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_J Immunol_ 2011; 187:6108-6119; Prepublished online 31 October 2011; doi: 10.4049/jimmunol.1100373

[http://www.jimmunol.org/content/187/11/6108](http://www.jimmunol.org/content/187/11/6108)
The Role for Decorin in Delayed-Type Hypersensitivity

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Decorin, a small leucine-rich proteoglycan, regulates extracellular matrix organization, growth factor-mediated signaling, and cell growth. Because decorin may directly modulate immune responses, we investigated its role in a mouse model of contact allergy (oxazolone-mediated delayed-type hypersensitivity [DTH]) in decorin-deficient (Dcn−/−) and wild-type mice. Dcn−/− mice showed a reduced ear swelling 24 h after oxazolone treatment with a concurrent attenuation of leukocyte infiltration. These findings were corroborated by reduced glucose metabolism, as determined by 18fluordeoxyglucose uptake in positron emission tomography scans. Unexpectedly, polymorphonuclear leukocyte numbers in Dcn−/− blood vessels were significantly increased and accompanied by large numbers of flattened leukocytes adherent to the endothelium. Intravital microscopy and flow chamber and static adhesion assays confirmed increased adhesion and reduced transmigration of Dcn−/− leukocytes. Circulating blood neutrophil numbers were significantly increased in Dcn−/− mice 24 h after DTH elicitation, but they were only moderately increased in wild-type mice. Expression of the proinflammatory cytokine TNF-α was reduced, whereas syndecan-1 and ICAM-1 were overexpressed in inflamed ears of Dcn−/− mice, indicating that these adhesion molecules could be responsible for increased leukocyte adhesion. Decorin treatment of endothelial cells increased tyrosine phosphorylation and reduced syndecan-1 expression. Notably, absence of syndecan-1 in a genetic background lacking decorin rescued the attenuated DTH phenotype of Dcn−/− mice. Collectively, these results implicated a role for decorin in mediating DTH responses by influencing polymorphonuclear leukocyte attachment to the endothelium. This occurs via two nonmutually exclusive mechanisms that involve a direct antiadhesive effect on polymorphonuclear leukocytes and a negative regulation of ICAM-1 and syndecan-1 expression. The Journal of Immunology, 2011, 187: 6108–6119.

Delayed-type hypersensitivity (DTH) is a common mouse model for allergic contact dermatitis (1), which allows the study of cell-mediated immune responses in vivo (2). The sensitization phase is characterized by covalent modification of surface proteins with the hapten oxazolone, followed by the uptake and processing by dendritic and Langerhans cells that migrate to lymph nodes and prime oxazolone-specific T cell populations (1, 2). During the elicitation phase, oxazolone exposure evokes recruitment and activation of primed T cells, followed by synthesis and local release of chemokines and cytokines, as well as mast cell degranulation. The release of vasoactive mediators leads to a massive leukocytic infiltration of the skin, a process that depends on cytokine gradients, and the synthesis and activation of adhesion molecules of the integrin, selectin, and cell adhesion molecule families (1, 2).

During inflammation, polymorphonuclear leukocytes follow chemotactic gradients to attach to activated endothelial cells, resulting in leukocyte diapedesis, penetration of the subendothelial matrix, and migration into areas of tissue damage (3, 4). This process involves coordinated signaling events mediated by proinflammatory cytokines and chemokines, as well as sequential interactions with multiple adhesion molecules, including selectins and their carbohydrate ligands, and integrins (3, 4). All of these steps are modulated by various types of proteoglycans (4, 5). Biochemical data demonstrated sequence-specific interactions of glycosaminoglycans with a variety of ligands relevant to inflammation (6). For example, mice deficient in syndecan-1 (Sdc1−/−) show increased leukocyte recruitment upon i.p. TNF-α stimulation, as well as during kidney inflammation, contact allergies, and colitis (7–9). Although a role for heparan sulfate in inflammation is increasingly recognized, the role of dermatan/chondroitin sulfate is less well investigated. Several observations indicated that dermatan sulfate can interact with various cytokines and chemokines (10), including fibroblast growth factor family members (11), and heparin cofactor II (6). Moreover, injection of mice with dermatan sulfate increases the soluble levels of circulating ICAM-1 (12). From a clinical perspective, the use of dermatan sulfate may be advantageous in comparison with heparin, because of the
lack of anticoagulant side effects. The most prominent dermatan sulfate proteoglycan in the skin is decorin, which is involved in collagen fibrillogenesis and extracellular matrix organization (13–15). Decorin also acts as a key signaling molecule that can modulate the activity of several tyrosine kinase receptors (16–18) and integrins (19). Furthermore, decorin inhibits growth of different tumor cell types in vitro (20) and in vivo (21), via interactions with the epithelial growth factor receptor. In vitro, endothelial cells synthesize decorin under inflammatory conditions (22) and Den−/− mice (23) show a delayed wound healing with enhanced blood vessel formation (24). In tubulointerstitial kidney fibrosis, decorin deficiency enhances apoptosis and increases inflammation (25). Based on these findings and considering the structural homology of heparan and dermatan sulfate, we hypothesized that decorin could play a role in modulating contact allergy. Thus, we investigated the role of decorin in delayed-type inflammation using an in vivo model of contact allergy and in vitro models of leukocyte recruitment like intravital microscopy and flow chamber assays on P-selectin, ICAM-1, and CXCL-1. To our knowledge, our results showed for the first time that decorin is expressed by polymorphonuclear leukocytes and mononuclear cells and that it influences the expression of adhesion molecules like ICAM-1 and SDC1. Combined with the antiadhesive properties of decorin, this regulation of adhesion molecules promotes leukocyte extravasation into the tissue.

Materials and Methods

Decorin-null mice and decorin/syndecan-1 double-deficient mice

Decorin-deficient mice (Dcn−/−) (23) and syndecan-1-deficient mice (Sdc1−/−) (26) were bred in the animal facility in accordance with the German Animal Protection Act and approved by the responsible Ethics Review Committee. Decorin/syndecan-1 double-deficient mice (Dcn−/−/Sdc1−/−) were generated by breeding and genotyped by genomic PCR, as previously described (23, 26).

DTH assay

DTH was carried out with 8–12-wk-old male Den−/− mice and the respective controls, as described previously (8). Briefly, mice were sensitized on abdominal shaved skin with 150 μl 2.5% oxazolone (Sigma, Deisenhofen, Germany) dissolved in acetone/ethanol (3:1 [vol/vol]). Mice were challenged with a Mitutoyo engineer’s micrometer, immediately before challenge, as controls were subjected to DTH and investigated 24 h later. Animals were excluded 3 mice because they did not respond to the oxazolone treatment. (Inveon, Siemens Medical Solutions), and a medium-resolution (25 μm/2 μm) CT acquisition was performed for each mouse. PET data were reconstructed into a single-image volume for each mouse, with a voxel size of 0.4 × 0.4 × 0.4 mm³. CT was reconstructed into a volume data set with a voxel size of 0.007 × 0.007 × 0.007 mm³. Image data sets were coregistered using extrinsic markers attached to the multimodal scanning bed and the image analysis software (Inveon Research Workplace, Siemens Medical Solutions). For quantification of regional FDG uptake, the outer ears were segmented three dimensionally using CT data, and the resulting volume of interest was applied to the coregistered PET data set. Measured data were corrected for partial volume effects, scatter, and background using a model-based approach. The FDG uptake was calculated as the percentage of injected dose per volume (%ID/ml) for each ear, and the net uptake in the inflamed left ear (∆%ID/ml) was calculated as (%ID/ml right ear) − (%ID/ml left ear).

