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Supplemental Information

Endothelial Heparan Sulfate Controls Chemokine Presentation in Recruitment of Lymphocytes and Dendritic Cells to Lymph Nodes

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Supplemental Experimental Procedure

Generation of *Tek-rtTA* transgenic mice. To construct the targeting vector for *Tek-rtTA* transgenic mouse generation, the 2.1 kb of the mouse Tie2 promoter and 10 kb of full enhancer were amplified by PCR from pT2HLacZpA11.7 vector (Schlaeger et al., 1997) provided by Dr. Thomas Sato and subcloned into the HindIII and XBaI/NotI sites of a pBluescript II SK(+) vector, respectively (Figure S1A). The 1.5 kb *rtTA* with a poly A sequence was amplified from commercial pTet-On vector (Clontech) and cloned into the EcoRI and SmaI sites of the same pBluescript vector (Stratagene). All these cloned DNA sequences were verified by complete sequencing. The targeting vector was injected in the blastocysts by the personnel in the Animal Facility of Sanford-Burnham Medical Research Institute, Three times injection generated a total 33 independent mouse clones (Figure S1C), among them clones 8, 19, 22, 24, 25, 27, 29 and 33 harbor the transgene. Clones 8 and 29 were selected to crossbreed with *Tet-Cre* and *Rosa26r* reporter lines or $Ext1^{flox/flox}$ for further characterization. After 2 week treatment with Dox in drinking water (2 mg/ml), the whole amount X-gal staining of various organs including heart, lung, aorta and lymph nodes was performed as described (Teng et al., 2002). Since both lines exhibited comparable efficiency in the recombination of β-galactosidase in *Rosa* and *Ext1* gene in $Ext1^{flox/flox}$ after Dox induction, clone 29 was chosen for most of the experiments described in this study. Unless specified, the mice that received a 3-week Dox treatment were subjected to analyses described in this study.

Reagents. The glycosaminoglycan-degrading enzymes, heparitinase, heparinase and chondroitinase ABC were from Seikagaku Co., Japan. Disparse I and Collagenase I were from Roche and Invitrogen, respectively. Recombinant mouse IL-4 and GM-CSF were from R&D. The following antibodies were used in the study: anti-heparan sulfate (HS) 10E4 (Seikagaku), antibody specific to digested HS proteoglycans 3G10 (Seikagaku), anti-chondroitin sulfate/dermatan sulfate (CS/DS) CS56 (Seikagaku, Japan), anti-digested

CS/DS proteoglycans 2B6 (Seikagaku, Japan), anti-mouse CD31 (390, BD), rat anti-perlecan (A7L6, Millipore), rabbit anti-mouse syndecan 4 (IBL, Japan), anti-mouse CD3 (17A2, BD), anti-mouse CD3ε (M20, Santa Cruz), anti-mouse B220 (RA3-6B2, BioLegend), anti-mouse PNAd (MECA-79, BD), FITC-conjugated MECA-79 (Santa Cruz), Alexa488-conjugated anti-mouse ICAM-1 (YN1/1.7.4, BioLegend), mouse CCL21 (R&D), mouse CCL19 (R&D), goat anti-mouse CXCL2 (R&D), biotinylated hamster anti-mouse CCL2 (eBioscience), biotinylated goat anti-mouse CCL21 (R&D), biotinylated goat anti-mouse CCL19 (R&D), PE-conjugated anti-mouse Podoplanin (8.1.1, BioLegend), PE-conjugated anti-mouse L-selectin (MEL14, BD), anti-mouse Gr1 (RB6-8C5, BD), PE-conjugated anti-mouse CD11c (Invitrogen), control mouse IgM (R6-60.2, BD), control mouse IgG (A85-1, BD), anti-rat Ig (MRK-1, BD). Alex488 or Alexa594-conjugated secondary antibodies (Invitrogen) were used for fluorescence microscopy.

Blood cell counting. Peripheral blood (200 μl) was collected from mouse eyes, and immediately transferred to EDTA-containing tubes (BD). Cell numbers of total white blood cells, lymphocytes, monocytes and granuocytes were analyzed by VETSCAN HMII (Abaxis) according to manufacturer's manual.

Lymphocyte homing. Lymphocytes from spleen and mesenteric lymph nodes of WT or mutant donor mice were labeled with Cell Tracker Green CMFDA or Orange CMRA (Molecular Probes), respectively, and were injected into the tail veins of adult WT and mutant recipient mice. After 1 h mice were killed, peripheral lymph nodes, mesenteric lymph nodes, Peyer's patches and spleens were collected. Lymphocyte suspensions were prepared from these organs and analyzed by Cytometer for cell numbers and by flow cytometry to determine the fraction of fluorescent cells. Numbers of homed cells were calculated by multiplying the total organ cell numbers by the fraction content of fluorescent cells. In some case, freshly isolated lymphocyte from donor mice were treated with a mixture of heparitinase and heparinase (25 mIU/ml each) at 37°C for 1 h before labeling with Cell Tracker and tail vein injection.

Lymphocyte proliferation. Single cell suspensions were prepared from inguinal lymph nodes of mice with or without abdomen skin sensitization of DNFB as described (Kawashima et al., 2005), and were cultured for 40 h in the presence or absence of 2 μg/ml of concanavalin A (EY Laboratories) or 200 μg/ml of DNBS (MP Biochemicals). Cell proliferation was measured with a Quick Cell Proliferation Assay Kit (BioVision). The absorbance at 650 nm obtained in the absence of Concanavalin A or DNBS was subtracted from that obtained in the presence of these agents.

Lymphocyte rolling. CHO cells stably expressing the following proteins and enzymes were generated as described (Mitoma et al., 2007) and were plated as monolayers in a flow chamber: CD34 and FucT-VII or CD34, FucT-VII, Core1-β3GnT, C2GnT1 and GlcNAc6ST-2. The monolayers were subjected to treatment with PBS containing 2 mM $CaCl_2$ or with PBS containing 2 mM $CaCl_2$ plus heparitinase and heparinase, or chondroitinase ABC, or all three enzymes together at 37°C for 1 h. Plates containing treated cells monolayers were then assembled in a parallel plate flow chamber on the stage of an inverted microscope equipped with a video microscopy, the chamber was perfused with 38C13 cells under controlled flow rates, and video scenes of rolling and tethered cells were recorded. Tethered cells that had a high rolling velocity near that of free-floating cells with multiple pauses in less than 0.5 s were counted and the rolling velocity of the tethered cells were measured. The effectiveness of the enzyme treatment on the cell monolayers was assessed with flow cytometry, using antibodies 10E4, 3G10, CS56 and 2B6 to stain the cells after harvesting them from the plate with PBS containing 3 mM EDTA.

