The TNF Family Member LIGHT is a Target for Asthmatic Airway Remodeling

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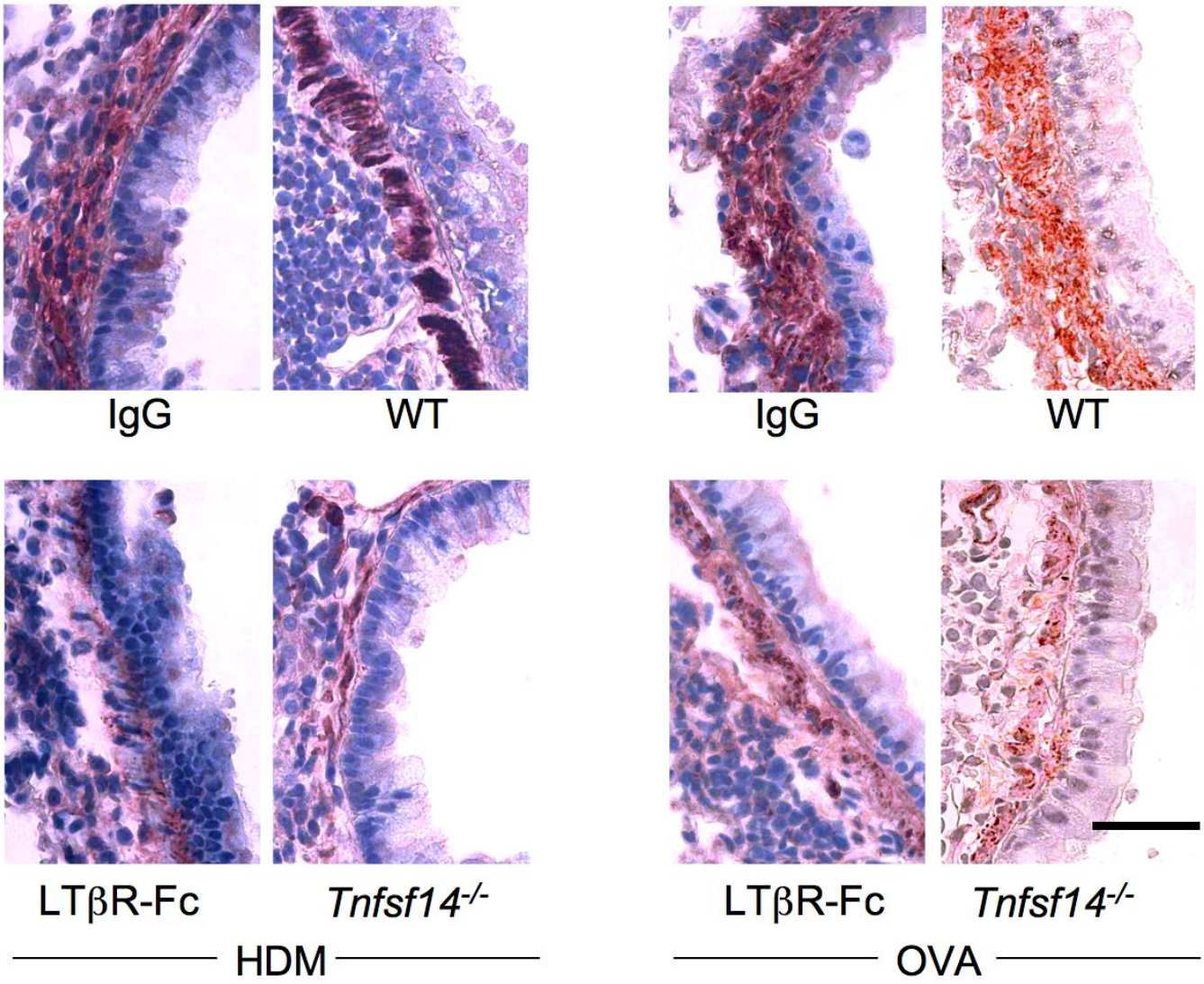
Supplementary Fig. 1



Reduction of peribronchial smooth muscle area in *Tnfsf14*$^{-/-}$ mice and after LTβR-Fc treatment. Mice from Figs. 1,2 and Supplementary Fig. 6 were chronically challenged with HDM (left) or OVA (right) as described in methods. Lung sections were stained for alpha smooth muscle actin. Scale bar, 50 μm. Untreated or IgG-treated WT mice (top row) and *Tnfsf14*$^{-/-}$ or LTβR-Fc treated WT mice (bottom row).

