 μ g protein, and any associated $\beta 5$ was detected by Western blot. $\beta 5$ migrates at ~ 95 kDa; 10 μ g input protein (prior to immunoprecipitation) was used as a loading control. Data were replicated three times and a representative example is shown.



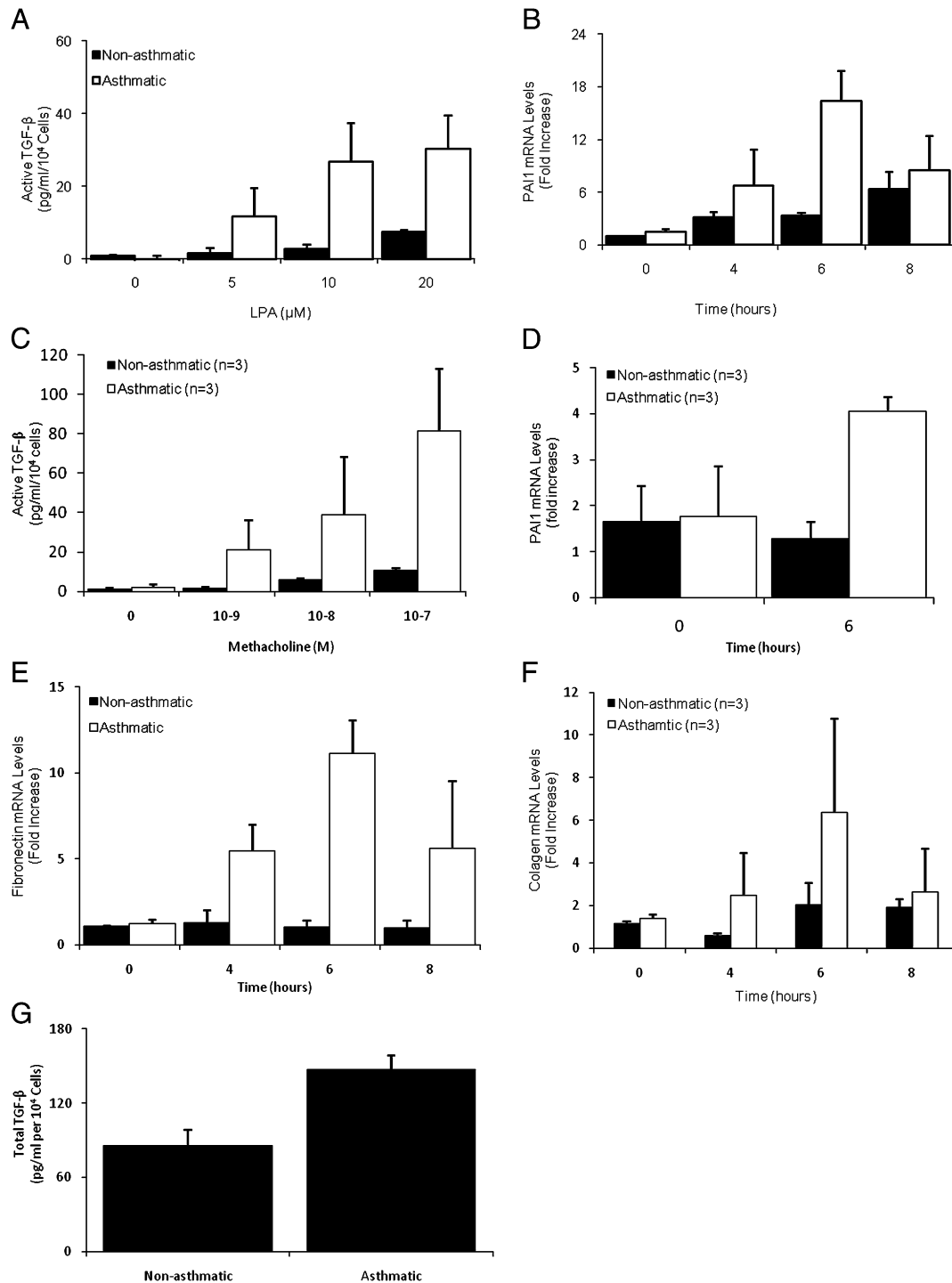


FIGURE 5. Asthmatic HASM cells activate more TGF- β than nonasthmatic HASM cells. **A**, A coculture of either nonasthmatic or asthmatic HASM cells with TMLCs was stimulated with increasing concentrations of LPA, and TGF- β activity was determined. Asthmatic cells activate more TGF- β in response to LPA than do nonasthmatic cells ($p < 0.005$). Data are expressed as mean TGF- β activity $\pm$ SEM; $n = 3$ nonasthmatic cell lines and $n = 3$ asthmatic cell lines. **B**, Nonasthmatic and asthmatic HASM cells were stimulated with $20 \mu\text{M}$ LPA and PAI1 mRNA levels were measured by quantitative RT-PCR. Asthmatic HASM cells expressed higher levels of PAI1 in response to LPA compared with nonasthmatic cells ($p < 0.05$). Data were normalized to housekeeping gene $\beta 2\text{-M}$ and expressed as fold increase in PAI1 over time $0 \pm$ SEM; $n = 3$ nonasthmatic and $n = 3$ asthmatic cell lines. **C**, A coculture of either nonasthmatic or asthmatic HASM cells with TMLCs was stimulated with increasing concentrations of methacholine, and TGF- β activity was determined. Asthmatic cells activate more TGF- β in response to methacholine than do nonasthmatic cells ($p < 0.005$). Data are expressed as mean TGF- β activity $\pm$ SEM; $n = 3$ nonasthmatic cell lines and $n = 3$ asthmatic cell lines. **D**, Nonasthmatic and asthmatic HASM cells were stimulated with $1 \mu\text{M}$ methacholine for 6 h, and PAI1 mRNA levels were measured by quantitative RT-PCR. Asthmatic cells expressed more PAI1 in response to methacholine than did nonasthmatic cells ($p < 0.05$). Data were normalized to housekeeping gene $\beta 2\text{-M}$ and expressed as fold increase in PAI1 over time $0 \pm$ SEM; $n = 3$ nonasthmatic and $n = 3$ asthmatic cell lines. **E**, Nonasthmatic and asthmatic HASM cells were stimulated with $20 \mu\text{M}$ LPA, and then fibronectin mRNA levels were measured. Asthmatic cells expressed more