Contact hypersensitivity. Mice were sensitized by 25 μl of 0.5% DNFB (Sigma) in acetone:olive oil (*v:v*, 4:1; vechicle) on the shaved abdominal skin on day 0 and day 1. On day 5, right ears were treated with 20 μl of 0.2% DNFB in PBS and the left ears with vehicle only. Swelling was measured with a thickness gauge before and 24 h after treatment (Kawashima et al., 2005). Cell numbers of draining inguinal lymph nodes and homing of lymphocytes to the draining lymph nodes of WT and mutant mice were assayed as described above on day 5 after the initial sensitization.

Skin painting Abdomen of mice were shaved and painted with 200 μl of fluorescein (Sigma, 5 mg/ml) in acetone: dibutyl phthalate (*v:v*, 1:1) . After 24 h, mice were killed and the cells of draining inguinal lymph nodes were counted and subjected to immunostaining with PE-conjugated CD11c on ice for 30 min. The stained cells were analyzed by flow cytometry to detect the fraction of $CD11c^+$ and $FITC^+$ double positive cells. In parallel, draining inguinal lymph nodes were imbedded in OTC compound, and the frozen sections of the tissues were examined by immunofluorescence microscopy.

Isolation of mouse lung endothelial cells. Isolation of mouse lung endothelial cells followed the protocols as described (Marelli-Berg et al., 2000). Mice lung were extensively perfused with PBS, dissected into Hanks-buffered salt solution (HBSS), cut into small pieces of 2 mm diameter, and digested with 0.5 mg/ml of Collagenase I at 37 °C for 90 min. The digest passed through a 70 μm cell strainer and incubated with rat anti-mouse CD31 on ice for 30 min. After washing with 0.5%FBS/PBS, cells were further incubated with goat anti-rat IgG-conjugated MACS beads (Miltenyi Biotec GmbH) on ice for additional 30 min followed

by magnetic cell sorting using a LS column according to the manufacturer's instruction. Bound cells were eluted with 0.5%FBS/PBS and washed with PBS twice. Cells were kept at –80 °C until use.

Isolation of MECA-79$^+$ lymph node HEV cells. Mouse peripheral and mesenteric lymph nodes were dissected from WT and mutant mice. Cells of dissected lymph nodes were gently squeezed out by glass slides, and the stroma tissues were treated with 0.2 mg/ml Disparse I and 0.2 mg/ml Collagenase I in RPMI medium at 37 °C for 90 min. The digest was passed through a 70 μm cell strainer and further treated with trypsin at 37 °C for 5 min. Digestion was stopped by addition of 10%FBS/RPMI, and cells were incubated with FITC-conjugated MECA-79 in 0.1%BSA/RPMI on ice for 30 min. Following a wash with 0.1%BSA/RPMI, the cells were further incubated with 20 μl anti-FITC-conjugated MACS beads (Miltenyi Biotec GmbH) on ice for additional 30 min followed by magnetic cell sorting as described above. Freshly isolated cells were kept at –80 °C until use.

Isolation of mouse dermal podoplanin$^+$ lymphatic endothelial cells. Mouse dermal lymphatic endothelial cells were isolated and cultured as described (Kriehuber et al., 2001) with slight modification. The skin of mouse trunk was shaved, peeled off and cut into 2 cm long strips, and digested with Collagenase I at 37 °C for 90 min. Dermal cells were striped from the epidermal skin, pass through a 70 μm cell strainer, cultured in endothelial growth medium (Lonza) in fibronectin-coated plate (10 μg/ml). Medium was changed every other day until reaching near confluency. Near confluent monolayer cells were dissociated with trypsin and incubated with PE-conjugated anti-mouse podoplanin on ice for 30 min. After a brief wash with 0.5%FBS/PBS, cells were further incubated with magnetic beads conjugated with anti-PE antibody (Miltenyi Biotec GmbH) followed by a magnetic cell sort as described above. Isolated cells were again cultured in fibronectin-coated dishes with the endothelial growth medium. Cells were splitted once reaching confluent, and only 1-3 time passengers were used in the experiments in the study.

RT-PCR and genomic PCR. Freshly isolated mouse lung endothelial cells, MECA-79$^+$ lymph node HEV cells and splenic lymphocytes were used for this analysis. Total RNA was extracted by Trizol reagent (Invitrogen) and reversely transcripted by Superscript II (Invitrogen). Genomic DNA was extracted by protease K digestion of the cells followed by an ethanol precipitation. The following forward and reverse primers were used for the PCR reactions: 5'-GACGTCTTGCTCCTCACCAC-3' and 5'-TGCGGTAGGACGAACAATCC-3'for Ext1; 5'-GAGAACAGGTACCCATGTTC-3' and 5'-GGAGTGTGGATGAGTTGAAG-3' for deleted Ext1; 5'-CCTGGCCAAGGTCATCCATGACA-3' and

5’-ATGAGGTCCACCACCCTGTTGCT-3’for Gapdh;
5’-TCTGACAACTTCTCTGGCTC-3’ and 5’-TGCGGATGAGATGTGACAGCTGCC-3’for syndecan-1; 5’-GTCCGCAGAGACGAGAACAGAGCT-3’ and
5’-TCAGGTGACTCAGTCTGAGCAGGT-3’for syndecan-2;
5’-CTCTGGCTACTTCGAGCAGGAGTC-3’ and
5’-CTGTGGCAGGTGCTGTGGCCATAG-3’for syndecan-3;
5’-GGAGAGTCGATTCGAGAGAC-3’ and 5’-TTGGACATGTCATCCCCCACGTCG-3’for syndecan-4; 5’-AGTGTCATTGGCGGTGTGCACGTG-3’ and
5’-GGCAGGAGTCATGGCCATCTTCTC-3’for glypican-1;
5’-CTCCTGCTGCTGGCTGAGAAACTC-3’ and
5’-CATACGGTTACAGGCAGTCCAGCC-3’for glypican-2;
5’-ACCACCATTGGCAATTGTGTGCC-3’ and
5’-CAATGATCTGGCTAAGGACCGGCT-3’for glypican-3;
5’-GCCTTCAGTGCTCGATTCAGACCT-3’ and
5’-TGGCTTGCTGGTGTCAACCTGGAC-3’for glypican-4;
5’-GAGCCTGATGCTCAGTAAGCCCTG-3’ and
5’-GTGTTGGCGTTCTCACTGGATGGC-3’for glypican-5;
5’-GATGCTGTACTGCCCATACTGCCG-3’ and
5’-TCTGGAGCTGAGCGAGCAGATCTG-3’for glypican-6; and
5’-GGACACTGCTCTGTTGTGAC-3’ and 5’-AGGCTCACACTGGACCACAT-3’for L-selectin. Reverse-transcripts of total RNA extract from mouse myoblast cell line C2C12 were used for controls for PCR reactions.

