Down-regulation of Caveolin-1, an Inhibitor of Transforming Growth Factor-β Signaling, in Acute Allergen-induced Airway Remodeling*

Claude Jourdan Le Saux, Kelsa Teeters, Shelley K. Miyasato, Peter R. Hoffmann, Oana Bollt, Vanessa Douet, Ralph V. Shohet, David H. Broide, and Elizabeth K. Tam

From the Departments of Cell and Molecular Biology and Medicine, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii 96813 and the Department of Medicine, University of California, San Diego, California 92093

Asthma can progress to subepithelial airway fibrosis, mediated in large part by transforming growth factor-β (TGF-β). The scaffolding protein caveolin-1 (cav1) can inhibit the activity of TGF-β, perhaps by forming membrane invaginations that enfold TGF-β receptors. The study goals were 1) to evaluate how allergen challenge affects lung expression of cav1 and the density of caveolae in vivo 2) to determine whether reduced cav1 expression is mediated by interleukin (IL)-4 and 3) to measure the effects of decreased expression of cav1 on TGF-β signaling. C57BL/6J, IL-4-deficient mice, and cav1-deficient mice, sensitized by intraperitoneal injections of phosphate-buffered saline or ovalbumin (OVA) at days 0 and 12, received intranasal phosphate-buffered saline or OVA challenges at days 24, 26, and 28. Additionally, another group of C57BL/6J mice received IL-4 by intratracheal instillation for 7 days. We confirmed that the OVA-allergen challenge increased eosinophilia and T-helper type 2-related cytokine levels (IL-4, IL-5, and IL-13) in bronchoalveolar lavage. Allergen challenge reduced lung cav1 mRNA abundance by 40%, cav1 protein by 30%, and the number of lung fibroblast caveolae by 50%. Administration of IL-4 in vivo also substantially decreased cav1 expression. In contrast, the allergen challenge did not decrease cav1 expression in IL-4-deficient mice. The reduced expression of cav1 was associated with activation of TGF-β signaling that was further enhanced in OVA-sensitized and challenged cav1-deficient mice. This study demonstrates a previously unknown modulation of TGF-β signaling by IL-4, via cav1, suggesting novel therapeutic targets for controlling the effects of TGF-β and thereby ameliorating pathological airway remodeling.

Asthma is often associated with structural changes in the bronchioles, commonly referred to as airway remodeling (1). These alterations result from injury and repair processes regulated by several mediators, such as T-helper type 2 (Th2) cytokines and transforming growth factor β (TGF-β). Cytokines modulate subepithelial fibrosis by regulating fibroblast proliferation and differentiation and by stimulating synthesis and stabilization of the proteins that constitute the extracellular matrix. In vitro, Th2-related cytokines regulate the secretion of connective tissue proteins, including collagens, fibronectin, and tenascin, all of which are involved in the thickening of the airway basement membrane (2–7). Interleukin-4 (IL-4) and IL-13 levels are notably increased in bronchoalveolar lavage (BAL) fluid of asthmatic patients (3, 8). Inhibitors of IL-4 and IL-13 prevent the development of fibrosis (9, 10). IL-4- and IL-13-deficient mice develop less subepithelial fibrosis and goblet cell hyperplasia after allergen challenge, compared with their normal counterparts (11, 12), indicating the importance of IL-4 and IL-13 in driving airway remodeling.

TGF-β, one of the most potent regulators of inflammation and connective tissue synthesis, plays an integral role in the development of airway remodeling ranging from fibroblast differentiation to increased deposition of connective tissue (13–15). Elevated levels of TGF-β and its signaling activity are associated with the development of airway remodeling during asthma and correlate with the thickening of the basement membrane (16–18). Previous work has shown that TGF-β effects are mediated via the Smad family of proteins (18–22). Upon ligand binding, TGF-β receptor activation leads to phosphorylation of Smad2 and Smad3. Phosphorylated Smad2 and Smad3 (p-Smad2 and p-Smad3) form a hetero-oligomeric complex with Smad4. The Smad complex then translocates to the nucleus where, together with DNA-binding cofactors, it regulates the transcription of target genes (21, 23).

