

Role of the Heparan Sulfate Proteoglycan Syndecan-1 (CD138) in Delayed-Type Hypersensitivity¹

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The cell surface heparan sulfate proteoglycan syndecan-1 (CD138) modulates the activity of chemokines, cytokines, integrins, and other adhesion molecules which play important roles in the regulation of inflammation. We have previously shown that syndecan-1-deficient murine leukocytes display increased interactions with endothelial cells and increased diapedesis in vivo and in vitro. In this study, we demonstrate that syndecan-1 has an important function as a negative modulator in the murine contact allergy model of oxazolone-mediated delayed-type hypersensitivity (DTH). Following elicitation of the DTH response, syndecan-1-deficient mice showed an increase in leukocyte recruitment, resulting in an increased and prolonged edema formation. Expression of the cytokines TNF- α and IL-6 of the chemokines CCL5/RANTES and CCL-3/MIP-1 α and of the adhesion molecule ICAM-1 were significantly increased in syndecan-1-deficient compared with wild-type mice. In wild-type mice, syndecan-1 mRNA and protein expression was reduced during the DTH response. The differentially increased adhesion of syndecan-1-deficient leukocytes to ICAM-1 was efficiently inhibited in vitro by CD18-blocking Abs, which emerges as one mechanistic explanation for the anti-inflammatory effects of syndecan-1. Collectively, our results show an important role of syndecan-1 in the contact DTH reaction, identifying syndecan-1 as a novel target in anti-inflammatory therapy. *The Journal of Immunology*, 2009, 182: 4985–4993.

The delayed-type hypersensitivity (DTH)³ response in mice is a widely used model of allergic contact dermatitis, allowing the study of cell-mediated immune responses in vivo (1, 2). In the sensitization phase, a hapten covalently binds to cell surface proteins, followed by uptake and processing by dendritic and Langerhans cells which migrate to draining lymph nodes and prime hapten-specific T cell populations (1, 3). During the elicitation phase, hapten exposure induces recruitment and activation of primed T cells, synthesis and local release of chemokines and activating cytokines, as well as mast cell degranulation and release of vasoactive mediators. These events promote a massive leukocyte infiltration into the skin, which relies on the presence of chemokine gradients, and synthesis and activation of adhesion

molecules of the integrin, selectin, and cell adhesion molecule protein families (1, 2). Inhibitor studies using blocking Abs directed against adhesion molecules and cytokines and the use of knockout (KO) mouse models have been invaluable tools in elucidating the mechanisms underlying the DTH reaction in vitro and in vivo (4–6).

In recent years, a role for the syndecan (Sdc) family of cell surface heparan sulfate proteoglycans in the regulation of inflammation has emerged (7–9). Sdcs consist of an ectodomain containing consensus sequences for glycosaminoglycan attachment, a single highly conserved transmembrane domain and a short cytoplasmic domain, which is capable of interacting with scaffolding proteins, thus providing a link to the cytoskeleton (7, 10). Highly conserved cytoplasmic phosphorylation sites participate in signaling events modulating cell adhesion and migration (11, 12). The major functional domain of the Sdc ectodomains are their heparan sulfate chains, which specifically bind to a large number of extracellular ligands active in morphogenesis, tissue repair, host defense, tumor development, and inflammation (10, 13–17). Sdcs play a major role as matrix and cell surface receptors, coreceptors for growth factor and chemokine signaling, internalization receptors, and as soluble paracrine effectors upon cleavage of their extracellular domain by matrix metalloproteinases (10, 18).

Among the Sdc, Sdc-1 is expressed earliest during embryonic development (10). In the adult, Sdc-1 is expressed in epithelia, endothelia, and several leukocyte subgroups including pre-B cells and plasma cells, macrophages, and hematopoietic stem cells (7, 17, 19). Using KO and transgenic mouse models, we and others could recently demonstrate a role for Sdc-1 in regulating inflammation in a variety of disease models: Sdc-1 KO mice show increased adhesion of leukocytes to retinal blood vessels under unstimulated conditions and massively increased leukocyte adhesion and transendothelial diapedesis following TNF- α stimulation of

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³ Abbreviations used in this paper: DTH, delayed-type hypersensitivity; KO, knockout; MPO, myeloperoxidase; PMN, polymorphonuclear cell; Sdc, syndecan; WT, wild type; GBM, glomerular basement membrane; Ct, threshold cycle.

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mesenteric blood vessels (20). These findings could be replicated in vitro using polymorphonuclear cells (PMNs) purified from the bone marrow of these mice and both murine and human endothelial cells (21, 22). In bleomycin-induced lung fibrosis, an essential role for matrilysin-shed Sdc-1 in the generation of a transepithelial gradient of the CXC chemokine KC was demonstrated, leading to survival benefit of Sdc-1 KO mice (23). Moreover, absence of Sdc-1 exacerbated allergic lung disease induced by intranasal administration of *Aspergillus fumigatus* allergens, resulting in significantly increased airway hyperresponsiveness, glycoprotein hypersecretion, and eosinophilia (24). The concentration of the Sdc-1-ectodomain-binding CC chemokines MCP-3/MARC/CCL7, eotaxin/CCL11, and TARC/CCL17 was significantly increased in the bronchoalveolar lavage of Sdc-1 KO compared with wild-type (WT) mice. Of note, shed Sdc-1 ectodomains suppressed Th2 cell accumulation in the lung, suggesting an endogenous mechanism by which the airway epithelium limits allergic airway inflammation and obstruction (24). Furthermore, increased leukocyte recruitment in Sdc-1 KO mice was observed in a model of myocardial infarction, concomitant with a differential up-regulation of the chemokine MCP-1/CCL2, exaggerated inflammation, and functionally adverse wound repair (22). Finally, in experimental antigenic basement membrane (anti-GBM) nephritis, glomerular leukocyte/macrophage influx was increased in Sdc-1 KO mice during the heterologous phase and influx of CD4⁺/CD8⁺ T cells was higher in the autologous phase, resulting in a more severe glomerular injury (25). Decreased Th1 and higher Th2 renal cytokine/chemokine expression in Sdc-1 KO mice indicated a shift of the Th1/Th2 balance toward a Th2 response in this model of kidney inflammation.

Because a modulation of chemokine function and leukocyte recruitment are key components of the DTH reaction (1, 2), the recent findings on a regulatory role of Sdc-1 in leukocyte recruitment prompted us to investigate Sdc-1 KO mice in the model of oxazolone-mediated DTH. We demonstrate that the absence of Sdc-1 results in a prolonged DTH reaction associated with changes in heparan sulfate epitope expression, increased leukocyte influx, and an increase in the expression of ICAM-1 and proinflammatory cytokines and chemokines. In vitro experiments indicate a role for Sdc-1 in modulating the functional interaction of ICAM-1 with its integrin counterreceptors LFA-1 and Mac-1. Our findings suggest a role for Sdc-1 as a negative regulator of contact allergies, with possible implications for Sdc-1- and heparan sulfate-targeted therapies.

Materials and Methods

Mice

Sdc-1 KO mice on a C57BL/6J background (26) that had been backcrossed for 11 generations (25) and their WT counterparts were bred, housed, and handled according to the guidelines of the local animal ethics committee, which approved this study (protocol nos. A101/2005 and G64/2003).

DTH assay

DTH reactions were induced in the ear skin of 5- to 6-wk-old C57BL/6 WT or Sdc-1 KO mice as follows: Mice were sensitized on the shaved abdominal skin with 150 μ l of 2.5% oxazolone (Sigma-Aldrich) dissolved in acetone/ethanol (3:1 (v/v)) applied topically. The DTH reaction was elicited 7 days later by challenging the mice with 20 μ l of 1.0% oxazolone in acetone/ethanol by topical administration of 10 μ l to each side of the ear. Thickness of a constant area (1 cm²) of the ear was measured with a Mitutoyo engineer's micrometer immediately before challenging, 24 h after challenge, and every other day thereafter for 3 days and after 7 days. Three to four mice were used per each experimental condition and time point, and statistical analysis was performed using the unpaired Student *t* test. All experiments were repeated at least three times with similar results. DTH reactions were significantly increased in Sdc-1 KO mice over WT

mice irrespective of gender. The increase in ear thickness over baseline levels (thickness of the ears treated with vehicle alone) was used as a parameter for the extent of inflammation.