Decorin purification

Decorin was purified from conditioned cell culture medium of human fibroblasts by anion-exchange chromatography and analyzed for purity on a silver gel, as described previously (27, 28).

Adhesion assay of polymorphonuclear leukocytes on endothelial cells

The murine endothelial cell line bEnd.3 was used for static leukocyte-adhesion assays, as previously described (9). About 20,000 endothelial cells/96-well plate were cultured overnight, followed by treatment with 5 nM TNF-α to stimulate endothelial cells, or were used directly for the experiment. Under these conditions, TNF-α treatment did not result in increased cell cytotoxicity (data not shown). Polymorphonuclear leukocytes of wild-type and Den−/− mice were prepared from bone marrow of tibias and femurs, as previously described (8). A total of 2 × 10⁵ cells/ml polymorphonuclear leukocytes in PBS/1% FCS was incubated with 1 μM fluorescent marker 2′,7-bis-(2-carboxylethyl)-5-carboxyfluorescein ace-toxyethyl ester (Molecular Probes, Eugene, OR) in DMSO for 20 min at 37°C. Equal labeling efficiency was controlled using standard curves. Labeled samples were centrifuged at 1500 rpm for 5 min before being resuspended in medium. Polymorphonuclear leukocytes were treated at 37°C for 30 min with 5 μg/ml decorin and the respective solvent controls. Thereafter, endothelial cells were incubated with pretreated polymorphonuclear leukocytes (2 × 10⁵/ml, 50 μl/well) for 10 min at 37°C. Wells were washed two times with PBS, and adherent polymorphonuclear leukocytes were lysed with lysis buffer (10 mM Tris/HCl, 0.1% SDS [w/v] pH 7.4) for 10 min on ice. The fluorescent intensity was quantified in a Spectrostar HR (Molecular Probes), excitation (485 nm; emission, 535 nm). The adhesion was reported as the percentage of adherent cells/mm². The results were expressed as mean ± SEM.

Histology and immunohistochemistry

Paraffin-embedded ears were cut in 5-μm sections, and every 20–30th section was stained with H&E. Sections were analyzed for polymorphonuclear leukocyte distribution in the tissue and in the blood vessels. The blood vessel size was also evaluated for wild-type and Den−/− mice. Free, round, flattened, and transmigrated polymorphonuclear leukocytes were analyzed by scoring adapted from Bixel et al. (29). Briefly, free or non-adherent and adherent polymorphonuclear leukocytes/10⁵ μm² of blood vessel surface area were evaluated by light microscopy with a color view soft imaging system (Sis, Münster, Germany). Furthermore, extravasated polymorphonuclear leukocytes/10⁵ μm² of inflamed tissue surface in wild-type and Den−/− were counted.

For immunohistochemistry, after rehydration the PFA-fixed sections were blocked with 10% BSA for 30 min at room temperature, following incubation with either rat anti-mouse syndecan-1 mAb (BD Biosciences, Franklin Lakes, NJ) or rat anti-mouse ICAM-1 mAb (BioLegend, Uithoorn, The Netherlands), both 1/100 with PBS containing 1% BSA, overnight at 4°C. The primary antibody was visualized with biotinylated Ab (Vectastain ABC, Vector Labs, Burlingame, MA), diluted 1/200 in 1% BSA/PBS, and visualized with diaminobenzidine reagent (Dako) and counterstained with hematoxylin. Sections were analyzed with an Olympus photomicroscope.

For immunofluorescence staining, the tissue was embedded in Tissue Tek OCT compound (Sakura Finetek, Tokyo, Japan). Five-micron tissue sections were fixed with ethanol for 10 min at −20°C, blocked with 1% BSA (Serva, Heidelberg, Germany) for 30 min at room temperature, followed by incubation with rat anti-mouse CD4 (clone L3T4; BD Pharmingen, Heidelberg, Germany), anti-mouse CD8 (clone 53–6;–7; BD Pharmingen, Heidelberg, Germany), rat-anti human F4/80 (Abcam,
Cambridge, U.K.), or rabbit-anti human decorin antiserum LF-113 (kindly provided by Dr. Larry Fisher, National Institutes of Health, Bethesda, MD) for 1 h at room temperature. Positive cells were detected using Cy3-labeled donkey anti-rat IgG (H+L) (Dianova, Hamburg, Germany) or Alexa Fluor 488-labeled goat-anti rabbit IgG (Invitrogen, Karlsruhe, Germany).

**Autoperfused flow chamber**

To investigate neutrophil arrest, we used a previously published flow chamber system (30, 31). Briefly, rectangular glass capillaries (20×200 μm, VitroCom) were coated with P-selectin (40 μg/ml), ICAM-1 (25 μg/ml), and CXC1L1 (25 μg/ml; all from R&D Systems) for 2 h and blocked for 1 h with casein (Thermo Fisher Scientific). The chamber was connected at one side to a PE 50 tubing and used to control the wall shear stress. The other side of the chamber was connected to a syringe filled with isolated PMNs resuspended in PBS containing 1 mM Mg2+ and 1 mM Ca2+. We perfused each chamber with PMNs for 6 min before we recorded representative fields of view for 30 s using an SW40/0.75 objective (Axio Scope, Carl Zeiss).

**Intravital microscopy**

We prepared cremaster muscle for intravital microscopy, as described previously (31, 32), and investigated leukocyte rolling flux fraction, rolling velocity, adhesion, and extravasation in postcapillary venules (20–40 μm diameter) 4 h after an intrascrotal injection of 50 ng IL-1β.