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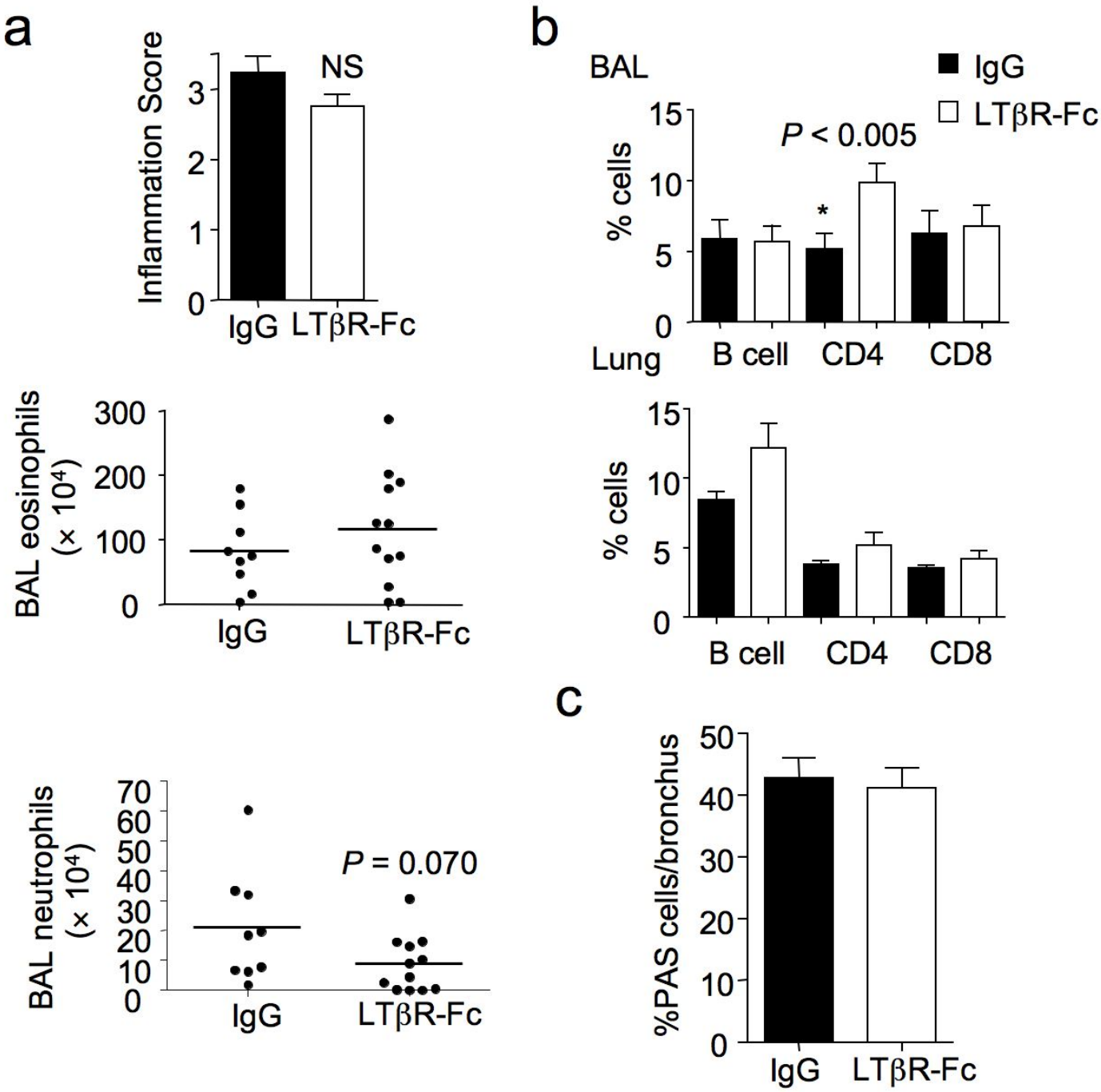
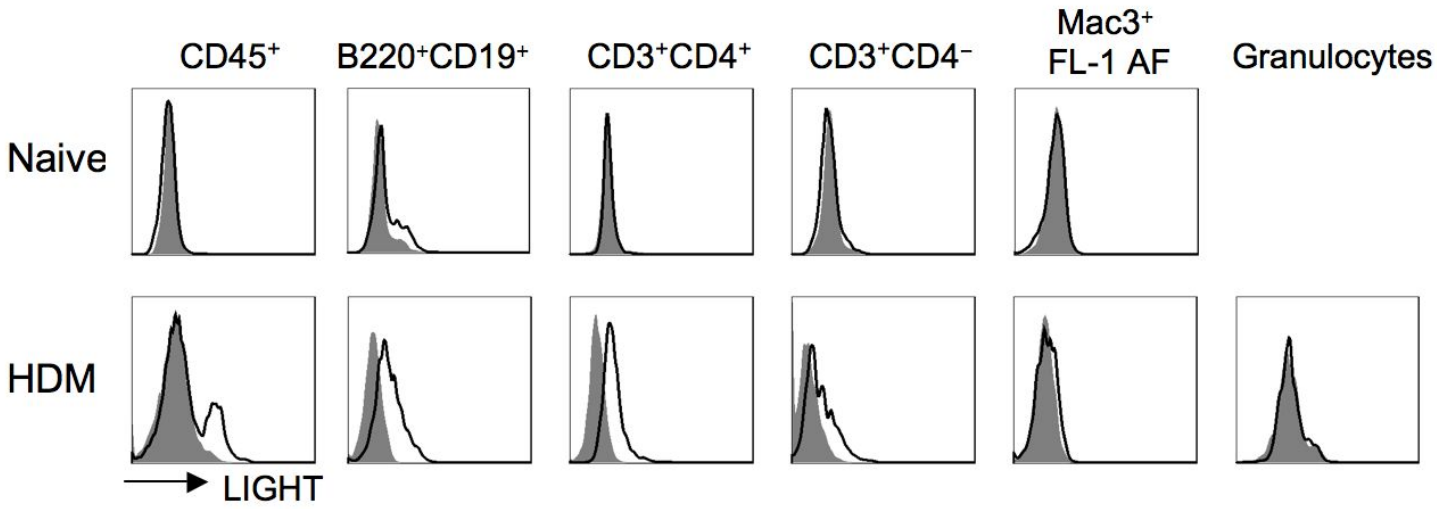
Supplementary Fig. 2