Whole mount X-gal staining. Mouse organs including heart, lung, aorta and peripheral lymph nodes were dissected and subjected to X-gal staining as described (Teng et al., 2002). Following overnight staining, organs were washed with PBS and photographed under a light microscopy.

Immunohistochemistry and flow cytometry. For immunofluorescence staining, frozen sections were fixed with acetone for 10 min, incubated with primary antibody in 1%BSA/PBS for 2 h at RT or overnight at 4 °C and then Alexa488- or Alexa594-conjugated secondary antibody in 1%BSA/PBS at RT for 1 h. In some cases, nuclear was stained with Hoechst before microscopic detection using an Olympus fluorescence microscopy. In case of CCL2 detection, anti-mouse CCL2 (10 μg) was injected to mice through tail veins. After 30 min circulation, lymph nodes were dissected and processed as described above.

For immunohistochemical staining, paraffin sections were deparaffined and rehydrated with xylene followed by a series of different concentrations of ethanol. Antigen retrieval was done by boiling the sections with 10 mM Tris-HCl, pH 8.0, containing 1 mM

EDTA for 10 min. Sections were sequentially incubated with primary antibody at RT for 60 min, biotinylated secondary antibody at RT for 30 min, and a ABC kit (Vector) at RT for 30 min. The detection was achieved using DAB kit (Vector), and the stained sections were photographed using an Olympus light microscopy.

For flow cytometric analysis, adherent cells were first dissociated with enzyme-free dissociation buffer (Chemicon), sequentially incubated with primary antibody on ice for 30 min and then fluorescent secondary antibody on ice for anther 30 min. Propidium iodide (Sigma) was included for excluding dead cells before analysis with a FACS Scan 3 color (BD). Treatment with enzyme-free dissociation buffer was omitted for non-adherent cells.

Immunoblotting. Antibody 3G10, which recognizes enzyme-digested HS (David et al., 1992), was used to blot the major HS PGs present in lymph nodes vasculatures. A mixture of enzymes including heparitinase (40 mIU), heparinase (40 mIU), and chondroitinase ABC (500 mIU) in 0.1%BSA/PBS was injected to WT mice through the tail veins to remove the long HS and CS/DS chains attached to protein core at the vascular endothelial cell surface. After 2 h, PLNs and MLNs were dissected, and lymphocytes in the lymph nodes were gently squeezed out by two glass slides. Remaining LN stroma tissues were lysized with Tris-HCl buffer containing 1% Triton X-100 (lysis buffer). Equal amounts of cell lysates were separated by SDS-PAGE and blotted by 3G10 as described above. Bound antibody was detected by HRP-conjugated secondary antibody (GE Health) followed by cheminoilluminence using ECL plus kit (GE Health). To verify the digestion efficacy, the lysate was also immunoprecipitated with 3G10, and the immunoprecipitate was further treated with the above enzyme mixture and blotted with 3G10 as described above.

For syndecan-4 blotting, freshly isolated lung endothelial cells from control and mutant mice as described above were lysized with Triton X-100-containing lysis buffer. Fifteen μg of cell lysate proteins was subjected to SDS-PAGE separation followed by immunoblotting using rabbit anti-mouse syndecan-4 antibody. The bound antibody was detected by HRP-conjugated secondary antibody followed by cheminoilluminence using ECL plus kit as described above. In some cases, pretreatment of the lysates with heparitinase and heparinase (HSases) or a mix of HSases and CSase ABC was conducted at 37 °C for 1 h. Blotting of β-actin was run as a loading control.

ELISA for Heparan Sulfate. For ELISA of HS on syndecan-4 in mouse lung endothelial cells, cells were lysed as described above, and 20 μg of cell lysate was load to wells of a 96 well plate, which was precoated with rabbit anti-mouse syndecan-4 at 4 °C for overnight. After 1 h incubation at RT, the wells were sequentially washed with the lysis buffer, blocked with 1%BSA/PBS (blocking buffer) at RT for 30 min, incubated with antibody 10E4 (x 100 dilution) in blocking buffer at RT for 1 h, washed with PBS, incubated with HRP-conjugated

anti-mouse IgM (Pierce, x 5,000 dilution) in blocking buffer at RT for 30 min, and then washed with PBS extensively. Bound HRP activity was detected by OPD (o-phenylenediamine dihydrochloride) and the absorbance at 490 nm was recorded after adding 3M H_2SO_4 to the OPD solution. Rabbit IgG (Sigma) was used for a negative control.

For cell-based ELISA of HS expression in cultured dermal lymphatic endothelial cells, cells derived from control or mutant mice were grown to confluent in 96 wells as described above. Wells were washed with PBS twice and then added with antibody 10E4 (x 200 dilution) in blocking buffer at 4 °C for 1h. After three times washes of PBS, wells were added HRP-conjugated mouse IgM (x 5,000 dilution) in blocking buffer and kept at 4 °C for 30 min. Wells were washed with PBS and further added with OPD solution, and absorbance at 490 nm was analyzed as described above.

ELISA for Chemokine Binding. Confluent cells of cultured dermal lymphatic endothelial cells grown in 96 wells were used after extensive PBS washing for chemokine binding. To remove endogenous chemokines, 100 μg/ml of heparin (Sigma) solution was added to each well and kept at RT for 10 min with gentle shaking. After washing off the exogenous heparin by PBS, recombinant mouse chemokines CCL21 or CCL19 (1 μg/ml) in blocking buffer was added to the wells and incubated at 4°C for 1 h. Wells were washed with PBS and further incubated with biotinylated rabbit anti-goat antibody (Vector, 0.5 μg/ml) in blocking buffer at 4°C for 30 min. After PBS washing HRP-avidin (Pierce, 1 μg/ml) in PBS was added to wells and kept at 4°C for additional 30 min. As described above, OPD solution was used to detect the bound HRP activity and the absorbance at 490 nm was recorded. For negative controls, chemokine was omitted. In some cases, heparin (100 μg/ml) was mixed with chemokine and added to wells for the analysis. In other cases, the monolayer cells were treated with heparitinase and heparinase (HSases) at 37 °C for 1h prior for the chemokine binding assay as described above.