Reverse transcription and quantitative TaqMan real-time PCR

After euthanasia of the animals, ears were excised and snap-frozen in liquid nitrogen, followed by preparation of total RNA using the RNeasy kit (Qiagen, Hilden, Germany). One microgram of total RNA was transcribed using the First Strand cDNA Synthesis Kit (Fermentas, St. Leon Rot, Germany). To detect Den, Sdc1, and Gapdh expression, conventional PCR was used with primer pairs for Den-sense 5’-CCT GTC GGC ACA AGT CTC TGG G-3’, Den-antisense 5’-TCG AAC ATG ACA CTG CTA CGC TG-3’ (24), Sdc1-sense 5’-GAC TCT GAC AAC TTC TCT GGC TCT-3’, Sdc1-antisense 5’-GCT GTG GTG ACT CTG ACT GTT G-3’ and Gapdh, cDNA corresponding to 25 ng total RNA was used as a template in the PCR consisting of Applied Biosystems MasterMix and predesigned TaqMan gene-expression systems (Applied Biosystems, Darmstadt, Germany), according to the manufacturer’s instructions. For detection of Sdc1, Tgfα, and Icam1 mRNA, primers Mm00448918_m1 (Sdc1 exons 2 and 3), Mm00443258_m1 (Tgfα exons 1 and 2), and Mm00516023_m1 (Icam1 exons 2 and 3) were used and normalized to the expression of mammalian 18S rRNA (Hs99999901_s1; all primers from Applied Biosystems). Quantitative real-time PCR was performed with an Applied Biosystems PRISM 7300 Sequence Detection System using the default thermal cycling conditions (10 min at 95˚C and 40 cycles of 15 s at 95˚C plus 1 min at 60˚C). Relative quantification was performed using the comparative cycle threshold method (33). Three to five biological replicates were used for each time point investigated. The Mann–Whitney U test was used for statistical analysis. A p value < 0.05 was considered statistically significant.

**FIGURE 1.** Reduced DTH reactivity in Den−/− mice compared with wild-type mice. Left ears of male wild-type and Den−/− mice were challenged with oxazolone. Right ears were treated with vehicle alone. A, Swelling of the left ears is expressed as the increase over baseline thickness of control ears. Den−/− mice showed significantly less swelling at 24 h after challenge (n = 45 [24 h]; n = 50 [48 h]; n = 15 [72 h]). Data are expressed as mean ± SEM. B, To visualize the reduced DTH reaction in three dimensions, combined PET/CT scans were performed 24 h after challenge to allow detection of maximal differences in metabolic activity between Den−/− and control animals. Arrows indicate the areas of interest in the horizontal images, and arrowheads indicate the areas of interest in the transaxial image. C, Quantification of 18FDG uptake of the treated ears showed a significant reduction in metabolic activity in Den−/− mice (n = 9; 0.85 ± 0.35 %ID/ml) compared with wild-type mice (n = 9, 1.53 ± 0.58 %ID/ml, p = 0.008). Data are expressed as mean ± SEM. D through G, Histological examination of oxazolone-induced DTH in the ears of wild-type and Den−/− mice. D, Quantification of infiltrated leukocytes in ear tissue shows reduced infiltration in Den−/− ears (n = 5 ears and 100 sections were quantified). E, Left panels, Representative histology (H&E) of wild-type and Den−/− ear section after 24 h of DTH. Scale bars, 50 μm. Den−/− ears have fewer leukocytes 24 h after treatment compared with wild-type ears. Right panels, Representative H&E staining of untreated wild-type and Den−/− ears. Scale bar, 200 μm. F, Quantification of leukocytes in blood vessels over the DTH time course reveals more leukocytes in Den−/− mice 24 h after challenge. ****p < 0.001, n = 5. Data are expressed as mean ± SEM. G, Representative histology (H&E staining) of wild-type and Den−/− blood vessels in ear sections after 24 h of DTH. Scale bar, 50 μm. ***p < 0.001.
Protein extraction, ELISA, and immunoblotting

For protein extraction, excised ears were snap-frozen in liquid nitrogen and homogenized, as described previously (8). Briefly, ears were homogenized on ice with 500 μl PBS containing 10 mM EDTA and a mixture of protease inhibitors. Samples were centrifuged for 10 min at 12,000 × g, and supernatant was collected. Total protein concentration was quantified by BCA-Lowry assay (Pierce, Rockford, IL). Protein extracts were used for ELISA or Western blotting. All protein samples were diluted to 1.5 mg/ml keratinocyte chemotactant (KC) or 1 mg/ml TNF-α, and the tissue concentrations of KC and TNF-α immunosassays were determined exactly as described by the manufacturer (R&D Systems, Wiesbaden, Germany). For Western blotting, ∼40 μg protein extracts of ears derived from DTH experiments or of bEnd.3 cells subjected to 24 h of TNF-α or decorin (5 μg/ml) stimulation were loaded on a 12% SDS-gel under nonreducing conditions. After blotting, the nitrocellulose membrane was blocked with 5% milk in TBST. The membrane was incubated with ICAM-1 Ab rat anti-mouse clone YN1/1.7.4 (BioLegend) or mouse anti P-tyrosine (P-Tyr; Cell Signaling) at 4˚C overnight. After washing the sections, HRP-labeled secondary anti-rat (Pierce, Rockland, PA) or anti-mouse (Calbiochem) Abs were used to detect ICAM-1 or P-tyrosine, respectively. Decorin was detected analogously following digestion of tissue extracts with Chondroitinase ABC (Seikagaku, Kogyo, Japan) for 2 h at 37˚C, using a polyclonal antiserum kindly provided by Dr. Larry Fisher, and HRP-labeled goat anti-rabbit IgG (Calbiochem) as a secondary Ab. The dot-blots for decorin were performed, as previously described (34), and analyzed densitometrically using ImageJ software (National Institutes of Health).

Statistical analysis

Statistical evaluation was performed with GraphPad Prism. If not mentioned, we used the Student t test, and p < 0.05 was considered significant.

Results

Decorin deficiency attenuates edema formation and leukocyte recruitment in oxazolone-mediated DTH

To study the role of decorin in a contact allergy model, we used oxazolone as a hapten and followed ear swelling as a readout of inflammation-induced edema formation for up to 72 h after challenging the mice. Dcn−/− mice showed a suppressed response to oxazolone, ∼25% less swelling, compared with wild-type (p < 0.001, Fig. 1A). Next, inflammatory activity of the ears was assessed noninvasively by PET/CT scanning, a strategy that allows direct visualization and quantification of tissue-metabolic activity by administration of a radioactive sugar, 18FDG, as surrogate marker for cellular glucose metabolism (35). We found that metabolism was reduced in Dcn−/− ears (Fig. 1B). Quantification of 18FDG uptake in the treated ears of different animals, normalized on the signal of the contralateral ear, showed that wild-type mice had an uptake of 18FDG of 1.53 ± 0.58%ID/ml. In contrast, Dcn−/–
mice showed a reduced tracer accumulation of 0.85 ± 0.35%ID/ml, equivalent to an ~40% reduction in metabolic activity (p < 0.001, Fig. 1C). These values are in good agreement with the measured reduction in ear swelling in Den−/− mice vis-à-vis wild-type mice. Additionally, ear volume was identified by CT scanning, confirming the results obtained by caliper measurements (data not shown).