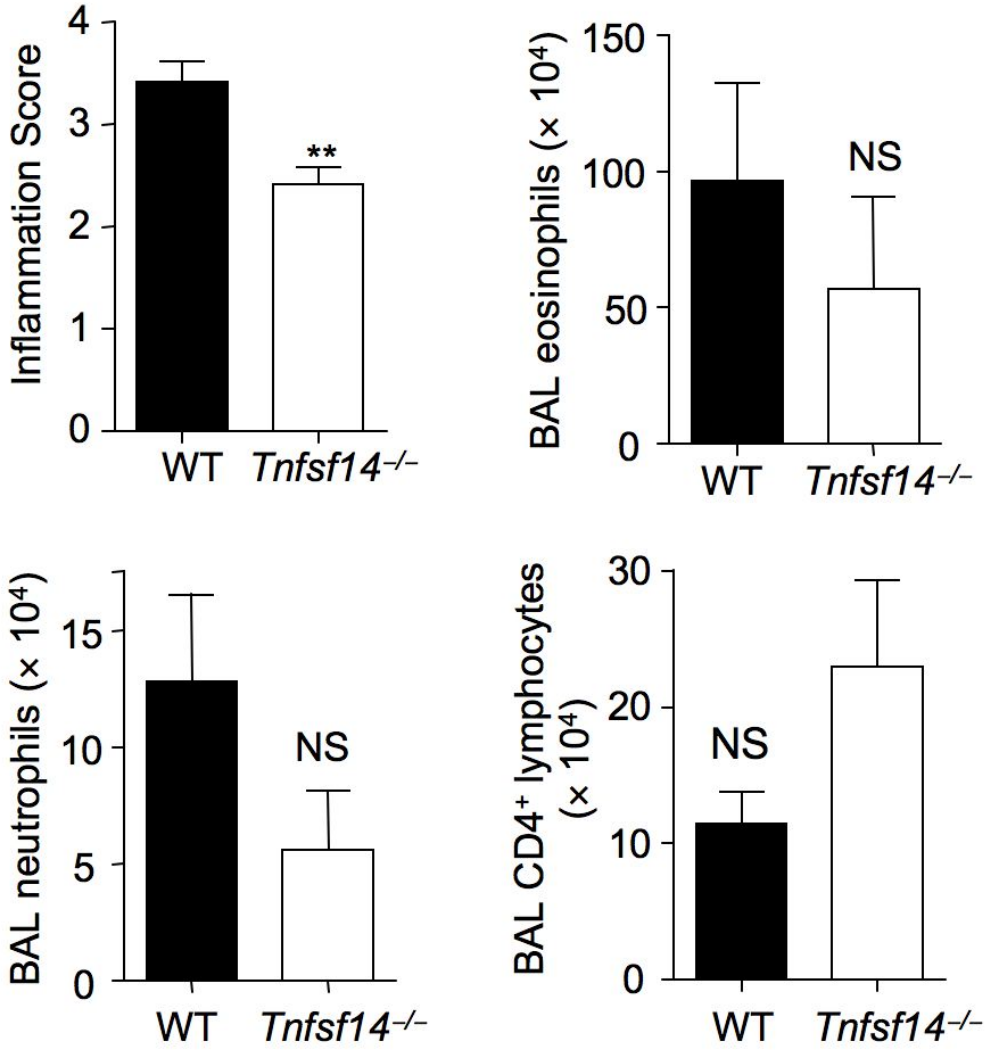
Lung cellular infiltration and mucus production after blockade of LIGHT/LTαβ. WT mice were given three challenges with HDM extract, once per week. LTβR-Fc or IgG was given 24 hours prior to each additional intranasal HDM challenge over the next four weeks (Fig. 1a). (**a**) Peribronchial infiltrate scored (top); BAL numbers of eosinophils (middle) and neutrophils (bottom). (**b**) Percentages of BAL (top) and lung (bottom) lymphocyte subsets, as described in methods. (**c**) Lung sections were stained with PAS and mucus-secreting (PAS positive) cells per bronchus enumerated. 9-12 mice per group for (**a**) and (**c**). 4 mice per group for (**b**), except BAL CD4 cells 9-12 mice per group. Data means +/- sem, Mann-Whitney, and/or values from individual mice.

Supplementary Fig. 3



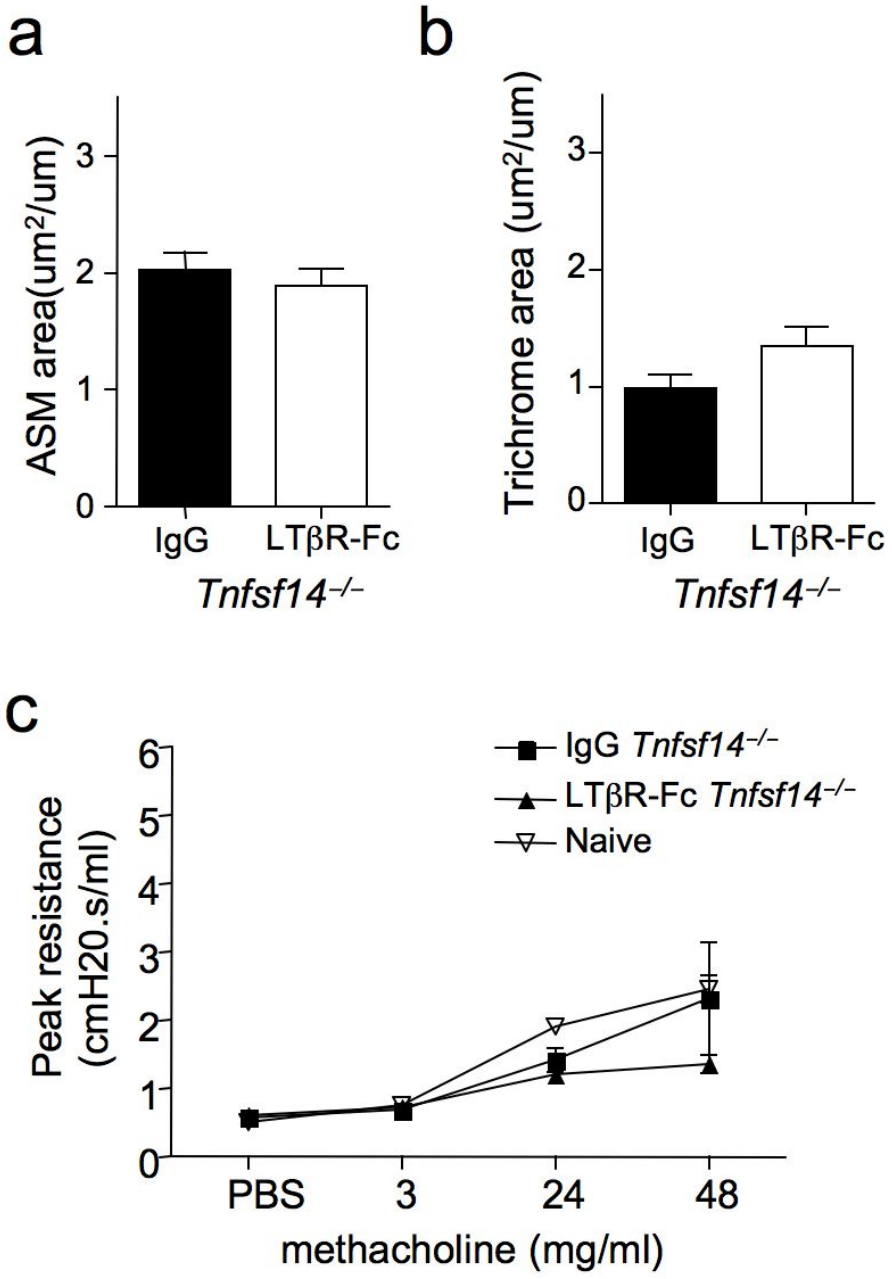
LIGHT expression on lung cells after HDM exposure. WT mice received intranasal HDM once per week for three weeks. One day after the last challenge, lungs cells were stained for CD45, CD4, CD3 (T cells), CD19, B220 (for B cells), and Mac3 with gating on autofluorescent cells (Macrophages). Granulocytes were gated on the characteristic FSC/SSC CD45+Mac3- population. LIGHT staining was performed as in methods (open black line). Control (solid grey) staining is of lung cells from *Tnfsf14*$^{-/-}$ mice (n=2 mice per group). HDM-challenged mice are compared to naïve mice.

