

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

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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)² 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).

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² The abbreviations used are: Th2, T-helper type 2; TGF, transforming growth factor; IL, interleukin; cav1, caveolin-1; PBS, phosphate-buffered saline; OVA, ovalbumin; BAL, bronchoalveolar lavage; STAT, signal transducers and activators of transcription.

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- β . We used a mouse model of airway inflammation to establish that allergic modulation of *cav1* expression is mediated by IL-4. Intratracheal 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- β 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-4^{tm1Nmt/J} (IL-4-deficient), C.129S2-Stat6^{tm1Gru/J} (STAT6-deficient), and Cav^{tm1Mls/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, Cav^{tm1Mls/J}, and C.129S2-Stat6^{tm1Gru/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, L-glutamine, and pyridoxine hydrochloride (Invitrogen) and incubated with stirring in 5% CO₂ at 37 °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/cm².

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- β (Sigma) or anti IL-4 antibody (catalog number AF-204NA; R & D Systems, Minneapolis, MN).

Allergen Challenge and Intratracheal Instillation of IL-4—Female 8-week old C57BL/6J (C57), IL-4-deficient, and *cav1*-deficient (*cav1* Δ) mice were sensitized by intraperitoneal injections on days 0 and 12 with 200 μ l of phosphate-buffered saline (PBS, negative control) or 50 μ g of OVA (Sigma-Aldrich) adsorbed onto 0.5 mg of alum in 200 μ l of PBS (n = 10 to 15 mice per group). Mice were challenged with intranasal administration of 50 μ l of either PBS or OVA (0.4 mg/ml) on days 24, 26, and 28 under brief isoflurane anesthesia. On day 29, the mice were exsanguinated by cardiac puncture, and BAL was performed. Right lung tissue was used for histology and electron microscopy, and left lung tissue was reserved for RNA and protein extraction.

To test whether *cav1* regulation was mediated by IL-4, female 8-week-old C57 mice were anesthetized by intraperitoneal injection of ketamine and xylazine prior to intratracheal inoc-

ulation with PBS or IL-4 (Prepro Tech, Rocky Hill, NJ)/anti-IL-4 (gift from Dr. Finkelman, University of Cincinnati) as described by Perkins *et al.* (27). BAL fluid, blood, RNA, and protein extracts were analyzed for cytokine levels, complete blood count, and mRNA abundance or immunoassay of TGF- β and connective tissue proteins, respectively.

Eosinophil Count, Cytokine Levels in BAL Fluid, and Serum OVA-specific IgE Level—Following intratracheal injection with 1 ml of PBS, BAL fluid was collected from each mouse and centrifuged at 1,000 $\times$ g at 20 °C for 10 min. The supernatant was stored at -80 °C until tested for cytokine levels. The cell pellet was resuspended in 100 μ l of PBS to determine cell counts. Cytospin preparations were made with a maximum of 2.5 $\times$ 10⁵ cells/slide using a Shandon Cytospin centrifuge (Shandon Lipshaw Inc., Pittsburgh, PA). Eosinophil counts were performed as described previously (28). IL-4 and IL-5 levels were measured in BAL fluid using mouse Th1/Th2 Cytokine Kit Cytometric Bead ArrayTM (BD Biosciences Pharmingen) according to the manufacturer's instructions. IL-13 levels were measured using IL-13 Quantikine ELISATM (R & D Systems, Minneapolis, MN). TGF- β 1 content of BAL fluid was measured using mouse/rat/porcine TGF- β 1 Quantikine ELISATM (R & D Systems catalog number MB100). Each sample was directly measured for active TGF- β 1. In addition, each sample was treated with acetic acid/urea and NaOH/HEPES according to the manufacturer's protocol, to activate latent TGF- β 1 to the immunoreactive form, allowing measurement of the total amount of TGF- β 1 in the samples.

