

A role for decorin in a murine model of allergen-induced asthma

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Marchica CL, Pinelli V, Borges M, Zumner J, Narayanan V, Iozzo RV, Ludwig MS. A role for decorin in a murine model of allergen-induced asthma. *Am J Physiol Lung Cell Mol Physiol* 300: L863–L873, 2011. First published March 4, 2011; doi:10.1152/ajplung.00300.2009.—Decorin (Dcn) is an extracellular matrix proteoglycan, which affects airway mechanics, airway-parenchymal interdependence, airway smooth muscle proliferation and apoptosis, and transforming growth factor- β bioavailability. As Dcn deposition is differentially altered in asthma, we questioned whether Dcn deficiency would impact the development of allergen-induced asthma in a mouse model. *Dcn*^{−/−} and *Dcn*^{+/+} mice (C57Bl/6) were sensitized with ovalbumin (OA) and challenged intranasally 3 days/wk $\times$ 3 wk. After OA challenge, mice were anesthetized, and respiratory mechanics measured under baseline conditions and after delivery of increasing concentrations of methacholine aerosol. Complex impedance was partitioned into airway resistance and tissue elastance and damping. Bronchoalveolar lavage was performed. Lungs were excised, and tissue sections evaluated for inflammatory cell influx, α -smooth muscle actin, collagen, biglycan, and Dcn deposition. Changes in TH-2 cytokine mRNA and protein were also measured. Airway resistance was increased in OA-challenged *Dcn*^{+/+} mice only ($P < 0.05$), whereas tissue elastance and damping were increased in both OA-challenged *Dcn*^{+/+} and *Dcn*^{−/−}, but more so in *Dcn*^{+/+} mice ($P < 0.001$). Inflammation and collagen staining within the airway wall were increased with OA in *Dcn*^{+/+} only ($P < 0.001$ and $P < 0.01$, respectively, vs. saline). IL-5 and IL-13 mRNA were increased in lung tissue of OA-challenged *Dcn*^{+/+} mice. Dcn deficiency resulted in more modest OA-induced hyperresponsiveness, evident at the level of the central airways and distal lung. Differences in physiology were accompanied by differences in inflammation and remodeling. These findings may be, in part, due to the well-described ability of Dcn to bind transforming growth factor- β and render it less bioavailable.

animal model; transforming growth factor- β ; proteoglycans; airway remodeling

THE BIOLOGICAL IMPORTANCE of proteoglycans (PGs), principal components of the extracellular matrix, in the lung has recently been studied. PGs have multiple biological functions, ranging from their role in determining lung mechanical properties to regulation of growth factors and cellular functions (1, 16, 23). The small leucine-rich PG (SLRP), decorin (Dcn), has been demonstrated to be of particular importance. Dcn plays a key role in the development and assembly of several tissues (13, 29) and has been shown to be involved in the stability and formation of collagen fibers (4, 6, 28). Dcn has been demonstrated to reversibly bind the core protein of the active form of transforming growth factor (TGF)- β , resulting in the reduction of its bioavailability (13, 30, 36).

Alterations in lung mechanics in *Dcn*^{−/−} vs. *Dcn*^{+/+} mice have been demonstrated (10). Lung mechanical behavior has been assessed by measuring complex impedance and quasi-static pressure-volume curves. Airway resistance (Raw) was decreased in *Dcn*^{−/−} mice, and both in vivo pressure-volume and in vitro length-stress curves showed increased compliance compared with *Dcn*^{+/+} mice (10). Lack of Dcn resulted in abnormal lung collagen deposition, and airway-parenchymal interdependence during induced constriction was also affected (31). Dcn also affects human airway smooth muscle (ASM) cells in culture. D'Antoni et al. (5) have shown that culturing human ASM cells on a Dcn matrix resulted in decreased cell number, due to decreases in proliferation and increases in apoptosis.

PGs have been shown to be altered in asthma. Moreover, PG deposition within the airway wall differs according to disease severity. We have postulated that the differential distribution of PGs may alter the degree of airway narrowing (26). Studies in mild, moderate, and severe asthmatic subjects have shown increases in lumican, biglycan (Bgn), and versican, compared with control subjects (8, 14, 26). Airway biopsies from fatal asthmatic patients have shown increases in Bgn and versican, but decreases in Dcn and lumican (7). Data obtained from animal models are similar. Pini and colleagues (27) demonstrated that Dcn and Bgn deposition were both increased in the airway wall of ovalbumin (OA)-challenged rats. The distribution differed: while Bgn was located within the ASM layer, Dcn was primarily detected external to the ASM, in the adventitial layer.

Thus there is considerable evidence that not only supports the potential role of PGs in asthma, but also demonstrates that alteration in their deposition and distribution results in functional consequences. We hypothesized that development of allergen-induced asthma would be enhanced in *Dcn*^{−/−} mice due to the observed effects of Dcn on ASM mass and the mechanical properties of the airway and lung tissues. Furthermore, the effects of Dcn on TGF- β bioavailability may influence remodeling and inflammation indirectly. Therefore, we evaluated lung mechanics under baseline conditions and after methacholine (MCh) challenge in both saline (Sal) and OA-challenged *Dcn*^{+/+} and *Dcn*^{−/−} mice and characterized inflammation and remodeling.

METHODS

Determination of Genotype

The original generation of the *Dcn*^{−/−} mice was as described previously by Danielson et al. (6). Briefly, the gene-targeting vector was achieved by introducing a phosphoglycerate kinase (PGK)-*neo* cassette within exon 2 of the murine *Dcn* gene, leading to disruption of the gene locus. The mice were backcrossed for at least nine generations into a C57Bl/6 genetic background. To generate additional *Dcn*^{−/−} mice, breeding was initiated between female C57Bl/6 mice, heterozygous for *Dcn* (*Dcn*^{+/-}) and C57Bl/6 *Dcn*^{−/−} males.

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The genetic makeup of the offspring was determined by extracting DNA from a piece of tail tissue. Briefly, 150 μ l of lysis buffer solution for tissue digestion containing 1 μ l proteinase K/100 μ l of lysis reagent were added to each DNA sample. Samples were heated for up to 24 h at 55°C, after which temperature was increased to 85°C for 45 min. Samples were then stored at -20°C until ready for use.

Polymerase chain reaction (PCR) was used for DNA analysis. As described by Danielson et al. (6), sense and antisense primers, which correspond to exon 2 of murine *Dcn*, as well as a third primer corresponding to the PGK promoter of the PGK-*neo* cassette, were employed. These sense and antisense primers displayed a 161-bp fragment, corresponding to the wild-type *Dcn* gene, whereas sense and PGK primers yielded a 250-bp fragment, signifying the knocked out *Dcn* gene. The total reaction mix of 13.21 μ l consisted of 1 μ l of DNA, 1 μ l of sense primer, 1 μ l of antisense primer or 1 μ l of PGK primer, 5.35 μ l of H₂O, 2 μ l of 10 $\times$ rxn buffer, 2 μ l of MgSO₄ (Invitrogen, Ontario, Canada), 0.8 μ l of dNTP mix (containing all 4 dNTPs; Onbio, Ontario, Canada), and 0.06 μ l of Taq polymerase (Invitrogen). The reaction conditions comprised the following: melting for 5 min at 95°C; 35 cycles of 1 min at 94°C, 30 s at 57°C, and 30 s at 72°C; followed by 10 min at 72°C; and cooling at 4°C. The products of the PCR reaction were then confirmed to be the correct size by running the samples on a 2.5% agarose gel (Agarose 'B' Low EEO, LAB MAT, Montreal, Canada) at 120 V for 25–30 min.

Animal Preparation

Dcn^{+/+} mice were obtained from Charles River Laboratories, and breeding initiated. Male and female mice were separated into four groups: *Dcn*^{+/+} Sal, *Dcn*^{+/+} OA, *Dcn*^{-/-} Sal, and *Dcn*^{-/-} OA mice. All mice were sensitized between 6 and 8 wk of age. Body weights at the time of experimental measurement ranged from 19 to 32.5 g and were not significantly different among groups (mean weights: Sal-challenged *Dcn*^{+/+}: 22.67 $\pm$ 0.88 g; OA-challenged *Dcn*^{+/+}: 22.94 $\pm$ 0.85 g; Sal-challenged *Dcn*^{-/-}: 27.32 $\pm$ 1.19 g; OA-challenged *Dcn*^{-/-}: 25.37 $\pm$ 1.66 g). All animals were housed in an animal facility at McGill University and were cared for in compliance with the Canadian Council of Animal Care's guide; protocols and procedures were evaluated and approved by the animal ethics committee of McGill University.