Supplementary Fig. 4



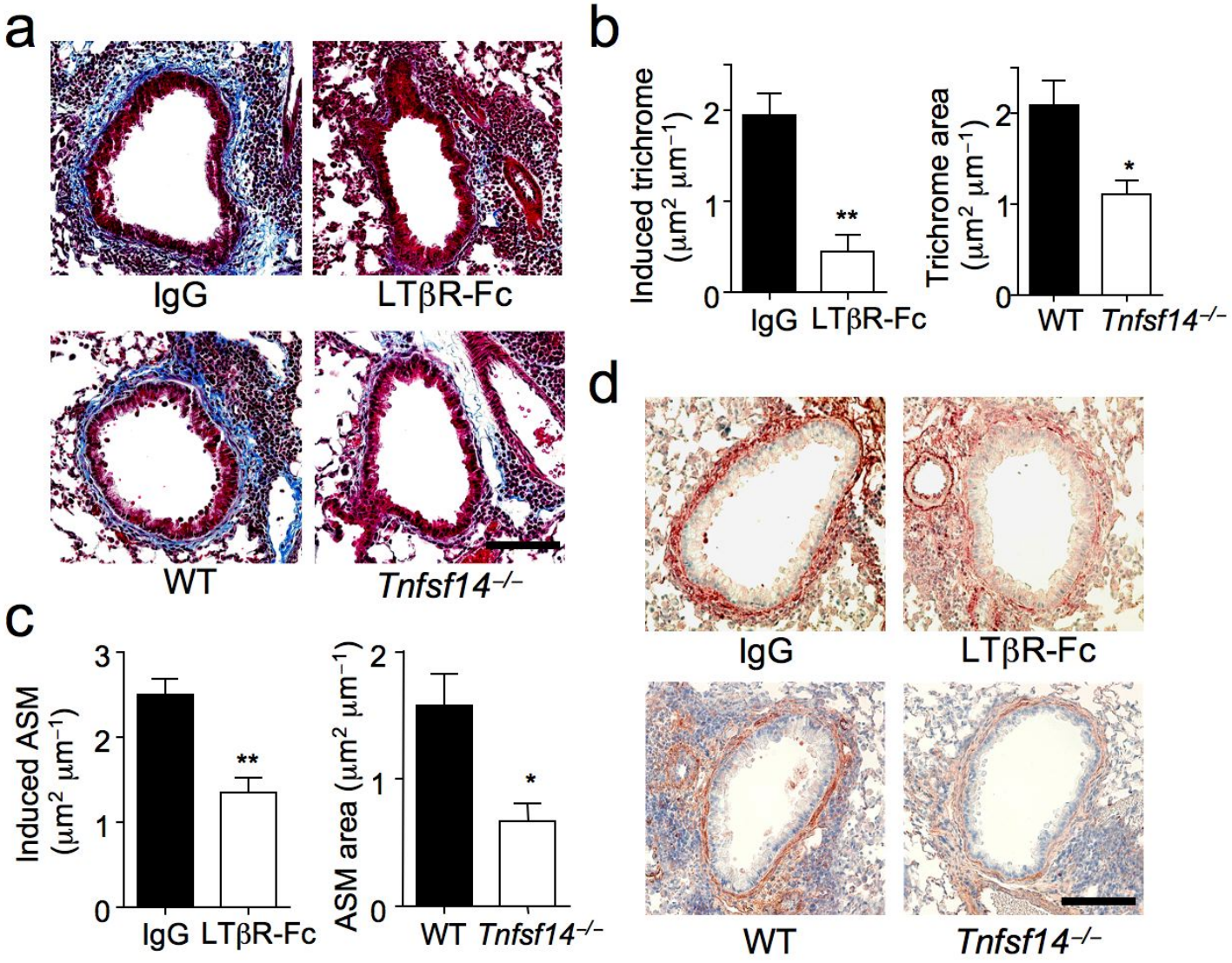
Peribronchial infiltrate, and eosinophil, neutrophil, and CD4⁺ cell numbers in *Tnfsf14*$^{-/-}$ mice after chronic HDM challenge. Lung sections from mice in Fig. 2 were stained with H&E and peribronchial infiltrates scored as described in methods (top left). BAL eosinophils, neutrophils, and CD4⁺ lymphocytes were analyzed by FACS and enumerated as in methods (top right, bottom left, bottom right, respectively). 6 mice per group, mean+/-SEM, Mann-Whitney test. $^{**}P < 0.005$, NS = no signiicant difference.