Gene and Protein Expression—Total RNA was extracted from cell or tissue samples using the RNeasyTM kit (Qiagen). RNA samples were treated with 0.05 unit/ml of DNase I (Qiagen) at 20 °C for 15 min. Total RNA (5 μ g) was converted into first strand cDNA using random hexamers (SuperScript First-Strand Synthesis System for RT-PCRTM; 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 (Sigma) containing 1 mM dithiothreitol, 1 $\times$ protease inhibitor mixture (Calbiochem, San Diego, CA), and 5 mM EDTA. Homogenates were centrifuged at 16,000 $\times$ g, and protein concentrations in supernatants were determined by the Bradford assay (Bradford reagent; Bio-Rad). Total protein (60 μ g) was combined with reducing Laemmli buffer, heated at 95 °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-Smad2 (51-1300; Zymed Laboratories, Inc. San Francisco, CA), 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-

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 (ImageJ 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 glycine (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 μ g/ml). PCR was performed on the immunoprecipitated complexes for the detection of the *cav1* promoter using forward primer 5'-TCCACTGAAGGACCTT-TCCAG-3' and reverse primer 5'-AGAGATATTTGCTTC-GAGGCC-3'. These primers specifically amplified the 137-bp region containing the putative STAT6-binding site within the *cav1* promoter. The samples were quantified by semi-quantitative real time PCR in duplicate from four independent immunoprecipitations.

The 137-bp PCR fragment containing the putative STAT6-binding site in *cav1* promoter was end-labeled using the Biotin 3' end DNA labeling kit (Pierce) as described by the manufacturer. The nucleo-protein binding was done in 11 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 2.5% glycerol, 0.05% Nonidet P-40, 5 mM MgCl₂, and 100 ng/ μ l Poly(dI-dC). 2.5 or 5 μ g of nuclear lysate was incubated with the radiolabeled probe for 30 min at 25 °C. For the oligonucleotide competition experiments, the extracts were preincubated with 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'-TTCTACGTTTTTCCCTA-GAACAGAATCCTAA-3' and 5'-TTAGGATTCTGTTCTAG-GGAAA AACGTAGAA-3' and STAT6 consensus binding site as previously described (30) 5'-GATCTTTTCTTATGAAC-3', and 5'-GATCGTTCATAAGAAA-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 Lumimeter 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 $\times$ 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 $\pm$ 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

TABLE 1

Eosinophils, Th2 cytokines, and TGF- β level in BAL of PBS- or OVA-challenged C57, IL-4-deficient, and *cav1*-deficient mice or PBS or IL-4 intratracheally injected C57.

IL, intratracheal injection

Strain	Treatment	Eosinophil	IL-4	IL-5	IL-13	TGF- β
		%	pg/ml	pg/ml	pg/ml	pg/ml
C57	PBS	5.6 $\pm$ 1.9	1.3 $\pm$ 0.5	4 $\pm$ 1	5.1 $\pm$ 1.2	76 $\pm$ 42
C57	OVA	59.7 $\pm$ 6	15.6 $\pm$ 4.6	265 $\pm$ 163	265 $\pm$ 77	738 $\pm$ 159
C57	PBS II	0.9 $\pm$ 0.6	14 $\pm$ 10	4.4 $\pm$ 3.6	3.8 $\pm$ 3.7	285 $\pm$ 232
C57	IL-4 II	43 $\pm$ 22	88 $\pm$ 8	44.8 $\pm$ 36.2	158 $\pm$ 66	1,142 $\pm$ 470
IL-4	PBS	0	2.8 $\pm$ 0.2	3.5 $\pm$ 0.5	1.4 $\pm$ 2	0
IL-4	OVA	2.1 $\pm$ 2.1	5.8 $\pm$ 2.9	3.4 $\pm$ 0.2	4.3 $\pm$ 3.4	91 $\pm$ 50
<i>cav1</i> Δ	PBS	0.74 $\pm$ 0.31	1.6 $\pm$ 0.7	2.5 $\pm$ 1.1	10.6 $\pm$ 1.9	284 $\pm$ 94
<i>cav1</i> Δ	OVA	56.7 $\pm$ 5.4	19.7 $\pm$ 7.3	99.3 $\pm$ 33.1	278 $\pm$ 86	920 $\pm$ 121