fibronectin after LPA stimulation than did nonasthmatic cells ($p < 0.005$). Data were normalized to housekeeping gene $\beta 2\text{-M}$ and expressed as fold increase in fibronectin over time $0 \pm$ SEM; $n = 3$ nonasthmatic and $n = 3$ asthmatic cell lines. **F**, Nonasthmatic and asthmatic HASM cells were stimulated with $20 \mu\text{M}$ LPA, and collagen mRNA levels were measured. There (Figure legend continues)

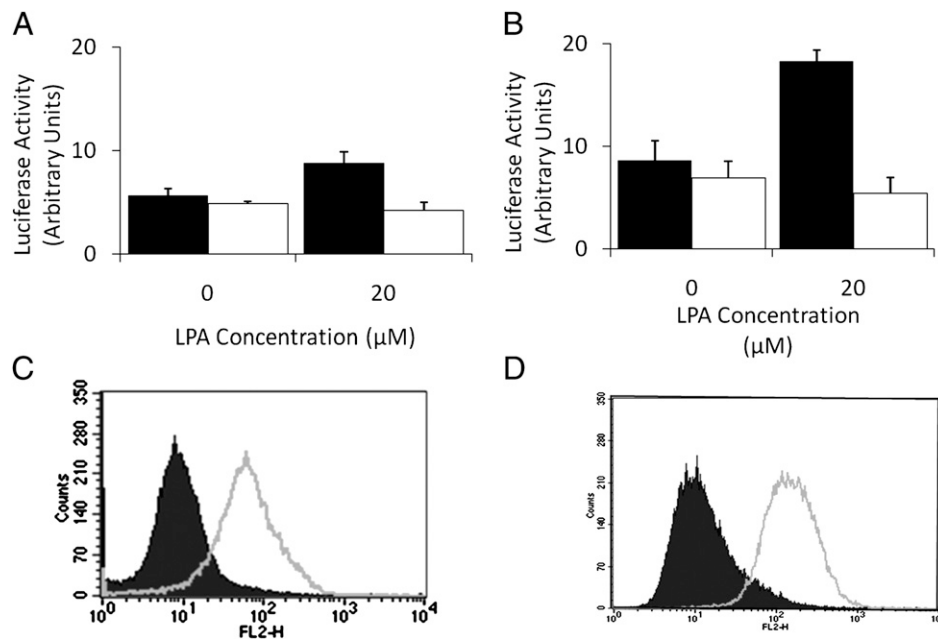


FIGURE 6. The $\alpha v\beta 5$ integrin mediates increased TGF- β activity in asthmatic cells, but not because of increased cell surface $\alpha v\beta 5$ integrin expression. *A*, Coculture of nonasthmatic HASM cells and TMLCs was stimulated with 20 μ M LPA with or without 10 μ g/ml anti- $\alpha v\beta 5$. Anti- $\alpha v\beta 5$ inhibited LPA-induced TGF- β activation ($p < 0.05$). Data are expressed as mean RLU $\pm$ SEM. Data were replicated three times and a representative example is shown. *B*, Coculture of asthmatic HASM cells and TMLCs was stimulated with 20 μ M LPA with or without 10 μ g/ml anti- $\alpha v\beta 5$. Anti- $\alpha v\beta 5$ inhibited TGF- β activation ($p < 0.01$). Data are expressed as mean RLU $\pm$ SEM. Data were replicated three times and a representative example is shown. *C*, Cell surface expression of integrin $\alpha v\beta 5$ on nonasthmatic HASM cells was assessed by flow cytometry. Data are expressed as mean PE fluorescence intensity. Data were replicated three times and a representative example is shown. *D*, Cell surface expression of integrin $\alpha v\beta 5$ on asthmatic HASM cells assessed by flow cytometry. Data are expressed as mean PE fluorescence intensity. Data were replicated three times and a representative example is shown.

determine whether the increased $\alpha v\beta 5$ integrin-mediated TGF- β activation was due to higher levels of the $\alpha v\beta 5$ integrin on the cell surface of asthmatic HASM cells, $\beta 5$ integrin subunit cell surface expression was measured by flow cytometry and compared with nonasthmatic cells (Fig. 6C, 6D). There was no difference in the mean fluorescent intensity of cell surface $\beta 5$ integrin between asthmatic HASM cells or nonasthmatic HASM cells.

