

The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling

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Individuals with chronic asthma show a progressive decline in lung function that is thought to be due to structural remodeling of the airways characterized by subepithelial fibrosis and smooth muscle hyperplasia. Here we show that the tumor necrosis factor (TNF) family member LIGHT is expressed on lung inflammatory cells after allergen exposure. Pharmacological inhibition of LIGHT using a fusion protein between the IgG Fc domain and lymphotoxin β receptor (LT β R) reduces lung fibrosis, smooth muscle hyperplasia and airway hyperresponsiveness in mouse models of chronic asthma, despite having little effect on airway eosinophilia. LIGHT-deficient mice also show a similar impairment in fibrosis and smooth muscle accumulation. Blockade of LIGHT suppresses expression of lung transforming growth factor- β (TGF- β) and interleukin-13 (IL-13), cytokines implicated in remodeling in humans, whereas exogenous administration of LIGHT to the airways induces fibrosis and smooth muscle hyperplasia. Thus, LIGHT may be targeted to prevent asthma-related airway remodeling.

Individuals with chronic asthma show evidence of structural remodeling of the airways, including accumulation of extracellular matrix proteins such as collagen and thickening of smooth muscle. Current therapies for asthma are beneficial in controlling symptoms and airway inflammation but have little effect on lung remodeling. For example, in bronchial biopsies from individuals with asthma similar levels of subepithelial fibrosis are seen after anti-inflammatory therapy with corticosteroids^{1,2}, suggesting that the mechanisms that regulate remodeling may be distinct from those that induce eosinophilia or other aspects of lung inflammation. The severity of asthma and level of lung function impairment are also associated with increased mass of peribronchial smooth muscle³. It has been suggested that airway remodeling is the result of a complex interplay between immune cells and these structural cells, driven by a network of cytokines and growth factors, notably TGF- β and IL-13 (refs. 4,5). Many of these soluble mediators are involved in immune responses as well as tissue repair. Thus, new targets for airway remodeling are needed for the development of therapeutics for diseases of the lung, including asthma.

The TNF superfamily consists of many membrane-bound and soluble proteins with proinflammatory effects on innate and adaptive immune responses. The TNF family ligand LIGHT (TNFSF14; homologous to lymphotoxins, shows inducible expression, competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes), is a homotrimer expressed on the surface of several immune cells. LIGHT binds the herpesvirus entry mediator (HVEM;

TNFRSF14) and also is a shared ligand with membrane lymphotoxin (LT $\alpha\beta$) for LT β R^{6,7}. As other TNF superfamily members are being recognized as key mediators in asthmatic inflammation, including OX40 ligand (TNFSF4)^{8,9}, as well as TNF itself¹⁰, we hypothesized that LIGHT might be involved in driving aspects of lung inflammation or have a role in airway remodeling. In line with this, a recent report found that sputum LIGHT levels in people with asthma correlated with decreased lung function¹¹. Using two mouse models of chronic asthma and a therapeutic blocking strategy, we now show a role for LIGHT in controlling the extent of airway remodeling with resultant regulation of the proremodeling cytokines IL-13 and TGF- β .

RESULTS

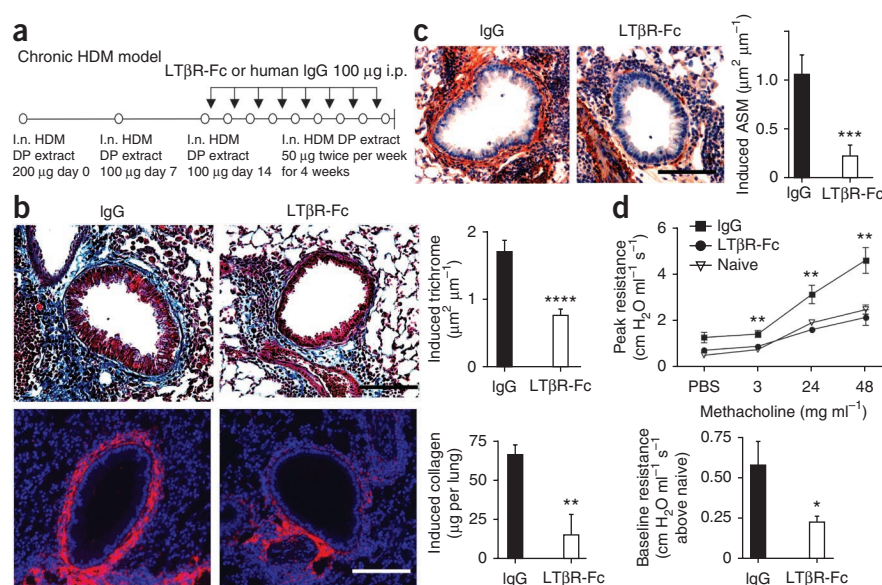
Blockade of LIGHT or LT $\alpha\beta$ reduces airway remodeling