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 TRANSFACT and MATINSPECTOR transcription factor data bases (www.genomatrix.de and transfac.gbf.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 α 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 ($3.8 \pm 2 \times 10^5$ versus $0.5 \pm 0 \times 10^5$ cell/ml), eosinophils, IL-4, IL-13, and TGF- β 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 ($n = 3 - 6$) (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- β Signaling Activation Is Associated with Reduced *cav1* Expression in Allergen-challenged Mice—TGF- β was detected at low levels in BAL fluid of PBS-sensitized and challenged mice

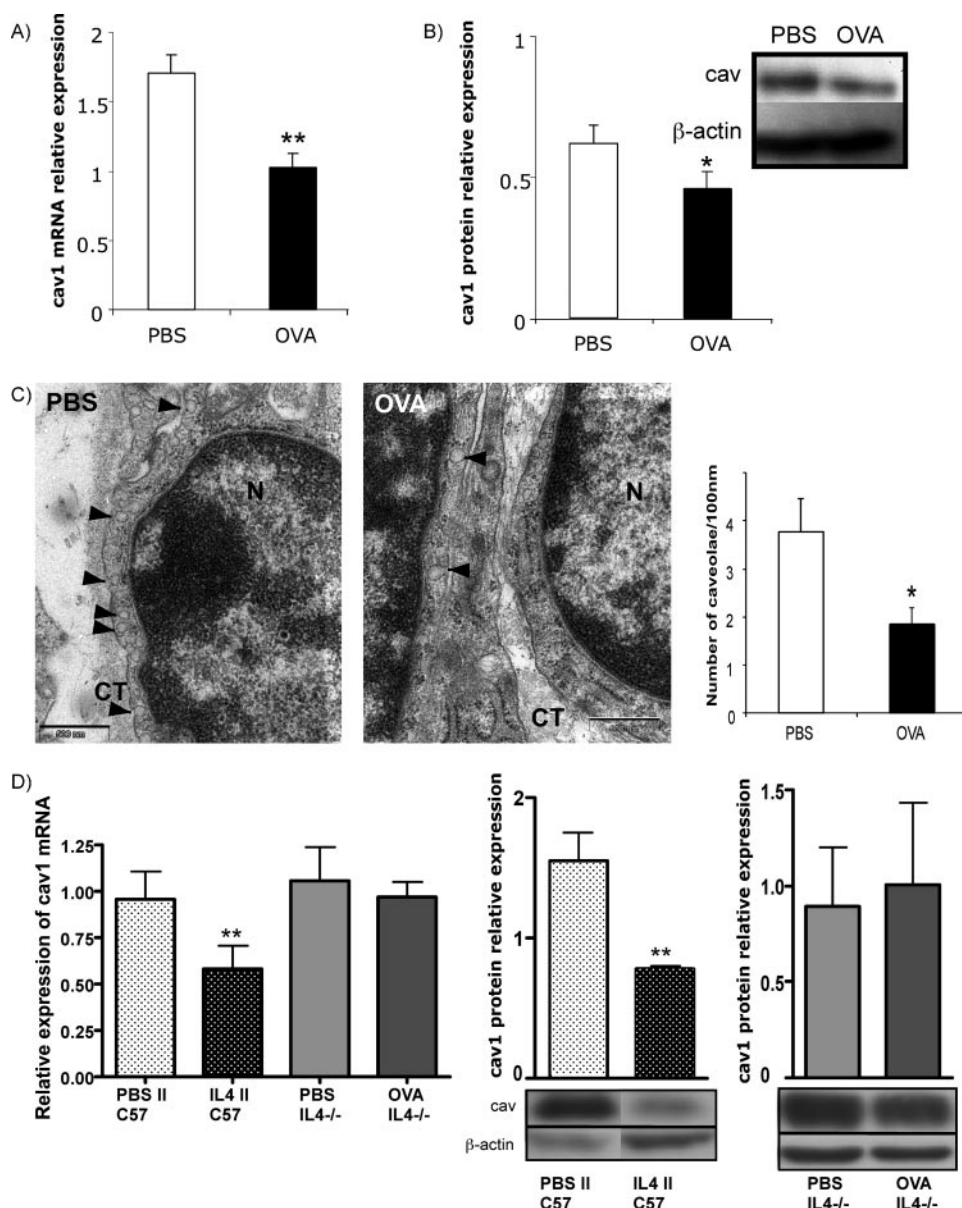


FIGURE 1. Caveolin-1 expression in whole lung. *cav1* gene expression in OVA-sensitized and challenged mice (OVA-1w, black bar) was decreased, compared with PBS-sensitized and challenged mice (PBS-1w, white bar). The bar represents the mean $\pm$ S.D. *, $p < 0.05$; **, $p < 0.01$. $n = 7$ PBS-sensitized 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. **B**, 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 carboxyl-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 *cav1* Δ 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 ($p < 0.005$, $n \geq 10$) (Table 1).

By contrast, OVA sensitization and challenge produced an increase in the level of p-Smad2 in *cav1* Δ 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 ele-

ments, was more pronounced in the *cav1* Δ lung fibroblasts regardless of the *in vitro* TGF- β $\pm$ 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 *cav1* Δ 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