Allergic Sensitization and Challenge Protocol

The sensitization and challenge protocol used in this study is shown in Fig. 1. Briefly, experimental groups were sensitized with an intraperitoneal (ip) injection of a Sal solution containing 10 μ g OA (Sigma Chemical, St. Louis, MO) with 1 mg of adjuvant aluminum hydroxide, Al(OH)₃ (Fisher Scientific, Ottawa, Ontario). Sensitization occurred on 2 separate days: day 0 and day 7. One week following the second sensitization, mice were challenged via an intranasal instillation of 10 μ g OA in 50 μ l of Sal three times per week for 3 wk under light halothane anesthesia. Two days following the final challenge, experiments were performed. Control mice were sensitized and challenged with Sal solution.

Measurement of In Vivo Lung Mechanics

Mice were injected ip with xylazine (10 mg/kg) and pentobarbital sodium (30 mg/kg). Mice were tracheostomized, and an 18-gauge metal cannula was inserted into the trachea and firmly tied. Subse-

quently, the animal was connected to a computer-controlled small-animal ventilator (Flexivent, Scireq, Montreal, Canada), and normal tidal breathing was initiated. Mice were mechanically ventilated at 150 breaths/min, and a positive end-expiratory pressure of 3 cmH₂O. Animals were then paralyzed with pancuronium bromide (1.2 mg/kg ip), administered ip, before the initiation of mechanical ventilation.

Respiratory mechanics were measured using the forced-oscillation technique. Briefly, a computer-generated volume signal, composed of a 2-s pseudorandom perturbation comprising waveforms of mutually primed frequencies (1–20.5 Hz), was delivered at the airway opening. Measurements of volume or piston displacement, as well as the cylinder pressure, were recorded during this signal perturbation. Data were obtained at baseline, as well as after the delivery of increasing concentrations of MCh aerosols (6.25, 12.5, 25, and 50 mg/ml) (Sigma), which were dissolved in sterile Sal and delivered to the airways via an ultrasonic nebulizer (Hudson RCI, Teleflex Medical) for a period of 10 s. Before data collection, two total lung capacity breaths of 30 cmH₂O were delivered to standardize volume history. Perturbations were delivered every 15 s for 5 min following MCh aerosol delivery. A total of six measurements were acquired for each variable during the MCh challenge, and the peak response was determined.

Measurement of Complex Impedance and the Constant-phase Model

The complex impedance model uses multiple-frequency perturbations, which permit the partitioning of the response into central (Raw) and peripheral [tissue damping (Gti), tissue elastance (Hti)] lung components. Input impedance spectra were derived from pressure and volume measurements made at the airway opening using the "forced oscillation technique." The impedance of the respiratory system (Zrs) was calculated using the following equation:

$$Z_{rs}(\omega) = P(\omega)/[dV(\omega)/dt]$$

where P denotes pressure, and V is volume (ml) corresponding to piston displacement. Both of these parameters are dependent on angular frequency (ω).

Input impedance data were then fit to the "constant phase model" (12) using a multiple linear regression.

$$Z_{rs} = R_{aw} + J\omega I + (G_{ti} + jH_{ti})/\omega^a$$

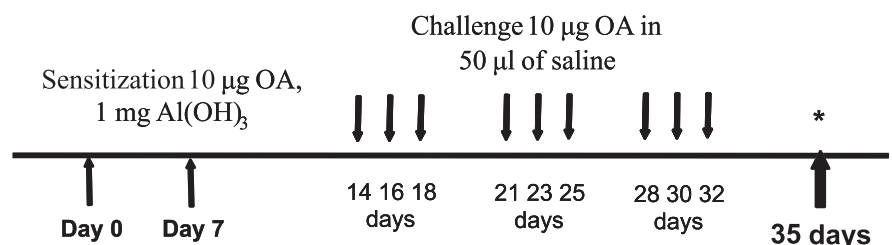
where I is airway inertance, $\alpha = (2/\pi)\tan^{-1}(H/G)$, and J represents the imaginary part.

For each parameter measured, the peak response, having a coefficient of determination >0.8, was determined and used in the statistical analyses.

Bronchoalveolar Lavage

Following lung mechanics, bronchoalveolar lavage (BAL) was carried out twice by instilling, and subsequently withdrawing, 1 ml of cold Sal via the tracheal cannula. A cytospin centrifuge was then used to deposit the BAL cell suspension on a glass slide, which was then fixed and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI). Differential cell counts were determined by analyzing the percentage of macrophages, eosinophils, neutrophils, and lymphocytes out of a total of 300 cells that were counted on BAL samples slides.

Fig. 1. Antigen sensitization and challenge protocol. Experimental groups were sensitized on two separate occasions, 1 wk apart, with ovalbumin (OA) and adjuvant aluminum hydroxide. This was followed by a total of 9 challenges by intranasal instillation of OA in saline (Sal; 3 days/wk for 3 wk). Physiological assessments were carried out on day 35. Control mice were sensitized and challenged with Sal alone.



Supernatant from the first BAL was collected and stored at -80°C . ELISA was performed to measure IL-13 and TGF- β_1 , according to the manufacturer's instructions (eBioscience, San Diego, CA). IL-4 and IL-5 were quantified by electrochemiluminescence using a MULTI-SPOT, 96-well plate (Meso Scale Discovery, Gaithersburg, MD).

Tissue Preparation and Fixation

Following the *in vivo* measurements, the chest wall was opened, and lungs were excised. Part of the right lobes of the lungs were snap-frozen in liquid nitrogen and stored for protein analysis, and the other part was stored in RNAlater (Qiagen, Mississauga, Ontario, Canada) for mRNA analysis. The left lung was inflated with 10% phosphate-buffered formalin at a transpulmonary pressure of 25 cmH $_2$ O for 25 min. The tissue was processed and subsequently embedded in paraffin blocks. Sagittal tissue sections with a thickness of 5 μm were cut sequentially, and staining was performed.

Immunohistochemistry

α -Smooth muscle actin. Antigen retrieval was initially performed on tissue sections using 0.01 M sodium citrate buffer (pH 6.0). Sections were then incubated overnight at 4°C in a humid chamber with the primary antibody α -smooth muscle actin (α -SMA) (1:250 dilution, rabbit anti-mouse α -SMA, Abcam Laboratories, Cambridge, MA).

Dcn and Bgn. Tissue sections were incubated overnight with the primary antibody for Dcn (rabbit anti-Dcn 1:800 dilution), or Bgn (rabbit anti-Bgn 1:500 dilution, both provided by Dr. Larry Fisher, National Institutes of Health).

The secondary antibody applied for all immunostaining was biotinylated goat anti-rabbit (1:100 dilution, Vector Laboratories), and immunoperoxidase was used for detection. Slides were counterstained using hematoxylin (Gill I Hematoxylin, Sigma Aldrich). All immunostaining was developed using diaminobenzidine (DAKO Cytomation, Carpinteria, CA). Positive stain for α -SMA, Dcn, and Bgn was visualized to be brown under a light microscope (Olympus BX51, Ontario, Canada).

Measurement of TGF- β_1 in Lung Tissue

Homogenates were prepared from whole lung tissue. Western blotting was performed using primary antibody for TGF- β_1 . Detection was performed using chemiluminescence. Membranes were stripped and reprobed using anti- β -actin antibodies.

RNA Extraction and Quantitative Real-time PCR

RNA was extracted from lung tissue using a commercial kit (RNeasy Mini Kit; Qiagen, Mississauga, Ontario, Canada). RNA quantity and quality were assessed by using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). Complementary DNA (cDNA) was made in a 20- μl reaction, with 250 ng of total RNA, oligo(dT) $_{12-18}$ primer, dNTP mix, RNaseOUT, and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions.

The mRNA levels of IL-4, -5, and -13, TGF- β_1 , and S9 were examined. The primers were designed using Primer3 (<http://frodo.wi.mit.edu/>). Amplicons were verified on 2.5% agarose gel to be the expected size.

Quantitative real-time PCR was performed in a 96-well plate with the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 10- μl reaction volume containing 0.5 μM of sense and antisense primers and 1 μl of cDNA. The plate was sealed with adhesive, and the run method was performed with three stages: holding, cycling, and melt curve stage. The holding stage was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, annealing temperature for 30 s, and 72°C for 30 s. The annealing temperature was 55°C for IL-4; 60°C for TGF- β_1 , IL-5, and IL-13;

and 64°C for S9. The melting curve stage was 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Each reaction was performed in duplicate with a negative control (RNase-free water). The comparative $\Delta\Delta\text{Ct}$ method was used for analysis, and mRNA levels of ribosomal protein S9 (reference gene) were used for normalization.