RANTES and ICAM-1 ELISA

Excised ears from mice subjected to a DTH reaction at different time points were snap frozen in liquid nitrogen and subsequently homogenized on ice in 500 μ l of PBS with 0.01 M EDTA and a proteinase inhibitor mixture (2 μ g/ml aprotinin (Sigma-Aldrich), 100 μ g/ml Pefabloc (Roche), and protease inhibitor mix (Sigma-Aldrich)) and 1 ml of 1.5% Triton X-100 in PBS. Samples were placed on a rotary shaker at 300 rpm on ice for 30 min, centrifuged at 12,000 $\times g$ for 10 min, and the supernatants were collected. Total protein concentration for each sample was quantified by a BCA Lowry assay (Pierce). All samples were diluted to 0.9 mg/ml (RANTES) or 0.09 mg/ml (ICAM-1), and the tissue concentrations of RANTES and ICAM-1 immunoassays were determined exactly as described by the manufacturer (R&D Systems).

Myeloperoxidase (MPO) assay

For determination of MPO activity, tissue extracts were prepared as described under *RANTES and ICAM-1 ELISA*. The protein concentration in all tissue extracts was adjusted to 0.9 mg/ml. MPO activity was determined using the EnzChek MPO Activity Assay Kit (Invitrogen) according to the manufacturer's instructions.

Quantification of circulating neutrophils

Leukocytes of peripheral blood, taken from the submandibular vein, were counted in an improved Neubauer hemacytometer following erythrocyte lysis and incubation with 0.1% crystal violet solution. These counts were multiplied with the percentages of granulocytes as determined by double staining with anti-CD45 and anti-Gr-1 Abs (BD Pharmingen) and flow cytometry, as previously described (27), to obtain the number of granulocytes per ml of blood.

Immunohistochemistry

Normal ear tissue obtained from WT and Sdc-1 KO mice were obtained and were formalin-fixed and paraffin-embedded using standard techniques. Consecutive sections of 2–3 μ m were cut from the paraffin blocks, dewaxed, and rehydrated. Ag retrieval was performed by hot water steaming in citrate buffer (pH 6, 30 min; DakoCytomation). Endogenous peroxidase activity was quenched with methanol/0.6% H₂O₂, followed by three washes with PBS. Following a blocking step with 10% Aurion BSAc solution in PBS (DakoCytomation) for 30 min, sections were incubated with the following primary Abs, diluted in PBS containing 1% BSA overnight at 4°C: rat anti-mouse Sdc-1 mAb (catalog no. 553712; BD Biosciences) with a dilution of 1/100, rabbit anti-mouse Sdc-2 monospecific antiserum (1/100; catalog no. sc-15348; Santa Cruz Biotechnology), rat anti-mouse Sdc-3 mAb (1/100; catalog no. MAB2734; R&D Systems), and rat anti-mouse Sdc-4 mAb (1/50; catalog no. 550350; BD Biosciences). Omission of the primary Ab or a rat IgG2a isotype control Ab (catalog no. 553930; BD Biosciences) served as negative controls. Following three washes with PBS, primary Abs were detected using the DakoCytomation mouse/rabbit EnVision plus-HRP systems and the AEC+ substrate (DakoCytomation), followed by counterstaining with Mayer's hemalum (Merck) and mounting in Kaiser's glycerol gelatin (Merck). Sections were observed using a Zeiss Axiovert 100 microscope equipped with a Zeiss Achroplan objective ($\times 32$ magnification) and documented using an Axiophot Mrc camera (Zeiss) and Zeiss Axiovision software. Composite figures were assembled using Adobe Photoshop 6.0 software without further adjustment of the original image parameters. Immunostaining with single chain Fv anti-GAG Abs was performed essentially as described elsewhere (28). Cryosections (3 μ m) of ear tissue from Sdc-1 KO and WT mice were dried and blocked for 10 min with PBS containing 2% BSA. The sections were subsequently incubated with the primary single-chain Fv Abs HS4C3 (28) or EV3C3 (29), the rat-anti-mouse Sdc-1 mAb 281-2, or its isotype control Ab (BD Biosciences) for 90 min at 22°C. Bound Abs were visualized using anti-VSV-tag Ab P5D4 followed by Alexa Fluor 488-labeled anti-mouse or rat IgG Abs (Invitrogen). Finally, sections were fixed in ethanol, air dried, and embedded in Mowiol. As a control, primary Abs were omitted or substituted by an irrelevant single chain Ab (MPB59). Sections were observed with a Leica DM6000B fluorescence microscope equipped with a Leica DFC480 camera.

Histomorphometric analysis

Histomorphometry was performed on digital images of H&E-stained ear tissue sections from three Sdc-1 KO and WT mice, each essentially as described by Staite et al. (4). The areas of microabscesses were calculated from user-defined outlines using Zeiss Axiovision software. Cell counts were performed inside user-defined areas located in the subdermal tissue, above the lateral cartilage layer. Microabscess areas and the average frequency of microabscesses per ear were calculated. Microabscesses were excluded from the analysis of infiltrating cells in the subdermal tissue. The number of cells that infiltrated the subdermal tissue and the total tissue (excluding microabscesses) were averaged for nine separate regions per ear. The cell density was expressed as cells per subdermal or total ear diameter (μm), respectively.

Quantitative TaqMan real-time PCR

After euthanasia of the animals, ears were excised and either snap frozen in liquid nitrogen or stored in RNAlater solution (Ambion), followed by preparation of total RNA using the RNeasy kit (Qiagen). Five hundred nanograms of total RNA was transcribed using the high capacity cDNA synthesis kit (Applied Biosystems). cDNA corresponding to 0.5 ng of total RNA was used as a template in the PCR consisting of Applied Biosystems MasterMix and predesigned TaqMan gene expression systems (Applied Biosystems) according to the manufacturer's instructions. For detection of Sdc-1, TNF- α , ICAM-1, CCL3/MIP-1 α , CCL2/MCP-1, and IL-6 mRNA, primers Mm00448918_m1 (Sdc-1 exons 2 and 3), Mm00443258_m1 (TNF- α exons 1 and 2), Mm00516023_m1 (ICAM-1 exons 2 and 3), Mm00441242_m1 (CCL2 exons 1 and 2), Mm00441258_m1 (CCL3 exons 1 and 2), and Mm00446190_m1 (IL-6 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 by 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 quantitation was performed using the comparative cycle threshold method. Three to five biological replicates were used for each time point investigated. For statistical analysis, Student's *t* test was used. A *p* < 0.05 was considered statistically significant.

ICAM-1 adhesion assay

Adhesion of Sdc-1 KO and WT mouse PMNs to recombinant ICAM-1 was performed essentially as described previously (30). Briefly, 10 $\mu\text{g}/\text{ml}$ ICAM-1 protein in PBS/1% FCS was coated onto 96-well flat-bottom plates (Maxisorp; Nunc). Murine PMNs were prepared from the bone marrow of Sdc-1 KO and WT mice by Histopaque gradient centrifugation exactly as previously described (21, 22). PMNs were fluorescently labeled with 2,7-bis-(2-carboxyethyl)-5-carboxyfluorescein acetoxymethyl ester (Invitrogen), washed, and incubated with the ICAM-1-coated plates at a concentration of 3×10^5 cells/well in sextuplets for 45 min at 4°C. Following washing with PBS, adhering cells were lysed and the fluorescence signal was quantified in a Spectramax fluorometer (excitation 485 nm, emission 535 nm; Molecular Devices). For inhibition experiments, PMNs were preincubated for 30 min with 10 $\mu\text{g}/\text{ml}$ of a monoclonal blocking Ab directed against mouse CD18 (31) (clone GAME-46; BD Pharmingen), followed by incubation in the presence of the Ab as described above. Plates were washed four times with PBS and adherent cells were fixed using 1% paraformaldehyde. Adhering cells were observed at 32-fold magnification using a Zeiss Axioskop microscope equipped with a charge-coupled device camera as described under *Immunohistochemistry*. Cell numbers were quantified and expressed as cells/ mm^2 with the assistance of Zeiss Axiovision imaging software. Statistical analysis was performed using Student's *t* test. A *p* < 0.05 was considered statistically significant.