Statistics. A two-tailed Student *t*-test was applied for statistical comparison of two groups. Each group consists of three to eight mice. A p value of 0.05 or less was considered significant. The numbers in this study are expressed as mean ± S.E. except that the ELISA data are expressed as mean ± S.D.

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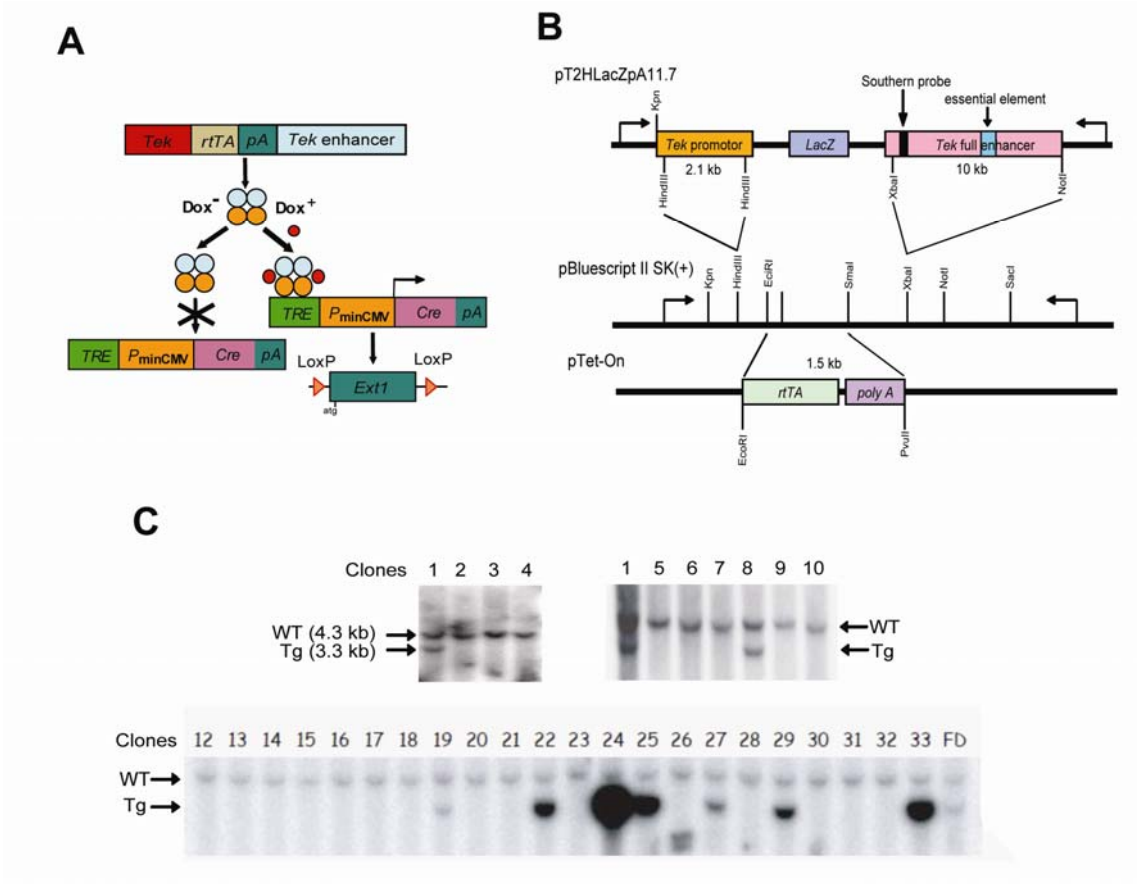

Figure S1. Scheme of animal model and generation of *Tek-rtTA* transgenic mice. (A) A scheme of inducible and conditional inactivation of *Ext1* in endothelial cells. Upon doxcycline administration, *Ext1* gene is inactivated in *Tek*-specific manner. (B) Construction of the transgenic vector. (C) Southern blots of transgenic mouse clones (see also Supplemental Experimental Procedures).