Next, we examined leukocyte infiltration, because invasion of leukocytes into the tissue is a pivotal step during DTH (1, 36). Quantification of total tissue leukocytes showed that, at 24 h postchallenge, Den−/− mice contained significantly less compared with wild-type mice (p < 0.001, Fig. 1D, 1E). The untreated control and Den−/− mouse ear tissues showed no difference (Fig. 1D, 1E, right panels). Histological examination of the treated ears revealed a different leukocyte distribution within the tissue of wild-type and Den−/− mice, which was particularly prominent in blood vessels (Fig. 1F). At 24 h after oxazolone treatment, the blood vessels of Den−/− treated ears contained significantly more leukocytes compared with wild-type ones (Fig. 1F, 1G). Analysis of the blood vessel size revealed no differences (data not shown). In contrast, 48 h after elicitation, leukocyte numbers in the blood vessels of Den−/− mice had increased significantly compared with wild-type mice (Fig. 1F), and leukocyte recruitment into the tissue resumed, suggesting a delayed leukocyte infiltration following DTH elicitation in the decorin-null background.

Den−/− leukocytes show increased adherence to the endothelial cell surface in vivo

The previous results raised the question about which mechanisms would prevent leukocytes from leaving the blood vessel lumen during inflammation in the absence of decorin. Therefore, the tissue sections were scored with respect to free luminal and attached and transmigrated leukocytes (29). Quantification of 35 sections from wild-type and Den−/− ear sections revealed no significant difference in the number of free leukocytes in the blood vessels (Fig. 2A–C). However, we found a significant increase in the number of flattened leukocytes in Den−/− ears (Fig. 2C, p < 0.001, n = 5). Although Den−/− leukocytes adhered more frequently to the endothelial cells, the number of transmigrated leukocytes decreased compared with wild-type ones 24 h after DTH elicitation (Fig. 2C). To investigate the molecular mechanism of this observation, we performed intravital microscopy of the cremaster muscle using chimeric mice reconstituted with wild-type or Den−/− bone marrow cells. We observed no differences in the rolling velocity of the leukocytes from both populations (data not shown), but the rolling flux fraction was increased in chimeric mice reconstituted with bone marrow from Den−/− mice (Fig. 2D, p < 0.05). Furthermore, these chimeric mice showed more adherent and transmigrated leukocytes compared with chimeric mice reconstituted with wild-type bone marrow leukocytes (Fig. 2E, 2F; p < 0.05). Moreover, detailed analysis of leukocyte subsets extravasated into the tissue was performed (Supplemental Fig. 1). Upon measuring peroxidase activity as a readout of neutrophil activity in ear protein extracts (8), no differences between genotypes were observed 24 h after DTH elicitation; however, after 48 h, a significant increase was observed in Den−/− mouse ears compared with wild-type ones (Supplemental Fig. 1A, 1B). F4/80 staining revealed no difference in macrophage numbers in the dermis between wild-type and Den−/− treated ears (Supplemental Fig. 1C, 1D). The immune fluorescence staining revealed that the numbers of CD4+ leukocytes were not differently affected in wild-type and Den−/− treated ears during DTH (Supplemental Fig. 1E, 1F). Similarly, the number of CD8+ cells was not significantly changed 24 h after oxazolone treatment (Supplemental Fig. 1G, 1H). Analysis of blood leukocyte numbers did not reveal a significant difference between Den−/− and wild-type mice (data not shown), as well as no changes in the differential blood analysis (Fig. 2G; n = 4). Interestingly, 24 h after oxazolone treatment, the number of neutrophils increased significantly in Den−/− mice compared with wild-type mice, whereas the number of lymphocytes decreased significantly (Fig. 2H, n = 5; p < 0.05).

ICAM-1 and Sdc1 are differentially expressed in Den−/− mice during DTH

Extravasation is a complex process, which requires sequential steps of leukocyte adhesion to endothelial cells followed by release of

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**FIGURE 3.** ICAM-1 expression is differentially altered in wild-type and Den−/− mice during DTH activity. **A.** Real-time PCR for ICAM-1 expression during the time course of DTH (n = 3–5). The p value was not statistically significant. **B and C.** Increased Icam1 expression in Den−/− mouse tissues is confirmed by Western blotting 24 h after DTH induction. **B.** Representative Western blot. Ponceau S staining was used as loading control (lower panel). **C.** Densitometric analysis (n = 4). *p = 0.039. **D.** Western blot analysis of ICAM-1 expression in plasma of wild-type and Den−/− mice prior to and 24 h after DTH elicitation; 20 μg of protein/lane was analyzed. GAPDH was used as a loading control.
leukocytes. The cell surface adhesion molecules ICAM-1 and SDC1 are known to be involved in the multistep leukocyte-adhesion cascade (3, 4). Therefore, expression of ICAM-1 was evaluated by quantitative real-time PCR (Fig. 3A, n = 3–5). No significant differences in ICAM-1 mRNA expression were observed between wild-type and Dcn−/− mice. In unstimulated control ears, ICAM-1 protein was not detectable by immunohistochemistry for either genotype; however, 24 h after elicitation, Dcn−/− ears expressed more ICAM-1 protein (Supplemental Fig. 2). This observation was confirmed by Western blotting; although ICAM-1 expression increased in wild-type mice during the DTH response, as expected, oxazolone-treated ears of Dcn−/− mice showed an even stronger increase compared with wild-type mice (Fig. 3B, 3C). ICAM-1 levels in the plasma were also increased in Dcn−/− mice compared with wild-type mice (Fig. 3D).

Syndecan-1 is an adhesion molecule with anti-inflammatory properties (9), which was recently identified as a novel modulator of DTH responses (8). Analysis of Sdc1 mRNA expression showed that the basal level in Dcn−/− mice was ∼2-fold higher than in wild-type ears (Fig. 4A, n = 3–5, p < 0.05). In oxazolone-treated ears of Dcn−/− mice, the expression of Sdc1 mRNA was significantly increased after 24 h compared with wild-type ears (Fig. 4A, n = 3–5; p < 0.05). In accordance with previous reports (4, 37, 38), Sdc1 is highly expressed in the epidermis. In addition, leukocytes were labeled for SDC1 in Dcn−/− and wild-type mice (Fig. 4B, arrows, Fig. 4C). Within the time course of DTH, at 24 h, Dcn−/− ears showed a significantly increased amount of SDC1+ leukocytes compared with wild-type ears (Fig. 4C, n = 5; p < 0.001). Forty-eight hours after oxazolone application, most of the SDC1 signal disappeared in Dcn−/− and wild-type leukocytes (data not shown). Thus, differentially increased expression of the adhesion molecules Icam1 and Sdc1 during the time course of DTH may contribute to the increased leukocyte numbers in Dcn−/− blood vessels.