Results

DTH reactions are prolonged and increased in Sdc-1 KO mice

We first studied the DTH reactivity in Sdc-1 KO mice on a C57BL/6 background (25, 26) and their WT counterparts. Mice were sensitized with the hapten oxazolone as described in *Materials and Methods* and the DTH reaction was elicited 7 days later by applying oxazolone onto the ears. Compared with WT mice, Sdc-1 KO mice showed an increased and prolonged DTH reaction, which was evident for up to 7 days, reaching significance at 48 and 72 h after DTH elicitation (Fig. 1A). Histological examination revealed edema formation, leukocyte recruitment, and formation of

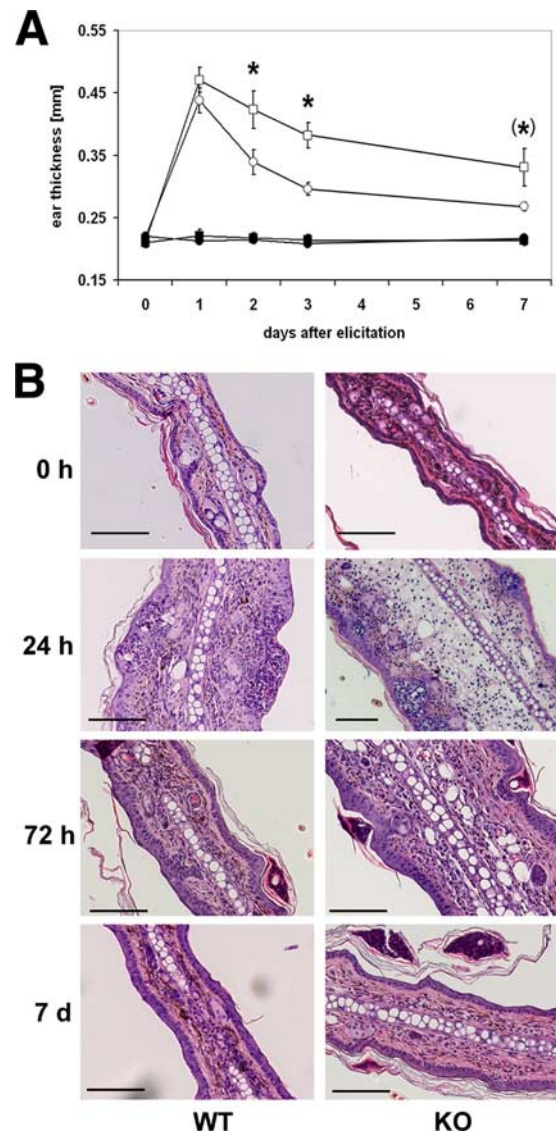


FIGURE 1. Increased and prolonged DTH reactivity in Sdc-1 KO mice. DTH reactions were elicited in the right ear of Sdc-1 KO mice and their WT counterparts using oxazolone. Left ears of the same mice were treated with vehicle alone. **A**, Swelling of the challenged ears is expressed in mm as the increase over baseline thickness (● and ■) of ears treated with vehicle alone. Sdc-1-KO mice (□) show a significantly increased and prolonged DTH reaction over WT (○) at 48 and 72 h after DTH elicitation. *, *p* < 0.05, *n* ≥ 5; (*), *p* = 0.06; data are expressed as mean ± SEM. The experiments were repeated five times with similar results. **B**, Histological features of oxazolone-induced DTH responses in the ears of WT and Sdc-1-deficient (KO) mice. Representative ear sections show increased leukocyte recruitment and interstitial edema formation in Sdc-1-KO mice. H&E staining; original magnification, $\times 32$. Scale bar, 100 μm . A higher magnification version of **B** is shown as supplemental Fig. 1.

neutrophil-containing microabscesses in the epidermis of WT mice 24 h after DTH elicitation, which was markedly reduced after 72 h and largely comparable to vehicle-treated ears after 7 days (Fig. 1B and supplemental Fig. 1⁴). In contrast, after 24 h, edema formation and leukocyte recruitment in the ear tissue of Sdc-1 KO mice were increased compared with WT, with increased edema formation still being evident 72 h after DTH elicitation (Fig. 1, A and B, and supplemental Fig. 1).

⁴ The online version of this article contains supplemental material.

Increased leukocyte recruitment during DTH in Sdc-1 KO mice

Because histological staining had revealed a differentially increased influx of leukocytes into the ear tissue of Sdc-1 KO mice after DTH elicitation, we wanted to independently confirm increased leukocyte recruitment. For this purpose, MPO activity was determined as a surrogate marker for the presence of neutrophils and monocytes (32) in ear tissue extracts of Sdc-1 KO and WT mice subjected to DTH. Baseline activity of MPO was significantly increased in the vehicle-treated ears of Sdc-1 KO mice compared with WT mice (Fig. 2A), whereas the number of circulating neutrophils did not significantly differ (Fig. 2B). A significant 50% increase of MPO activity over WT was observed in Sdc-1 KO mice 24 h after DTH elicitation (Fig. 2A). Histomorphometric analysis revealed a differentially altered distribution of leukocytes in inflamed ear tissue of Sdc-1 KO and WT mice 24 h after DTH elicitation: a significantly increased number of microabscesses and increased leukocyte numbers localizing to microabscesses were detected in Sdc-1 KO compared with WT mouse ears (Fig. 2C). Moreover, an increased proportion of Sdc-1 KO leukocytes localized to subdermal areas compared with WT tissue (NS), concomitant with a significantly increased ratio of the subdermal to total ear diameter, indicative of increased edema formation (Figs. 1 and 2D).

Changes in Sdc-1 and heparan sulfate epitope expression in DTH

Because the increased DTH reactivity in Sdc-1 KO mice indicated a role for Sdc-1 in the regulation of contact allergies, we studied its expression in WT mice during the course of DTH. Immunohistological staining of unchallenged WT mouse ears confirmed a strong expression of Sdc-1 protein in the epidermis and hair follicles and a moderate expression in blood vessels (Fig. 3A). Twenty-four hours after elicitation of the DTH response Sdc-1 staining was still prominent in hair follicles, yet reduced in the epidermal epithelium, in particular in the vicinity of microabscesses. The epidermal layer was still present next to the abscesses. Leukocytes in microabscesses stained moderately positive for Sdc-1 (Fig. 3B). After 48 h, epithelial Sdc-1 staining was weak compared with unchallenged ears, whereas subdermal tissue layers and leukocyte-containing microabscesses stained positive for Sdc-1 (Fig. 3C). After 72 h, the distribution of Sdc-1 was similar to unchallenged tissue; however, staining intensity was markedly reduced (Fig. 3D). Quantitative real-time PCR analysis confirmed a decrease in Sdc-1 expression at the mRNA level in ear tissue of WT mice during the course of the DTH reaction (Fig. 3E). Of note, 48 and 72 h after DTH elicitation, Sdc-1 expression was significantly reduced by 60–70% compared with unchallenged controls. Similar to our previous findings in a myocardial infarction model (22), no differential expression of the other three members of the Sdc family was seen between Sdc-1 KO and WT mice (data not shown). Sdc-1 modulates the activity of a variety of proteins relevant to inflammation via interactions with its heparan sulfate chains (7–9), which are capable of forming specific ligand binding sites depending on posttranslational modifications such as epimerization and *N*- and *O*-sulfation (10, 13). To uncover potential structural changes in heparan sulfate during the course of the DTH reaction, we used the heparan sulfate-specific single-chain Ab EV3C3 (29) and the Ab HS4C3 (28), which preferentially recognizes a 3-*O*-sulfated heparan sulfate motif and which is able to compete with the anti-thrombin III binding site of heparin (33), for the immunolocalization of specific heparan sulfate epitopes in