Airway Morphometry

Morphometric analyses were performed on airways that had intact epithelial lining, as well as a minimum-to-maximum internal diameter ($D2/D1 \geq 0.5$). Stained slides were viewed under the Olympus light microscope (Olympus, Ontario, Canada), and images were captured with either the Cool-Snap PROc color camera (Media Cybernetics, Baltimore, MD) or the Olympus Qcolor5 Camera (Olympus, Center Valley, PA). Measurements were obtained using the Image pro-PLUS software (version 5.2, Media Cybernetics L.P., Silver Spring, MD). A total of $n = 8$ mice were sampled at random from each group (i.e., 8 Sal control, 8 OA challenged). Generally, five airways were examined per mouse, with the exception of Periodic acid Schiff (PAS) data, which sampled three airways.

Tissue inflammation. To quantify tissue inflammation, the number of inflammatory cells, including granulocytes and mononuclear cells, was counted within the airway wall area (WA) around the airways. The WA was characterized as the area extending from the basement membrane to the peribronchial area, defined by the alveolar attachments. Tissue inflammation was expressed as the number of inflammatory cells per WA.

Epithelial thickness and area. Measurements of epithelial area and basement membrane length were gathered using the Image pro-PLUS software (version 5.2, Media Cybernetics L.P.). Epithelial thickness was subsequently calculated by dividing the epithelial area by the basement membrane length.

Mucus staining. PAS staining was used to visualize mucus within the airway epithelium. The tissue section was scanned, and three airways were selected at random. Positive staining appeared pink under a light microscope and was quantified by outlining the area of the positive stain within the epithelial area. Results were expressed as the percent (%) positive area divided by total epithelial area.

ASM. To quantify ASM, the following measurements were made: basement membrane perimeter (P_{bm}), and total area of smooth muscle bundles present around the airway (ASM). Results were expressed as the area of smooth muscle divided by P_{bm} squared (P_{bm}^2). ASM was quantified by two separate operators between which a correlation was established (R^2 value = 0.98).

Collagen, Dcn, and Bgn. For each airway, WA was delineated. Analysis of positive staining for collagen, Dcn, and Bgn was limited to this area. Using the Image Pro-Plus software, the color of the positive stain was selected, and an area measurement was generated. Results were expressed as the area of positive stain/WA.

Data Analysis

Respiratory physiology data were generated using the Flexivent software (Scireq, Montreal, Canada). A two-way ANOVA for repeated measures with Bonferroni post hoc analyses was applied to determine whether *in vivo* mechanical parameters were altered during the MCh response curve in OA-challenged mice compared with Sal controls and in *Dcn* $^{-/-}$ vs. *Dcn* $^{+/+}$ groups. One-way ANOVA with Bonferroni posttests was used for all histological analyses. A P value < 0.05 was considered statistically significant. Values are depicted as means $\pm$ SE.

RESULTS

Dynamic Measurements of In Vivo Lung Mechanics

Lung mechanics were partitioned into central (Raw) and distal (Gti, Hti) lung components (Fig. 2, A–C, respectively). Baseline

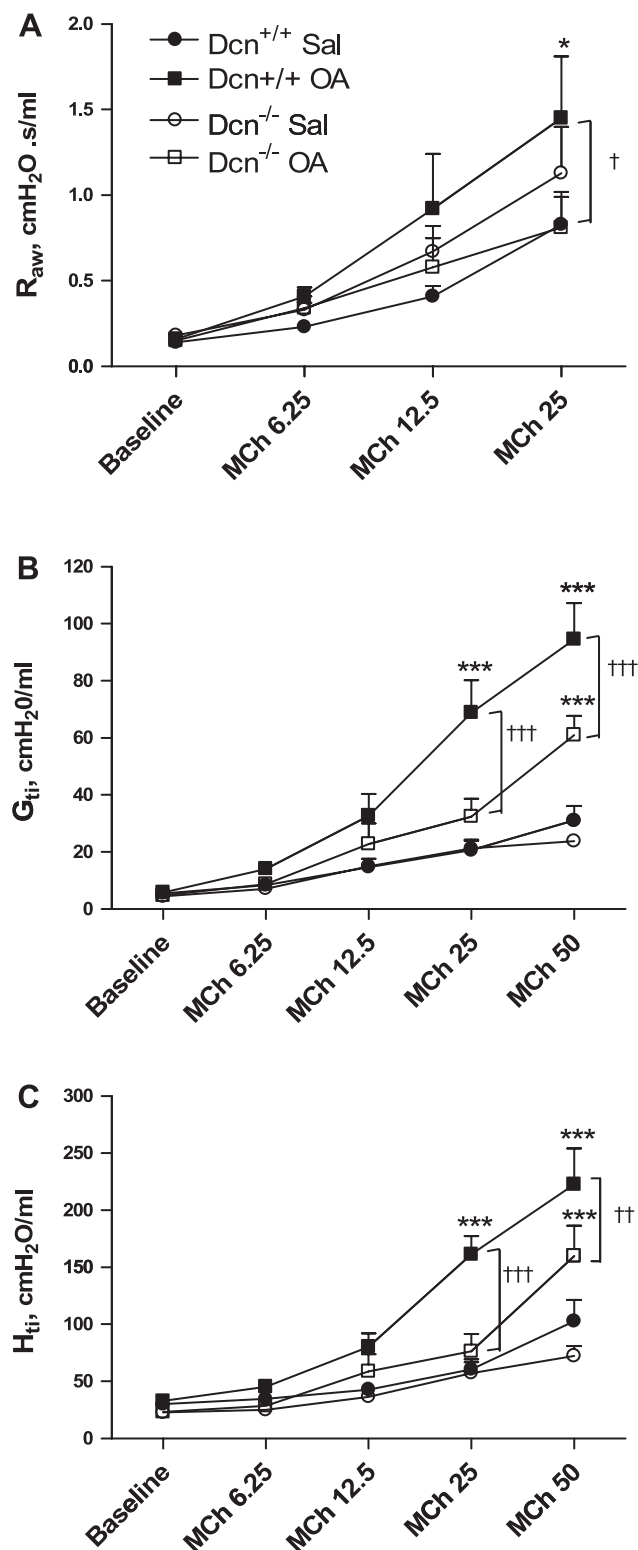
measurements of lung mechanics are shown in Table 1. Raw and Gti were not significantly different among the four groups. However, Hti was significantly lower in OA-challenged $Dcn^{-/-}$ mice compared with OA-challenged $Dcn^{+/+}$ mice.

During MCh aerosolization, OA-challenged $Dcn^{+/+}$ mice showed a significantly greater increase in Raw compared with

Table 1. Baseline respiratory mechanics

	$Dcn^{+/+}$		$Dcn^{-/-}$	
	Sal	OA	Sal	OA
Raw, cmH ₂ O·ml ⁻¹ ·s	0.142 ± 0.015	0.158 ± 0.01	0.184 ± 0.028	0.149 ± 0.016
Gti, cmH ₂ O/ml	5.57 ± 0.285	5.80 ± 0.40	4.48 ± 0.196	4.77 ± 0.226
Hti, cmH ₂ O/ml	30.21 ± 1.81	32.89 ± 2.85	22.87 ± 1.08	23.41 ± 1.41*

Values are means ± SE. Dcn, decorin; Sal, saline; OA, ovalbumin; Raw, airway resistance; Gti, tissue damping; Hti, tissue elastance. * $P < 0.05$, $Dcn^{-/-}$ OA vs. $Dcn^{+/+}$ OA.



Sal-challenged $Dcn^{+/+}$ mice ($P < 0.05$ at 25 mg/ml MCh). However, in $Dcn^{-/-}$ mice, airway responsiveness during MCh aerosolization was not different in the Sal-challenged vs. OA-challenged animals. Furthermore, OA $Dcn^{-/-}$ mice had a significantly lower Raw response at a dose of 25 mg/ml of MCh compared with OA-challenged $Dcn^{+/+}$ mice (0.81 ± 0.18 vs. 1.45 ± 0.36 , $P < 0.05$). Gti and Hti were significantly increased during MCh aerosolization in both $Dcn^{+/+}$, as well as $Dcn^{-/-}$, OA-challenged mice, compared with their respective Sal controls. However, $Dcn^{-/-}$ mice showed significantly less OA-induced distal lung hyperresponsiveness during MCh aerosolization than $Dcn^{+/+}$ mice. Responses for Gti and Hti were significantly lower during MCh aerosolization in the OA-challenged $Dcn^{-/-}$ mice compared with OA-challenged $Dcn^{+/+}$ mice at MCh doses of 25 (Gti and Hti, $P < 0.001$) and 50 mg/ml (Gti, $P < 0.001$; Hti, $P < 0.01$).