Cytokine expression is dysregulated in Dcn−/− mice during DTH

KC (CXCL-1) is an early response gene expressed by keratinocytes, monocytes, and macrophages. CXCL-1 expression is upregulated by cutaneous contact with allergens (39), and its synthesis can be induced by the proinflammatory cytokine TNF-α in keratinocytes (40) and leukocytes (41) and during DTH in mice (42). ELISA data showed that KC was upregulated during the time course of DTH, peaking at 24 h after DTH. Although Dcn−/− mice showed a lesser degree of upregulation compared with wild-type controls, this difference was not significant (Fig. 5A). Furthermore, we analyzed Ccl2 mRNA expression and obtained a similar increase for both genotypes (Supplemental Fig. 3A). Moreover, lymph nodes were analyzed for the occurrence of CD4+ and CD8+ cells by flow cytometry (Supplemental Fig. 3B, 3C). After sensitization, the amount of CD4+ and CD8+ cells was not significantly different in wild-type and Dcn−/− lymph nodes (Supplemental Fig. 3B, 3C). In contrast, basal TNF-α levels in Dcn−/− mice were significantly lower compared with wild-type controls (Fig. 5B).

Dcn−/− polymorphonuclear leukocytes show increased adhesion to endothelial cells

Using RT-PCR, we initially determined that decorin was expressed by both polymorphonuclear leukocytes and monocytes isolated from wild-type mice bone marrow but not by the correspond-

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Sdc-1 expression is differentially altered in wild-type and Dcn−/− mice during DTH activity. A, Quantitative real-time PCR analysis of Sdc1 mRNA expression in ears of wild-type and Dcn−/− mice during the time course of DTH. Sdc1 expression is significantly upregulated in Dcn−/− mice relative to wild-type mice under inflammatory conditions (n = 3–6). B, SDC1 expression was confirmed by staining of ear sections with a specific Ab for syndecan-1. Scale bar, 100 μm. C, Quantification of SDC1+ leukocytes shows a significant difference between wild-type and Dcn−/− mice 24 h after elicitation (n = 5). *p < 0.05, **p < 0.001.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Differential expression of TNF-α and KC/CXCL11 in ear tissue of Dcn−/− mice compared with wild-type mice 24–72 h after DTH induction. A, ELISA of KC/CXCL1 levels in treated and untreated ear tissues of Dcn−/− and wild-type mice 24, 48, and 72 h after DTH induction. Data are mean ± SEM (nwild-type = 3; nDcn−/− = 4), each in triplicates. The p value was not statistically significant. B, ELISA of TNF-α level in ear tissues of Dcn−/− and wild-type mice after DTH induction. Data are mean ± SD (nwild-type = 3; nDcn−/− = 4). *p < 0.05.
ing decorin-deficient cells (Fig. 6A). We then performed static-adhesion experiments with fluorescently labeled polymorphonuclear leukocytes and murine bEnd.3 endothelial cells (43), with or without TNF-α stimulation. In line with its antiadhesive effect, decorin proteoglycan purified from skin fibroblasts blocked adhesion of wild-type neutrophils to the unstimulated endothelial cells (Fig. 6B, n = 10). Interestingly, the Dcn−/− polymorphonuclear leukocytes did not respond to the decorin treatment (Fig. 6B, p < 0.001, versus wild-type).

During DTH, endothelial cells are activated, leading to the expression of adhesion molecules and chemokines promoting leukocyte recruitment to the endothelium (3, 4). To simulate inflammatory conditions in vitro, bEnd.3 endothelial cells were incubated for 16 h with recombinant murine TNF-α (5 nM), followed by adhesion measurements. Under TNF-α-stimulated conditions, adhesion of both wild-type and Dcn−/− polymorphonuclear leukocytes to endothelial cells was higher compared with unstimulated conditions (Fig. 6B, 6C). Of note, Dcn−/− polymorphonuclear leukocytes showed a significantly increased adhesion to TNF-α–activated bEnd.3 cells (p < 0.05) (Fig. 6C). Addition of exogenous decorin did not result in an inhibitory effect, suggesting a differential modulation of adhesion ligands in unstimulated and TNF-α–activated endothelium. These results could be confirmed under flow conditions (Fig. 6D, p < 0.05). The adhesion of Dcn−/− cells to the coated ligands ICAM-1, P-selectin, and CXCL-1 was significantly increased compared with wild-type leukocytes.

Sdc1 deficiency rescues the DTH phenotype of Dcn−/− mice

The results described above suggested that Sdc1 expression could directly contribute to the attenuated DTH phenotype of Dcn−/− mice. To this end, we generated Dcn−/−/Sdc1−/− double-knockout mice by mating. Dcn−/−/Sdc1−/− mice were healthy and viable and showed no overt developmental phenotype. The 8–10-wk-old male Dcn−/−/Sdc1−/− mice had a similar weight (23.2 ± 1.2 g) to the wild-type (21.7 ± 1.4 g) and the single knock-out mice (n = 8). Genomic PCR showed no Dcn band at 162 bp for Dcn−/− and Dcn−/−/Sdc1−/− mice, but a knock-out band was visible at 250 bp (Fig. 7A). The Sdc1 genomic PCR showed the 450-bp knock-out signal for the Sdc1−/− and Dcn−/−/Sdc1−/− mice and the wild-type signal at 250 bp (Fig. 7B). The absence of DCN and SDC1 protein expression in Dcn−/−/Sdc1−/− mice was confirmed by Western blotting (Fig. 7C) and immunohistochemistry (Fig. 7D, 7E), respectively. When we performed another set of DTH experiments, the results showed that the ear swelling phenotype was completely rescued in Dcn−/−/Sdc1−/− mice, thereby validating our results described in the preceding sections (Fig. 7F). Real-time PCR (Fig. 7G) and Western blot analysis (Fig. 7H, 7I) revealed that ICAM-1 expression levels in Dcn−/−/Sdc1−/− mice were comparable to wild-type mice and significantly decreased relative to Dcn−/− mice. In untreated control ears, TNF-α protein expression, as determined by ELISA, was significantly lower in Dcn−/− and Dcn−/−/Sdc1−/− mice compared with wild-type mice (Fig. 7J). Under DTH conditions (24 h), TNF-α expression was decreased in Dcn−/−/Sdc1−/− mice compared with wild-type mice (n = 4, p = 0.0571), closely resembling the response of Dcn−/− mice (Fig. 7J). Interestingly, the adhesion under flow conditions revealed a significant increase for Dcn−/− and Dcn−/−/Sdc1−/− leukocytes compared with wild-type ones (Fig. 6D).

