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

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Marchica CL, Pinelli V, Borges M, Zummer 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 × 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−/− mice (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.

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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 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.
The genetic makeup of the offspring was determined by extracting DNA from a piece of tail tissue. Briefly, 150 µl of lysis buffer solution for tissue digestion containing 1 µl proteinase K/100 µl 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 µl consisted of 1 µl of DNA, 1 µl of sense primer, 1 µl of antisense primer or 1 µl of PGK primer, 5.35 µl of H2O, 2 µl of 10× rxn buffer, 2 µl of MgSO4 (Invitrogen, Ontario, Canada), 0.8 µl of dNTP mix (containing all 4 dNTPs; Ontbio, Ontario, Canada), and 0.06 µl 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). The reaction mix was then purified using the QIAquick PCR Purification Kit (Invitrogen). The reaction conditions comprised the following: melt-min. Samples were then stored at 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.

The sensitization and challenge protocol used in this study is shown in Fig. 1. Antigen sensitization and challenge protocol. The sensitization and challenge protocol used in this study is shown in Fig. 1. Antigen sensitization and challenge protocol. The sensitization and challenge protocol used in this study is shown in Fig. 1. Antigen sensitization and challenge protocol.

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, 22.67 ± 0.88 g; OA, 22.94 ± 0.85 g; Sal-challenged Dcn−/−, 23.72 ± 1.19 g; OA-challenged Dcn−/−, 25.37 ± 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

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<table>
<thead>
<tr>
<th>Sensitization 10 µg OA, 1 mg Al(OH)3</th>
<th>Challenge 10 µg OA in 50 µl of saline</th>
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<tr>
<td>Day 0 14 16 18 days</td>
<td>Day 7 21 23 25 days</td>
</tr>
<tr>
<td>28 30 32 days</td>
<td>35 days</td>
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Supernatant from the first BAL was collected and stored at −80°C. ELISA was performed to measure IL-13 and TGF-β1, according to the manufacturer’s instructions (eBioscience, San Diego, CA) for mRNA analysis. The left lung was inflated with 10% phosphate-buffered formalin at a transpulmonary pressure of 25 cmH2O 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-SM 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 on tissue sections using 0.01 M sodium citrate buffer (pH 6.0).

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 1 Hematoxylin, Sigma Aldrich). All immunostaining was developed using diaminobenzidine (DAKOCTymation, 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-β1 in Lung Tissue**

Homogenates were prepared from whole lung tissue. Western blotting was performed using primary antibody for TGF-β1. 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-β1, 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-β1, 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 ΔΔCt 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) ≥ 0.5. Stained slides were viewed under the Olympus light microscope (Olympus, Ontario, Canada), and images were captured with either the Cool-Snap PROcf 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 (Pbm), and total area of smooth muscle bundles present around the airway (ASM). Results were expressed as the area of smooth muscle divided by Pbm squared (Pbm^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 ± 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 Sal-challenged Dcn−/− mice, indicating increased airway resistance (Raw; Fig. 2A). 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 × 10^3 ± 1.1 × 10^3 vs. 8.0 × 10^3 ± 5.2 × 10^2, P < 0.001).

### Table 1. Baseline respiratory mechanics

<table>
<thead>
<tr>
<th></th>
<th>Dcn+/+ Sal</th>
<th>Dcn+/+ OA</th>
<th>Dcn−/− Sal</th>
<th>Dcn−/− OA</th>
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<tr>
<td>Raw, cmH2O·ml⁻¹·s</td>
<td>0.142 ± 0.015</td>
<td>0.158 ± 0.011</td>
<td>0.184 ± 0.028</td>
<td>0.149 ± 0.016</td>
</tr>
<tr>
<td>Gti, cmH2O/ml</td>
<td>5.57 ± 0.285</td>
<td>5.80 ± 0.400</td>
<td>4.48 ± 0.196</td>
<td>4.77 ± 0.226</td>
</tr>
<tr>
<td>Hti, cmH2O/ml</td>
<td>30.21 ± 1.81</td>
<td>32.89 ± 2.95</td>
<td>22.87 ± 1.98</td>
<td>23.41 ± 1.41*</td>
</tr>
</tbody>
</table>