Airway Inflammation and BAL Analysis

We determined the degree of peribronchial inflammation and found a notable increase in inflammatory cells around airways of OA-challenged $Dcn^{+/+}$ mice (Fig. 3B) compared with the $Dcn^{+/+}$ Sal (Fig. 3A) group. OA $Dcn^{-/-}$ airways did not demonstrate any differences in terms of inflammatory infiltrate compared with $Dcn^{-/-}$ Sal mice (Fig. 3, D and C, respectively). Quantitative analysis of the tissue inflammation, expressed as the number of inflammatory cells/WA (Fig. 3E), revealed that the inflammatory cell infiltrate was significantly increased in the OA-exposed $Dcn^{+/+}$ group vs. $Dcn^{+/+}$ Sal ($12.8 \times 10^3 \pm 1.1 \times 10^3$ vs. $7.8 \times 10^3 \pm 5.58 \times 10^2$, $P < 0.001$). In contrast, OA challenge did not induce tissue inflammation in the $Dcn^{-/-}$ mice. Moreover, the amount of inflammation present within the WA of OA $Dcn^{+/+}$ mice was significantly higher than that observed around OA $Dcn^{-/-}$ airways ($12.8 \times 10^3 \pm 1.1 \times 10^3$ vs. $8.0 \times 10^3 \pm 5.2 \times 10^2$, $P < 0.001$).

Fig. 2. Lung physiology measurements evaluating changes in central airway resistance (Raw; A), tissue damping (Gti; B), and tissue elastance (Hti; C) during methacholine (MCh) aerosol challenges in OA-challenged and Sal control decorin (Dcn) $^{+/+}$ and $Dcn^{-/-}$ mice. A: Raw was significantly increased in the $Dcn^{+/+}$ OA group only, compared with Sal-challenged mice. Moreover, the Raw response observed in OA $Dcn^{-/-}$ mice was significantly lower than that of OA-challenged $Dcn^{+/+}$ mice at MCh dose of 25 mg/ml. B and C: Gti and Hti responses were significantly increased in $Dcn^{+/+}$ as well as $Dcn^{-/-}$ OA-exposed mice compared with Sal, although this response was apparent at a lower dose of MCh (25 mg/ml) in the $Dcn^{+/+}$ group. In addition, Gti and Hti responses were significantly higher in $Dcn^{+/+}$ than in $Dcn^{-/-}$ OA groups at MCh of 25 and 50 mg/ml. Values are means ± SE. * $P < 0.05$, OA vs. Sal. *** $P < 0.001$, OA vs. Sal. † $P < 0.05$, $Dcn^{-/-}$ vs. $Dcn^{+/+}$. †† $P < 0.01$, $Dcn^{-/-}$ vs. $Dcn^{+/+}$. ††† $P < 0.001$, $Dcn^{-/-}$ vs. $Dcn^{+/+}$.

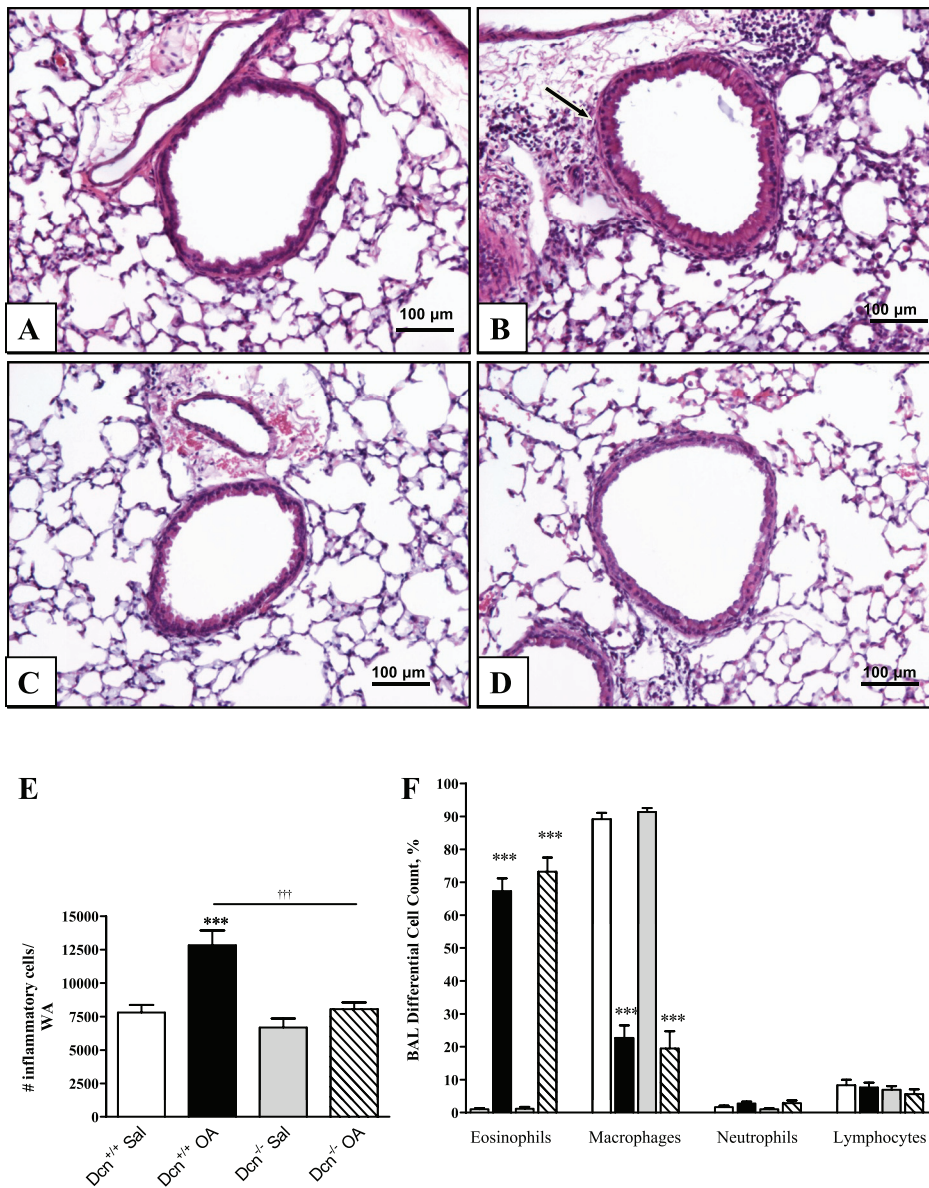


Fig. 3. A–D: photomicrographs depicting tissue inflammation (hematoxylin and eosin). Magnification $\times 100$ μm . A: *Dcn*^{+/+} Sal control. B: *Dcn*^{+/+} OA challenged. C: *Dcn*^{-/-} Sal control. D: *Dcn*^{-/-} OA challenged. Tissue inflammation, determined by the presence of inflammatory cells (as displayed by the arrow), is present in greater amounts in the OA-challenged *Dcn*^{+/+} group (B) compared with Sal control (A). Furthermore, no significant inflammation is seen with OA in *Dcn*^{-/-} (D). E: tissue inflammation quantified in the airway wall as the number of inflammatory cells/wall area (WA) (mm^2). Tissue inflammation was shown to be significantly increased in OA-challenged *Dcn*^{+/+} mice only, compared with *Dcn*^{+/+} Sal mice ($***P < 0.001$). The increase observed in OA-exposed *Dcn*^{+/+} mice was, in addition, significantly higher than the level of tissue inflammation present in the OA *Dcn*^{-/-} group ($\dagger\dagger\dagger P < 0.001$). F: bronchoalveolar lavage (BAL) cell differential. Percentage of eosinophils, macrophages, neutrophils, and lymphocytes determined out of a total of 300 cells counted. OA-challenged mice demonstrated significant increases in the percentage of eosinophil ($***P < 0.001$), along with concomitant decreases in the percentage of macrophages ($***P < 0.001$). No significant changes were observed in the percentage of neutrophils or lymphocytes across all four groups. Values are means $\pm$ SE.