Effect of decorin treatment on endothelial cells

It is well established that decorin can function as a signaling molecule (14, 18) (e.g., by interacting with various tyrosine kinase

FIGURE 6. Polymorphonuclear leukocytes and mononuclear cells express decorin and show increased adhesion to endothelial cells in the absence of decorin. A, RT-PCR analysis confirms expression of Den mRNA in wild-type bone marrow mononuclear cells (upper panel) and polymorphonuclear leukocytes (middle panel) (n = 3). Dcn−/− (n = 2) mononuclear cells and polymorphonuclear leukocytes did not express decorin. M, 100-bp ladder; neg., negative control with no cDNA. B, Static adhesion experiments of polymorphonuclear leukocytes to unstimulated bEnd.3 endothelial cells. The adhesion of wild-type polymorphonuclear leukocytes was significantly inhibited by exogenous human decorin (5 μg/ml), whereas decorin treatment had no effect on Den−− leukocyte adhesion. Data represent the mean ± SEM of five experiments. C, Adhesion of polymorphonuclear leukocytes purified from Dcn−/− or wild-type mice bone marrow to TNF-α–stimulated endothelial cells. Dcn−/− polymorphonuclear leukocytes show significantly increased adhesion compared with wild-type. Data represent the mean ± SEM of five experiments. D, Adhesion assay under flow condition of wild-type, Dcn−/−, and Dcn−/−/Sdc1−/− cells to the coated ligands ICAM-1, P-selectin, and CXCL-1. Adhesion is significantly increased for Dcn−/− leukocytes compared with wild-type ones. The leukocytes of Dcn−/−/Sdc1−/− cells showed a further increased adhesion compared with Dcn−/− cells. Data represent the mean ± SEM (n = 3). *p < 0.05, ***p < 0.001.
FIGURE 7. Absence of Sdc1 rescues the anti-inflammatory phenotype of Dcn\(^{-/}\) mice. A and B, Genotyping of Dcn\(^{-/}\)/Sdc1\(^{-/}\) knockout mice. A, Genomic PCR shows no Dcn signal (162 bp) for Dcn\(^{-/}\) and Dcn\(^{-/}\)/Sdc1\(^{-/}\) mice, but it reveals the respective knock-out signal of 250 bp. B, Genomic PCR shows no Sdc1 signal (250 bp) for Sdc1\(^{-/}\) and Dcn\(^{-/}\)/Sdc1\(^{-/}\) mice, but it reveals the respective knock-out signal of 450 bp. C, Western blotting reveals the expression of DCN in extracts of wild-type (WT) and Sdc1\(^{-/}\) mouse ear tissue and its absence in Dcn\(^{-/}\) and Dcn\(^{-/}\)/Sdc1\(^{-/}\) mouse ear tissue. D, Immunohistochemistry demonstrates the presence of SDC1 expression in the epithelial layer of wild-type mouse ears and the absence of SDC1 expression in Dcn\(^{-/}\)/Sdc1\(^{-/}\) mouse ears (original magnification \(\times 20\)). E, Immunofluorescence microscopy demonstrates the presence of DCN expression in the interstitial matrix of wild-type mouse ears, and absence of DCN expression in Dcn\(^{-/}\)/Sdc1\(^{-/}\) mouse ears (original magnification \(\times 20\)). F, Dcn\(^{-/}\)/Sdc1\(^{-/}\), wild-type, and Dcn\(^{-/}\) mice were used in a DTH assay, as described in Figure 1, to evaluate the contribution of Sdc1 to the phenotype of Dcn\(^{-/}\) mice. Dcn\(^{-/}\) mice show a reduced response to DTH compared with wild-type mice (compare with Fig. 1). Dcn\(^{-/}\)/Sdc1\(^{-/}\) mice respond (Figure legend continues)
receptors, such as EGFR, the insulin-like growth factor receptor I, and c-Met (16, 18, 19). Thus, we determined the effects of exogenous decorin and TNF-α treatment on overall tyrosine phosphorylation in bEnd.3 endothelial cells. Both decorin and TNF-α significantly increased generalized Tyr phosphorylation (Fig. 8A). Furthermore, TNF-α–evoked phosphorylation was significantly potentiated by decorin (Fig. 8B, n = 3, p < 0.01). Quantitative real-time PCR analysis revealed that Sdc1 mRNA expression was significantly reduced by TNF-α and decorin treatment (Fig. 8C, n = 3, p < 0.01) but showed no additive effect for TNF-α + decorin treatment. Dot-blot analysis confirmed significant down-regulation of DSC1 protein expression by decorin treatment in TNF-α–stimulated bEnd.3 cells (Fig. 8D, n = 3, p < 0.05). In contrast to the observed transcriptional downregulation, TNF-α mono treatment did not result in significantly altered SDC1 protein levels (Fig. 8D).

Discussion

Decorin is a well-established regulator of matrix assembly and growth factor activity (14, 44). The function of decorin is diverse and exerts both pro- and antiinflammatory conditions, depending on the experimental system (14, 23, 24, 44). In this study, we expanded the spectrum of decorin functions to the field of allergic skin inflammation, establishing a novel role in leukocyte recruitment during DTH responses. Successful sensitization and upregulated early cytokine expression (Ccl2) showed that decorin-deficient mice responded to the allergen. However, compared with wild-type mice, lack of decorin resulted in an attenuated edema and metabolic activity at the site of inflammation, concomitant with a reduction in polymorphonuclear leukocytes transmigrating into the inflamed tissue. Histological studies showed that the reduced leukocyte diapedesis in Dcn−/− mice was associated with increased numbers of flattened, adherent polymorphonuclear leukocytes in the blood vessels, as well as an increased percentage of circulating polymorphonuclear leukocytes. Intravital microscopy experiments demonstrated that chimeric mice reconstituted with Dcn−/− bone marrow cells displayed an increased leukocyte adhesion and a reduced number of transmigrated leukocytes. Similarly, in vitro, adhesion of Dcn−/− polymorphonuclear leukocytes to endothelial cells was increased compared with wild-type ones, and wild-type adhesion could be inhibited by addition of human decorin. Overall, our data suggested that decorin directly modulates leukocyte adhesion to the endothelium and affects diapedesis of leukocytes into the inflamed tissue during DTH. Because the in vivo adhesion phenotype could be replicated in vitro using Dcn−/− polymorphonuclear leukocytes, part of the phenotype appears to depend on decorin expression by polymorphonuclear leukocytes, in accordance with the antiadhesive properties of decorin (45). Our flow chamber assays identified increased adhesion of Dcn−/− polymorphonuclear leukocytes to ICAM-1, P-Selectin, and CXCL-1 as relevant ligands for this process. Interestingly, polymorphonuclear leukocytes synthesized decorin but obviously not to a sufficient degree to block adhesion completely as we demonstrated with exogenous decorin. Increased numbers of circulating polymorphonuclear cells in Dcn−/− mice may be indicative of a lack of inhibitory signals on leukocyte recruitment triggered by successful diapedesis, which is inhibited at the 24-h time point.

Under fibrotic conditions, genetic deficiency of decorin results in an increased inflammatory reaction, likely mediated by the compensatory upregulation of biglycan (25), a known proinflammatory proteoglycan that acts by activating TLR4 (46). In our model, biglycan expression did not appreciably change in either wild-type or decorin-null–treated ears (data not shown), indicating that there are tissue-specific changes. Notably, biglycan can stimulate the synthesis of TNF-α and MIP-2 (46), whereas TNF-α was reduced in our Dcn−/− model. Therefore, we concluded that biglycan is not compensating for decorin under contact allergy conditions at the analyzed time points. Moreover, our findings are supported by a recent study on the role of decorin in a mouse model of allergen-induced asthma: Marchica et al. (47) demonstrated reduced allergic inflammation in Dcn−/− mice relative to wild-type mice, which could partially be attributed to differentially reduced amounts of TGF-β in the bronchoalveolar lavage of the allergen-stimulated decorin-deficient mice.