ASM cells express $\alpha v\beta 5$ integrins in vivo and activate TGF- β , which promotes ASM hypertrophy in an OVA model of asthma

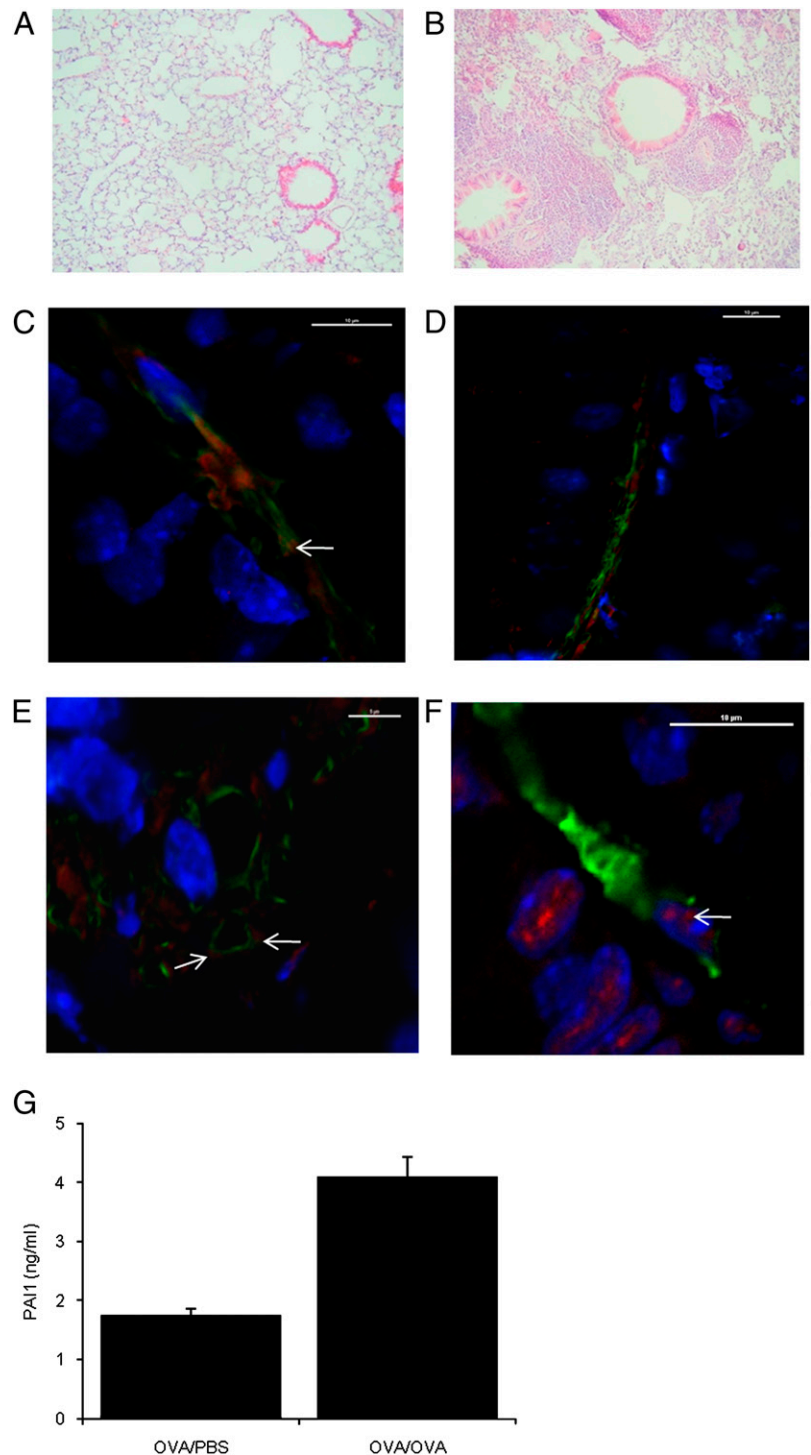
To investigate the role of the $\alpha v\beta 5$ integrin in vivo, we used the OVA model of airway remodeling. Histologic sections from the saline-challenged mice showed normal lung architecture, few inflammatory cells, and an intact epithelial layer (Fig. 7A). However, histologic sections from mice sensitized and challenged with OVA showed widespread airway inflammation and evidence of airway remodeling, such as a thickened ASM layer (Fig. 7B). The ASM layer stained positive for both α SMA and $\alpha v\beta 5$ in saline- and OVA-treated animals, with $\alpha v\beta 5$ integrins localized at the ends of actin fibers at cell-cell contacts and on the cell surface (Fig. 7C, 7D). When smooth muscle bundles from OVA-treated animals were visualized in cross section, it was apparent that ASM cells express $\alpha v\beta 5$ integrin on the external surface of the actin fibers at the cell surface (Fig. 7E). To confirm that ASM cells can activate TGF- β , histologic sections were dual labeled with Abs against α SMA and P-Smad2. ASM cells, as demonstrated by α SMA ex-

pression, showed nuclear expression of P-Smad2 (Fig. 7F, arrow). Global levels of TGF- β activation in the lung were elevated in OVA-treated mice, as estimated by measuring levels of the TGF- β inducible protein PAI1 in murine lung homogenates by sandwich ELISA (Fig. 7G).