TGF-β-mediated phosphorylation of Smad2 can be prevented by caveolin-1 (cav1), one of three members of the caveolin protein family (24). Cav1 plays a crucial role in the formation of caveolae, 50–100-nm wide omega-shaped plasma membrane invaginations that are found in fibroblasts, muscle cells, capillary endothelium, and type I pneumocytes (25). Within caveolae, cav1 modulates many signaling proteins, including those in the TGF-β pathway (26). The scaffolding domain (residues 82–101) of cav1 confers this regulation by binding and releasing these proteins in a controlled fashion (26).
We hypothesized that the effects of cytokines, released during allergic inflammation, may regulate the expression and function of cav1 and thereby indirectly affect the signaling and pathological effects of TGF-\(\beta\). We used a mouse model of airway inflammation to establish that allergic modulation of cav1 expression is mediated by IL-4. Intratrahecal instillation of IL-4 results in cav1 down-regulation in contrast to no change in cav1 expression in allergen-challenged IL-4-deficient mice. We then showed that TGF-\(\beta\) signaling is exaggerated by this IL-4-activating allergen challenge in cav1-deficient mice compared with wild-type mice.

**MATERIALS AND METHODS**

**Mice**—C57Bl/6J, C57Bl/6-IL-4tm1Nmt/J (IL-4-deficient), C.129S2-Stat6tm1Gru/J (STAT6-deficient), and Cavtm1Mls/J (cav1-deficient) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The genetic background for the cav1 knock-out mice is a mixture of C57Bl/6J and 129S6/SvEv. The resulting chimeric animals were then crossed to C57Bl/6J mice for six generations. The animal experiments were conducted using a protocol approved by the Institutional Animal Care and Use Committee of the University of Hawaii.

**Cell Isolation and Culture**—Mouse lung fibroblasts were extracted from C57Bl/6J, Cavtm1Mls/J, and C.129S2-Stat6tm1Gru/J mice. Lung tissues were digested with 0.0025% Trypsin (Invitrogen) and 1 mg/ml collagenase type I (Invitrogen) in Dulbecco’s modified Eagle’s medium with high glucose, \(\gamma\)-glutamine, and pyridoxine hydrochloride (Invitrogen) and incubated with stirring in 5% \(CO_2\) at 37\(^\circ\)C for 30 min. The digestion medium was removed, and the cells were placed into 20 ml of Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. The digestion was repeated on the remaining tissue for a total of three digestion cycles. The cell suspension was filtered through one thickness of sterile gauze and centrifuged at 500 \(\times\) \(g\) for 10 min. The cells were resuspended in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, counted with a hemocytometer, and plated at 50,000 cells/cm\(^2\).

Lung fibroblasts were used at confluence and between passages 3 and 5. The cells were treated for 48 h with 10 ng/ml of recombinant IL-4 (Chemicon International, Temecula, CA) and/or 2 ng/ml of TGF-\(\beta\) (Sigma) or anti IL-4 antibody (catalog number AF-204NA; R & D Systems, Minneapolis, MN). IL-4 and IL-5 levels were measured in BAL fluid using mouse Th1/Th2 Cytokine Kit Cytometric Bead Array \(^\text{TM}\) (BD Biosciences PharMingen) according to the manufacturer’s instructions. IL-13 levels were measured using IL-13 Quantikine ELISA \(^\text{TM}\) (R & D Systems, Minneapolis, MN). TGF-\(\beta\) content of BAL fluid was measured using mouse/rat/porcine TGF-\(\beta\) Quantikine ELISA \(^\text{TM}\) (R & D Systems catalog number MB100). Each sample was directly measured for active TGF-\(\beta\). In addition, each sample was treated with acetic acid/urea and NaOH/HEPES according to the manufacturer’s protocol, to activate latent TGF-\(\beta\) to the immunoreactive form, allowing measurement of the total amount of TGF-\(\beta\)1 in the samples.