The resolution of inflammation depends on the localization and activation of lymphocytes and macrophages. Apparently, altered macrophage recruitment was not the cause of the reduced DTH response in Dcn−/− mice. In contrast, it was shown that lumican, another member of the small leucine-rich proteoglycan family, is necessary for peritoneal polymorphonuclear leukocytes to extravasate into inflamed tissue in a β2 integrin-dependent manner (48).

Increased adhesion of Dcn−/− polymorphonuclear leukocytes resembles previous observations in Dcn−/− embryonic fibroblasts, for which an involvement of increased α2β1 integrin on the cell surface was demonstrated (49, 50). Similarly, an increased expression of adhesion molecules was observed in the current study, because the expression of the endothelial cell adhesion molecule ICAM-1, and of DSC1, a novel player in DTH regulation and leukocyte recruitment (8), was upregulated in Dcn−/− mice vis-à-vis wild-type mice. Of note, dermatan sulfate, the major carbohydrate moiety of skin-derived decorin, was shown to be involved in the induction of soluble ICAM-1 in an animal model (12). Similarly, under DTH conditions, endothelial cells expressed ICAM-1, which increased during the course of DTH. In decorin-deficient mice, differential ICAM-1 upregulation during DTH may be a potential compensatory mechanism activated in an attempt to increase the amount of polymorphonuclear leukocytes available for diapedesis. Dressler et al. (51) showed that ICAM-1 is increased in endothelial cells near inflammation infiltration during wound healing. Importantly, our data identified ICAM-1 as a relevant ligand for the increased recruitment of Dcn−/− neutrophils compared with wild-type ones. However, in the absence of decorin, leukocyte diapedesis, rather than adhesion, appears to be inhibited, suggesting that decorin also modulates downstream steps of the leukocyte-adhesion cascade (3, 4). Although speculative at this point, the strong increase in soluble ICAM-1 in the serum of Dcn−/− mice may be linked to this phenomenon, because it was shown that soluble ICAM-1 depletion from serum results in increased leukocyte diapedesis in vitro (52).

to oxazolone similarly to wild-type mice. Compared with Dcn−/− mice, the response is significantly different, indicating that Dcn−/− mice contributed to the DTH response in vivo (n = 5). G, Quantitative real-time PCR analysis for ICAM-1 expression in ear tissue of wild-type, Dcn−/−, and Dcn−/−/Sdc1−/− mice 24 h after DTH elicitation. Data represent the mean ± SEM (n ≥ 3). H and I, Western blot analysis of ICAM-1 expression in ear tissue of wild-type, Dcn−/−, Sdc1−/−, and Dcn−/−/Sdc1−/− mice at 24 h after DTH elicitation. H, Semi-quantitative densitometric analysis of ICAM-1 normalized to total protein expression. Data represent the mean ± SEM (n ≥ 4). I, Representative Western blot. J, TNF-α ELISA of ear tissue extracts of wild-type, Dcn−/−, and Dcn−/−/Sdc1−/− mice at 24 h after DTH elicitation. Data represent the mean ± SEM (n ≥ 4). *p < 0.05, **p < 0.01.
Part of the phenotype reported in this study could be linked to the increased expression of SDC1 in \( \text{Den}^{-/-} \) mice, because we recently demonstrated increased DTH reactions in \( \text{Sdc1}^{-/-} \) mice (8). Both \( \text{Sdc1}^{-/-} \) and \( \text{Den}^{-/-} \) polymorphonuclear leukocytes show increased adhesion to endothelial cells in vitro. However, additional mechanisms, such as the coreceptor role of the heparan sulfate chains of SDC1 in chemokine signaling (4, 5), or the proposed effect of SDC1 on edema formation during inflammation (reviewed in Ref. 4) may be of relevance in this context. ICAM-1 expression was similar to wild-type levels in an \( \text{Sdc1}^{-/-} \) background, suggesting a potential mechanistic contribution of SDC1 to upregulated ICAM-1 expression in the absence of decorin. An important contribution of SDC1 to the inflammatory phenotype of \( \text{Den}^{-/-} \) mice is further suggested by the observation that genetic ablation of \( \text{Sdc1} \) in the \( \text{Den}^{-/-} \) background efficiently rescued the ear swelling phenotype of \( \text{Den}^{-/-} \) mice during DTH at the 24-h time point. Because both \( \text{Sdc1} \) and \( \text{Den}^{-/-} \) deficiency promote leukocyte adhesion to endothelium and ICAM-1, reduced leukocyte recruitment does not appear to be the mechanism behind this finding. However, our intravital microscopy data demonstrated that, although \( \text{Den}^{-/-} \) showed increased leukocyte adhesion, leukocyte transmigration was significantly inhibited. In contrast, a previous study showed that \( \text{Sdc1}^{-/-} \) mice exhibit both increased leukocyte adhesion and transmigration during intravital microscopy (37). Therefore, our data strongly suggested that the absence of \( \text{Den} \) on the leukocytes plays a pivotal role in reducing allergic inflammation during DTH and that the absence of \( \text{Sdc1} \) relieves the block on diapedesis imposed on \( \text{Den}^{-/-} \) leukocytes. Diapedesis phenotypes similar to our findings have been reported in the case of a loss of PECAM-1 or CD99 function (reviewed in Ref. 53). In fact, impaired diapedesis, combined with a lack of feedback regulation indicating the presence of leukocytes in the inflamed tissue, may be a reason for the increased circulating leukocyte numbers in \( \text{Den}^{-/-} \) mice, despite leukocyte hyperadhesiveness. \( \text{Sdc1}^{-/-} \) derived heparan sulfate chains may, in fact, bind to PECAM-1, possibly at a heparin-binding domain located between the IgG-like domains 2 and 3 (54) and, thereby, may contribute to endothelial cell–endothelial cell integrity (55). Further work will be required to clarify the exact role of glycosaminoglycans in PECAM-1 function. Finally, the differential downregulation of TNF-\( \alpha \) expression observed in \( \text{Den}^{-/-} \) mice was also present in an \( \text{Sdc1}^{-/-} \) background, suggesting that decorin may either act upstream of SDC1 or that its effect on TNF-\( \alpha \) expression may be independent from SDC1.