To assess the importance of $\alpha v\beta 5$ integrin-mediated TGF- β activation on airway remodeling, an $\alpha v\beta 5$ blocking Ab (ALULA) was administered to mice undergoing OVA-induced airway remodeling and the ASM layer around smaller airways was assessed. There was minimal staining for α SMA around the smaller airways of saline treated mice (Fig. 8A), and the $\alpha v\beta 5$ blocking Ab had no effect in saline-treated animals (Fig. 8B). In sections from mice treated with OVA and an isotype control Ab, there was significant thickening of the ASM layer (Fig. 8C), and this was reduced in animals receiving an $\alpha v\beta 5$ blocking Ab (Fig. 8D). The thickness of the ASM layer was quantified, confirming that mice treated with OVA plus an anti- $\alpha v\beta 5$ Ab had significantly less α SMA staining around the airways compared with animals treated with OVA and an isotype control Ab (Fig. 8E). However, there appeared to be enhanced inflammation on histologic sections from mice with OVA-induced remodeling treated with the $\alpha v\beta 5$ integrin blocking Ab, compared with mice treated with isotype control (Fig. 8G, 8H). Therefore, the inflammatory cell infiltrate was quantified on BAL fluid (BALF). OVA treatment increased the total number of inflammatory cells present in BALF (Fig. 8F), which appeared to be enhanced in animals treated with an anti-

was no significant difference in response between asthmatic and nonasthmatic cells. Data were normalized to housekeeping gene $\beta 2$ -M and expressed as fold increase in fibronectin over time $0 \pm$ SEM; $n = 3$ nonasthmatic and $n = 3$ asthmatic cell lines. *G*, The amount of total TGF- β sequestered extracellularly under basal conditions by nonasthmatic ($n = 3$) and asthmatic ($n = 3$) HASM cells was determined by acid treatment of cells to activate all TGF- β present. TGF- β activity was determined using TMLCs. Asthmatic HASM cells expressed more total TGF- β than did nonasthmatic HASM cells ($p < 0.005$). Data are expressed as mean TGF- β concentration per 10^4 cells $\pm$ SEM.

FIGURE 7. A, H&E stain of a lung section from a saline-treated animal (original magnification $\times 20$). B, H&E stain of lung section from an OVA-treated animal (magnification $\times 20$). C, Expression of $\alpha\text{v}\beta 5$ by murine ASM in saline-treated mice was assessed using an αSMA Ab with an FITC-labeled secondary Ab and an $\alpha\text{v}\beta 5$ Ab with a DyLight649-labeled secondary Ab. Nuclei were stained with DAPI. Images were analyzed by confocal microscopy and imaged through a single plane to confirm the same cell coexpressing $\alpha\text{v}\beta 5$ integrin and smooth muscle cell actin (original magnification $\times 100$). $\alpha\text{v}\beta 5$ integrins are localized at the end of actin fibers (arrow). D, Expression of $\alpha\text{v}\beta 5$ by murine ASM in OVA-treated mice was assessed using an FITC-labeled αSMA Ab and a DyLight649-labeled $\alpha\text{v}\beta 5$ Ab. Nuclei were stained with DAPI (original magnification $\times 100$). E, Expression of $\alpha\text{v}\beta 5$ by murine ASM in OVA-treated mice was assessed using a FITC-labeled αSMA Ab and a DyLight649-labeled $\alpha\text{v}\beta 5$ Ab. Cross-sectional views of smooth muscle cell shows $\alpha\text{v}\beta 5$ staining at the apices of the triangular actin skeleton (arrows). Nuclei were stained with DAPI. F, Activation of TGF- β in vivo in an OVA model of asthma was confirmed by staining histologic sections using an αSMA Ab with an FITC-labeled secondary Ab and a phospho-Smad2 Ab with a DyLight594-labeled secondary Ab. Nuclei were stained with DAPI (original magnification $\times 100$). Costaining of a smooth muscle cell nuclei expressing phospho-Smad2 is indicated by an arrow. Images were analyzed by confocal microscopy and imaged through a single plane to confirm the same cell coexpressing nuclear phospho-Smad2 and αSMA . G, Expression of murine PAI1 protein in lung homogenates from mice challenged with saline ($n = 4$) or OVA ($n = 4$) was determined by sandwich ELISA. PAI1 levels were higher in OVA-challenged mice ($p < 0.05$). Data are expressed as mean PAI1 concentration (ng/ml) $\pm$ SEM.



$\alpha\text{v}\beta 5$ blocking Ab; however, these differences were not statistically significant. There was no difference in the differential cell counts between isotype control and $\alpha\text{v}\beta 5$ Ab treated animals (data not shown).

Itgb5^{-/-} mice develop less allergen-induced ASM thickening

To further elucidate the role of $\alpha\text{v}\beta 5$ in the development of airway remodeling, we used an *A. fumigatus* model of allergic airway remodeling in asthma in *itgb5*^{-/-} mice. Both *itgb5*^{-/-} and control animals, following saline challenge, had minimal αSMA -positive cells surrounding their airways (Fig. 9A, 9B). Treatment of control mice with *A. fumigatus* caused thickening of the ASM layer (Fig. 9C), which was reduced in *A. fumigatus* exposed *itgb5*^{-/-} animals

(Fig. 9D). This reduction in ASM thickness was statistically significant when αSMA staining was quantified (Fig. 9E). Similar to the observation in OVA exposed mice, *A. fumigatus* caused an increase in the total number of inflammatory cells present in control mice ($p < 0.005$), which was further enhanced in *itgb5*^{-/-} mice exposed to *A. fumigatus* ($p < 0.05$).