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 in the 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 × 10^3 ± 1.1 × 10^3 vs. 7.8 × 10^3 ± 5.58 × 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 × 10^3 ± 1.1 × 10^3 vs. 8.0 × 10^3 ± 5.2 × 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 < 0.01, Dcn−/− vs. Dcn+/+. †††P < 0.001, Dcn−/− vs. Dcn+/+. **P < 0.01, Dcn−/− vs. Dcn+/+. †††P < 0.001, Dcn−/− vs. Dcn+/+. 

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Specifically, cells in the BAL of OA-challenged mice demonstrated a significant increase in the percentage of eosinophils compared with Sal control in 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 (†††P < 0.001). No significant changes were observed in the percentage of neutrophils or lymphocytes across all four groups. Values are means ± SE.

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 was 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

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 ± 4.36% in OA vs. 1.17 ± 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.

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

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Representative micrographs showing airway mucus production are seen in Fig. 5, A and B. Positive staining for mucus expression was 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
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 \textit{Dcn}^{+/+} and \textit{Dcn}^{-/-} OA-challenged mice. As shown in Fig. 6A, the area of positive stain was significantly increased in both OA-challenged \textit{Dcn}^{+/+} and \textit{Dcn}^{-/-} mice compared with their Sal controls (\textit{Dcn}^{+/+} 19.21 ± 2.91 vs. 0.43 ± 0.22%; and \textit{Dcn}^{-/-} 16.69 ± 3.44 vs. 0.187 ± 0.15%, \textit{P} < 0.001). Mucus expression was similar between OA \textit{Dcn}^{+/+} and \textit{Dcn}^{-/-} mice.

**ASM**

Photomicrographs of staining for \(\alpha\)-SMA in the airways of \textit{Dcn}^{+/+} Sal and OA mice are shown in Fig. 5 (\textit{C} and \textit{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 \( \text{Dcn}^{+/+} \) (C, E, and I) and \( \text{Dcn}^{-/-} \) (A and G), as well as OA-challenged \( \text{Dcn}^{+/+} \) (D, F, and J) and \( \text{Dcn}^{-/-} \) (B and H) mice. Staining is shown of airways with periodic acid Schiff (PAS) (A and B), primary antibody for \( \alpha \)-smooth muscle actin (\( \alpha \)-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 \( \text{Dcn}^{-/-} \) mice. Dcn-positive stain, also shown in brown, is demonstrated to be increased with OA challenge in \( \text{Dcn}^{+/+} \) mice. Magnification \( \times 100 \, \mu \text{m} \).
stained bundles surrounding the airways. As depicted in Fig. 6B, ASM mass was not increased with OA in either Den+/+ or Den−/− mice. There was a modest trend toward an increase in the area of ASM per Pbm with OA challenge in Den+/+ mice.

Collagen Deposition

Collagen deposition within the WA of the airways was also evaluated. Figure 5, E and F, shows Den+/+ Sal and OA tissue sections, respectively, stained with picrosirius red, which was used to visualize collagen. Sal control Den+/+ and Den−/− mice showed relatively similar amounts of collagen staining (Fig. 6C). Collagen deposition was found to be significantly increased in OA-challenged Den+/+ mice compared with Den+/+ Sal controls (0.523 ± 0.02 vs. 0.401 ± 0.032, \( P < 0.05 \)). Analysis of the positively stained area in OA-challenged Den−/− 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 Den+/+ OA-exposed mice was significantly higher compared with that in OA Den−/− mice (\( P < 0.01; 0.523 ± 0.02 \) vs. 0.367 ± 0.03).

Bgn and Den

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 Den+/+ 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 Den−/− mice (0.532 ± 0.05 vs. 0.285 ± 0.05, \( P < 0.001 \)) (Fig. 6D).