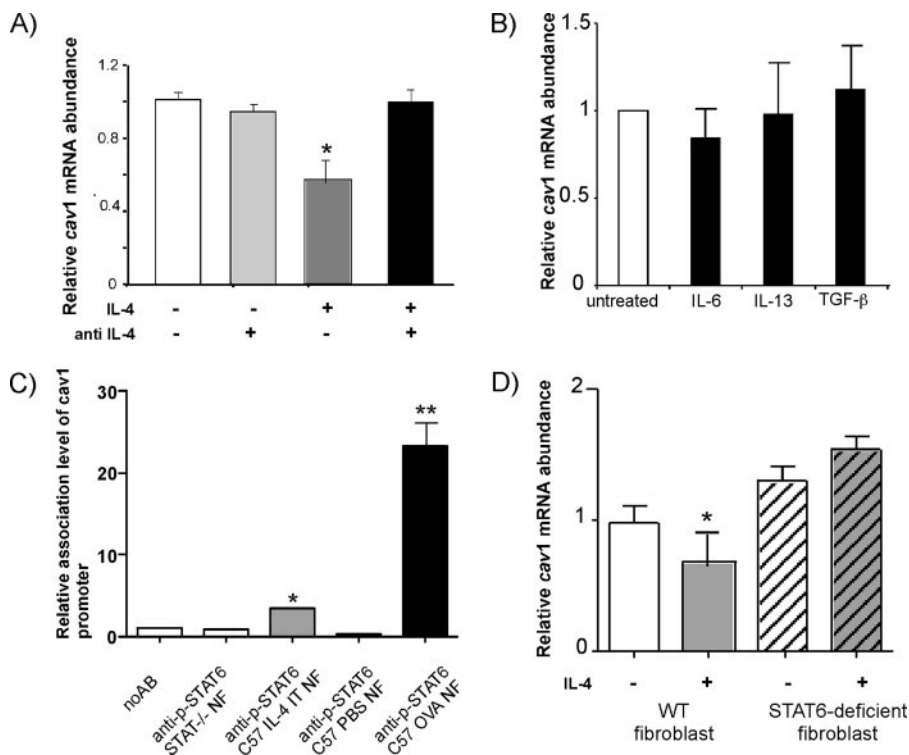


FIGURE 2. *cav1* expression is IL-4 dependent via STAT6 mediation. *A*, *cav1* mRNA level. Total RNA was extracted from lung fibroblasts cultured with or without anti IL-4 neutralizing antibody, IL-4, or IL-4 + anti-IL-4 fibroblasts. Units are normalized to 18 S RNA. Standard deviations are indicated, $n = 9$ for each group. The data are representative of three independent experiments. *B*, modulation of *cav1* gene expression by various cytokines. Total RNA was extracted from lung fibroblasts cultured with IL-6 (0.1 ng/ml), IL-13 (10 ng/ml), or TGF- β (2 ng/ml) for 48 h. *cav1* gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase gene expression. The values are the means $\pm$ S.D. ($n = 6$). The data are representative of two independent experiments. *C*, specific *in vivo* binding of STAT-6 to *cav1*-promoter in response to OVA allergen protocol and IL-4 injection in C57Bl/6J mice. Formaldehyde cross-linked DNA-protein complexes extracted from STAT-6-deficient, IL-4 intratracheally injected, PBS-challenged, or OVA-challenged mouse lung tissues were immunoprecipitated with control rabbit IgG (noAB) or anti-p-STAT6 antibody (anti-p-STAT6). The immunoprecipitated chromatin fragments were quantified by qPCR using primers specific to the *cav1* promoter region containing the STAT6-binding element. The results were normalized to the total input of DNA used before immunoprecipitation, and the negative control assay was performed without anti-p-STAT6 antibody (noAB). The data represent the means of independent immunoprecipitations using nuclear extracts from five mice, quantified in duplicate assays. The standard errors are indicated. *D*, *cav1* expression is not regulated by IL-4 in STAT6-deficient lung fibroblasts. Total RNA was extracted from C57 and STAT6-deficient mouse lung fibroblasts cultured with IL-4 (10 ng/ml) for 48 h. *cav1* gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase gene expression. The values are the means $\pm$ S.D.; $n = 3$ –9. The data are representative of three independent experiments. WT, wild type.

fibrotic response on trichrome-stained lung tissue sections in PBS- and OVA-challenged C57 and *cav1* Δ mice. As expected we saw an increase in the total fibrotic score in OVA-challenged compared with PBS-challenged mice for both strains ($p \leq 0.01$, $n = 5$ –9). Importantly, OVA-challenged *cav1* Δ mice presented a substantially greater increase than did the OVA-challenged C57 mice ($p \leq 0.05$, $n = 5$ –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 *cav1* Δ mice presented an 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). In a previous study, the minimal region for transcriptional regulation of *cav1* in mouse fibroblast 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 cyclin D1 and D3 (38). *cav1* Δ 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