**Gene and Protein Expression**—Total RNA was extracted from cell or tissue samples using the RNeasy \(^\text{TM}\) kit (Qiagen). RNA samples were treated with 0.05 unit/ml of DNase I (Qiagen) at 20\(^\circ\)C for 15 min. Total RNA (5 \(ug\)) was converted into first strand cDNA using random hexamers (SuperScript First-Strand Synthesis System for RT-PCR \(^\text{TM}\); Invitrogen). The level of expression of cav1 was detected by semi-quantitative real time PCR using commercially available cav1 primers (Applied Biosystems, Foster City, CA) and the TaqMan system (Applied Biosystems). Total protein was extracted by homogenizing 0.5 g of frozen lung tissues on ice in 10 ml of CellLytic MT buffer (Calbiochem, San Diego, CA), and 5 mM EDTA. Homogenates were centrifuged at 16,000 \(g\) at 20\(^\circ\)C for 10 min, cooled on ice, and loaded into wells of a 10% polyacrylamide gel (Bio-Rad). The protein was transferred to polyvinylidene difluoride membrane and blotted with primary antibodies including anti-cav1 (RDI-caveol1abm, Fitzgerald, Concord, MA), anti-\(\beta\)-actin (Sigma, St. Louis, MO), and anti-p-Smad2 (3104; Cell Signaling, Danvers, MA). Appropriate ECL-peroxidase-linked secondary antibodies were detected using ECL Plus (Amersham Biosciences). For densitometry, digital images of autoradiographic film were captured using Gel Logic 200 and Kodak MI software (Kodak Scientific Imaging Systems, Rochester, NY). This software was used to measure the mean intensity from regions of interest corresponding to bands (e.g. cav1) to be measured. The inten-
Enhancement of TGF-β Signaling Mediated by IL-4

sity of the target bands was normalized to that of the β-actin band to obtain a relative level of proteins of interest on an unsaturated exposure.

**Electron Microscopy**—Right middle lobe lung tissue or cell culture samples were fixed by immersion in 4% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.35. The fixed samples were then washed in 0.1 M cacodylate buffer, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in a graded ethanol series, and embedded in LX-112 epoxy resin. Ultrathin sections (70–80 nm) were collected on copper grids, stained with uranyl acetate and lead citrate, viewed, and digitally photographed on a LEO912 EFTEM at 100 kV. For each sample, at least 70 sections were counted by investigators blinded to the identity of the sections, and the number of caveolae was reported per nm of cell membrane (Image software, rsb.info.nih.gov/ij).

**Chromatin Immunoprecipitation and Electrophoretic Mobility Shift Assay**—Gene expression is often regulated by IL-4 through a STAT6-dependent mechanism. To demonstrate that the STAT-6 binding element of the cav1 promoter binds STAT6 in vivo, we used chromatin immunoprecipitation according to a standard protocol (29) with modifications. Mouse lung tissues (25 mg) were incubated in 1% formaldehyde for 15 min at room temperature. Fixation was stopped by dilution with glycerine (0.125 M). The cells were washed with cold PBS and incubated in 0.6% Nonidet P-40, 10 mM KCl, 10 mM HEPES, pH 7.9, 10 mM EDTA, and protease inhibitor mixture for 15 min at 4 °C. The homogenate was centrifuged for 10 min at 1,500 rpm, and the pellets were incubated at 4 °C for 15 min in 50 mM HEPES, 2.5% glycerol, 410 mM NaCl, 0.03 mM MgCl₂, 0.2 mM EDTA, and protease inhibitor mixture. The pellets were resuspended in chromatin immunoprecipitation sonication buffer (1% Triton X-100, 0.1% deoxycholate, 50 mM Tris, 150 mM NaCl, 5 mM EDTA, protease inhibitor) and incubated at 4 °C for 10 min. The soluble chromatin was sonicated (10–15 pulses at 5-s/pulse) to shear the genomic DNA. Immunoprecipitations were performed with anti-p-STAT6 antibody (Upstate, Temecula, CA) or with normal rabbit IgG (noAb). Cross-linking was reversed in the immunoprecipitated complexes by incubation in 200 mM NaCl at 65 °C for 12 h with proteinase K treatment (150 μg/ml). PCR was performed on the immunoprecipitated complexes for the detection of the cav1 promoter using forward primer 5'-TTCTACGTTTTCCCTAAAGACAGAATCTCAA-3' and reverse primer 5'-TTAGGATCTGGTCTAGGGAAAACGTAGAA-3' and STAT6 consensus binding site as previously described (30) 5'-GATCTTTCTTTAGAAC-3', and 5'-GATCGTCTCATAGAAA-3'.

**TGF-β Signaling Pathway Activation**—The activation of the TGF-β pathway was evaluated by measuring the level of expression of p-Smad2 (Western blot) in vivo and by measuring the level of expression of the luciferase gene reporter under the regulation of Smad response element in vitro (plasmid provided by Dr. Aristidis Moustakas, Ludwig Institute for Cancer Research, Uppsala, Sweden).