Supplementary Fig. 5



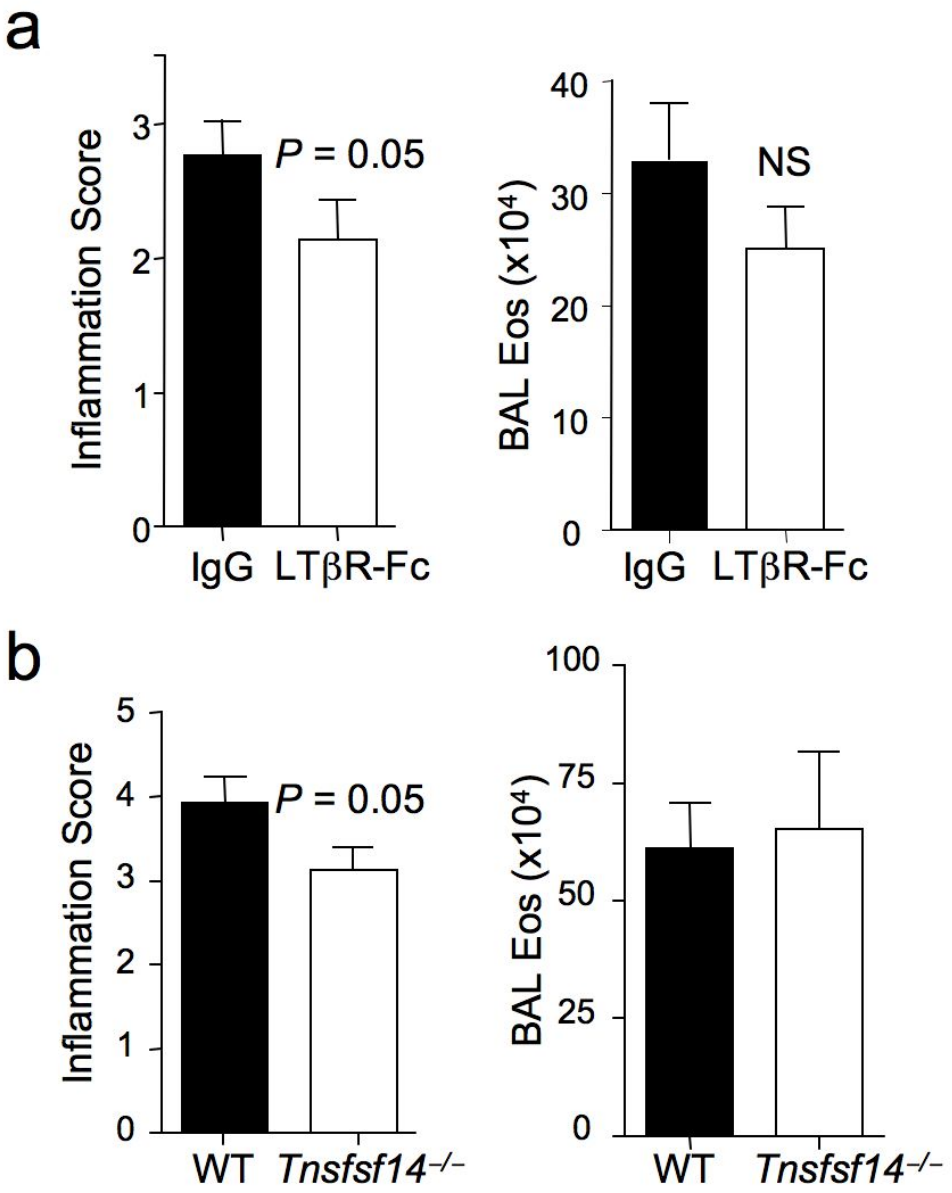
LTβR-Fc treatment in *Tnfsf14*$^{-/-}$ mice does not further suppress remodeling and AHR. *Tnfsf14*$^{-/-}$ mice were immunized and acutely challenged on days 24, 26, and 28 followed by intranasal OVA challenge, twice per week for 4 weeks. Mice then received LTβR-Fc (100ug) or human IgG i.p. beginning on day 29, and given 24 hours prior to each additional challenge. Lung sections were stained and scored for (**a**) alpha smooth muscle actin, and (**b**) fibrosis (trichrome). Some mice underwent invasive resistance tests with increasing doses of methacholine, in comparison to unimmunized naïve mice (**c**). N=6-7 mice in each group, 35-36 airways per group scored, mean+/-SEM, Mann-Whitney test.