asthma and 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

Enhancement of TGF- β Signaling Mediated by IL-4

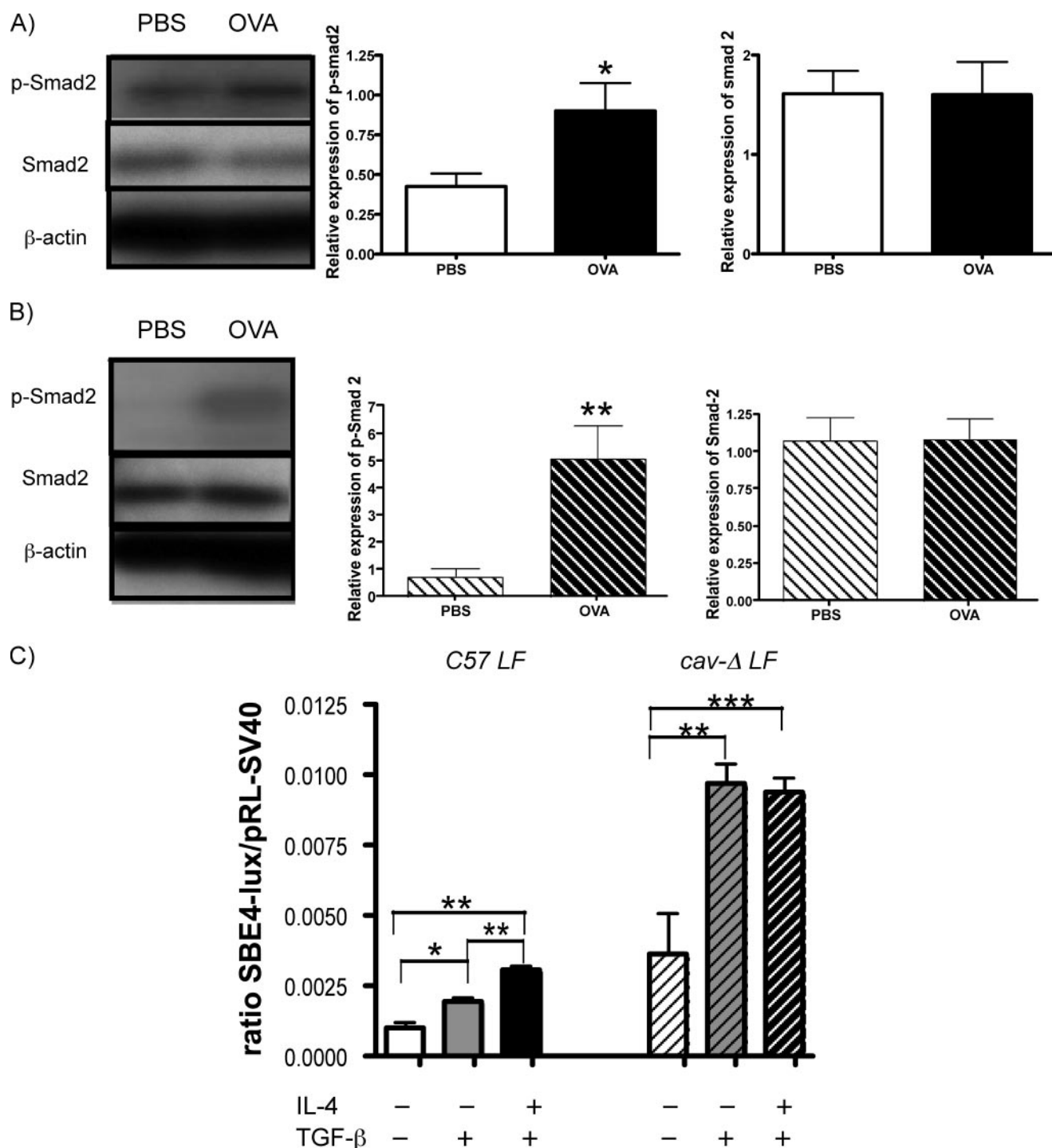


FIGURE 3. Acute OVA-allergen challenge protocol increased TGF- β activity. *A*, representative Western blot and analysis of Smad-2 and p-Smad2 in PBS-sensitized challenged (white bar) and OVA-sensitized and challenged mice (black bar). The bar represents the mean of the relative level of expression of Smad2 or p-Smad2 after normalization to β -actin. *B*, representative Western blot of Smad2 and P-Smad2 and Western blot analysis of Smad-2 and P-Smad2 in PBS-sensitized (dashed white bar) and OVA-sensitized *cav1Δ* mice (dashed black bar). The bar represents the mean of the relative level of expression of Smad2 or P-smad2 after normalization to β -actin. *, $p < 0.05$ and **, $p < 0.001$. $n = 6$ for each group and each strain. *C*, enhanced Smad response element transcriptional activity. The Smad response element construct was transfected into lung fibroblasts extracted from C57 and *cav1Δ*. These fibroblasts were transfected with 1 μ g of plasmid and 0.1 μ g of pRL-SV40 plasmid for control purposes and cultured for 24 h in presence or absence of IL-4. The cells were then treated with 10 ng/ml of TGF- β for 30 min. Luciferase activity was normalized to the *Renilla* luciferase activity. Each value represents the mean $\pm$ S.D. of at least three independent transfection experiments, each performed in triplicate.