We used a model of house dust mite (HDM)-induced chronic asthma to test the effects of blocking the interactions of LIGHT or LT $\alpha\beta$ with HVEM and LT β R. Wild-type (WT) mice develop acute airway inflammation after three challenges with HDM extract¹² and then undergo a fibrotic response in the lung, together with other structural changes reminiscent of those found in human asthma, when challenges are extended to twice per week for several weeks. We used a fusion protein of Fc with the extracellular portion of the lymphotoxin β receptor (LT β R-Fc) that can prevent LIGHT-LT β R, LIGHT-HVEM and LT $\alpha\beta$ -LT β R interactions¹³. We administered LT β R-Fc after development of acute airway inflammation, 24 h before each additional intranasal HDM challenge (Fig. 1a). Both increased peribronchial smooth muscle

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Figure 1 Blockade of LIGHT or LT α inhibits airway remodeling and AHR induced by HDM. (a) Protocol for HDM-induced remodeling. WT mice were given three intranasal (i.n.) challenges with HDM extract, once per week. LT β R-Fc or IgG was given 24 h before each additional intranasal HDM challenge over the next 4 weeks. i.p., intraperitoneal. (b) Lung sections were stained for Masson's trichrome (top left and middle) and collagen-1 (bottom left and middle) and scored for the extent of fibrosis (top right, $n = 54$ –75 airways per group). Induced total lung collagen was measured (bottom right, pooled from four mice per group, two experiments shown). (c) Lung sections stained for α -smooth muscle actin (left) and scored for extent of induced peribronchial smooth muscle (right, $n = 49$ –70 airways per group). Induced reflects levels above those detected in mice receiving three intranasal challenges before LT β R-Fc treatment. (d) Peak airway resistance with increasing doses of methacholine and baseline resistance without methacholine exposure (six or seven mice per group). * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$, means $\pm$ s.e.m., Mann-Whitney test. Data are from two or three independent experiments. Scale bars, 100 μ m.



area and lung fibrosis were induced in control mice after chronic HDM exposure (Fig. 1b,c), but mice receiving LT β R-Fc showed much less fibrosis, as measured by peribronchial trichrome staining¹⁴, airway collagen-1 expression and assays for total lung collagen (Fig. 1b). α -smooth muscle actin expression was also significantly lower in the LT β R-Fc-treated mice (Fig. 1c and Supplementary Fig. 1).

We next performed invasive pulmonary function testing. After exposure to nebulized methacholine, mice treated with LT β R-Fc had reduced airway hyperresponsiveness (AHR) as compared with IgG-treated mice (Fig. 1d). We also found a decrease in baseline airway resistance (Fig. 1d), which is probably related to the reduction in peribronchial smooth muscle and airway fibrosis¹⁵. Bronchoalveolar lavage fluid (BALF) eosinophil numbers, and the overall extent of peribronchial cellular infiltrates scored by lung histology, were similar in both LT β R-Fc and control IgG groups (Supplementary Fig. 2a). BALF neutrophil, B cell and CD4⁺ and CD8⁺ T lymphocyte numbers in the lung, and the number of bronchial epithelial cells staining for mucus (periodic acid-Schiff), were not significantly different after LT β R-Fc treatment (Supplementary Fig. 2). Thus, we found the reduction in remodeling features and AHR after LT β R-Fc treatment were independent of effects on airway eosinophilia and histological infiltration detected after chronic allergen challenge.

LIGHT is required for airway remodeling

LIGHT expression was induced on lung CD45⁺ cells after acute HDM challenge (Supplementary Fig. 3). We found LIGHT on B and T cells but not macrophages or granulocytes. To directly investigate the role of LIGHT, we subjected LIGHT-deficient (*Tnfsf14*^{-/-}) mice¹⁶ to the chronic HDM protocol. Similarly to mice treated with LT β R-Fc, *Tnfsf14*^{-/-} mice had reduced peribronchial fibrosis, collagen-1 and α -smooth muscle actin expression, total lung collagen abundance (Fig. 2a,b), and showed reduced AHR as compared with WT mice (Fig. 2c). LIGHT-deficient mice had only a modest reduction in lung inflammatory infiltrates, as determined by histologic scoring, and no statistical difference in airway eosinophilia (Supplementary Fig. 4).

Because there is no reagent that can specifically block the interaction of LIGHT with HVEM and LT β R without affecting LT α -LT β R

interactions, we treated *Tnfsf14*^{-/-} mice with LT β R-Fc to determine whether LT α is active in the absence of LIGHT. LT β R-Fc- and control IgG-treated *Tnfsf14*^{-/-} mice had similar fibrosis and smooth muscle hyperplasia (Supplementary Fig. 5a,b) and no difference in AHR (Supplementary Fig. 5c). Although LT α and LIGHT could act sequentially, the data from the LIGHT-deficient mice suggests that LIGHT is the primary mediator of the remodeling process.