To determine whether Den deposition was altered with OA challenge in Den+/+ mice, immunostaining for this PG was also performed (Fig. 5, I and J). OA challenge significantly increased the amount of Den 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 Den was also verified using immunohistochemical staining on Den−/− tissue sections (data not shown).

TGF-β1

Western blot analysis of TGF-β1 levels in lung tissue homogenates demonstrated that TGF-β1 was increased with OA in both Den+/+ and Den−/− mice. While these increases did

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Fig. 6. Quantification of airway mucous (PAS), α-SMA, collagen, and Bgn immunostaining. A: mucus expression was significantly increased with OA in both Den+/+ and Den−/− mice (***\( P < 0.001 \)). B: Den+/+ Sal, Den−/− Sal, and Den−/− OA-exposed mice demonstrated approximately the same amount of positive staining for α-SMA, whereas OA-challenged Den+/+ mice exhibited a slightly increased amount of α-SMA staining. C: OA challenge significantly increased collagen deposition around the airways of Den+/+ mice only, compared with Den+/+ Sal controls (**\( P < 0.05 \)). Collagen deposition was not observed to be altered between OA and Sal Den−/− mice. In addition, the amount of collagen deposition present in OA Den+/+ mice was significantly higher than that present in OA Den−/− mice (††\( P < 0.01 \)). D: increased Bgn deposition was observed with OA challenge in both Den+/+ and Den−/− mice compared with their respective Sal controls. This increase, however, was found to be statistically significant when comparing OA vs. Sal Den−/− mice only (***\( P < 0.001 \)). Values are means ± SE. Pbm, 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-β1 in the BAL fluid was decreased in the OA-challenged Den−/− mice compared with OA-challenged Den+/+ mice (P < 0.001) (Fig. 7C). Finally, the mRNA expression of TGF-β1 was not found to differ among the groups (Fig. 7D).

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 Den−/− compared with OA-challenged Den+/+ 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 Den−/− mice, nor was TH-2 cytokine message increased. Finally, only in OA-challenged Den+/+ mice was collagen deposition increased. The more modest airway remodeling observed in the Den−/− 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, Den−/− mice were shown to exhibit lower baseline Raw compared with Den+/+ 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 Den−/− vs. OA-challenged Den+/+ 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 Den+/+ and Den−/− mice and showed no differences. We report a similar observation here. Responsiveness of the airways during a MCh-response curve was assessed in both Den−/− OA-challenged and Sal controls and compared with the responsiveness demonstrated by Den+/+ OA-challenged and Sal mice. OA-challenged Den−/− mice demonstrated more modest AHR compared with Den+/+ 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 Den+/+ and Den−/− 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 Den-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 Den−/− mice. Cytokine analysis showed that message for the TH-2 cytokines, IL-5 and IL-13, was increased in OA-challenged Den+/+ 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 Den+/+ and Den−/− groups. OA challenge significantly increased the percentage of eosinophils in Den−/− mice compared with Sal controls, as was also observed in Den+/+ mice. The observation that eosinophils were the predominant cells present within the BAL of both OA-exposed Den+/+ and Den−/− 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 Den−/− OA-exposed vs. Sal mice. However, the more modest response in these animals compared with Den+/+ 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 Den−/− group may be the reduced degree of airway remodeling. A trend to an increased area of ASM was shown in OA-exposed Den+/+ mice, whereas Den−/− mice challenged with OA did not exhibit any changes in ASM area compared with Sal controls. In addition, OA Den−/− mice did not show any increases in the deposition of collagen, which was found to be significantly increased in the Den+/+ 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 Den−/− 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 Den+/+ and Den−/− lung tissues from unchallenged mice. Our evidence suggests that exposure to OA in Den−/− 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 Den−/− 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 eosinophil 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 Den−/− mice.

In the present study, Western blot analysis showed that TGF-β1 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 Den−/− OA vs. Den+/+ 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.

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

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