Supplementary Fig. 6



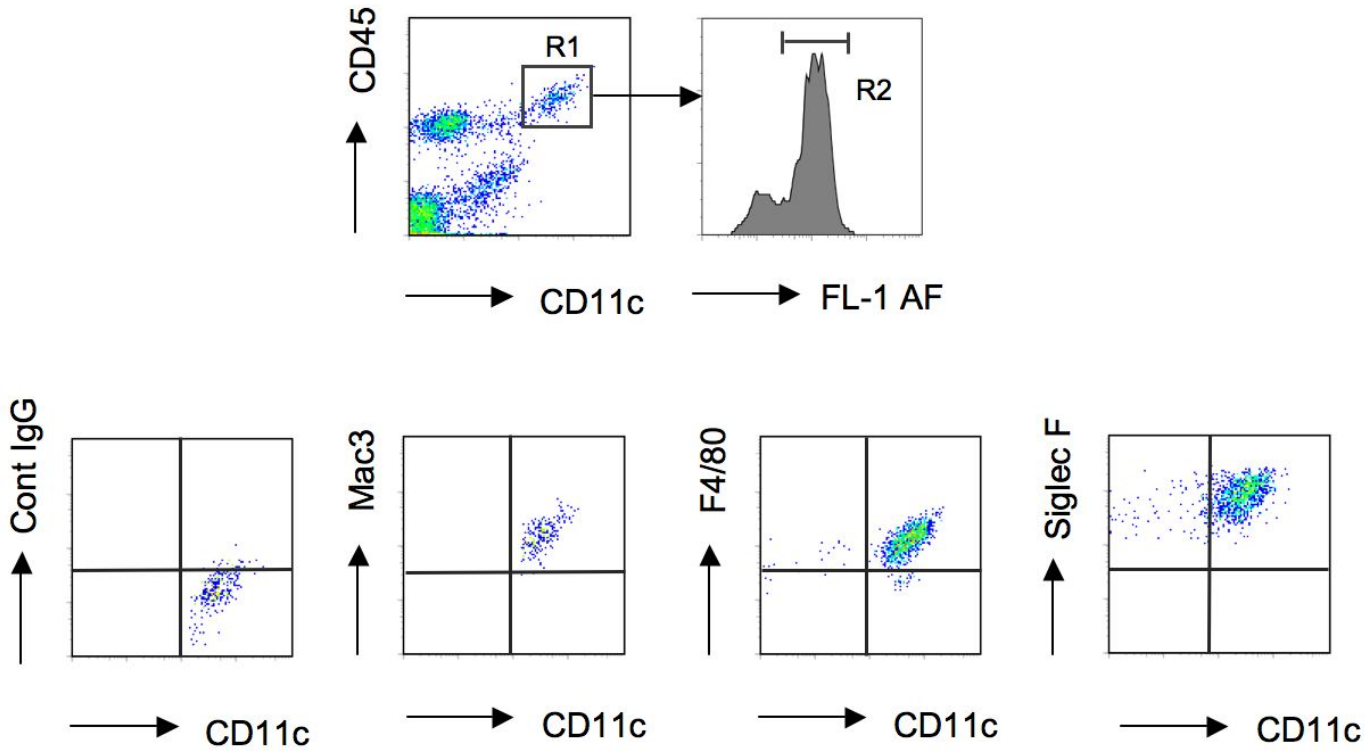
LIGHT is required for airway remodeling induced by chronic exposure to OVA. WT or *Tnfsf14*$^{-/-}$ mice were immunized and acutely challenged on days 24, 26, and 28 followed by chronic intranasal OVA challenge, twice per week for 4 weeks. Some WT mice received LTβR-Fc (100μg) or human IgG i.p. beginning on day 29 and then 24 hours prior to each additional antigen challenge. (**a-b**) Lung sections were stained with Masson's trichrome (a) and scored for fibrosis (b, n=45-48 airways per group, mean +/- sem, Mann-Whitney test). (**c-d**) Lung sections were stained for α-smooth muscle actin (d) and scored for extent of peribronchial smooth muscle (c, n=35-36 airways per group for WT and *Tnfsf14*$^{-/-}$, n=51-56 airways per group for IgG and LTβR-Fc, mean +/- sem, Mann-Whitney test). Data are representative of two experiments. *P < 0.005, **P < 0.0001. Scale bars, 100 μm.

Supplementary Fig. 7



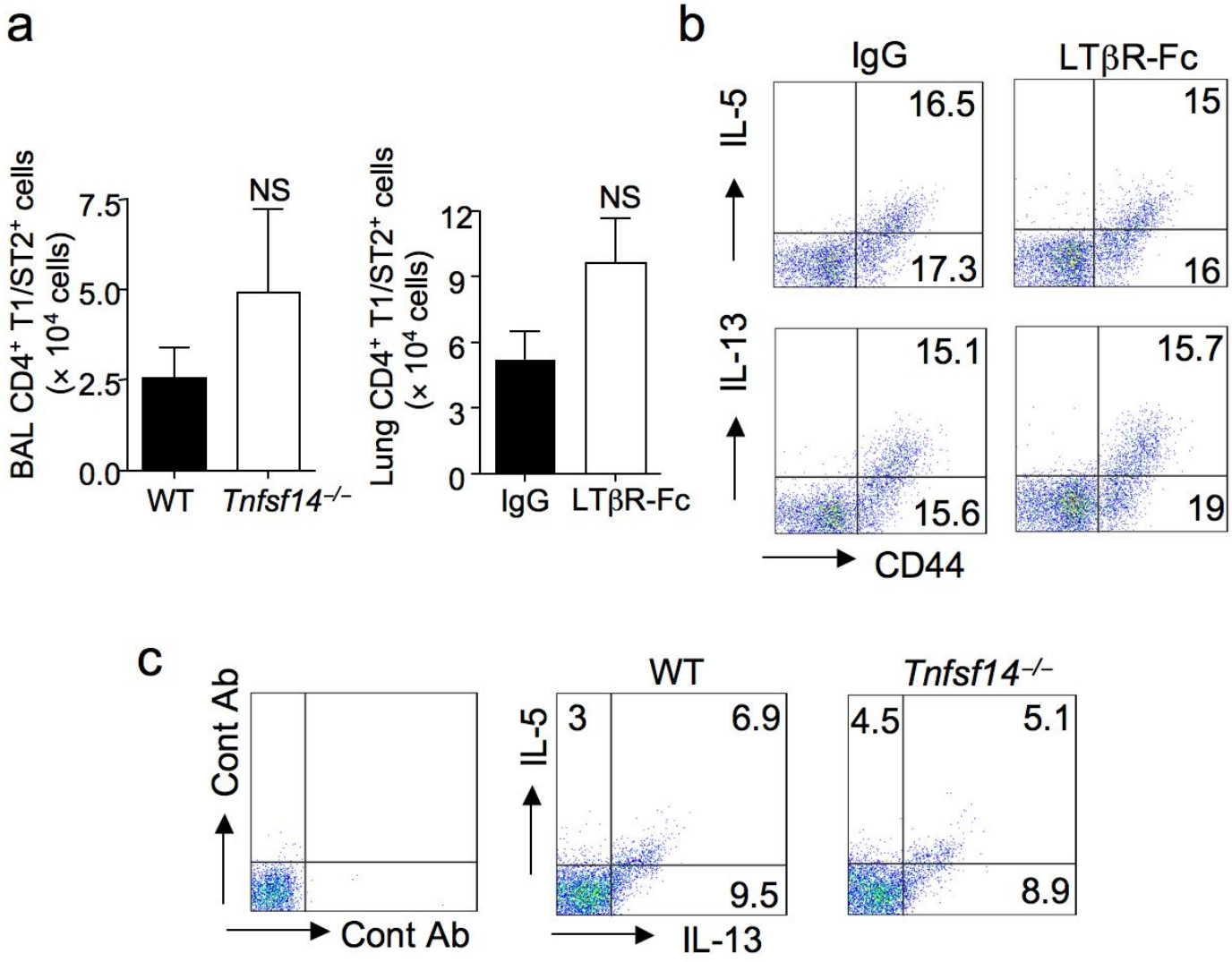
Peribronchial infiltrates and BAL eosinophil numbers after chronic OVA challenge. Lung sections from mice in Fig. 3 were stained with H&E and scored for peribronchial infiltrate (left). BAL eosinophils were enumerated (right). (**a**) WT mice receiving IgG or LTβR-Fc, 14 mice per group for BAL eosinophils, and 46-48 airways scored per group, and (**b**) WT versus *Tnsfsf14*$^{-/-}$ mice, 6 mice per group for BAL eosinophils and 48 airways scored per group. Mean +/- sem, Mann-Whitney test.