Additional information regarding the relative cellular constituents of the inflammatory cell infiltrate was derived from the quantitative analysis of the BAL cell differential (Fig. 3F). Although tissue inflammation was not increased with OA challenge compared with Sal control in *Dcn*^{-/-} mice (Fig. 3E), the distribution of cells in the BAL did markedly change. Specifically, cells in the BAL of OA-challenged *Dcn*^{-/-} mice were predominantly eosinophils ($73.17 \pm 4.36\%$ in OA vs. $1.17 \pm 0.55\%$ in Sal *Dcn*^{-/-}, $P < 0.001$), similar to what was observed in the *Dcn*^{+/+} group. Along with the significant increase in the percentage of BAL eosinophils, OA challenge resulted in a significant decrease in the percentage of macrophages ($P < 0.001$). Neutrophils and lymphocytes percentages were comparable across all four groups.

Cytokine Analysis

The amount of TH-2 cytokines is shown in Fig. 4. The levels of IL-4, IL-5, and IL-13 in the BAL were not statistically

different among groups (Fig. 4, A–C, respectively). mRNA expression of IL-5 and IL-13 was significantly increased in the lung tissue of OA-challenged *Dcn*^{+/+} mice compared with Sal-challenged *Dcn*^{+/+} mice ($P < 0.05$) (Fig. 4, B and C). There was no difference in the expression of IL-4 between the two groups (Fig. 4A). OA challenge did not induce an increase in the mRNA expression of the TH-2 cytokines in *Dcn*^{-/-} mice.

Remodeling Epithelial Thickness and Area

There were no significant differences in epithelial thickness or epithelial area among the four groups (data not shown).

Goblet Cell Hyperplasia

Representative micrographs showing airway mucus production are seen in Fig. 5, A and B. Positive staining for mucus expression is visualized as dark pink on stained lung tissue sections. Both *Dcn*^{+/+} and *Dcn*^{-/-} groups displayed similar patterns of mucus expression; Sal-challenged groups displayed minimal positive

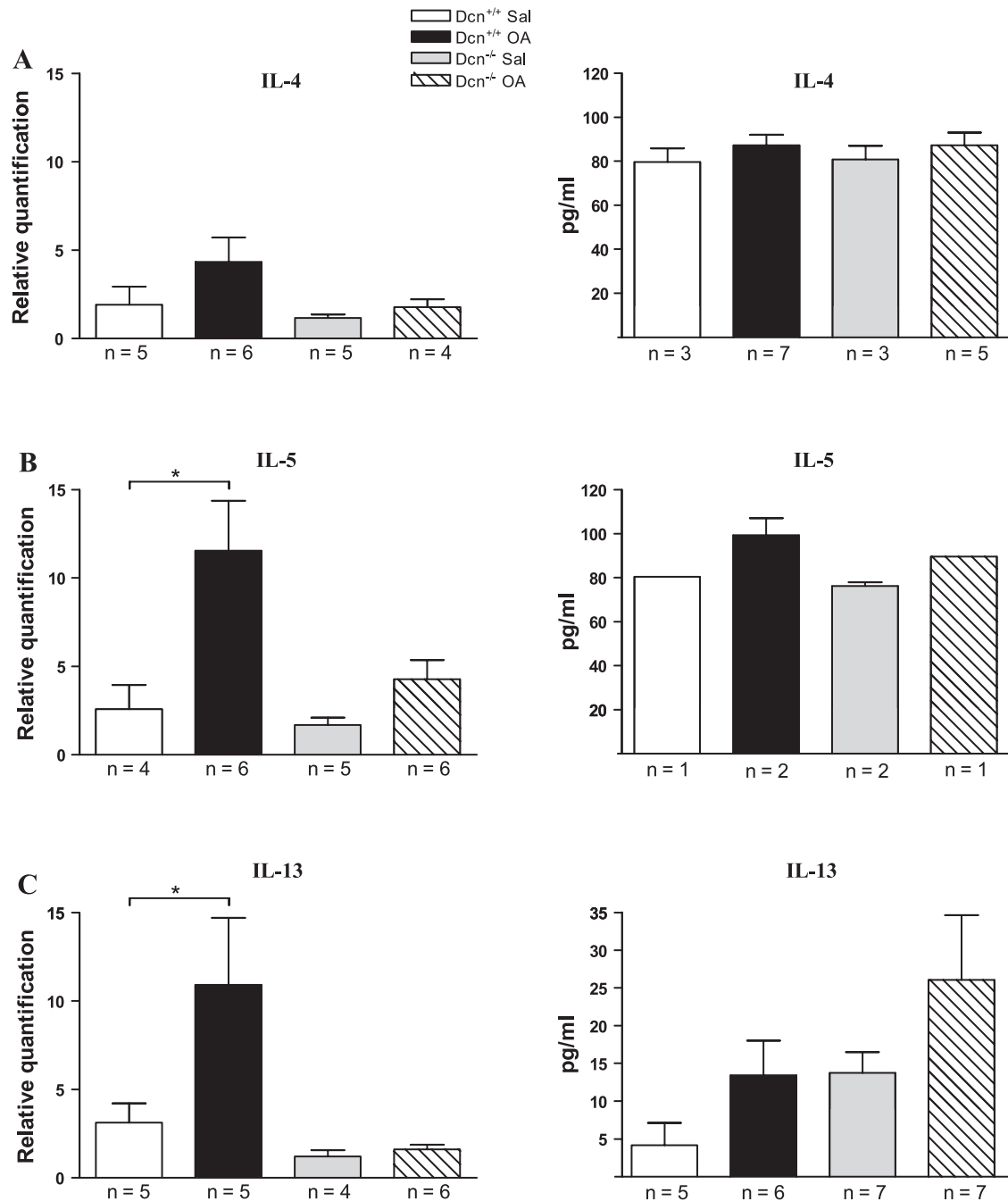


Fig. 4. TH-2 cytokines. Levels of TH-2 cytokines, IL-4 (A), IL-5 (B), and IL-13 (C), of mRNA message were evaluated in lung tissue using quantitative PCR (left), as well as protein levels in BAL, as quantified using ELISA (right). IL-4 mRNA expression (A, left) was not found to be significantly different between the experimental groups. IL-5 (B, left) and IL-13 (C, left) were significantly increased in the lung tissue of OA-challenged $Dcn^{+/+}$ mice compared with Sal-challenged $Dcn^{+/+}$ mice (* $P < 0.05$). The OA challenged did not induce an increase in the mRNA expression of these TH-2 cytokine $Dcn^{-/-}$ mice. IL-4 (A, right) and IL-5 (B, right) BAL levels were not significantly different among groups. C: IL-13 was not significantly different among the four experimental groups. Values are means $\pm$ SE; n, no. of animals.

staining within the epithelial lining of airways, whereas substantial positive staining can be seen with OA challenge. To determine the extent of the increase in mucus production, the area of PAS-positive staining was quantified within the epithelium. Tissue sections stained with PAS demonstrated an increase in mucus expression within the bronchial epithelium of both $Dcn^{+/+}$ and $Dcn^{-/-}$ OA-challenged mice. As shown in Fig. 6A, the area of positive stain was significantly increased in both OA-challenged $Dcn^{+/+}$ and $Dcn^{-/-}$ mice compared with their Sal controls

($Dcn^{+/+}$ 19.21 ± 2.91 vs. $0.43 \pm 0.22\%$; and $Dcn^{-/-}$ 16.69 ± 3.44 vs. $0.187 \pm 0.15\%$, $P < 0.001$). Mucus expression was similar between OA $Dcn^{-/-}$ and $Dcn^{+/+}$ mice.