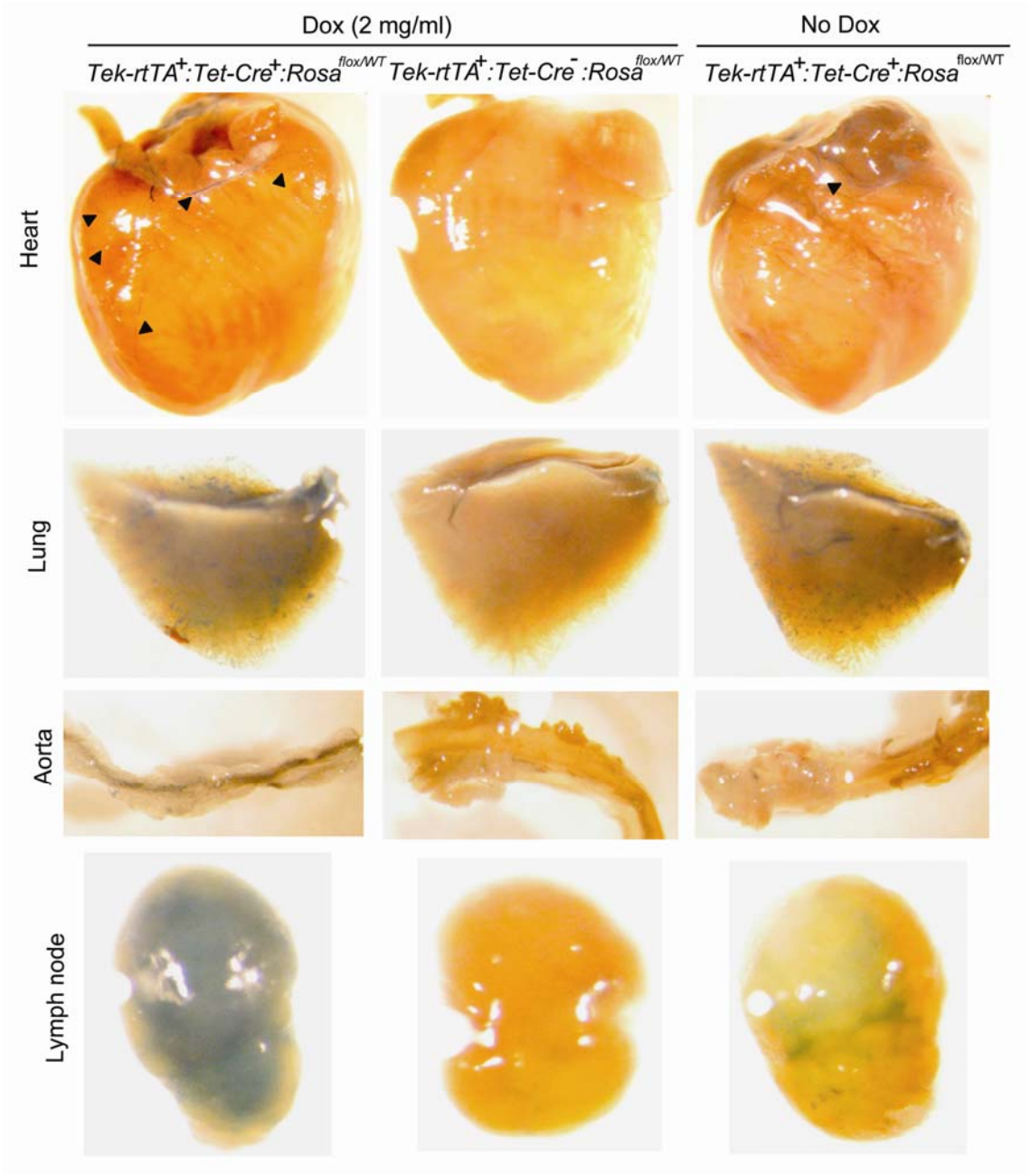

Figure S2. Whole amount X-gal staining of mouse organs. Mice of indicated genotypes were treated with doxcycline at 2 mg/ml in drinking water for 2 weeks. Note both vasculatures and lymphocytes were positive in triple transgenic mice with doxcycline (Dox) induction. A baseline leakage in some vasculatures of triple transgenic mice without doxcycline treatment was also observed.

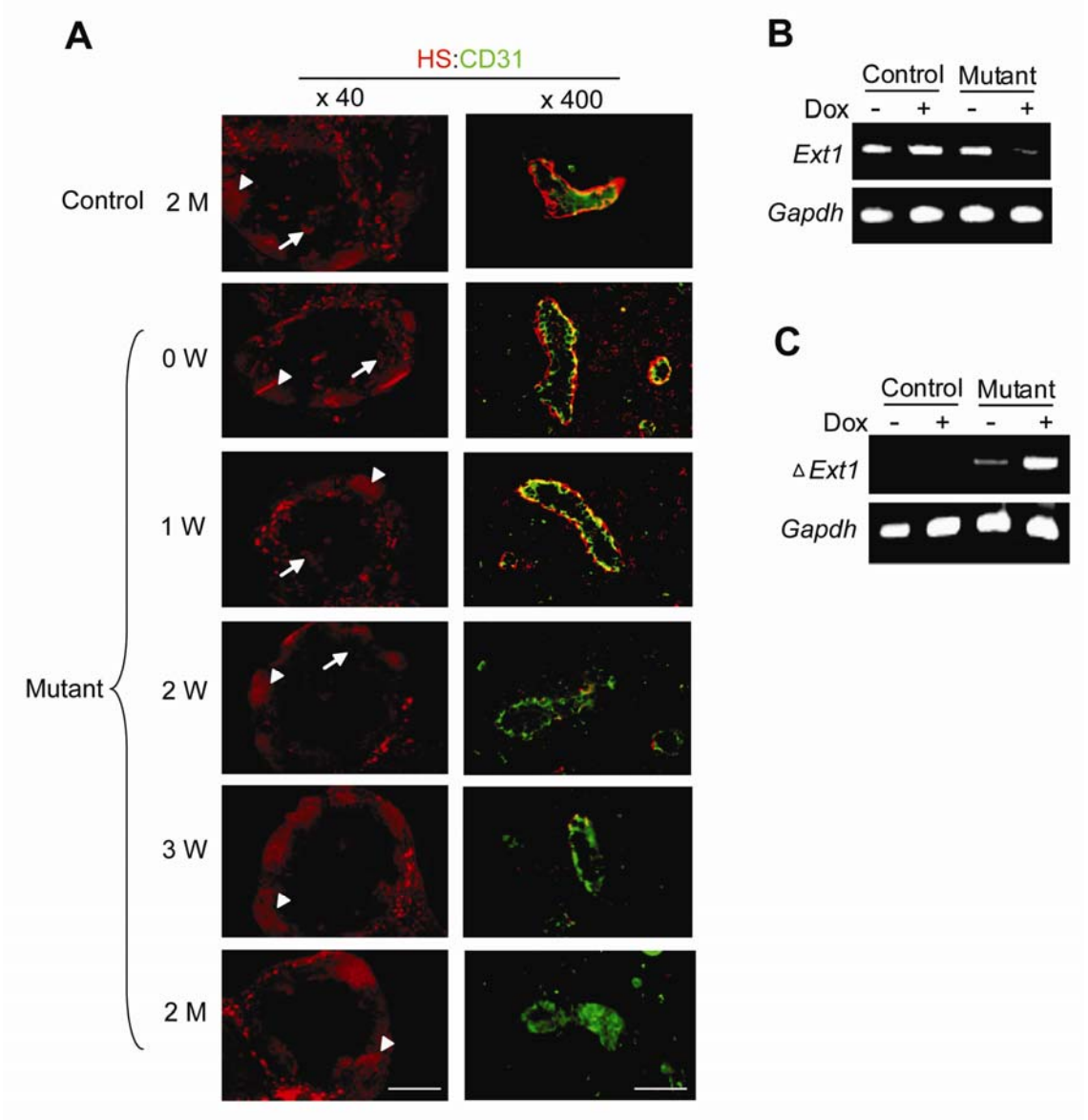

Figure S3. Efficacy of heparan sulfate deletion in mutant mice with doxcycline induction. (A) *Tek-rtTA*$^{+}$:*Tet-cre*$^{+}$:*Ext1*$^{flox/flox}$ mice were treated with doxcycline (2 mg/ml in drinking water) for different periods as indicated (W, week; M. month). After the treatment, lymph nodes were dissected and heparan sulfate expression was examined by immunostaining with mAb 10E4, which is mouse IgM. B cell follicoles are positive because of the cross-reactivity of IgM-specific secondary antibody. Co-staining with CD31 was also included. Arrow, high endothelial venules; arrowheads, IgM positive B cell follicoles. (B, C) PCR analyses of *Ext1* expression (B) and *Ext1* deletion (C) of splenic lymphocytes from mutant mice that received 3-week doxcycline treatment. *Bars* represent 500 μm in x 40 and 50 μm in x 400 amplifications, respectively.