Discussion

The aims of this study were to investigate whether contraction agonists could induce TGF- β activation in HASM cells and to explore the mechanism of activation and its relevance to asthmatic airway remodeling. We have shown that LPA and methacholine activate TGF- β in HASMs, and we have identified a novel

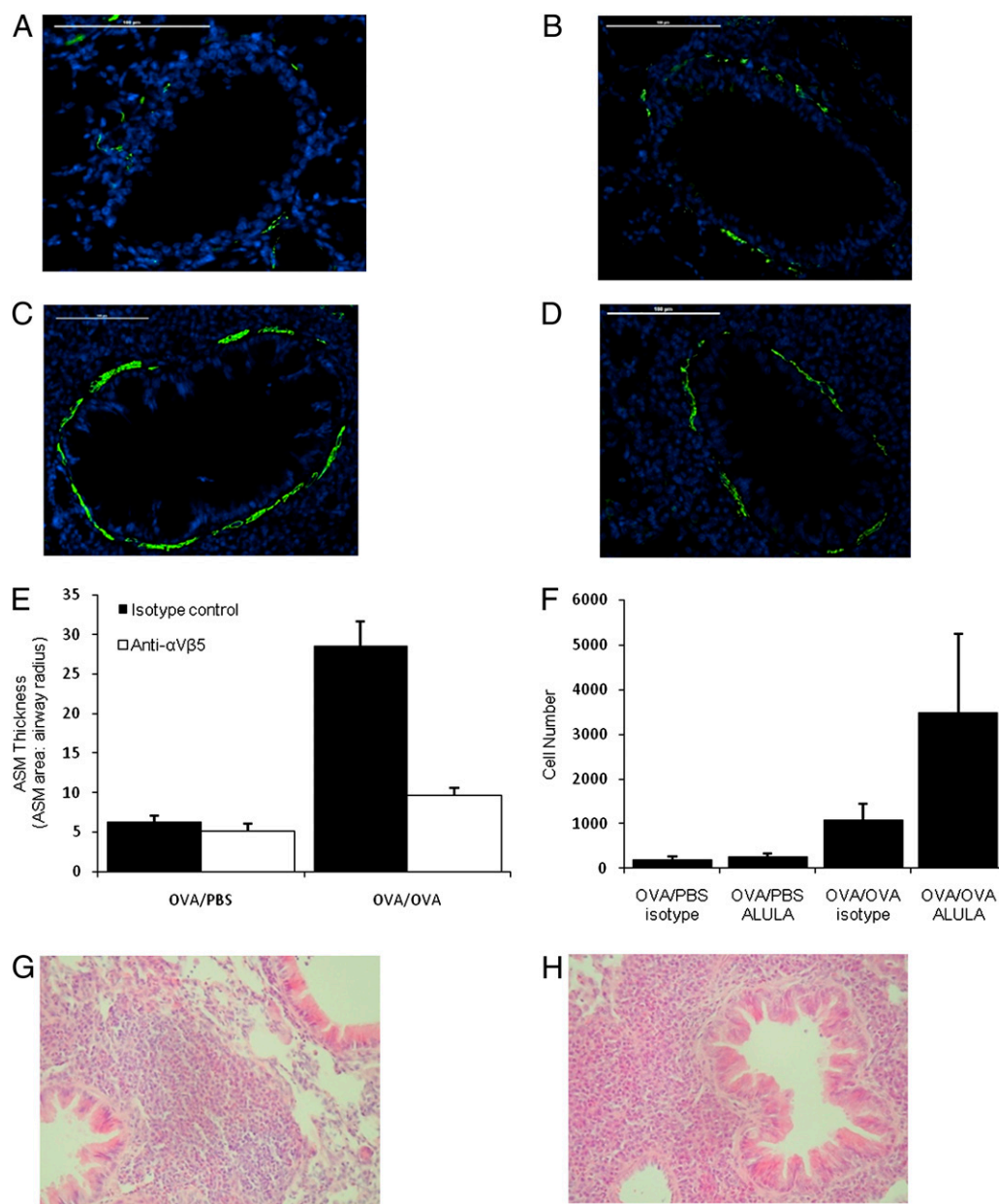


FIGURE 8. A, Histologic sections from mice treated with saline and an isotype control Ab were stained with FITC-labeled anti- α SMA Ab (green) and a DAPI nuclear stain (original magnification $\times 100$). B, Histologic sections from mice treated with saline and an $\alpha\text{v}\beta 5$ Ab were stained with FITC-labeled anti- α SMA Ab (green) and a DAPI nuclear stain (original magnification $\times 100$). C, Histologic sections from mice treated with OVA and an isotype control Ab were stained with FITC-labeled anti- α SMA Ab (green) and a DAPI nuclear stain (original magnification $\times 100$). D, Histologic sections from mice treated with OVA and an $\alpha\text{v}\beta 5$ Ab were stained with FITC-labeled anti- α SMA Ab (green) and a DAPI nuclear stain (original magnification $\times 100$). E, Treatment with an anti- $\alpha\text{v}\beta 5$ Ab reduces OVA-induced increases in ASM thickness. ASM area was quantified by measuring area of α SMA staining around airways using Nikon NIS Elements software. Data were expressed as a mean ratio of ASM area: airway radius $\pm$ SEM. F, The number of inflammatory cells present in BALF cytopins was counted. Data are shown as mean cell numbers $\pm$ SEM. G, Histologic sections from mice treated with OVA and an isotype control Ab were stained with H&E (original magnification $\times 20$). H, Histologic sections from mice treated with OVA and an $\alpha\text{v}\beta 5$ Ab were stained with H&E (original magnification $\times 20$).

mechanism of TGF- β activation in these cells involving the cytoskeleton, talin, and the $\alpha\text{v}\beta 5$ integrin. We have shown that HASM cells isolated from asthmatic patients activated more TGF- β via this pathway than did cells isolated from nonasthmatic individuals. To our knowledge, this is the first time that this mechanism of increased TGF- β activation has been described in cells from patients with asthma. Furthermore, we show that murine ASM cells express the $\alpha\text{v}\beta 5$ integrin *in vivo* and can activate TGF- β in an OVA model of asthmatic airway remodeling. Finally, we show that impaired $\alpha\text{v}\beta 5$ integrin function *in vivo*, through both pharmacologic inhibition and gene deletion, results in re-

duced allergen-induced ASM thickness but increased airway inflammation. These data identify a key mechanism that dissociates inflammation from airway remodeling in asthma and supports the novel hypothesis that recurrent bronchoconstriction might lead to contraction-induced TGF- β activation by ASM cells promoting the development of asthmatic airway remodeling.

To our knowledge, this study is the first to describe $\alpha\text{v}\beta 5$ integrin-mediated TGF- β activation in ASM cells. Activation of TGF- β by the $\alpha\text{v}\beta 5$ integrin occurs in scleroderma fibroblasts promoting the transition of fibroblasts into fibrogenic myofibroblasts (44). Furthermore, myofibroblasts activate TGF- β from