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 re-

sponses 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

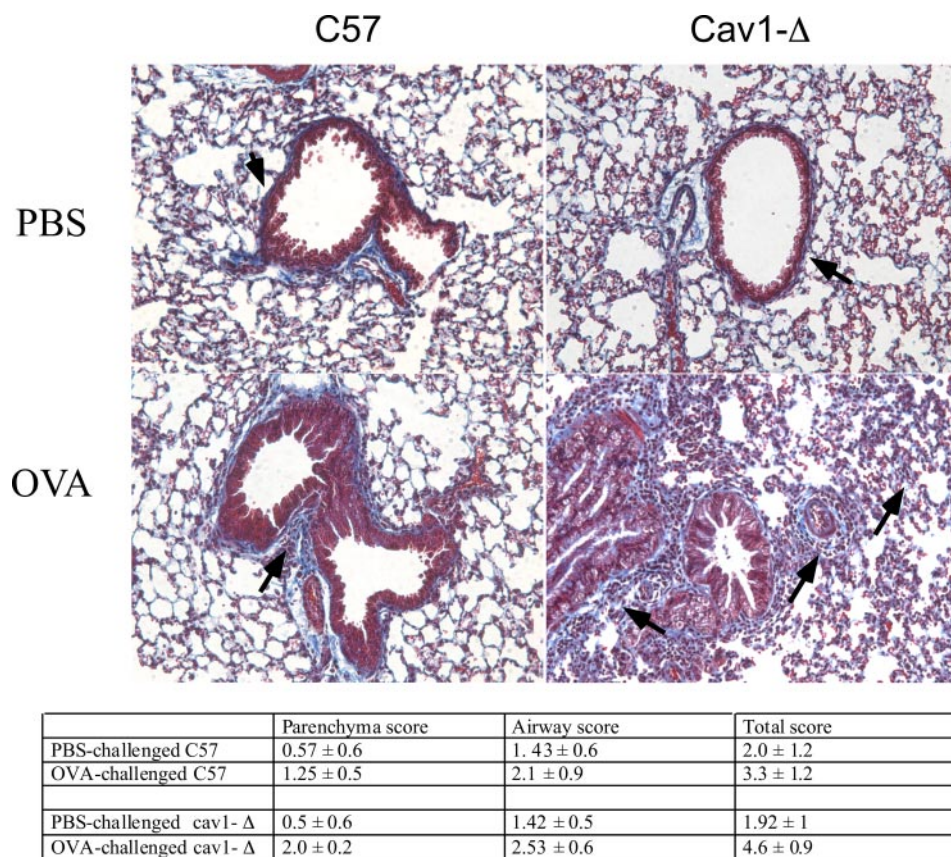


FIGURE 4. Comparison of trichrome staining of lung sections from PBS- and OVA-sensitized and challenged C57 and *cav1* Δ mice. Comparison between OVA-challenged tissues illustrates the increased collagen deposition (blue stain) in *cav1* Δ mice. Original magnification was $\times 200$. The table in the lower part of the figure shows the results of the grading from parenchymal and peribronchial and/or perivascular collagen deposition.

shift assay. We also showed that IL-4 stimulation of STAT6-deficient lung fibroblasts did not result in the down-regulation of *cav1* gene expression seen in wild-type fibroblasts. Our data also indicate that only in the presence of IL-4 (intratracheal inoculation or OVA challenge) does the STAT6 transcription factor bind to the *cav1* promoter. The STAT6 activation pathway is necessary for the induction of expression of IL-4-responsive genes. These data are consistent with a required role for IL-4 in caveolin regulation. The regulation of *cav1* is a novel role for IL-4. In our model, increased levels of IL-4 were accompanied by decreased amounts of *cav1* and caveolae number (Fig. 1). The decreased abundance of mRNA was proportional to the decrease in protein and caveolae, suggesting that regulation of *cav1* expression occurred mainly at the level of transcription. In addition, the reduced abundance of *cav1* mRNA in IL-4-stimulated mice was comparable with the level in OVA-sensitized and challenged mice. This result supports the notion that *cav1* expression is regulated by IL-4.

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 *cav1* Δ 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 *cav1* Δ 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 *cav1* Δ 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 develop-

ment 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.

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.

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