ASM

Photomicrographs of staining for α -SMA in the airways of $Dcn^{+/+}$ Sal and OA mice are shown in Fig. 5 (C and D, respectively). Smooth muscle is evidenced by the brown-

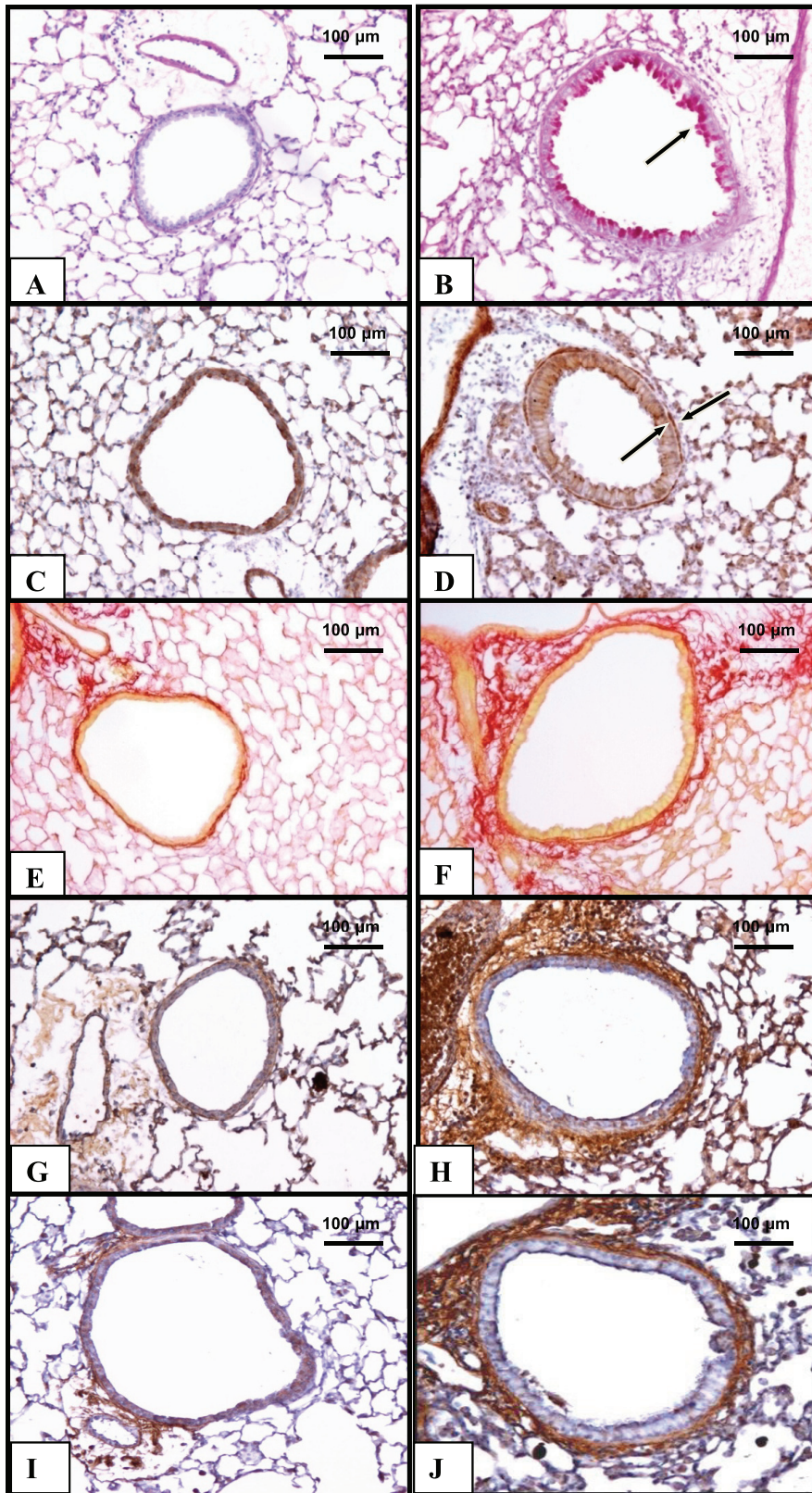
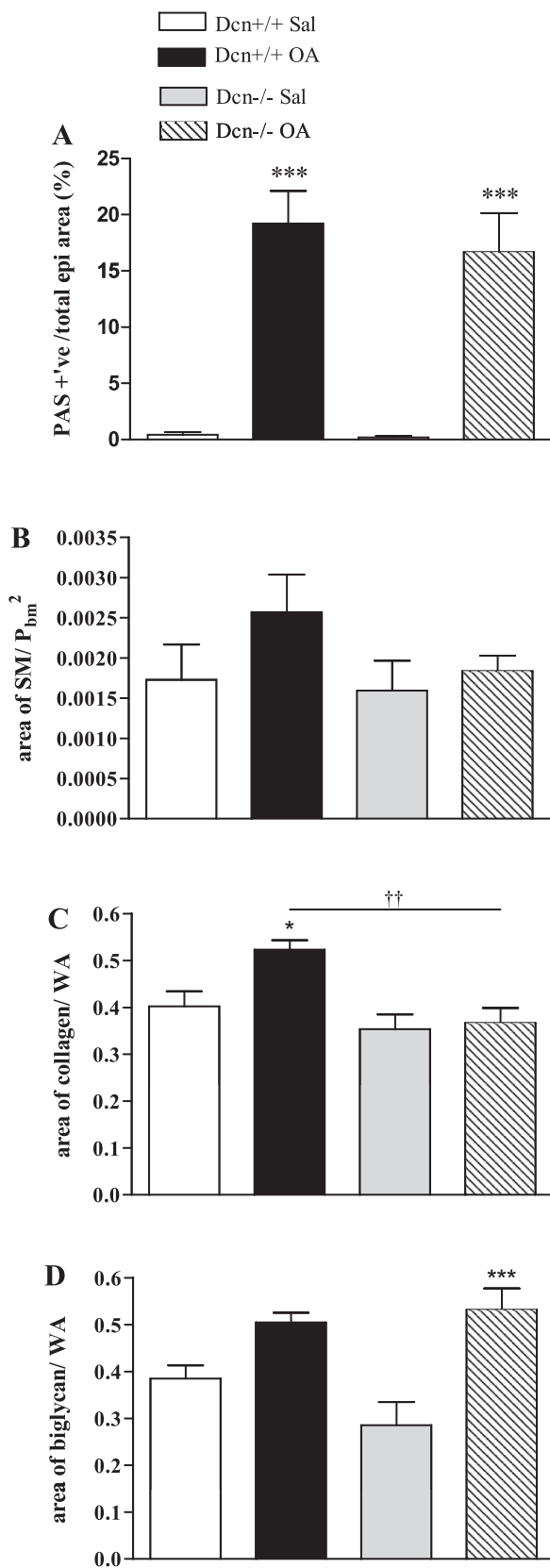


Fig. 5. Representative photomicrographs of histochemical and immunohistochemical staining in the airway wall of Sal-challenged *Dcn*^{+/+} (C, E, and I) and *Dcn*^{-/-} (A and G), as well as OA-challenged *Dcn*^{+/+} (D, F, and J) and *Dcn*^{-/-} (B and H) mice. Staining is shown of airways with periodic acid Schiff (PAS) (A and B), primary antibody for α -smooth muscle actin (α -SMA; C and D), picrosirius red (E and F), primary antibody for biglycan (Bgn) (G and H), and primary antibody for Dcn (I and J). Arrows in B and D identify goblets cells positive for mucus expression and smooth muscle (SM) bundles, respectively. Photomicrographs (E and F) reveal positive staining for collagen fibers shown in red, present around the airways. Positive staining for Bgn, displayed in brown, shows increased Bgn deposition with OA challenge in *Dcn*^{-/-} mice. Dcn-positive stain, also shown in brown, is demonstrated to be increased with OA challenge in *Dcn*^{+/+} mice. Magnification $\times 100$ μ m.



stained bundles surrounding the airways. As depicted in Fig. 6B, ASM mass was not increased with OA in either *Dcn*^{+/+} or *Dcn*^{-/-} mice. There was a modest trend toward an increase in the area of ASM per P_{bm}^2 with OA challenge in *Dcn*^{+/+} mice.

Collagen Deposition

Collagen deposition within the WA of the airways was also evaluated. Figure 5, E and F, shows *Dcn*^{+/+} Sal and OA tissue sections, respectively, stained with picrosirius red, which was used to visualize collagen. Sal control *Dcn*^{+/+} and *Dcn*^{-/-} mice showed relatively similar amounts of collagen staining (Fig. 6C). Collagen deposition was found to be significantly increased in OA-challenged *Dcn*^{+/+} mice compared with *Dcn*^{+/+} Sal controls (0.523 ± 0.02 vs. 0.401 ± 0.032 , $P < 0.05$). Analysis of the positively stained area in OA-challenged *Dcn*^{-/-} tissue sections, on the other hand, showed no increase in collagen deposition compared with Sal controls (0.367 ± 0.032 vs. 0.353 ± 0.032). Of note, the amount of collagen deposition observed in *Dcn*^{+/+} OA-exposed mice was significantly higher compared with that in OA *Dcn*^{-/-} mice ($P < 0.01$; 0.523 ± 0.02 vs. 0.367 ± 0.03).

Bgn and Dcn

Next, we determined the deposition of Bgn, another member of class I SLRPs with high homology to Dcn (Fig. 5, G and H). The amount of Bgn present within the WA was not statistically different in *Dcn*^{+/+} OA-challenged mice with respect to their Sal controls (0.504 ± 0.02 vs. 0.384 ± 0.03). However, Bgn staining was significantly increased with OA challenge in *Dcn*^{-/-} mice (0.532 ± 0.05 vs. 0.285 ± 0.05 , $P < 0.001$) (Fig. 6D).