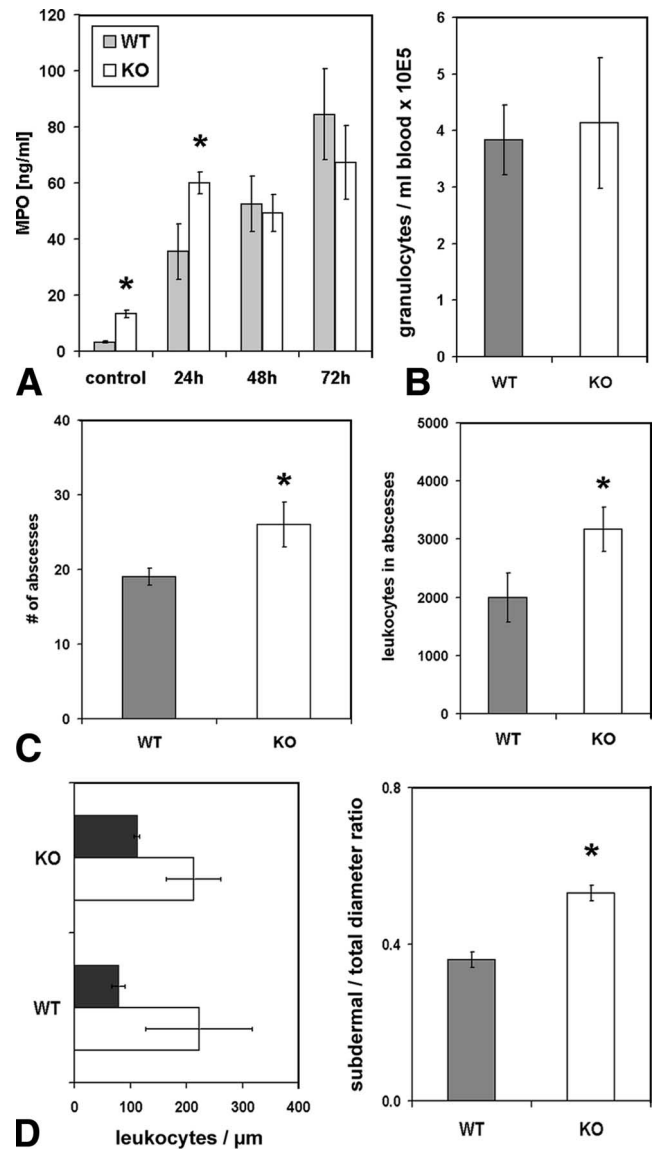


FIGURE 2. MPO activity, circulating neutrophil counts, and morphometric analysis of Sdc-1 KO and WT mouse tissues subjected to DTH. **A**, Increased MPO activity in Sdc-1 KO vs WT ear tissue. A DTH response was elicited in WT and Sdc-1 KO mice as described in Fig. 1, and tissue lysates were prepared from the ear tissue as described in *Materials and Methods*. MPO activity was determined as a surrogate marker for neutrophil and monocyte influx. MPO activity in Sdc-1 KO mice was significantly increased relative to WT mice before and 24 h after DTH elicitation. *, $p < 0.05$, $n \geq 4$; error bars, SEM. **B**, The number of circulating neutrophils in the blood of Sdc-1 KO ($n = 9$) and WT ($n = 13$) mice was determined in three independent experiments by flow cytometry as described in *Materials and Methods*. Circulating numbers of neutrophils do not significantly differ between Sdc-1 KO and WT mice. Error bars, SEM. **C** and **D**, Morphometric analysis of leukocyte distribution in Sdc-1 KO and WT mouse ear tissue sections 24 h after DTH elicitation. **C**, Number of microabscesses/ear section (left panel) and total leukocyte numbers in microabscesses (right panel). *, $p < 0.05$, $n = 3$; error bars, SD. **D**, Increased edema formation (subdermal to total diameter ratio, right panel) and subdermal leukocyte distribution (left panel) in Sdc-1 KO vs WT mice. *, $p < 0.05$, $n = 3$; error bars, SEM. Left panel, ■ = dermal leukocytes; □ = total leukocytes.

cryosections of vehicle- and oxazolone-treated ears of Sdc-1 KO and WT mice (Fig. 4). Similar to our observations in paraffin-embedded tissue sections, Sdc-1 immunoreactivity was high in

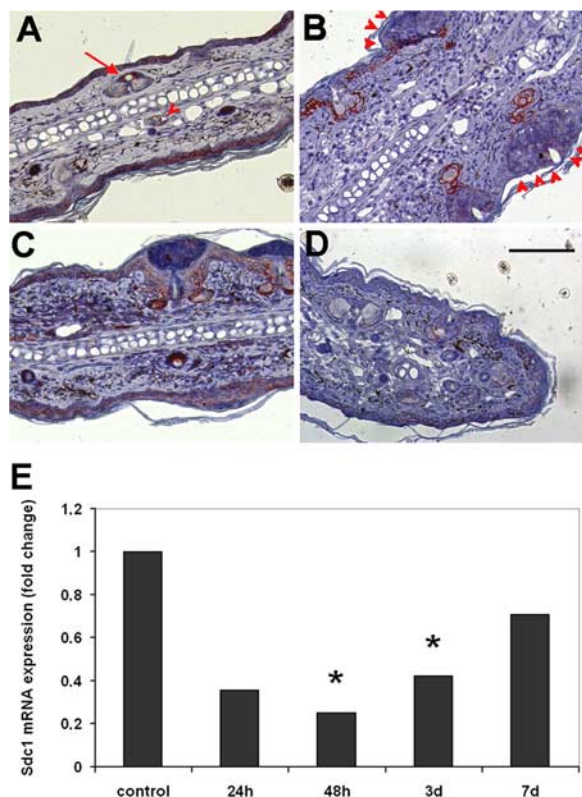


FIGURE 3. Decreased Sdc-1 expression in WT mice during the course of the DTH reaction. A DTH reaction was elicited in WT mice as described in Fig. 1 and Sdc-1 expression was monitored by immunohistochemistry of paraffin-embedded tissue sections (A–D) and by quantitative PCR (E). A–D, Ear tissue was harvested from vehicle-treated (A) and oxazolone-treated WT mice after 24 h (B), 48 h (C), and 72 h (D) and processed for immunohistochemical analysis of Sdc-1 expression (reddish AEC staining). In unchallenged ears, Sdc-1 expression was prominent in the epithelium of the skin, hair follicles (marked by arrow), and blood vessels (arrowhead) (A). Twenty-four hours after elicitation of the DTH response (B), Sdc-1 staining is still prominent in hair follicles, yet reduced in the epidermis in the vicinity of microabscesses (marked by arrowheads). Note Sdc-1 staining of leukocytes in microabscesses. After 48 h (C), epithelial Sdc-1 staining is weak compared with unchallenged ears, whereas subdermal tissue layers and leukocyte-containing microabscesses stain positive for Sdc-1. After 72 h (D), the distribution of Sdc-1 is similar to unchallenged tissue (A), but staining intensity is markedly reduced. Original magnification, $\times 32$; scale bar, 100 μ m (applies to all panels). E, Quantitative real-time PCR analysis of Sdc-1 mRNA expression in ear tissue of WT mice subjected to a DTH reaction. Δ Ct values were calculated relative to 18S mRNA expression expressed as fold change as calculated by the $2^{-\Delta\Delta C_t}$ method. Forty-eight and 72 h after DTH elicitation, Sdc-1 expression was significantly reduced by 60–70% compared with unchallenged controls. *, $p < 0.05$, $n \geq 4$. Δ Ct values are shown in supplemental Fig. 2.

hair follicles and in keratinocytes and reduced in the keratinocyte layer upon DTH elicitation (Fig. 4A). In unchallenged WT and Sdc-1 KO mouse ears, HS4C3 epitope staining was strongest in the basal lamina separating the epidermis and the dermis, in the basal lamina of blood vessels, in hair follicles, and in the central cartilage layer of the ear (Fig. 4). It was not present on keratinocyte membranes, yet was weakly induced in these cells upon DTH elicitation. During DTH, HS4C3 staining decreased in the basement membranes located underneath abscesses. EV3C3 reactivity was highest in the basal lamina and blood vessels (Fig. 4). EV3C3 immunoreactivity appeared to be slightly reduced in the keratinocyte layer of Sdc-1 KO vs WT