\( \text{Sdc1} \) is expressed in polymorphonuclear leukocytes and mononuclear cells, indicating that it might play a role in adhesion of these cell types. Consistent with the observed upregulation of \( \text{Sdc1} \) in \( \text{Den}^{-/-} \) mice, \( \text{bEnd.3} \) cells showed decreased expression of \( \text{Sdc1} \) mRNA and protein upon decorin treatment. Of note, decorin treatment of \( \text{bEnd.3} \) cells resulted in an activation of general tyrosine phosphorylation, which was enhanced synergistically by TNF-\( \alpha \) treatment. These findings are in accordance with the established role for decorin as a modulator of receptor tyrosine kinase activation (16–18) and open up an additional mechanistic level of decorin function in contact allergic reactions. Although our bone marrow transplantation and in vitro adhesion assays suggested a major contribution of decorin-deficient leukocytes to the DTH phenotype, its influence on endothelial receptor tyrosine kinase signaling suggests that an endothelial contribution cannot be fully excluded. TNF-\( \alpha \) treatment leads to significant downregulation of \( \text{Sdc1} \) mRNA expression, whereas its protein expression was not significantly altered. We can only speculate whether \( \text{Sdc1} \) expression is subject to posttranscriptional regulation in our experimental system, similar to previous reports on cAMP-dependent modulation of SDC1 levels (56).

In addition to altered adhesion molecule expression, cytokine expression was dysregulated during DTH in \( \text{Den}^{-/-} \) mice. Ex-
pression of proinflammatory TNF-α was reduced in Den−/− ears 24 h after DTH elicitation. Cellular adhesion molecules and C-X-C chemokines, such as KC/CXCL-1 and MIP-2, regulate tissue leukocyte accumulation in a multitude of inflammatory states. However, although TNF-α induces KC synthesis in keratinocytes during DTH reactions (40, 42), the reduction in KC expression in leukocyte accumulation in a multitude of inflammatory states. 24 h after DTH elicitation. Cellular adhesion molecules and C-ICAM-1 and anti-inflammatory SDC1, as well as exerts a protec-

Acknowledgments

We thank Margrit Bahl, Anne Forberg, Birgit Pers, and Christine Bätz for expert technical assistance; Dr. Deirdre Coombe for discussions; and Dr. Larry Fisher for the generous gift of decorin antiserum.

Disclosures

The authors have no financial conflicts of interest.

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Figure S1

**Leukocyte subtypes in oxazolone-treated ear tissue during DTH.** A, quantification of neutrophils on HE-stained ear section indicates no difference between Wild-type and Dcn⁻/⁻ mice in tissue and blood vessels. B, Same result is reported by peroxidase activity assay in treated ears as marker for granulocytes. We determined peroxidase activity of protein samples as previously described (Goka AK, Farthing MJ. The use of 3,3',5,5'-tetramethylbenzidine as a peroxidase substrate in microplate enzyme-linked immunosorbent assay. J Immunoassay. 1987,8:29-41). Briefly, 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) was used as a sensitive chromogen substrate for peroxidase. For equal protein loading, a bicinchoninic acid protein assay kit (Pierce) was used to calculate protein concentration in each sample. 15 µg of sample and 100 µl of TMB solution were added to a microtiter plate to start the reaction. After 20 min incubation at room temperature the reaction was stopped with 50 µl 2M H₂SO₄. Absorbance at 450 nm was measured as indicator for peroxidase activity. Each OD was normalized to the OD value of untreated tissue samples of wild-type. 24 h after DTH induction no difference in peroxidase activity is detectable between Wild-type and Dcn⁻/⁻ mice. Whereas after 48 h a significant increased peroxidase activity, respectively neutrophil infiltration, is visible in Dcn⁻/⁻ mice; n > 4, * p < 0.05.

C, representative fluorescence staining for the macrophage marker F4/80 on paraffin sections of treated ears 24 h after DTH induction. F4/80⁺ cells are shown in red and nuclei in blue. Positive cells were visualized with a primary rat anti-mouse F4/80 antibody (CI:A3-1), secondary antibody donkey anti-rat IgG (H+L)-Cy3 and counted per HPF [20x]. Quantification D shows no difference of infiltrated macrophages between Wild-type and Dcn⁻/⁻ mice; n = 3. E, fluorescence staining on frozen ear sections of Wild-type and Dcn⁻/⁻ mice for CD4⁺ cells (rat anti-mouse CD4; RM4-5) confirm no change of CD4⁺ cell infiltration 24 h after DTH induction (F); n = 3. G, CD8α⁺ staining (rat anti-mouse CD8α; Ly-2) of frozen ear sections shows a non significantly decreased infiltration of CD8α⁺ cells into treated ear tissue of Dcn⁻/⁻ mice compared to wild-type (H) 24 h after induction; n = 3. All fluorescence pictures shown at the same magnification, scale bar = 50 µm.

Figure S2

**ICAM-1 staining of tissue sections.** Paraffin embedded ears were cut in 5-µm sections and subjected to immunohistochemistry. Wild-type and Dcn⁻/⁻ treated and untreated ears were analysed. After rehydration the PFA-fixed sections were blocked with 10% BSA for 30 minutes at room temperature, following incubation with rat anti-mouse ICAM-1 monoclonal antibody (Biolegend, Uithoorn, NL) diluted 1/100 with PBS containing 1% BSA over night at 4°C; omission of the primary antibody served as a negative control. A secondary peroxidase conjugated affinity purified anti-Rat IgG (Dako, Glostrup, DK) diluted 1/1000 with PBS containing 1% BSA, anti-rabbit IgG (Amersham, Braunschweig, Germany) was applied for 30 minutes at room temperature. After washing with PBS, sections were incubated for 5 minutes with DAB reagent (Dako) and counterstained with hematoxylin. Sections were analyzed with an Olympus photomicroscope. ICAM-1 protein in unstimulated control ears was not detectable by immunohistochemistry for both genotypes. However, 24 h after elicitation Dcn⁻/⁻
ears express more ICAM-1 protein (n=5 mice, compare also to immunoblotting results in main manuscript).

Figure S3

**Sensibilisation of mice and analysis of T cells by FACS.** A, qRT-PCR analysis time course for the expression of the cytokine Ccl2. The results show that 24h after oxazolone treatment Ccl2 expression in Wild-type and Dcn⁻/⁻ ears is increased, indicating an immune response to the allergen in both genotypes. B, C, 8-12 weeks old C57Bl/6 and Dcn⁻/⁻ mice were sensitized by epicutaneous treatment with 150 µl 2.5% oxazolone (Sigma-Aldrich) dissolved in acetone/ethanol (3:1 v/v) on the shaved abdomen (Kharabi Masouleh B et al. J Immunol. 2009; 182:4985-93). 5 days after sensitisation skin-draining (inguinal, axillary and brachial) lymph nodes were removed, passed through cell strainers (70 µm, BD Biosciences) in PBS, pH 7.4, containing 2% FCS. Cells were counted and 10⁵ cells were stained 30 min on ice with anti-CD4-FITC (clone YTS 191.1.2) and anti-CD8a-PE (clone YTS 169.4) (EuroBioSciences) (Cobbold SP et al. Nature. 1984, 312:548-51). Samples were acquired using a FACS Calibur flow cytometer and analyzed with CellQuest software (BD Biosciences). There are no significant differences in the distribution of CD4⁺ and CD8⁺ cells in naïve or sensibilised lymph nodes.