To determine whether Dcn deposition was altered with OA challenge in *Dcn*^{+/+} mice, immunostaining for this PG was also performed (Fig. 5, I and J). OA challenge significantly increased the amount of Dcn present within the WA of airways in these mice (0.500 ± 0.03 vs. 0.187 ± 0.04 , $P < 0.001$) (data not shown). The absence of Dcn was also verified using immunohistochemical staining on *Dcn*^{-/-} tissue sections (data not shown).

TGF- β_1

Western blot analysis of TGF- β_1 levels in lung tissue homogenates demonstrated that TGF- β_1 was increased with OA in both *Dcn*^{+/+} and *Dcn*^{-/-} mice. While these increases did

Fig. 6. Quantification of airway mucous (PAS), α -SMA, collagen, and Bgn immunostaining. A: mucus expression was significantly increased with OA in both *Dcn*^{+/+} and *Dcn*^{-/-} mice ($***P < 0.001$). B: *Dcn*^{+/+} Sal, *Dcn*^{-/-} Sal, and *Dcn*^{-/-} OA-exposed mice demonstrated approximately the same amount of positive staining for α -SMA, whereas OA-challenged *Dcn*^{+/+} mice exhibited a slightly increased amount of α -SMA staining. C: OA challenge significantly increased collagen deposition around the airways of *Dcn*^{+/+} mice only, compared with *Dcn*^{+/+} Sal controls ($*P < 0.05$). Collagen deposition was not observed to be altered between OA and Sal *Dcn*^{-/-} mice. In addition, the amount of collagen deposition present in OA *Dcn*^{+/+} mice was significantly higher than that present in OA *Dcn*^{-/-} mice ($\dagger\dagger P < 0.01$). D: increased Bgn deposition was observed with OA challenge in both *Dcn*^{+/+} and *Dcn*^{-/-} mice compared with their respective Sal controls. This increase, however, was found to be statistically significant when comparing OA vs. Sal *Dcn*^{-/-} mice only ($***P < 0.001$). Values are means $\pm$ SE. P_{bm}^2 , basement membrane perimeter squared.

not reach statistical significance, this was likely due to large interanimal variation (Fig. 7, A and B). The level of TGF- β_1 in the BAL fluid was decreased in the OA-challenged *Dcn*^{-/-} mice compared with OA-challenged *Dcn*^{+/+} mice ($P < 0.001$) (Fig. 7C). Finally, the mRNA expression of TGF- β_1 was not found to differ among the groups (Fig. 7D).

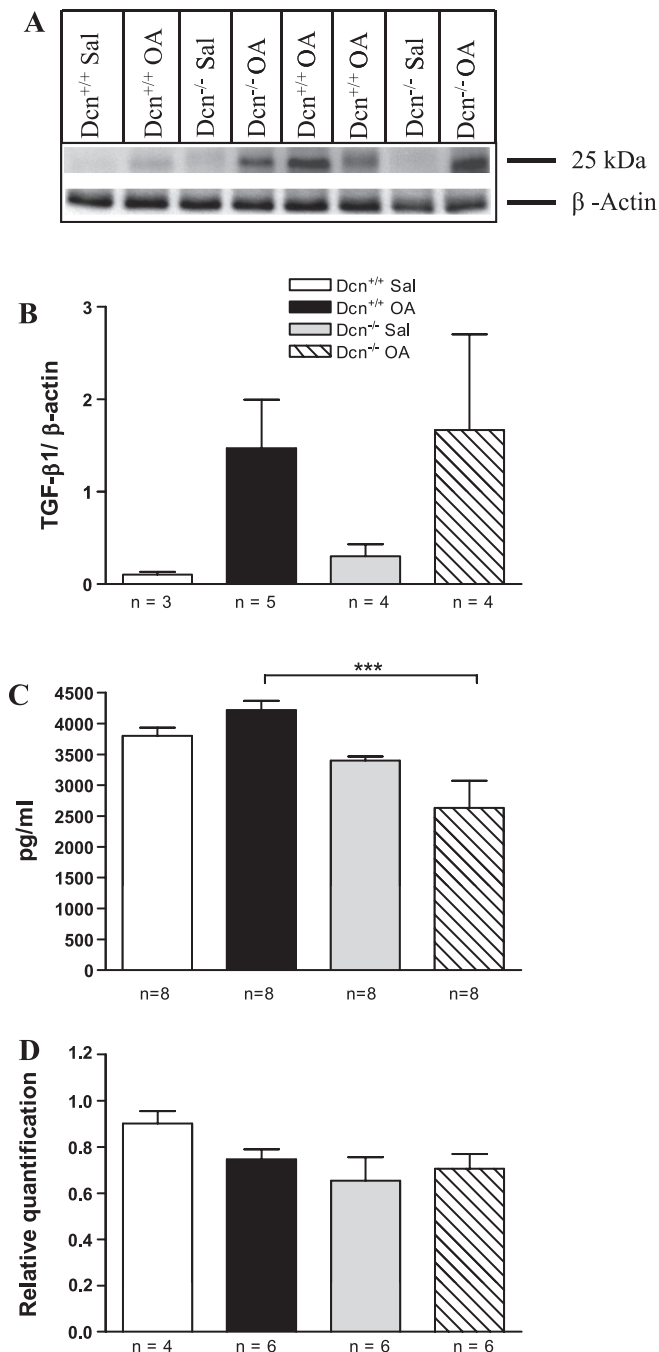


Fig. 7. Transforming growth factor (TGF- β) levels. A: total lung proteins were extracted and analyzed by Western blot using a specific antibody for TGF- β_1 . B: densitometric analysis of Western blot corrected for β -actin levels shows increases in TGF- β_1 in OA-challenged mice vs. Sal controls, although this did not reach statistical significance. C: BAL levels of TGF- β_1 , using ELISA, were decreased in the OA-challenged *Dcn*^{-/-} mice compared with OA-challenged *Dcn*^{+/+} mice ($***P < 0.001$). D: TGF- β_1 mRNA expression in extracted lung tissue, as quantified using quantitative PCR, was not found to be significantly different among the experimental groups. Values are means $\pm$ SE.

DISCUSSION

The main finding of this work is that the development of allergen-induced asthma in C57Bl/6 mice is significantly altered in the absence of Dcn, as evidenced by significant differences in lung mechanics, inflammation, and airway remodeling in OA-challenged *Dcn*^{-/-} compared with OA-challenged *Dcn*^{+/+} mice. In particular, after OA challenge, Dcn-deficient mice demonstrated less hyperresponsiveness than wild-type mice. Furthermore, OA challenge did not induce significant tissue inflammation in *Dcn*^{-/-} mice, nor was TH-2 cytokine message increased. Finally, only in OA-challenged *Dcn*^{+/+} mice was collagen deposition increased. The more modest airway remodeling observed in the *Dcn*^{-/-} animals may be a consequence of the relatively decreased inflammation. These findings are contrary to our initial hypothesis that Dcn deficiency would lead to enhanced allergen-induced airway responsiveness.

The presence of airway remodeling is well recognized as an important feature of asthma (18). There is now considerable data showing alteration of various PGs within the asthmatic airway wall (7, 14, 26). In particular, the SLRP, Dcn, has been shown to be differentially altered in this disease, depending on disease severity. In the present study, we extend these observations to evaluate the functional effects of Dcn in a murine model of allergic asthma.

We applied the complex impedance model to assess airway hyperresponsiveness (AHR) during the MCh-response curve, as this allowed separation of the total response into central (Raw) and distal lung components, Gti and Hti. The latter represents the responses in the distal airways, as well as the parenchymal tissues and, as such, gives a more complete picture of the physiological changes occurring throughout the lung, especially during induced constriction, and better reflects the site of the asthmatic response in human disease (12, 24). During MCh challenge, Gti also reflects small airway constriction, as peripheral airway inhomogeneities and small airway closure become increasingly significant (17, 19). This is of particular relevance in clinical asthma, as this disease is characterized by a heterogeneous constriction of the peripheral airways (11, 20). Thus separation of the lung response into central and peripheral mechanical indexes adds an additional level of understanding into the physiological mechanism contributing to this multifactorial disease.