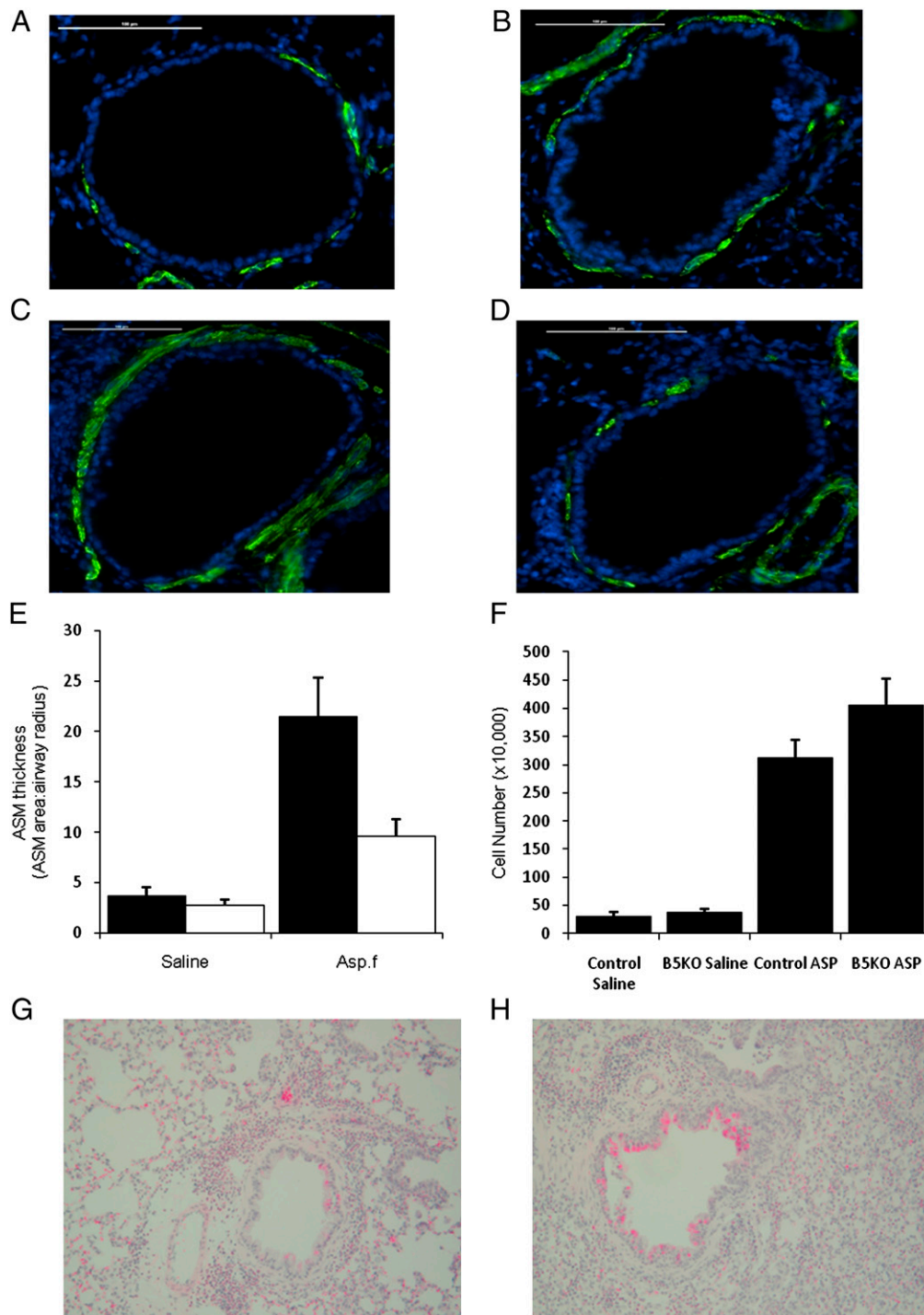


FIGURE 9. *A*, Histologic sections from control mice treated with saline were stained with FITC-labeled anti- α SMA. The sections were counterstained with a DAPI nuclear stain (original magnification $\times 100$). *B*, Histologic sections from *itgb5*^{-/-} mice treated with saline were stained with FITC-labeled anti- α SMA Ab. The sections were counterstained with a DAPI nuclear stain (original magnification $\times 100$). *C*, Histologic sections from control mice treated with *A. fumigatus* were stained with FITC-labeled anti- α SMA Ab. The sections were counterstained with a DAPI nuclear stain (original magnification $\times 100$). *D*, Histologic sections from *itgb5*^{-/-} mice treated with *A. fumigatus* were stained with FITC-labeled anti- α SMA Ab. The sections were counterstained with a DAPI nuclear stain (original magnification $\times 100$). *E*, ASM area was quantified by measuring area of α SMA staining around airways using Nikon NIS Elements software. Data were expressed as a mean ratio of ASM area: airway radius $\pm$ SEM. $\beta 5$ knockout mice had reduced *A. fumigatus*-induced ASM compared with wild type mice ($p < 0.01$). *F*, The number of inflammatory cells present in BALF was quantified. *A. fumigatus* treatment increased the number of inflammatory cells present. BALF from $\beta 5$ knockout mice had more inflammatory cells present compared with BALF from wild type mice ($p < 0.05$). Data are shown as mean cell numbers $\pm$ SEM. *G*, Histologic sections from control mice treated with *A. fumigatus* were stained with H&E (original magnification, $\times 20$). *H*, Histologic sections from *itgb5*^{-/-} mice treated with *A. fumigatus* were stained with H&E (original magnification, $\times 20$).

extracellular stores by transmitting contractile force via the $\alpha\beta 5$ integrin to latent TGF- β , which is inhibited by $\alpha\beta 5$ function-blocking Abs (30). Smooth muscle cells are contractile cells, and

both LPA and methacholine augment ASM contraction via effects on rhoA and rho kinase (45–47). Our data demonstrate that contraction-induced TGF- β activation was inhibited by blocking