Mouse fibroblasts were extracted from lung tissue and were maintained in Dulbecco’s modified Eagle’s medium, with 10% fetal bovine serum. Transient transfections were performed using the GeneJammer transfection reagent (Stratagene, La Jolla, CA) as described by the manufacturer. The cells were harvested for luciferase activity assays after a 24-h incubation period. For luciferase activity assays, a Turner Designs Lumimunometer model TD-20/20 genetic reporter system (Sunnyvale, CA) was used.

**Evaluation of Fibrosis**—Lung tissues were fixed in 4% paraformaldehyde solution, embedded in paraffin, and stained with trichrome stain. All of the samples were processed as a batch under identical conditions. Scoring was performed by investigators blinded to the coding of the samples. Two grading scales were established prior to the observation of the lung sections under 200× power. The grading scale for trichrome stain observed around the airways and blood vessels was as follows: 1 indicates a marginal peribronchial and perivascular blue trichrome stain; 2 indicates a slight increase in peribronchial and/or perivascular trichrome stain; 3 indicates an increase in trichrome stain in all airways and all vessels; and 4 indicates dramatically increased stain in all airways and vessels. We used the following grading scale for parenchymal fibrosis: 0 indicates no to marginal stain; 1 indicates slight and consistent increase; 2 indicates increased, uniform stain throughout the parenchyma; and 3 indicates, dramatic increase. The final score for each sample reflected the grading using both scales.

**Statistical Analysis**—The results were expressed as the means ± S.D. A two-tailed Student’s t test was used to determine statistical significance between compared groups. A p value <0.05 was considered significant. Data entry, management, and statistical analysis were performed using Prism software (GraphPad Software, San Diego, CA).

**RESULTS**

To determine whether cav1 participates in airway remodeling based on its potential interaction with the TGF-β signaling pathway, we studied cav1 expression and caveolae in the well established OVA-allergen challenge model (31).

**OVA-Allergen Challenge Causes an Inflammatory Response Associated with Reduced Expression of cav1**—We confirmed the expected pulmonary inflammation in the OVA-sensitized and challenged mice in our experiments by comparing various amounts of unlabeled competitor oligonucleotide on ice for 20 min before the radiolabeled probe was added. The following competitors were used in these experiments: Putative STAT6 binding site on cav1 promoter, 5'-TTCTACGTTTTCCCTAAAGACAGAATCTCAA-3' and 5'-TTAGGATCTGGTCTAGGGAAAACGTAGAA-3' and STAT6 consensus binding site as previously described (30) 5'-GATCTTTCTTTAGAAC-3', and 5'-GATCGTCTCATAGAAA-3'.
inflammatory parameters in these samples to PBS-treated control mice. After allergen exposure, BAL cell count increased 4-fold (significantly different from baseline, \( p < 0.01 \)). One-week of OVA-allergen challenge caused a BAL eosinophilia (\( p < 0.001, n \geq 10 \); Table 1). In addition, IL-4, IL-5, and IL-13 levels were increased in BAL fluid from OVA-sensitized and challenged mice, \( (p < 0.001, n \geq 10 \); Table 1).

We then investigated the expression of cav1 and formation of caveolae. As determined by qPCR, the amount of cav1 mRNA in the lung of OVA-sensitized and challenged animals was reduced by 40% compared with PBS-sensitized and challenged mice \( (p < 0.001) \). Western blot assays showed a decrease in cav1 protein (30% reduction, \( p < 0.05 \)), which corresponded to a decreased number of caveolae, as observed by transmission electron microscopy (50% reduction, \( p < 0.05 \)) (Fig. 1).

In addition, a group of eight C57 females was sensitized with OVA and challenged with PBS, as described under “Materials and Methods.” The inflammatory response (BAL eosinophilia, TH2-related cytokine levels) and the level of cav1 expression were similar to those measured in PBS-sensitized and challenged mice. Therefore, we did not further include these OVA-sensitized, PBS-challenged mice in our analysis.

**IL-4 Down-regulates the Expression of cav1** — A 2,600-bp promoter region of the murine cav1 gene was analyzed with the TRANSFAC and MATINSPECTOR transcription factor data bases (www.genomatrix.de and transfac.gdb.de). The *in silico* analysis revealed several STAT family binding sites (STAT3 at \(-2,351 \) to \(-2,333 \) bp, STAT6 at \(-1,880 \) to \(-1,861 \) bp, and STAT5 at \(-1,221 \) to \(-1,202 \) bp). Both STAT3 and STAT5 are activated by a wide variety of cytokines, growth factors, and other stimuli. STAT6 is the primary signal transducer specifically activated in response to IL-4 or IL-13 stimulation (32).