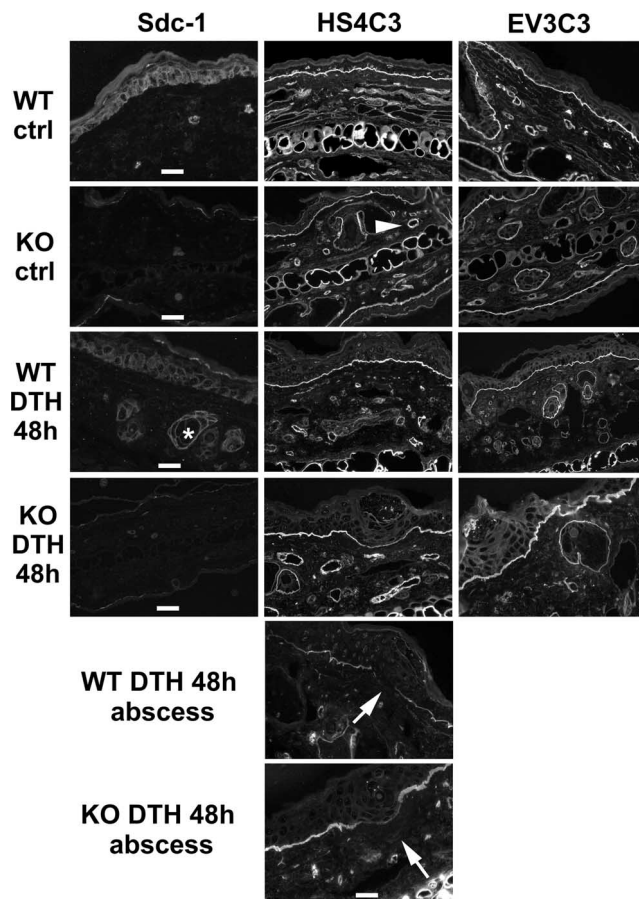


FIGURE 4. Elicitation of a DTH response results in altered heparan sulfate epitope reactivity. Cryosections of WT and Sdc-1 KO ear tissue before (ctrl) or 48 h after (DTH 48 h) elicitation of a DTH response were stained with a mAb directed against Sdc-1 or with the heparan sulfate epitope Abs HS4C3 and EV3C3, respectively. Distribution was visualized by immunofluorescence microscopy after incubation with secondary Abs coupled to fluorescent Alexa Fluor 488 dyes. In WT mice, Sdc-1 is mainly expressed in keratinocytes and hair follicles (asterisk). Only a slight autofluorescence is observed on top of the epidermal cell layer (KO-Sdc-1). HS4C3 immunoreactivity is strong in the basement membrane of the epithelium, in blood vessels (arrowhead) and the cartilage layer, and slightly up-regulated in keratinocytes following DTH. Decreased staining of the basement membranes underlying abscesses is observed after DTH (arrows). EV3C3 immunoreactivity is strongest in the basal lamina and slightly increased in keratinocytes of WT and Sdc-1 KO mice subjected to DTH.

mice. At the peak of the DTH response, EV3C3 immunoreactivity was slightly up-regulated in keratinocytes of both WT and Sdc-1 KO mice (Fig. 4).

Up-regulation of cytokine and chemokine expression during DTH in Sdc-1 KO mice

We next investigated whether increased leukocyte recruitment in Sdc-1 KO mice resulted in increased chemokine expression in extracts of control and oxazolone-treated ears. Elicitation of a DTH response led to a 67% increased induction of the Sdc-1-binding chemokine RANTES (34), in Sdc-1 KO mice over WT after 48 h, as determined by ELISA (Fig. 5A). We further investigated expression of proinflammatory cytokines and chemokines by quantitative real-time PCR. Expression of TNF- α , a key player in the induction of local inflammation in the elicitation phase of DTH (6), was significantly increased 9.5-fold in Sdc-1 KO compared with

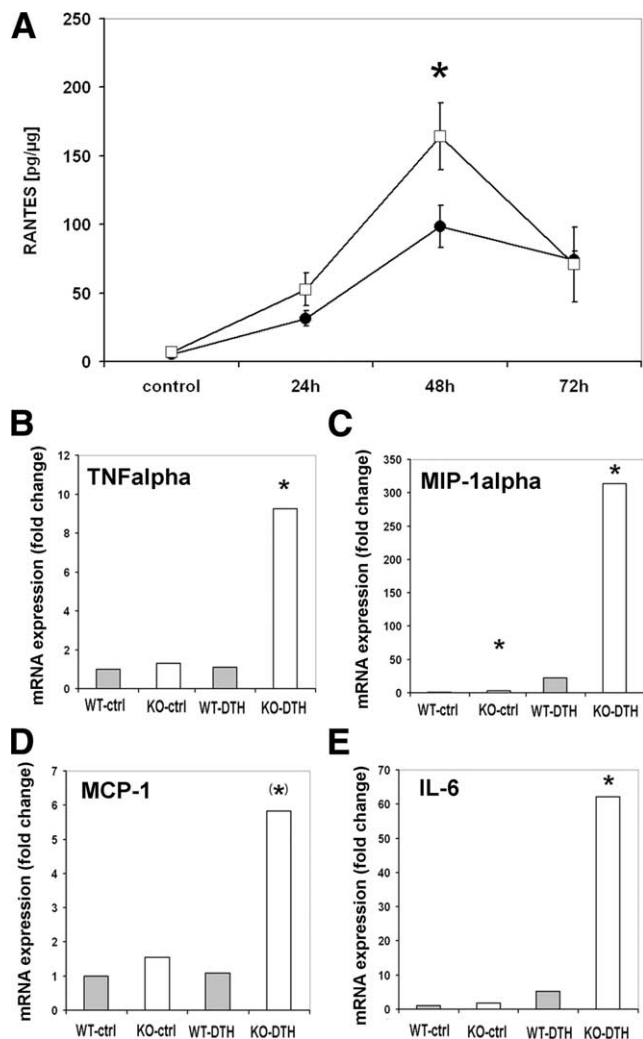


FIGURE 5. Increased RANTES, TNF- α , CCL-3/MIP-1 α , CCL-2/MCP-1, and IL-6 expression in Sdc-1 KO mice during the DTH response. **A**, RANTES ELISA. Cell lysates were prepared from the ears of Sdc-1 KO and WT mice before and after elicitation of a DTH response as described in the legend of Fig. 1, followed by a quantification of RANTES expression by ELISA. RANTES expression was increased by 67% in Sdc-1 KO ($\square$) vs WT mice ($\bullet$) 48 h after DTH elicitation. *, $p < 0.05$, $n \geq 4$; error bars, SEM. **B–E**, Differentially increased chemokine and cytokine expression in Sdc-1 KO vs WT mice during DTH. mRNA was prepared from ear tissue of control mice (ctrl) and of mice subjected to oxazolone-induced DTH elicitation for 72 h (DTH). Following transcription into cDNA, the expression of selected cytokines and chemokines was determined by quantitative real-time PCR (cf Fig. 3B). Data are expressed as fold change as calculated by the $2^{-\Delta\Delta C_t}$ method. **B**, Increased TNF- α expression in Sdc-1 KO vs WT. **C**, Increased MIP-1 α expression in Sdc-1 KO vs WT mice. **D**, Increased MCP-1 expression in Sdc-1 KO vs WT mice. **E**, Increased IL-6 expression in Sdc-1 KO vs WT mice. *, $p < 0.05$; (*), $p = 0.06$; $n \geq 4$; error bars, SEM. ΔC_t values are shown in supplemental Fig. 2.

WT mice after DTH elicitation (Fig. 5B). Expression of MIP-1 α /CCL3, a heparan sulfate-binding chemokine involved in edema formation in DTH (35–37), was significantly increased 325-fold and expression of MCP-1/CCL2 was increased 5.8-fold ($p = 0.06$) in Sdc-1 KO mice over WT after DTH elicitation (Fig. 5, C and D). Moreover, a significant 63-fold increase in IL-6 expression was observed 72 h after DTH elicitation in Sdc-1 KO mice (Fig. 5E).