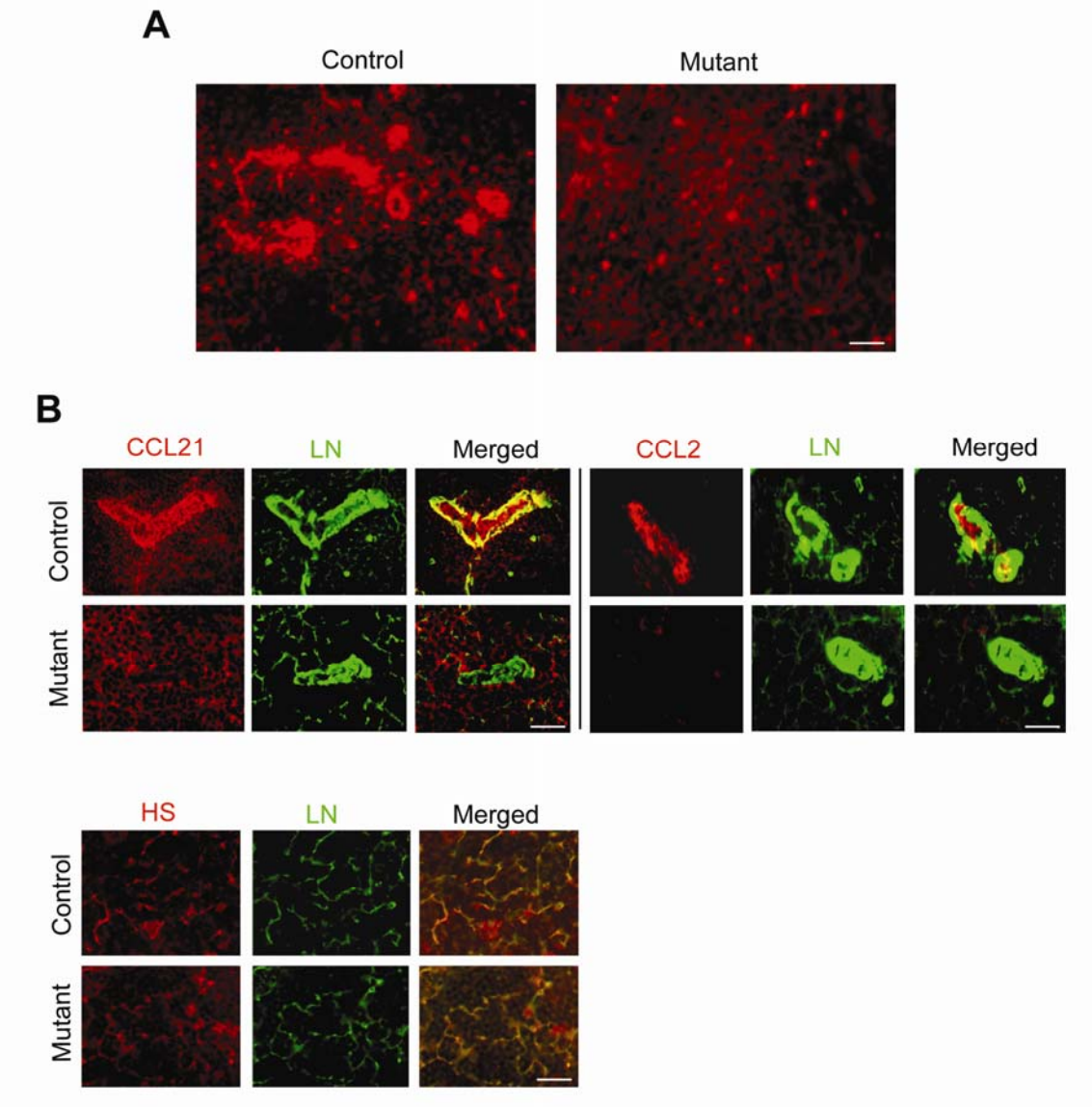

Figure S4. Expression of chemokines and heparan sulfate in lymph nodes. (A) Endogenous expression and localization of chemokine CCL21 in T cell zone of lymph nodes from control mice (left) and *Ext1* mutant mice (right) are visualized by immunostaining. Note the stromal tissues in lymph nodes are also positive for CCL21. (B) Co-immunostaining of CCL21, CCL2 and heparan sulfate (HS) with laminin (LN) in lymph nodes. Note that expression of heparan sulfate in LN^+ fibroblastic reticular network (bottom panels of B) is not altered in the mutant mice. *Bars*, 50 μm in A and the upper panels of B, and 25 μm in the bottom panels of C.