We first analyzed whether cav1 expression was mediated by IL-4 or IL-13. Lung fibroblasts were treated with IL-4, IL-13, and anti-IL-4 receptor \( \alpha \) antibody. *In vitro* studies indicated that cav1 gene was IL-4-mediated and not IL-13-mediated (Fig. 2, A and B). We then investigated the *in vivo* regulation of cav1 gene expression in C57 mice receiving intratracheal IL-4 and in allergen-challenged IL-4-deficient mice. After seven daily inoculations, we measured an expected increase in total cell count \((3.8 \pm 2 \times 10^8 \text{ versus } 0.5 \pm 0 \times 10^3 \text{ cell/ml})\), eosinophils, IL-4, IL-13, and TGF-\( \beta \) level in BAL fluid of IL-4-treated mice, compared with PBS-treated animals (Table 1). Cav1 mRNA and cav1 protein levels were reduced by 40% in the lungs of mice treated with intratracheal instillation of IL-4, compared with control mice \( (n = 6) \) (Fig. 1). By contrast, the abundance of cav1 mRNA was not diminished in OVA-sensitized and challenged IL-4-deficient mice compared with PBS-sensitized and challenged mice \( (n = 3 \times 6) \) (Fig. 1). As expected IL-4-deficient mice did not develop a strong immune response after allergen challenge (Table 1).

Given the known association between STAT6-dependent pathways and IL-4, we sought to determine whether STAT6 binds to the cav1 promoter in *vivo*, as a possible mechanism for IL-4 regulation of cav1 transcription. Chromatin immunoprecipitation was performed with anti-phospho-STAT6 antibody (anti-p-STAT6) on chromatin fragments extracted from mouse lungs that had been previously stimulated with IL-4, PBS, or OVA. DNA precipitated with anti-p-STAT6 yielded PCR-amplified cav1 promoter fragments (Fig. 2C). Anti-p-STAT6 immunoprecipitated chromatin from lungs of STAT6-deficient mice did not reveal PCR amplification of the cav1 promoter. 3.4- and 24-fold enrichment of cav1 promoter fragment was measured by qPCR in the IL-4-stimulated and in OVA-sensitized and challenged samples, respectively, compared with PBS-control lung tissues. STAT6 specifically bound to the cav1 promoter in *vivo* in response to IL-4 treatment and OVA-allergen challenge. To confirm binding of STAT6 to the cav1 promoter, electrophoretic mobility shift assay experiments were conducted. Incubation with nuclear extracts derived from OVA-challenged C57 lungs generated a band representing delayed migration of the labeled promoter fragment of the cav1 gene that contains the putative STAT6-binding site. Binding to this fragment was competed away by pretreatment of nuclear lysate with double-stranded oligonucleotides representing the putative STAT6 binding site on the cav1 promoter, as well as a canonical consensus STAT6-binding site (data not shown). To demonstrate the applicability of these binding studies in cell culture, we took advantage of a STAT6-deficient mouse strain. Lung fibroblasts were isolated from these animals, and their response to IL-4 stimulation was compared with fibroblasts from normal mice. In the absence of STAT6 transcription factor, cav1 gene expression was not down-regulated in IL-4-stimulated STAT6-deficient lung fibroblasts (Fig. 2D). These data suggest that the negative regulation of cav1 in allergen-simulated lungs is IL-4-dependent and most likely via STAT6-mediated signaling.

**TGF-\( \beta \) SignalingActivation Is Associated with Reduced cav1 Expression in Allergen-challenged Mice** — TGF-\( \beta \) was detected at low levels in BAL fluid of PBS-sensitized and challenged mice.
Enhancement of TGF-β Signaling Mediated by IL-4