the $\alpha\text{v}\beta 5$ integrin, and LPA-induced TGF- β activation was inhibited by the inhibitor of actin assembly (i.e., cytochalasin D) and the $\beta 2$ adrenergic receptor agonist formoterol. These data suggest that contraction agonists induced $\alpha\text{v}\beta 5$ integrin-mediated TGF- β activation via smooth muscle cell contraction. It is possible that formoterol inhibited TGF- β activation via effects on the cAMP pathway independent of its bronchodilator effects, although the effects of cytochalasin D and observations in myofibroblasts (29) would suggest that cytoskeletal changes are central to $\alpha\text{v}\beta 5$ integrin-mediated TGF- β activation.

Further evidence to support the role of the cytoskeleton in $\alpha\text{v}\beta 5$ integrin mediated TGF- β activation comes from data obtained from experiments using constructs encoding wild type and a naturally occurring polymorphism in the $\beta 5$ integrin subunit (43). This polymorphism leads to a 9-bp deletion within the NxxY talin binding region of the $\beta 5$ subunit's cytoplasmic domain and results in the loss of 3 aa (FNK^{767–769}) from the cytoplasmic tail. This polymorphism has not previously been shown to have any functional effect (43), but our data demonstrated that the polymorphism resulted in a $\beta 5$ subunit that was unable to activate TGF- β . Talin is a cytoplasmic protein capable of binding to the cytoplasmic domains of β integrin subunits and is fundamental for inside-out activation of integrins (48). A role for talin and inside-out activation of integrins in integrin-mediated TGF- β activation has been suggested (49). The data presented in this study show that talin was still able to interact with the polymorphic $\beta 5$ subunit; however, the rod domain interactions were aberrant. The functional role of the talin rod domain is largely unclear; however, this would suggest that the rod domain of talin could be central for mediating cytoskeletal forces that promote integrin mediated TGF- β activation.