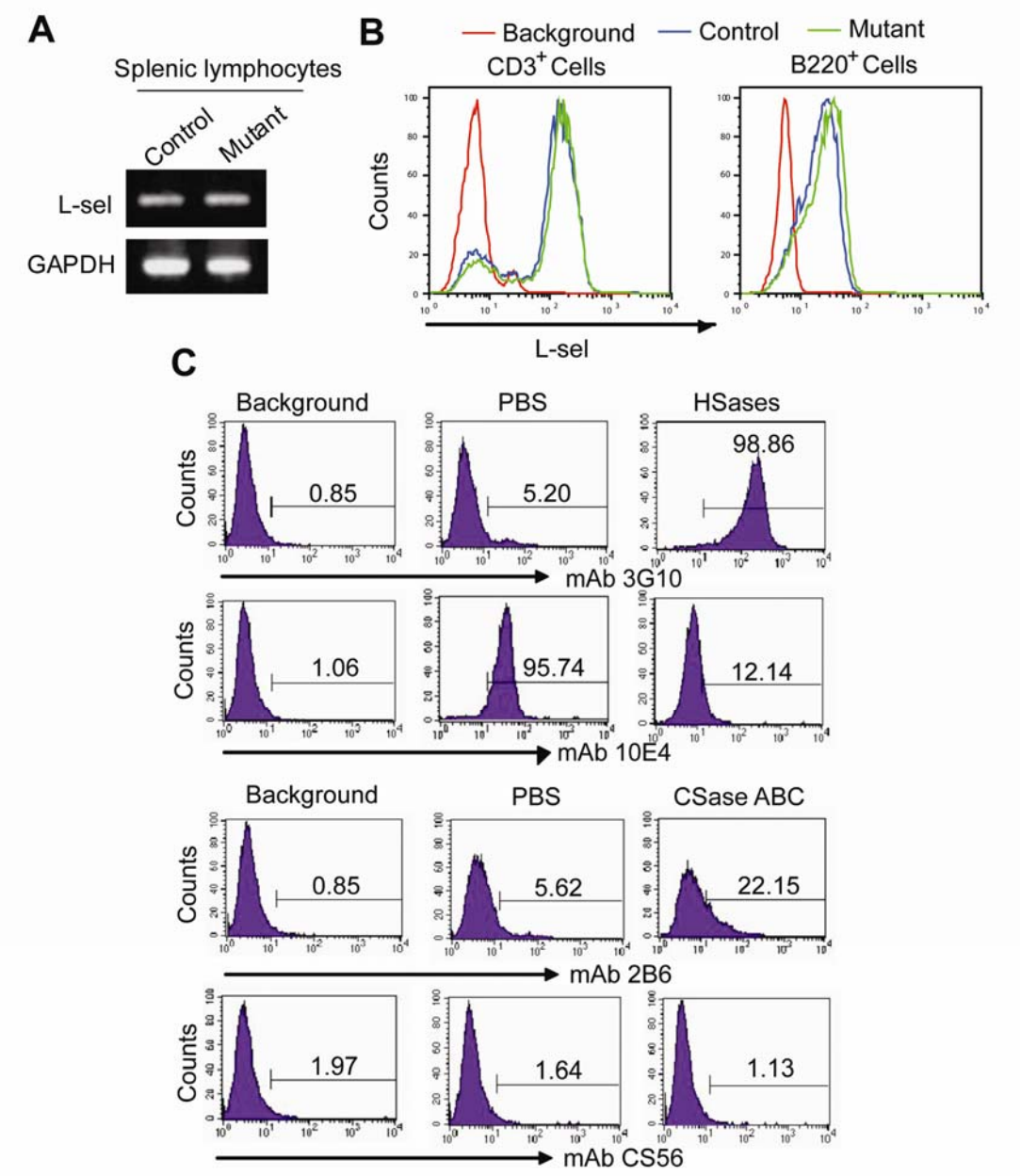

Figure S5. L-selectin expression in splenic lymphocytes and efficacy of enzymatic treatments. (A, B) Analyses of L-selectin expression in splenic T and B cells of control and mutant mice at mRNA level (A) and protein level (B) by RT-PCR and flow cytometry, respectively. (C) Flow cytometric analyses of engineered CHO cells that received treatments with heparitinase and heparinase (HSases) and/or chondroitinase ABC (CSase ABC). MAbs 3G10 and 10E4 recognize digested and intact heparan sulfate, respectively; mAbs 2B6 and CS56 recognize digested chondroitin sulfate and intact chondroitin sulfate, respectively. CHO cells were stably transfected with cDNAs encoding CD34, FuT-VII, Core1β3GnT, Core2GnT1 and GlcNAc6ST-2.