FIGURE 1. Caveolin-1 expression in whole lung. cav1 gene expression in OVA-sensitized and challenged mice (OVA-1w, black bar) was decreased, compared with PBS-sensitized and challenged mice (PBS-1w, white bar). The bar represents the mean ± S.D. *p < 0.05; **p < 0.01. n = 7 PBS-sensitized and challenged mice and 10 OVA-sensitized and challenged mice. cav1 protein levels were reduced, resulting in fewer caveolae on the surface of fibroblasts. A, cav1 gene expression. Gene expression was assessed by TaqMan assay and normalized to 18S, β, level of cav1 protein expression. In the inset, a representative WB gel of the expression of cav1 and β-actin in PBS- and OVA-sensitized and challenged C57. C, caveolae formation. Representative electron micrograph of fibroblasts in lung tissue. The arrows indicate the plasma membrane; arrowheads show numerous caveolae on the plasma membrane of PBS-sensitized and challenged in contrast to the smoother appearance of plasma membrane of fibroblasts from OVA-sensitized and challenged animals. D, cav1 gene and protein expression in PBS- (white spotted bar) and IL4- (black spotted bar) intratracheally inoculated C57 mice, and in PBS-sensitized and challenged IL4-deficient mice (PBS IL4−/−, light gray bar) and OVA-sensitized and challenged IL4-deficient mice (OVA IL4−/−, dark gray bar).

but was markedly increased in OVA-sensitized and challenged mice (Table 1; p < 0.01).

Smad2 is a downstream effector for TGF-β and provides an indication of the extent to which TGF-β signaling is activated. Smad2 activation is recognized by its carboxy-terminal phosphorylation. Therefore, we investigated the effect of OVA challenge on TGF-β activation by measuring the level of p-Smad2 in lung tissues by Western blot assays. Although no difference was detected in total lung Smad2, a substantial increase in the level of p-Smad2 was identified in the lungs of OVA-sensitized and challenged mice, indicating activation of the TGF-β signaling pathway (Fig. 3A). Similarly, the p-Smad2 level was increased in mice receiving intratracheal IL-4 but not in allergen-challenged IL-4-deficient mice.

These results suggested an association between the down-regulation of cav1 expression and the activation of TGF-β signaling. We next tested whether the absence of cav1 would result in enhanced TGF-β signaling activity. To test this hypothesis, cav1-deficient mice were PBS- or OVA-sensitized and challenged.

Enhanced TGF-β Signaling in OVA-Allergen-challenged cav1-deficient Mice—Following allergen exposure, the inflammatory responses of cav1Δ mice were similar to those in C57 mice (wild-type controls). One week of OVA-allergen challenge caused a 4-fold increase in BAL total cell count, and BAL eosinophilia compared with that seen in PBS control group (p < 0.001) (Table 1). In addition, BAL IL-4, IL-5, IL-13, and TGF-β levels were increased in OVA-sensitized challenged animals compared with the PBS control group (p < 0.005, n = 10) (Table 1).

By contrast, OVA sensitization and challenge produced an increase in the level of p-Smad2 in cav1Δ mice as compared with C57 (Fig. 3B). The level of unphosphorylated Smad2 was similar between the two groups of animals (Fig. 3). These results indicated that in the absence of cav1 expression, TGF-β signaling was enhanced in OVA-sensitized and challenged mice.

In addition, the activity of transfected reporter constructs, containing TGF-β-inducible Smad response elements, was more pronounced in the cav1Δ lung fibroblasts regardless of the in vitro TGF-β ± IL-4 treatment. Interestingly, in the TGF-β + IL-4 C57 lung fibroblasts, the activity was enhanced compared with TGF-β treatment only (Fig. 3C). These data confirmed that in cav1Δ or with reduced expression (C57 lung fibroblasts treated with IL-4), TGF-β signaling was enhanced, resulting in an increase in TGF-β-Smad-dependent gene expression.

To define whether enhanced activation of TGF-β signaling results in enhanced TGF-β-induced lung fibrosis, we scored the
enhanced activation of the TGF-β signaling pathway compared with C57 mice, resulting in a more pronounced collagen deposition.

Most studies on the transcriptional regulation of cav gene have been related to their role in cholesterol trafficking or cancer (33–36). However, in lung epithelial cells, ETS1, PEA3, and ERM binding factors play critical roles in cav1 expression (39). cav1 is down-regulated in lungs of mice with pulmonary hypertension, associated with increased activation of STAT3 and cyclins D1 and D3 (38). cav1α mice also present increased activation of STAT3 and cyclins D1 and D3 (38). Regulation of the cav1 gene in inflammatory processes has not been previously explored. A Th2 cytokine production is characteristic of the inflammatory response in fibrotic response on trichrome-stained lung tissue sections in PBS- and OVA-challenged C57 and cav1Δ mice. As expected, we saw an increase in the total fibrotic score in OVA-challenged compared with PBS-challenged mice for both strains (p = 0.01, n = 5–9). Importantly, OVA-challenged cav1Δ mice presented a substantially greater increase than did the OVA-challenged C57 mice (p = 0.05, n = 5–9) (Fig. 4). Interestingly, the increased collagen deposition was observed around the vessels and in the parenchyma as well as the airways.