Supplementary Fig. 8



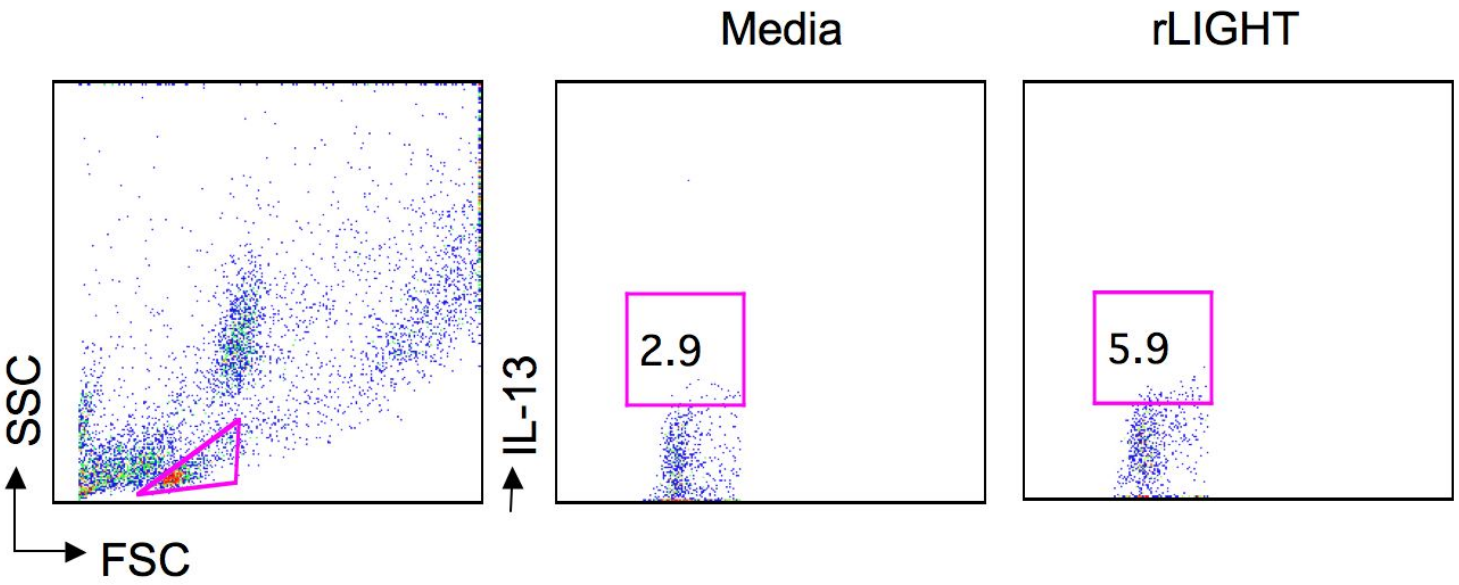
Characterization of lung macrophages and dendritic cells (DC) by flow cytometry. Single-cell suspensions were prepared from collagenase/DNase digested lungs, and stained with CD45 for hematopoietic cells and CD11c. Two peaks of autofluorescence (AF) can be distinguished within CD45$^+$ CD11c$^+$/hi cells, with lung DC displaying low AF and lung macrophages displaying high AF in the FL1 channel (top). High AF lung macrophages were further characterized by expression of Mac3, F4/80 and Siglec F (bottom).

Supplementary Fig. 9



Airway Th2 cell recruitment and antigen specific MLN cytokine production in *Tnfsf14*$^{-/-}$ and LTβR-Fc treated mice. (**a**) BAL or lung cells were stained for CD4 and T1/ST2 and enumerated from mice from Figs. 1 & 2 (left, 6 mice per group, right 3-4 mice per group, mean+/- sem, t-test, both non-significant). (**b**) Mediastinal lymph node cells from mice in Fig. 1 were cultured for 48 hr with 10ug/ml HDM and stained with CD4, CD44, and intracellular IL-5 (top) and IL-13 (bottom). (**c**) Mediastinal lymph node cells from mice in Fig. 2 were cultured for 48 hr with 10ug/ml HDM and stained with CD4, control antibody (left), IL-5, and IL-13. Plots shown are gated on CD4+ cells. Data (b & c) are from pooled MLN from 4-6 mice per group. *NS* = not significant.

Supplementary Fig. 10



BAL lymphocytes do not express IL-13 after stimulation with recombinant LIGHT. WT mice were immunized with OVA/alum on day 0 and challenged with intranasal OVA on day 7 and 8 as in Fig. 6d. Lung cells were cultured with rLIGHT or media. FSC/SCC gated lymphocytes (left) were analyzed for intracellular IL-13 expression (middle and right).

Supplemental Methods

Mouse models of airway remodeling and interventions. For OVA experiments, mice were given intraperitoneal (i.p.) injections on days 0 and 12 with 50 μg of OVA (Sigma) adsorbed to 0.5 mg of Alum (Pierce). Intranasal challenges of 20 μg of OVA in 20 μl PBS were given on days 24, 26, and 28. Further intranasal challenges using the same OVA dose were then performed two times per week for four weeks to allow progressive airway remodeling.