To our knowledge, these data show for the first time that HASM cells from asthmatic patients activated more TGF- β than did cells from smooth muscle from nonasthmatic individuals in response to both LPA and methacholine stimulation. It is possible that the enhanced TGF- β activity is merely due to the enhanced total TGF- β secreted by HASM cells from asthmatic patients; however, we do not favor this possibility. The level of total TGF- β in HASM cells from nonasthmatics and asthmatics was in considerable excess of levels of active TGF- β following stimulation with LPA, as found in other cell systems (15, 32). The enhanced TGF- β activity detected in asthmatic cells was completely inhibited by blocking the $\alpha\text{v}\beta 5$ integrin, suggesting that the augmented TGF- β activity observed in asthmatic cells was completely dependent on the $\alpha\text{v}\beta 5$ integrin. However, no increase in cell surface expression of $\alpha\text{v}\beta 5$ integrin was found in asthmatic HASM cells compared with nonasthmatic cells. Several studies have suggested that asthmatic HASM cells has intrinsic hypercontractility compared with nonasthmatic HASM cells (50–52). Future work will investigate whether hypercontractility of asthmatic HASM cells is responsible for the enhanced TGF- β activation in response to contraction agonists.

It has previously been shown in in vivo models that TGF- β activity is increased in asthma (20, 53), but the cell type responsible for the increased activation and the mechanism of activation have not been investigated. By assessing lung sections from a murine OVA model of asthma, we have shown that ASM cells express $\alpha\text{v}\beta 5$ and can activate TGF- β in vivo, suggesting that ASM cells are an important source of TGF- β in asthma. A role for TGF- β activating integrins in asthmatic airway remodeling has been suggested because blocking all TGF- β binding integrin function using an RGDS (Arg-Gly-Asp-Ser) peptide can prevent airway remodeling in an OVA asthma model (54), and fibroblast-specific deletion of the *itgb8* integrin also reduces OVA-induced

airway remodeling (26). Whereas deletion of fibroblast *itgb8* reduces TGF- β -induced dendritic cell migration and inflammation leading to reduced airway remodeling (26), our data provide evidence that blockade of smooth muscle cell $\alpha\text{v}\beta 5$ integrins significantly reduces allergen-induced increases in ASM regardless of effects on inflammation. The finding that ASM thickness was not completely reduced to levels comparable with saline-treated animals implies that $\alpha\text{v}\beta 5$ -mediated TGF- β activation is not the sole mechanism of TGF- β activation promoting increased ASM cell mass. Although $\alpha\text{v}\beta 8$ integrin-mediated TGF- β activation contributes to the development of asthmatic airway remodeling via effects on inflammation, its effects on ASM were not investigated (26). Furthermore, the mechanism of TGF- β activation by the $\alpha\text{v}\beta 8$ integrin does not require cytoskeletal integrity, and thus will not be directly affected by bronchoconstriction.

Although it is clear that controlling inflammatory responses are important in asthmatic airway remodeling (26, 55, 56), there is also a positive correlation between airway responsiveness to methacholine and subepithelial fibrosis (57). More recently it has been shown that recurrent bronchoconstriction promotes airway remodeling independent of airway inflammation (4). Furthermore, the muscarinic receptor antagonist tiotropium, which inhibits methacholine-induced contraction, reduces allergen-induced increases in ASM mass (58). The mechanism responsible for bronchoconstriction-induced airway remodeling has not been determined, but our data suggest that bronchoconstriction results in $\alpha\text{v}\beta 5$ integrin-induced TGF- β activation by HASM cells, which then drives structural changes associated with airway remodeling. In addition to reducing allergen-induced increases in ASM mass, blockade or loss of $\alpha\text{v}\beta 5$ appeared to increase airway inflammation, at least in the *Aspergillus* model. The anti-inflammatory effects of TGF- β are well documented (27, 59, 60); therefore, it is possible that inhibiting TGF- β activity on immune and inflammatory cells may exacerbate the inflammation associated with allergen challenge as observed in our studies. Additional studies are required to investigate a possible role for $\alpha\text{v}\beta 5$ in regulating airway inflammation.

These data demonstrate that contraction agonists can lead to TGF- β activation in smooth muscle cells via an $\alpha\text{v}\beta 5$ integrin-mediated pathway in vitro, and that this mechanism is accentuated in asthmatic HASM cells. Furthermore, inhibiting the function of the $\alpha\text{v}\beta 5$ integrin reduces ASM mass in vivo, without reducing inflammation. It is conceivable that this mechanism is responsible for the bronchoconstriction-induced airway remodeling observed by other groups (4, 57). Thus, while global inhibition of $\alpha\text{v}\beta 5$ integrin-mediated TGF- β activation is unlikely to be a useful therapeutic strategy in asthma because of the effects on the inflammatory response, defining cell-specific pathways of $\alpha\text{v}\beta 5$ integrin-mediated TGF- β activation, through careful in vitro cell biology, may aid the development of novel therapies for airway remodeling in patients with poorly controlled disease.

Acknowledgments

We thank Prof. E. Kawahara for providing the $\beta 5$ constructs and Prof. Daniel Rifkin for supplying the TMLC reporter cells, Katherine Huang for assistance with the *A. fumigatus* in vivo model, Amha Atakilit for producing and supplying the $\alpha\text{v}\beta 5$ blocking Ab for the in vivo studies, the nurses at Glenfield Hospital, Leicester and City Hospital, Nottingham for subject recruitment, and Ruth Saunders for isolation and characterization of primary HASM cells.

Disclosures

G.J. has a research agreement with GlaxoSmithKline to study pulmonary fibrosis and D.S. has a sponsored research agreement with Stromedix. The other authors have no financial conflicts of interest.

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