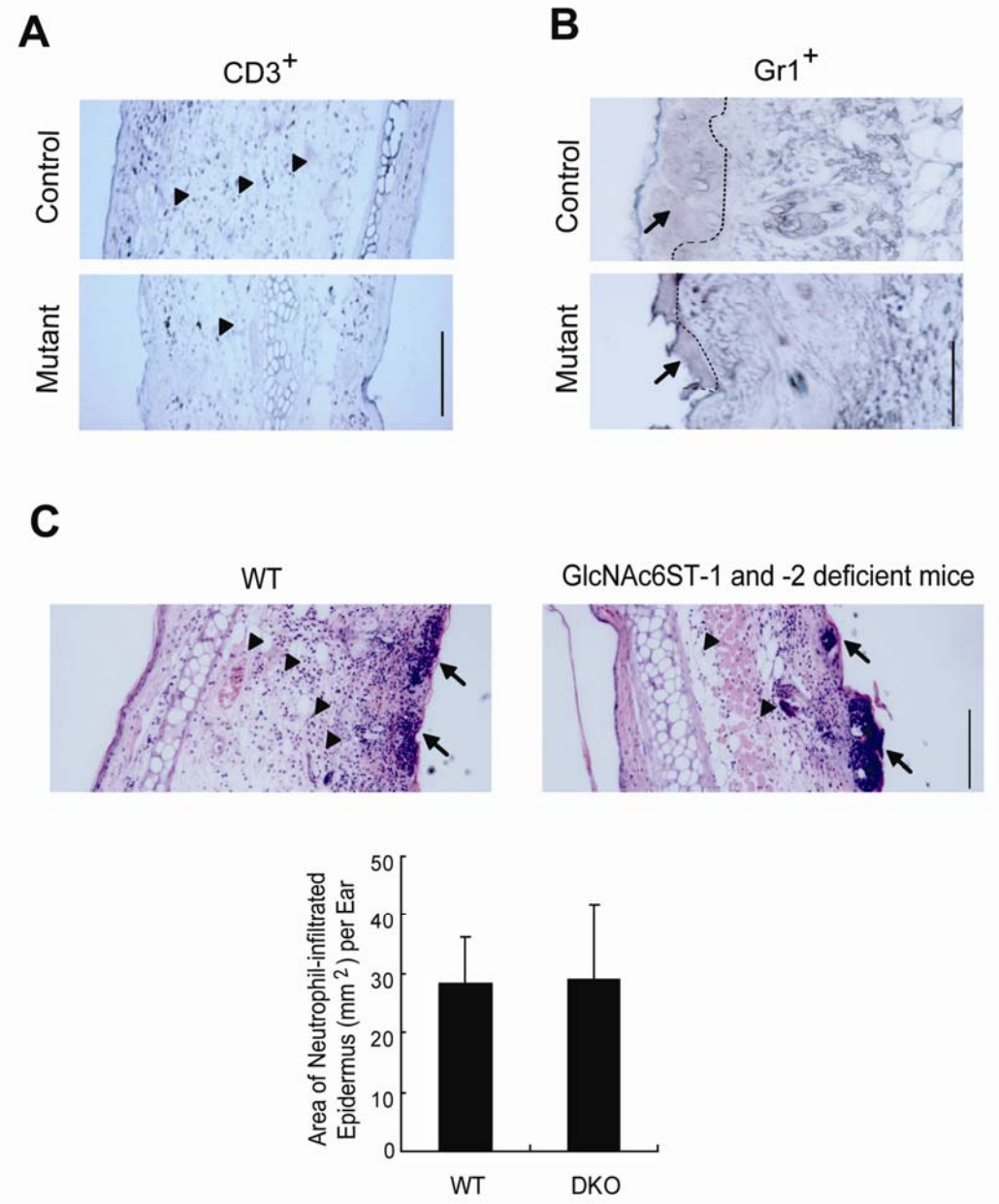

Figure S6. Histochemistry of inflamed mouse ears. (A, B) Immunostaining of $CD3^+$ T cells (A) and $Gr1^+$ neutrophils (B) of DNFB-stimulated ears in control and mutant mice. Note that $CD3^+$ cells denoted by arrowheads distributed in the inner parts of the ears, while infiltrated neutrophils were mostly confined in the epithermal layer of the ears (denoted by arrows and dashed areas). (C) Hematoxyline-and-eosin staining of the ears from WT and GlcNAc6ST-1 and –2 double knockout mice in contact hypersensitivity assay. Arrowheads denote infiltrated mononuclear cells and arrows indicate the infiltrated neutrophils. A quantitative analysis of the ear epithermal infiltration of neutrophils is shown in the bottom panels of C. *Bars*, 200 μm in A and 100 μm in B and 250 μm in C.

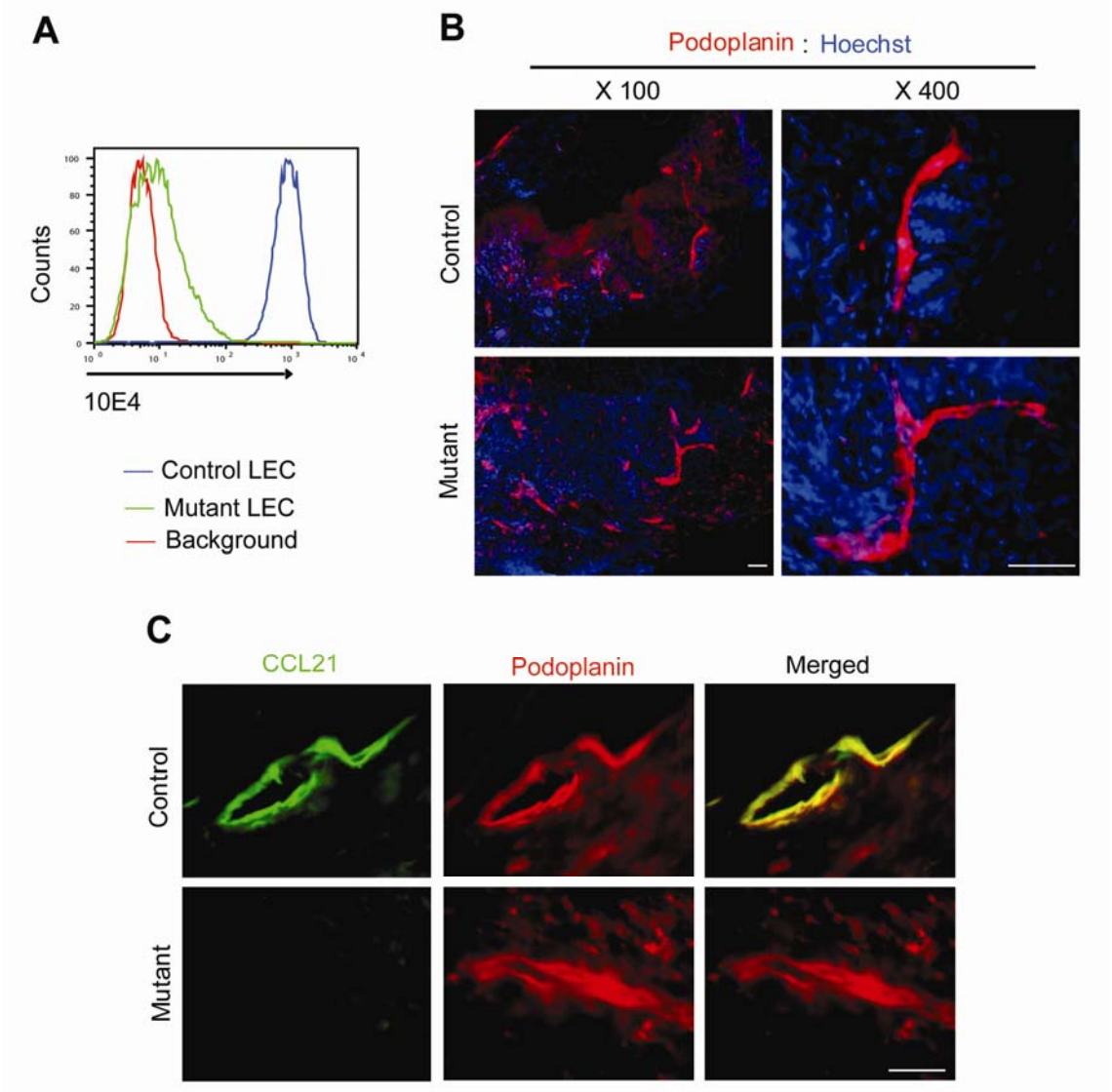

Figure S7. Heparan sulfate expression and chemokine CCL21 presentation in skin lymphatic vessels. (A) Flow cytometric analysis of cultured lymphatic endothelial cells (LEC) isolated from the skins of control and *Ext1* mutant mice. (B) Podoplanin staining of the DNFB-treated skins of control and mutant mice. Two amplifications are shown. Note that no structural alteration was visible in the mutant mice. (C) CCL21 staining of DNFB-treated skins of control and mutant mice. Note a diminished presentation of CCL21 in podoplanin-positive vessels in mutant mouse skin. *Bars*, 50 μm.