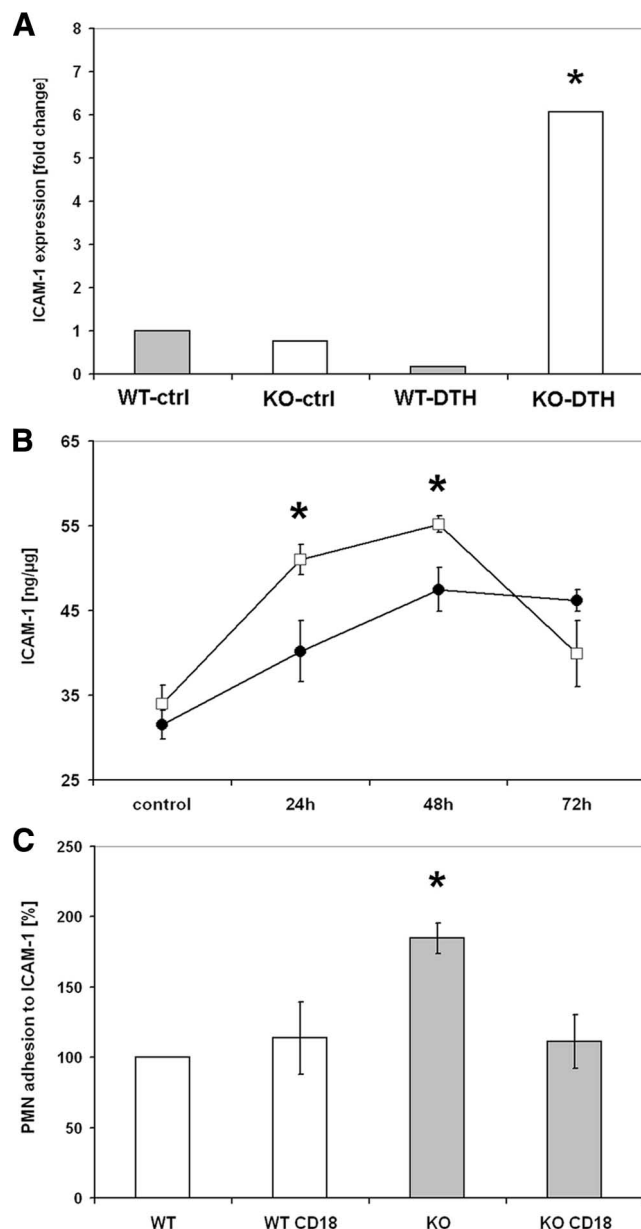


FIGURE 6. Sdc-1 deficiency promotes PMN adhesion to ICAM-1 in a CD18-dependent manner. **A**, Quantitative real-time PCR analysis of ICAM-1 expression in Sdc-1 KO and WT mice reveals a significant 6.3-fold increase of ICAM-1 mRNA expression in Sdc-1 KO mice subjected to DTH (cf Fig. 5). **B**, Cell lysates were prepared from the ears of Sdc-1 KO ($\square$) and WT ($\bullet$) mice before and after elicitation of a DTH response as described in the legend of Fig. 1, followed by a quantification of ICAM-1 protein expression by ELISA. ICAM-1 expression in Sdc-1 KO mice was significantly increased by 27 and 16% at 24 and 48 h after DTH elicitation, respectively. **C**, PMNs were purified from the bone marrow of Sdc-1 KO and WT mice and binding to recombinant ICAM-1 was quantified using an ELISA-style assay as described in *Materials and Methods*. The significant 1.9-fold increase in the adhesion of Sdc-1 KO PMNs to ICAM-1 could be efficiently blocked with Abs directed against the leukocyte integrin CD18. *, $p < 0.05$, $n \geq 4$; error bars, SEM.

Sdc-1 deficiency leads to up-regulation of ICAM-1 expression and increased PMN adhesion to ICAM-1 in a CD18-dependent manner

TNF- α induced up-regulation of the vascular leukocyte adhesion receptor ICAM-1 plays an important role in mediating leukocyte recruitment following elicitation of a DTH response (5, 38). Since

TNF- α was differentially up-regulated in the inflamed tissue of Sdc-1 KO mice, we studied ICAM-1 expression by quantitative real-time PCR. ICAM-1 mRNA expression was significantly up-regulated 6.3 times in Sdc-1 KO over WT upon DTH elicitation (Fig. 6A). In accordance with previous confocal immunofluorescence microscopy data (8), ELISA analysis of tissue extracts revealed a significant differential increase in the amounts of ICAM-1 protein in Sdc-1 KO vs WT mouse ears 24 and 48 h after DTH elicitation (Fig. 6B). Because ICAM-1 is an important adhesion receptor for neutrophils, we investigated *in vitro* whether Sdc-1 deficiency may cause an altered interaction of ICAM-1 with its leukocyte β_2 integrin counterreceptor CD18. PMNs isolated from the bone marrow of Sdc-1 KO mice showed an increased adhesive behavior toward ICAM-1. The number of PMNs increased 1.9-fold compared with WT in the adhesion to ICAM-1. Of note, this increased adhesion could be efficiently blocked with an Ab directed against CD18 (Fig. 6C) known to block the interaction of ICAM-1 with the leukocyte integrins LFA-1 and Mac-1, respectively (31).

Discussion

Via its heparan sulfate chains, Sdc-1 binds to and modulates the activity of a wide range of molecules relevant to inflammation, such as chemokines, selectins, integrins, and other adhesion molecules (7, 13). The present *in vivo* study on the role of Sdc-1 in oxazolone-mediated DTH confirms the role for Sdc-1 as a negative regulator of inflammatory responses and identifies aberrant leukocyte function in Sdc-1 KO mice as a key factor in this process.

In accordance with previous findings of increased endothelial recruitment of leukocytes in the vasculature of Sdc-1 KO mice (20), MPO activity was elevated in the vehicle-treated ears of Sdc-1 KO mice and further increased over WT after elicitation of the DTH response (Fig. 2). Increased baseline activity could not be attributed to systemically increased circulating neutrophil numbers (Fig. 2B); however, it is in accordance with increased recruitment of Sdc-1 KO leukocytes to unstimulated retinal blood vessels (20) and of Sdc-1 KO PMNs and monocytes to unstimulated HUVEC cells (21). Both total number of microabscesses and leukocyte numbers within these microabscesses were significantly increased in the Sdc-1 KO mice. Moreover, increased edema formation was observed in these mice along with an increased subdermal leukocyte distribution (Figs. 1 and 2). This finding is in line with our previously stated hypothesis that Sdc-1 may modulate vascular permeability via its interaction with kininogens or CAP37 (7) and with an increase in paracellular protein leakage in Sdc-1 KO vs WT mice in a mouse model of protein-losing enteropathy (39). Increased leukocyte recruitment in the absence of Sdc-1 was consistently observed in different contexts of inflammation and repair, including allergic lung inflammation (24), anti-GBM nephritis (25), myocardial infarction (22), and upon TNF- α -stimulation *in vivo* (20). We are now providing further mechanistic clues elucidating the role of Sdc-1 in leukocyte recruitment: It has previously been shown that soluble heparin, an extensively sulfated and epimerized form of heparan sulfate, can bind to the leukocyte integrin Mac-1 on stimulated monocytes and granulocytes and is capable of inhibiting binding of ICAM-1 to Mac-1 (40). We were recently able to show that different heparans can reduce increased adhesion of Sdc-1 KO PMNs to human and murine endothelial cells (21, 22).

Using an established inhibitory Ab directed against CD18, the common β_2 integrin chain of LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), we could abolish increased adhesion of Sdc-1 KO PMNs to ICAM-1 (Fig. 6). Our data suggest that in WT mice, Sdc-1 may negatively regulate the interaction of ICAM-1 and

CD18, possibly via its heparan sulfate chain (40), leading to increased leukocyte recruitment to ICAM-1 in the up-regulation of ICAM-1 expression (Fig. 6), similar to our previous observations in the renal cortex of Sdc-1 KO mice during anti-GBM nephritis (25). The antiadhesive function of Sdc-1 may also be the underlying cause of increased baseline MPO and MIP-1 α levels in Sdc-1 KO mice (Figs. 2 and 5). A negative regulatory role for the heparan sulfate chains of Sdc-1 in DTH is in line with recent findings of a DTH-promoting function of the TNF- α -induced heparan sulfate degrading enzyme heparanase (16) and with the proposed role of an inhibitory function of cell-associated heparan sulfate on the interaction of TNF- α and IFN- γ with their receptors in a model of protein-losing enteropathy (39). In accordance with our findings of increased DTH responses accompanied by increased ICAM-1 expression and function in Sdc-1 KO mice, genetic or Ab-mediated ablation of ICAM-1 function leads to reduced delayed-type and immediate contact hypersensitivity responses in mice due to reduced recruitment of dendritic cells, mast cells, and neutrophils (5, 41).