DISCUSSION

We hypothesized that cav1 would be down-regulated and associated with increased TGF-β signaling in an IL-4-driven mouse model of acute pulmonary allergen challenge. We confirmed that increased levels of Th2 cytokines, specifically IL-4, were associated with decreased expression of cav1 and activation of TGF-β signaling. The decreased expression of cav1 was not identified in allergen-challenged IL-4-deficient mice. Moreover, administration of IL-4 resulted in a down-regulation of cav1 expression, likely mediated by STAT6 binding on the cav1 promoter. We confirmed in vivo that cav1 down-regulation was IL-4-mediated. In addition, OVA-sensitized and challenged cav1Δ mice presented an enhanced activation of the TGF-β signaling pathway compared with C57 mice, resulting in a more pronounced collagen deposition.

In a previous study, the minimal region for transcriptional regulation of cav1 in mouse fibroblasts is located within the first few kilobases upstream of the initiation translation codon (37). In human fibroblasts, the cav1 promoter is regulated by Sp1, p53, and E2F/DP-1, located between −395 and −139 bp (35, 38). However, in lung epithelial cells, ETS1, PEA3, and ERM binding factors play critical roles in cav1 expression (39). cav1 is down-regulated in lungs of mice with pulmonary hypertension, associated with increased activation of STAT3 and cyclins D1 and D3 (38). cav1α mice also present increased activation of STAT3 and cyclins D1 and D3 (38). Regulation of the cav1 gene in inflammatory processes has not been previously explored. A Th2 cytokine production is characteristic of the inflammatory response in the OVA-allergen-challenged mouse model. In this context, cav1 mRNA and protein abundance are reduced, suggesting that the transcription of cav1 could be mediated by Th2 cytokines. Analysis of the cav1 promoter region revealed several putative transcription binding elements associated with cytokine regulation, including motifs previously shown to bind STAT1, STAT3, and STAT6. The STAT6 transcription factor is specifically regulated by IL-4/IL-13, whereas STAT1 and STAT3 respond to a wide spectrum of cytokines. Although IL-4 and IL-13 signal through common receptors and transducers, IL-4, but not IL-13, induces the down-regulation of cav1 as shown in our in vitro experiments. Previous studies of allergen-induced asthma mouse models have also demonstrated distinct roles for these two cytokines (40). Human lung fibroblasts express IL-4 and IL-13 receptor subunits. The expression of these receptors may be altered in pathological conditions, thereby contributing to their differential effects (41). IL-4 exerts its biological activity by binding to the IL-4 receptor.
complex and activating two separate signal transduction pathways, the insulin receptor substrate and STAT6. The insulin receptor substrate pathway is important for activating IL-4-mediated mitogenic signals, whereas IL-4 transcriptional responses are associated with STAT6 (32). Interestingly, the cav1 promoter contains a STAT6-binding site that we demonstrated to be functional in vivo. The binding of STAT6 to that precise binding site was also confirmed by electrophoretic mobility...
Enhancement of TGF-β Signaling Mediated by IL-4

The regulation of the TGF-β signaling pathway by cav1 has been demonstrated in various fibrotic disorders. More recently, reduced cav1 expression was described in association with increased TGF-β activity in interstitial pulmonary fibrosis (49). Interstitial pulmonary fibrosis is a persistent inflammation associated with fibrosis. Severe asthmatic patients also have persistent inflammation associated with subepithelial fibrosis. However, the regulation of cav1 in interstitial pulmonary fibrosis has not yet been associated with pro-inflammatory mediators as shown in this study. In children with a renal syndrome of tubulo-interstitial obstruction/fibrosis, increased levels of TGF-β and decreased expression of cav1 have also been shown (50). In vitro studies have demonstrated that in renal fibrosis, IL-6 was involved in the enhanced TGF-β signaling pathway but not in the transcriptional regulation of cav1 expression (51).