There are certain technical issues that warrant discussion. The in vivo studies were performed using the Flexivent small-animal ventilator, which has certain mechanical limitations. In partitioning the responsiveness of the airways into central and distal lung components, the power detection threshold of the system made it difficult to obtain representative values for Raw at the highest concentration of MCh (50 mg/ml), because of the degree of bronchoconstriction. As a consequence, the volume signal was no longer equally distributed across the lung, and the assumptions underlying the quantification of Raw using the constant phase model no longer apply. As a result, we obtained negative values for Raw, which were excluded from our analysis.

Lung mechanics have been shown to be influenced by Dcn. Dcn deficiency has been shown to affect Raw, airway and lung compliance, and airway-parenchymal interdependence under baseline conditions (10, 31). In previous studies from this laboratory, *Dcn*^{-/-} mice were shown to exhibit lower baseline Raw compared with *Dcn*^{+/+} mice. In the present study, no differences

in baseline Raw were observed. This may relate to the current protocol of sensitization and repeated challenge before physiological measurement. On the other hand, Hti was significantly lower in OA-challenged *Dcn*^{-/-} vs. OA-challenged *Dcn*^{+/+} mice. These results are consistent with the increased compliance in parenchymal tissues reported by Fust et al. (10). Salerno et al. (31) examined the effect of MCh-induced bronchoconstriction in *Dcn*^{+/+} and *Dcn*^{-/-} mice and showed no differences. We report a similar observation here. Responsiveness of the airways during a MCh-response curve was assessed in both *Dcn*^{-/-} OA-challenged and Sal controls and compared with the responsiveness demonstrated by *Dcn*^{+/+} OA-challenged and Sal mice. OA-challenged *Dcn*^{-/-} mice demonstrated more modest AHR compared with *Dcn*^{+/+} OA-exposed mice. It is possible that the differences in baseline mechanical behavior may have contributed to the current finding of differing mechanical responses to allergen-driven disease. For example, baseline differences in elastance between *Dcn*^{+/+} and *Dcn*^{-/-} mice could give rise to differences in lung volume, which could potentially affect airway diameter and airway-parenchymal interactions during induced constriction. However, there were also substantial differences in the inflammatory response between the *Dcn*-deficient and replete mice, which we believe also play a significant role in the differences in airway responsiveness.

Assessment of inflammation present within the peribronchial area of airways demonstrated that OA challenge significantly increased the inflammatory cell number/WA in wild-type mice. OA challenge did not induce tissue inflammation in *Dcn*^{-/-} mice. Cytokine analysis showed that message for the TH-2 cytokines, IL-5 and IL-13, was increased in OA-challenged *Dcn*^{+/+} mice only. Levels of protein in the BAL were not increased, which may relate to the time point at which the physiological experiments were performed. Protein levels in the BAL may have recovered 3 days after the last OA challenge, whereas message may remain elevated over a longer time frame (15, 32). Analysis of BAL cell differential revealed a similar pattern of cell distribution between *Dcn*^{+/+} and *Dcn*^{-/-} groups. OA challenge significantly increased the percentage of eosinophils in *Dcn*^{-/-} mice compared with Sal controls, as was also observed in *Dcn*^{+/+} mice. The observation that eosinophils were the predominant cells present within the BAL of both OA-exposed *Dcn*^{+/+} and *Dcn*^{-/-} groups may explain the observed increase in PAS-positive staining within the epithelial layer of these airways (3). The role of eosinophils in promoting increased mucus and goblet cell hypersecretion has been demonstrated by Lee et al. (22). Mucus hypersecretion may also play a role in airway obstruction and increases in respiratory resistance, which may partly account for the observed increases in airway responsiveness in *Dcn*^{-/-} OA-exposed vs. Sal mice. However, the more modest response in these animals compared with *Dcn*^{+/+} OA-challenged mice suggests that increased mucus production is not sufficient to drive the entire lung response (35).

The consequence of inflammatory exudate within the peribronchial region also has important implications in the mechanical behavior of the airways themselves. It has been postulated that the presence of edema or inflammation within the adventitial layer will uncouple the airways from the surrounding parenchyma, resulting in a decreased elastic load on ASM (25). The load acting in opposition to ASM shortening, and the way in which this load is altered during constriction of the airways, are critical determinants of airway narrowing (25).

Another factor that may contribute to the more modest hyperresponsiveness in the OA-challenged *Dcn*^{-/-} group may be the reduced degree of airway remodeling. A trend to an increased area of ASM was shown in OA-exposed *Dcn*^{+/+} mice, whereas *Dcn*^{-/-} mice challenged with OA did not exhibit any changes in ASM area compared with Sal controls. In addition, OA *Dcn*^{-/-} mice did not show any increases in the deposition of collagen, which was found to be significantly increased in the *Dcn*^{+/+} OA-exposed mice compared with their respective Sal controls. Wagers et al. (34) have shown that inflammation-induced AHR in the mouse can be explained by epithelial thickening and airway closure alone. However, in our model, we found no evidence of increases in epithelial thickening in either of the OA-challenged groups.

The amount of Bgn was significantly increased with OA in *Dcn*^{-/-} mice only, which suggests a specific upregulation of this PG, which is structurally similar to Dcn. Fust et al. (10) reported no differences in Bgn in *Dcn*^{+/+} and *Dcn*^{-/-} lung tissues from unchallenged mice. Our evidence suggests that exposure to OA in *Dcn*^{-/-} mice may trigger increased Bgn deposition. This may also have important implications in the generation of AHR. Although not evaluated in this study, Pini et al. (27) have shown that Bgn and Dcn are differentially located within the airway wall in a rat model of asthma. Whereas Dcn is observed within the adventitia, Bgn is present primarily within the ASM layer. The presence of increased Bgn within the ASM layer and surrounding the ASM bundles themselves may provide a parallel elastic impedance or radial constraint to ASM shortening and, consequently, a limitation in airway narrowing (25). Furthermore, absence of Dcn within the adventitia may result in a better coupling of the airways and parenchyma. Various investigators (25) have suggested that increased remodeling or thickening of the adventitial layer results in reduced transmission of interdependence forces acting against ASM shortening (25).

Lack of Dcn may also result in more bioavailable TGF- β , a growth factor that has been demonstrated to function as both an anti-inflammatory and/or pro-remodeling molecule, depending on the setting (21). We suggest that the more modest inflammation observed in *Dcn*^{-/-} OA-exposed animals may reflect the relative bioavailability of TGF- β . Alcorn and coworkers (2) demonstrated in a Balb/c model of allergic asthma that the presence of anti-TGF- β antibodies resulted in enhancement of AHR in mice exposed to aerosolized OA. They concluded that TGF- β may have a suppressive role in the development of antigen-induced AHR. Fattouh et al. (9) showed that TGF- β neutralization (using a pan-neutralizing TGF- β antibody) resulted in increased eosinophilic infiltration and enhanced airway responsiveness in a house dust mite-driven allergic model of asthma. Although a different mouse strain and challenge protocol were used compared with that in the study presented here, the evidence regarding the role of TGF- β in modulating AHR may explain the more modest AHR observed in the OA-exposed *Dcn*^{-/-} mice.

In the present study, Western blot analysis showed that TGF- β ₁ levels in extracted lung tissue tended to an increase with OA; however, no differences were found between *Dcn*-deficient and -replete mice. It was not possible to directly determine bioavailable TGF- β , because whole lung tissue homogenates were analyzed. On the other hand, BAL levels of TGF- β were shown to be decreased in *Dcn*^{-/-} OA vs. *Dcn*^{+/+} OA-challenged mice. Finally, no difference in message was detected among the four groups of mice. We did not measure active TGF- β , nor down-

stream signaling of TGF- β , which may be more pertinent indexes to test our hypothesis. On the other hand, other mechanisms may also play a role in the more modest response to allergen challenge in the *Dcn*^{-/-} mice, such as Dcn effects on cell migration (33), or as yet undocumented effects of Dcn on smooth muscle contractility or immunological responsiveness.

In conclusion, this study has demonstrated that lung responsiveness and inflammation, as well as certain indexes of remodeling, were more modest in OA-challenged *Dcn*^{-/-} vs. *Dcn*^{+/+} mice. *Dcn*^{-/-} OA mice exhibited more modest AHR, no significant increase in airway inflammatory infiltrate, nor an enhanced TH-2 cytokine response. Furthermore, these animals did not display increases in airway collagen deposition compared with *Dcn*^{+/+} OA mice. The data in the present study provides important information on the effect of Dcn deficiency in the context of allergic asthma. Further experiments to determine the mechanistic basis of this differential response are warranted.

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DISCLOSURE

No conflicts of interest, financial or otherwise, are declared by the author(s).

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