In contrast to our finding of an anti-inflammatory function of Sdc-1, a proinflammatory role of heparan sulfate has also been described previously (7, 13, 15). With respect to the well-characterized modulation of chemokine function by heparan sulfate, Sdc-1 can act both as an inhibitor or stimulator, depending on the cellular context and on the cell surface localization (8, 9). An additional major target of the proinflammatory action of heparan sulfate is the step of selectin-mediated leukocyte rolling. Apart from several *in vitro* studies demonstrating reduced leukocyte recruitment and L- and P-selectin binding in the presence of different heparan sulfate species (reviewed in Refs. 7 and 13), a recent study has confirmed this effect *in vivo* (15). Mice containing an endothelial-specific knockout of the heparan sulfate biosynthetic enzyme NDST-1, which mediates the pivotal initial step of heparan sulfation (10), displayed severe defects in leukocyte recruitment in a variety of inflammation assays, due to impaired L-selectin function, chemokine gradient formation, and chemokine transcytosis, which lead to decreased leukocyte rolling (15). Although the study requires cautious interpretation, since cell surface heparan sulfate was not absent, but rather structurally modified, it clearly shows a proinflammatory role of endothelial heparan sulfate at an early step of leukocyte recruitment. In contrast, our data suggest a modulation of the later step of integrin-dependent tight leukocyte-endothelial interactions by Sdc-1 (20–22), which occurs at the leukocyte level. Intravital microscopy studies in Sdc-1 KO mice have confirmed a role for Sdc-1 in regulating the integrin-dependent tight adhesion and the absence of an effect on selectin-mediated leukocyte rolling (20).

Increased leukocyte recruitment during the DTH reaction was accompanied by an increase in several proinflammatory cytokines and chemokines in the inflamed ear tissue of Sdc-1 over WT mice (Figs. 5 and 6). Although this increase will at least be in part due to the higher quantity of chemokine-producing leukocytes in Sdc-1 KO tissues, it is also known that Sdc-1 is directly involved in modulating cytokine and chemokine function by promoting chemokine dimerization and via facilitation of receptor interactions (7, 9, 35, 42). To date, interactions of Sdc-1 with the chemokines RANTES/CCL5 (19, 34), eotaxin/CCL11, TARC/CCL17, MARC/CCL7 (24), IL-8/CXCL8 (43), and KC/CXCL1 (23) have been demonstrated. In the DTH model, loss of Sdc-1 leads to a dysregulation of proinflammatory cytokine and chemokine expression, including several key players in the DTH reaction. Among the differentially up-regulated factors, local release of TNF- α has been regarded a critical factor for the optimal generation of DTH

reactions, inducing the expression of ICAM-1 and various chemokines which promote leukocyte recruitment and Langerhans cell migration, respectively (38, 44). TNF- α deficiency in vivo leads to reduced oxazolone-mediated DTH responses (45). RANTES/CCL5 is induced in allergic contact dermatitis, facilitating leukocyte recruitment to inflammatory sites (46). RANTES/CCL5 binds to and can induce shedding of Sdc-1 (34) and was differentially up-regulated in Sdc-1 KO mice in our DTH model. Similarly, the heparan sulfate-binding chemokine MIP-1 α /CCL3 (47) was differentially up-regulated. MIP-1 α /CCL3 is a chemoattractant for neutrophils, monocytes, eosinophils, basophils, and lymphocytes. It can induce a dose-dependent increase in neutrophil recruitment in a DTH reaction (48) and regulates T lymphocyte trafficking into lymph nodes in dinitrofluorobenzene-mediated hypersensitivity responses (36). Moreover, studies in MIP-1 α /CCL3 KO mice indicate reduced neutrophil recruitment during the cutaneous Arthus reaction (49) and a role for this chemokine in mediating mast cell-mediated immediate hypersensitivity reactions (50). Similarly, MCP-1/CCL2 is involved in ear swelling during the elicitation phase of the DTH reaction (37) and was up-regulated in the Sdc-1 KO mice ($p = 0.06$). Thus, the combined increase in proinflammatory cytokine and chemokine expression in these mice leads to a complex amplification of the DTH response. Similar to the DTH model, a differential increase of chemokine expression over WT has been noted in Sdc-1 KO mice in other disease models of inflammation and repair (22, 24, 25). However, these increases are highly context-dependent and reflect the chemokine spectrum associated with the particular disease. For example, MCP-1 expression is increased in Sdc-1 KO mice during myocardial infarction, whereas other chemokines were not significantly different from WT (22). Like in the DTH model, MCP-1 and IL-6 mRNA expression are increased in the renal cortex of Sdc-1 KO mice during the early stages of anti-GBM nephritis (25); however, in contrast to DTH, TNF- α and MIP-1 α were down-regulated compared with WT mice.

We observed a significant down-regulation of Sdc-1 mRNA expression in WT mice at 48 and 72 h after elicitation of the DTH response. These time points coincide with the significant increase of edema formation in Sdc-1 KO mice (Fig. 1), further emphasizing a regulatory role for Sdc-1 in this process. Apart from transcriptional regulation, down-regulation of Sdc-1 protein levels (Fig. 3) could have also been mediated by Sdc-1 ectodomain shedding (10, 16, 18), possibly induced by heparanase or different (metallo)proteinases, which are up-regulated during DTH (10, 23, 24, 51). In addition, we observed changes in the tissue distribution of the heparan sulfate epitopes recognized by the EV3C3 and HS4C3 Abs, which were slightly up-regulated in the keratinocyte layer upon DTH elicitation (Fig. 4). Moreover, HS4C3 reactivity was reduced in the basement membranes underlying abscesses, possibly indicating increased heparanase activity (52). Although these findings suggest a potential involvement of specific heparan sulfate epitopes in allergic inflammation, the absence of Sdc-1 had no substantial differential effect on HS4C3 and EV3C3 epitope expression, in accordance with identical organ-specific heparan sulfate disaccharide patterns in Sdc-1 KO and WT mice (53).

In summary, we have demonstrated a novel role for Sdc-1 as a modulator of DTH responses. Absence of Sdc-1 allowed for a more efficient interaction of ICAM-1 and its β_2 integrin counter-receptors, leading to increased leukocyte recruitment. The associated increase in proinflammatory cytokine and chemokine expression promoted increased ICAM-1 expression, increased and prolonged edema formation, and inflammation during DTH. The heparan sulfate proteoglycan Sdc-1 emerges as a novel target in the treatment of contact allergies.

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Disclosures

The authors have no financial conflict of interest.