Airway hyperresponsiveness. Mice were anesthetized, canulated via the trachea, administered increasing doses of methacholine, and airway resistance measured. Peak value is the highest resistance at a particular dose out of 5-6 values.

Airway Inflammation Analysis. Bronchoalveolar lavage (BAL) was performed by intratracheal insertion of catheter and lavaging five times with 0.8-0.9 ml of 2% filtered BSA (Sigma). The right hilum was tied off and lung was isolated for cellular analysis by FACS or snap frozen and homogenized for cytokine analysis by ELISA. The left lung was instilled with 0.4 ml of 4% paraformaldehyde (PFA) and placed in PFA overnight for histology. Paraformaldehyde fixed lung sections were stained with Hematoxylin and eosin (H&E). Slides were blinded and peribronchial regions, 6-8 per mouse, were evaluated at 200x and inflammatory

infiltrates around airways were graded for severity (0, normal; 1, <3 cell diameter thick; 2, 3-10 cells thick; 3, >10 cells thick) and extent (0, normal; 1, <10% of sample; 2, 10-25%; 3, >25%). Scores were calculated by multiplying severity by extent (max 9).

Lung Cytokines. ELISA of supernatants for IL-13 (ebioscience, R&D) and free TGF-β1 without acidification (Promega, R&D) was performed according to instructions. ELISA plates were read with SpectraMax plate reader and analyzed with SoftMax Pro software (Molecular Devices).

Airway remodeling analysis. Paraformaldehyde fixed lung sections were stained with Masson's Trichrome and alpha smooth muscle actin (Sigma) and analyzed as in Methods. Results are expressed as the area of staining per micrometer length of basement membrane of bronchioles. At least 6 bronchi were counted in each slide.

Flow Cytometry. After Fc block, lung or BAL cells were stained for 30 minutes with various combinations of Allophycocianin (APC) or FITC-conjugated CD45, CD4, CD3, CD19, B220, PE-conjugated Siglec-F, APC or FITC-conjugated CD11c (BD Biosciences), PE conjugated and FITC conjugated Mac-3 (Serotec), Biotinylated- anti-LAP (R&D), Biotinylated- anti-mouse lymphotoxin beta receptor (eBiosciences), Biotinylated T1/ST2 (mdbiosciences) and isotype control

antibodies. For IL-13, surface staining was performed with the indicated fluorochrome-conjugated mAbs. The cells were then resuspended in fixation/permeabilization solution (BD Cytofix/Cytoperm kit; BD Biosciences), and intracellular cytokine staining was performed in parallel with with isotype control antibodies according to the manufacturer's protocol. The samples were analyzed with a FACSCalibur flow cytometer (BD Immunocytometry Systems). Further analyses were performed with Flow Jo software (Tree Star).

Macrophage isolation and culture conditions. Purified macrophages were cultured in complete media in the presence of 5 μg/ml control Rat IgG or agonistic antibody to LTβR antibody (4H8) for 24 or 48 hrs. In order to activate latent TGF-β, HDM extract (10 μg/ml based on protein) was added during the last eight hours of culture. In some experiments, LAP^+ and LAP^- cells were further sorted from purified $SiglecF^+CD11c^+$ autofluorescent lung macrophages. For rLIGHT in vitro studies, macrophages were stained with anti-MAC3 APC (eBioscience) and then anti-APC mirobeads and purified with autoMACS pro (miltenyibiotec). The purified MQ were then cultured with PBS or 25ng/ml of rLIGHT (R&D systems) with or without 10 mM of ERK inhibitor U0126 (Invivogen) for 24 hrs and processed for intracellular TGF-beta (R&D systems).

Lymph node culture. Mediastinal lymph nodes were removed from mice undergoing chronic HDM challenges and pooled in groups in RPMI (Gibco).

Cells were purified using 70μm filter (BD falcon) and cultured (0.1 million per well) in 96 well plate U bottom in the presence of HDM (10 μg/ml) for 48 hrs. Golgi Plug (BD) was added for the last 8 hours of culture and surface staining for CD4, CD44, IL-5, and IL-13 was performed.

Quantitative real-time PCR. Macrophages were collected from the culture, and their total RNA was isolated using TRIzol reagent (Invitrogen). Single-strand cDNA was prepared by reverse transcribing 5 μg of total RNA using the SuperScript III kit (Invitrogen). For quantitation of TGF-β, cDNA samples were amplified in IQ SYRB Green Supermix (Bio-Rad Laboratories) using the following primer pair for TGF-β: forward, 5′ -TGA CGT CAC TGG AGT TGT ACG G -3′; reverse, 5′- GGT TCA TGT CAT GGA TGG TGC-3′. All samples were run in triplicate, and the mean values were used for quantification.

Immunostaining. For LTβR immunohistochemistry, a primary mAb directed against LTβR (Santa Cruz) was used with immunoperoxidase development. Sections visualized with a Nikon microscope. For Mac-3 and LAP immunofluorescent staining, lungs of WT mice undergoing chronic OVA challenges were embedded in OCT compound (Skaura Finetek) and frozen by liquid nitrogen. 6mm sections were dried, acetone fixed, incubated with normal goat IgG for blocking, followed by incubation with biotinylated conjugated antibody to LAP (R&D) and PE-conjugated antibody to Mac-3 (Serotec). After

washing, streptavidin-APC (BD Pharmingen) was added followed by DAPI staining, mounting and analysis using Marianas fluorescence microscope with Slidebook software (Intelligent Imaging Innovations). For collagen-1 staining, lung samples were de-paraffinized by sequential placement in xylene and ethanol and stained with a polyclonal Collagen-1 antibody (Millipore, USA) at 1:500 concentration. Tyramide Signal Aplification Kit #41 (Invitrogen, USA) was used to provide the fluorescent signal amplification with subsequent DAPI staining (Vector Laboratories). Lung airways were visualized using a DM2500 microscope (Leica Microsystems).