Cav1 is one of the main structural and functional proteins of caveolae, which serve to compartmentalize and integrate a wide range of signal transduction processes as well as to transport small molecules (42–47). In this well established model of pulmonary inflammation, we have shown elevated levels of total TGF-β in BAL fluid of the OVA-sensitized and challenged mice. We also demonstrated the activation of TGF-β signaling (increased ratio p-Smad2/Smad2. The level of expression of p-Smad2 in OVA-challenged cav1Δ mice was greater than wild-type mice for comparable levels of BAL TGF-β. Moreover, the TGF-β activation of Smad was more pronounced in cav1Δ lung fibroblasts or IL-4-treated lung fibroblasts, suggesting a new pathway by which TGF-β Smad-dependent gene expression is increased in these cells. TGF-β signaling after allergen challenge was further increased in cav1Δ mice. Thus, cav1 plays an important role in the increased rate of TGF-β-dependent remodeling in the allergic lung.

TGF-β is one of the most potent regulators of inflammation, and this growth factor plays an important role in promoting airway remodeling (14). Anti-TGF-β antibody treatment prevents the progression of airway remodeling following allergen challenge in mice without affecting established airway inflammation and Th2 cytokine production (48). TGF-β levels are elevated in BAL fluid of asthmatic patients and further increased after exposure to allergen (3, 16). Both expression levels of TGF-β and signaling activity are associated with the development of airway remodeling in asthma and correlate with the thickening of the basement membrane (16–18). TGF-β activity, however, is usually associated with the chronic stage of asthma. In this study, we demonstrated that an increased level of TGF-β was detectable in an acute allergen challenge model.

The regulation of the TGF-β signaling pathway by cav1 has been demonstrated in various fibrotic disorders. More recently, reduced cav1 expression was described in association with increased TGF-β activity in interstitial pulmonary fibrosis (49). Interstitial pulmonary fibrosis is a persistent inflammation associated with fibrosis. Severe asthmatic patients also have persistent inflammation associated with subepithelial fibrosis. However, the regulation of cav1 in interstitial pulmonary fibrosis has not yet been associated with pro-inflammatory mediators as shown in this study. In children with a renal syndrome of tubulo-interstitial obstruction/fibrosis, increased levels of TGF-β and decreased expression of cav1 have also been shown (50). In vitro studies have demonstrated that in renal fibrosis, IL-6 was involved in the enhanced TGF-β signaling pathway but not in the transcriptional regulation of cav1 expression (51). Indeed, Zhang and colleagues (51) indicated that the localization of TGF-β receptors in the presence of IL-6 was within the clathrin vesicles promoting TGF-β signaling. In the absence of IL-6, TGF-β receptors were associated within cav1-positive vesicles inhibiting TGF-β signaling.
Enhancement of TGF-β Signaling Mediated by IL-4

Our data support the hypothesis that IL-4 reduces the expression of cav1, a TGF-β inhibitor. Limiting pro-fibrotic effects of TGF-β by modulating caveolin may provide a therapeutic benefit in diseases such as asthma. Understanding the regulation of caveolae and its physiological consequences is an important step toward ameliorating airway remodeling and fibrosis.

Acknowledgments—We thank Dr. Stephen Wasserman for helpful discussion and scientific advice. We are also grateful to Dr. Fukun Hoffmann, Dr. Zoia Stoytcheva, and Joyce Pike for technical assistance.

REFERENCES

1. Bousquet, J., Jeffery, P. K., Busse, W. W., Johnson, M., and Vignola, A. M. (2000) Am. J. Respir. Crit. Care Med. 161, 1720–1745
2. Fujita, Y., Fukuda, K., Kumagai, N., and Nishida, T. (2003) Exp. Eye Res. 76, 107–114
3. Minshall, E. M., Leung, D. Y., Martin, R. J., Song, Y. L., Cameron, L., Ernst, P., and Hamid, Q. (1999) Am. J. Respir. Cell Mol. Biol. 21, 775–785
4. Minshall, E. M., Leung, D. Y., Martin, R. J., Song, Y. L., Cameron, L., Ernst, P., and Hamid, Q. (1999) Am. J. Respir. Cell Mol. Biol. 21, 775–785
5. Shima, K., and Kojima, M. (2003) Am. J. Respir. Crit. Care Med. 168, 959–967
6. Akamatsu, H., Okada, T., Okumura, K., Ogawa, H., Ra, C., Fukuda, T., and Chakir, J. (2003) Chest 124, (Suppl. 3) 777–777
7. Minshall, E. M., Leung, D. Y., Martin, R. J., Song, Y. L., Cameron, L., Ernst, P., and Hamid, Q. (1999) Am. J. Respir. Cell Mol. Biol. 21, 775–785