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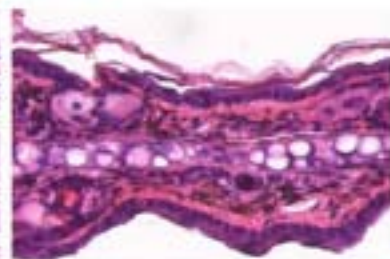
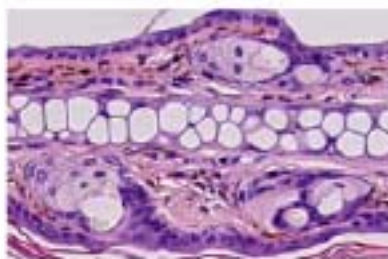
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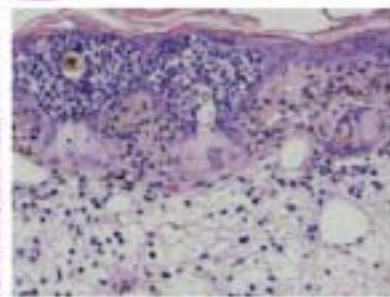
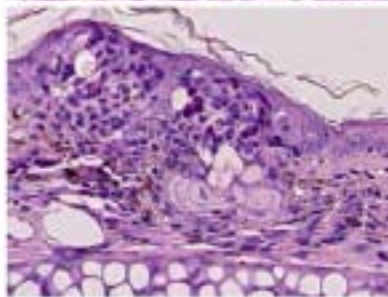
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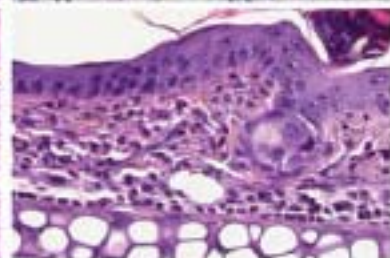
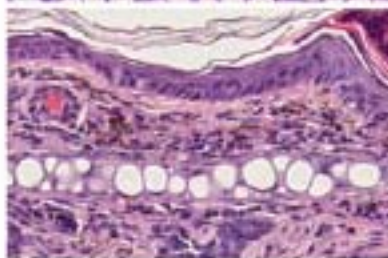
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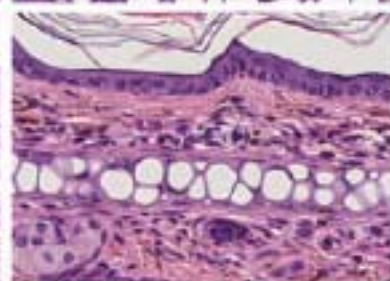
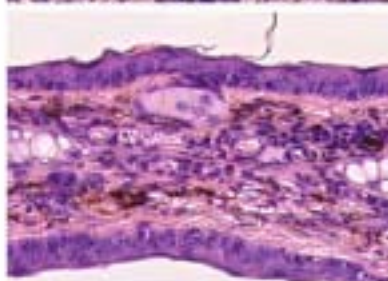
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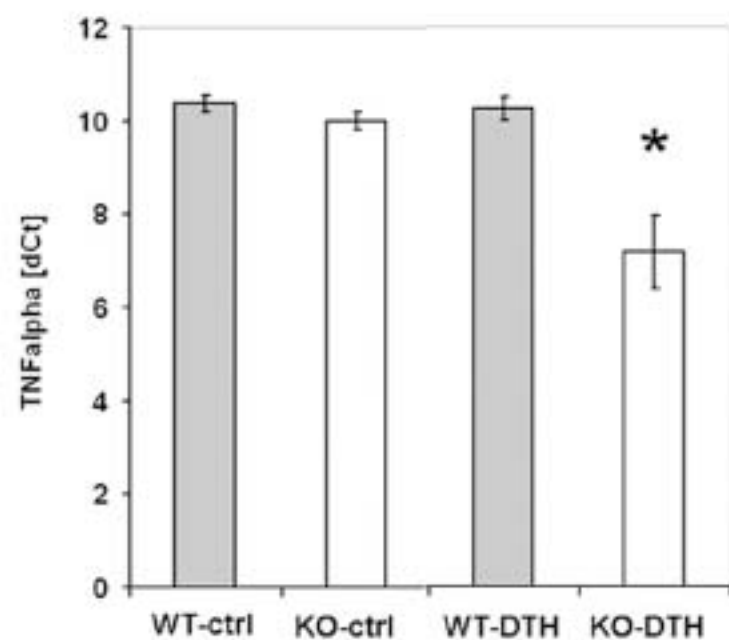
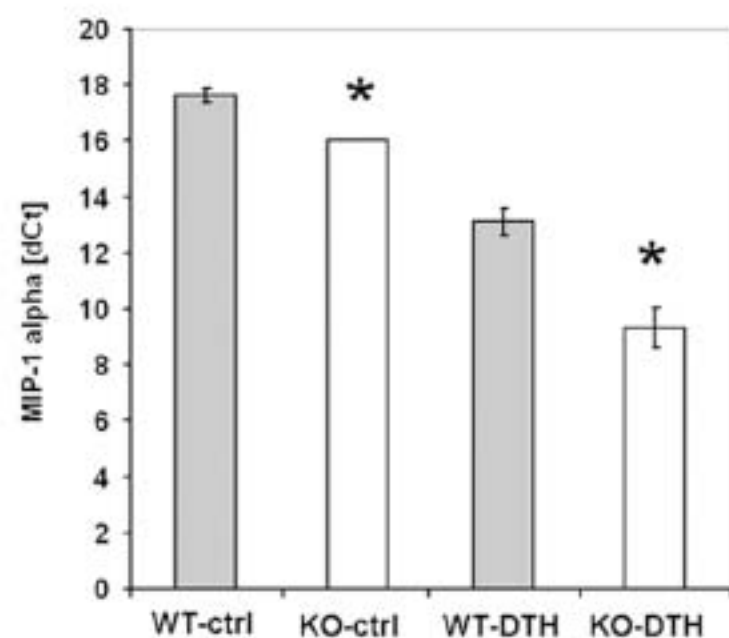
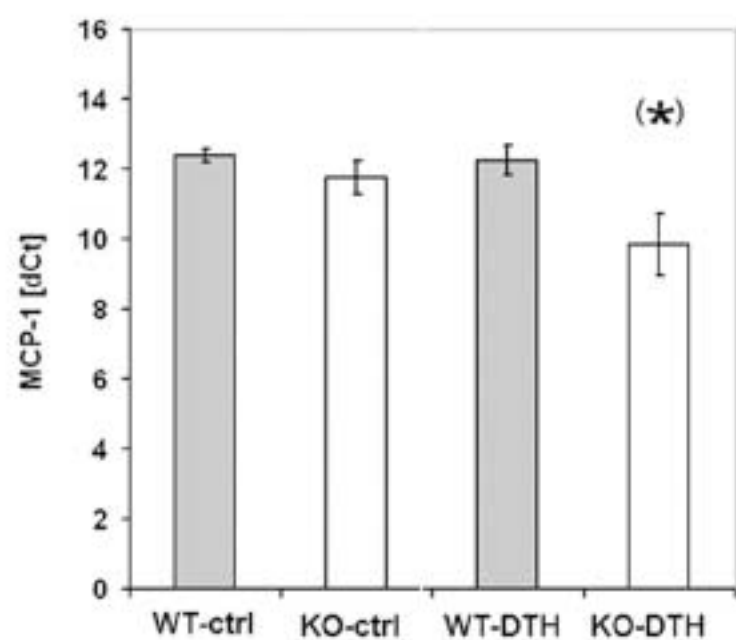
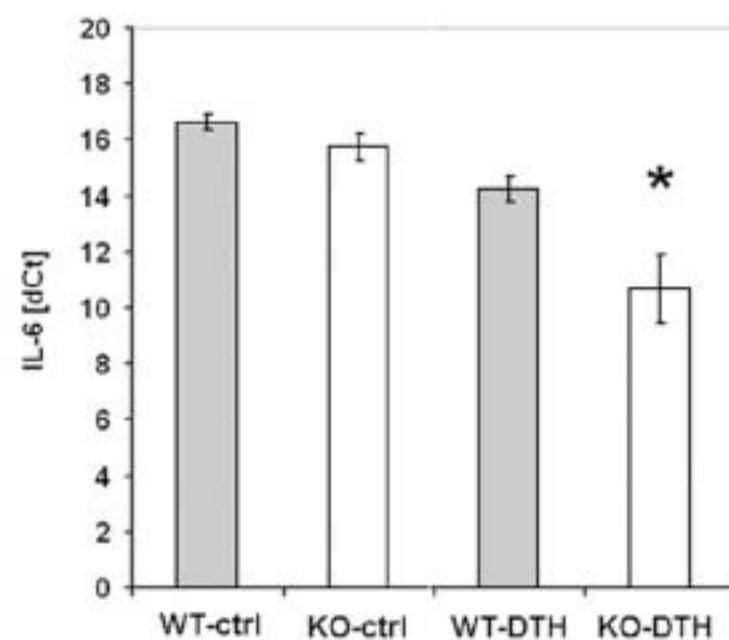
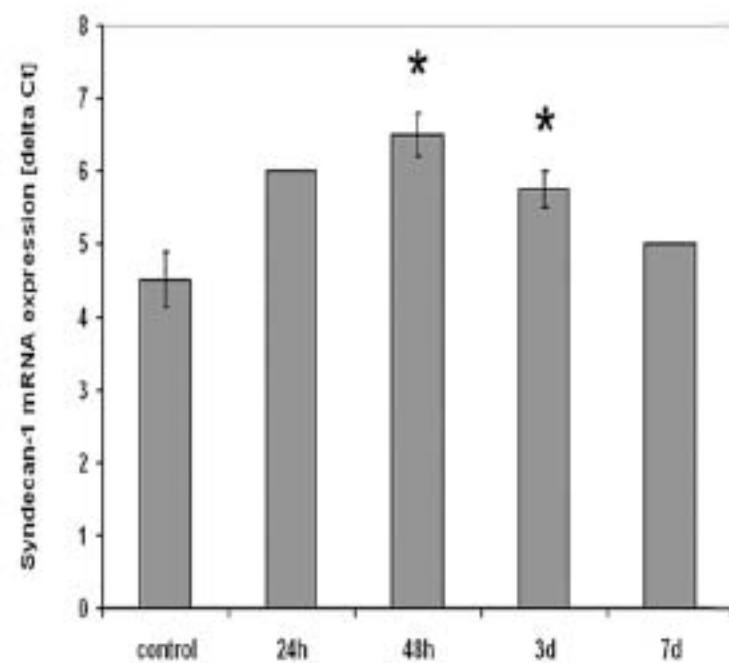


72 h



7 d



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