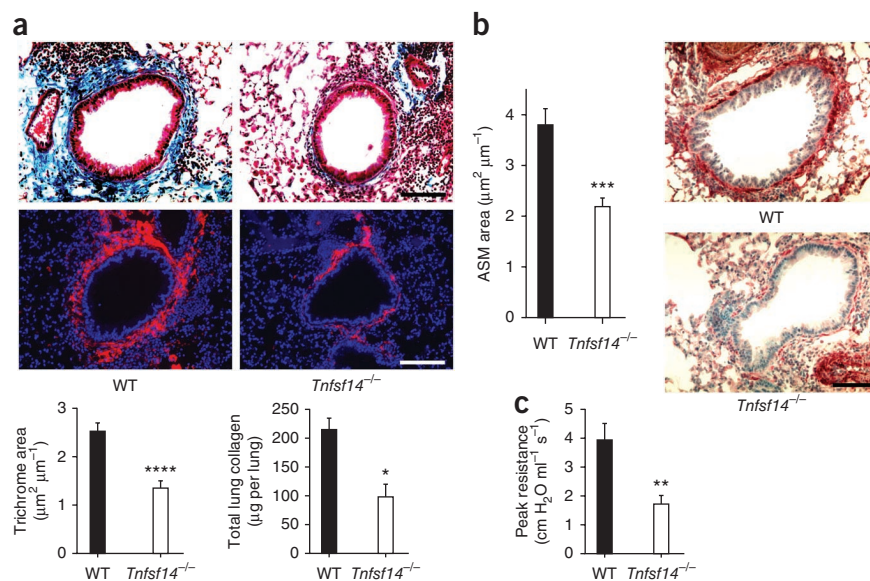
To confirm our findings in another model, we used ovalbumin (OVA) as the antigen for sensitization and chronic repetitive airway challenges. Mice treated with LT β R-Fc after acute inflammation was established had a strong reduction of induced fibrosis and smooth muscle actin expression (Supplementary Fig. 6). Scoring revealed only a modest reduction of inflammatory lung infiltrates, and airway eosinophilia was not different in LT β R-Fc-treated or *Tnfsf14*^{-/-} mice compared to controls (Supplementary Fig. 7). Thus, LIGHT is crucial for the induction of airway remodeling in both HDM- and OVA-induced chronic asthma models.

LIGHT regulates lung TGF- β

Studies of human and mouse cells have suggested that TGF- β is a principle mediator of airway remodeling, and its expression is elevated in lung samples from individuals with asthma⁵. TGF- β can induce epithelial-mesenchymal transition (EMT), differentiation of myofibroblasts, production of extracellular matrix proteins, including collagen-1, and smooth muscle cell proliferation^{5,17}. We detected a significantly increased concentration of lung TGF- β 1 after chronic HDM or OVA challenges (Fig. 3a). However, significantly less free TGF- β 1 was expressed in lung homogenates from WT C57BL/6 mice treated with LT β R-Fc, or from *Tnfsf14*^{-/-} mice (Fig. 3a).

Several cell types might produce TGF- β 1 in the lung and be targets of LIGHT. Immunohistochemistry revealed strong expression of LT β R in cells close to the subepithelial region, where smooth muscle hyperplasia and fibrosis occur during airway remodeling (Fig. 3b). This was consistent with the normal location of lung macrophages. Flow cytometry analysis revealed a population of cells expressing CD11c and Mac-3 that also strongly expressed LT β R (Fig. 3c). Further characterization confirmed that these were macrophages coexpressing

Figure 2 LIGHT-deficient mice are resistant to airway remodeling induced by HDM. WT and *Tnfsf14*^{-/-} mice received HDM intranasally once per week for 3 weeks, then twice per week for 4 weeks. Mice were killed 1 day after the last challenge. (a) Lung sections stained with Masson's trichrome (top) and collagen-1 (middle) and scoring for fibrosis (bottom left, $n = 35$ –36 airways per group, means $\pm$ s.e.m., Mann-Whitney test). Total lung collagen was also measured (bottom right, eight mice per group, means $\pm$ s.e.m., Mann-Whitney test). (b) Peribronchial smooth muscle area (left, $n = 34$ –35 airways per group, means $\pm$ s.e.m., Mann-Whitney test) and lung sections stained for α -smooth muscle actin (right). Levels reflect those above lung measurements from naive mice (a and b). (c) Invasive lung function test and resistance after challenge with 48 mg ml⁻¹ methacholine (means $\pm$ s.e.m., Mann-Whitney test from six or seven mice per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Scale bars, 100 μ m.



F4/80 and Siglec-F (**Supplementary Fig. 8**)^{18,19}. Macrophages have been implicated in many lung remodeling diseases, including asthma, chronic obstructive pulmonary disease and hypersensitivity pneumonitis^{20–22}, and it has been suggested that they promote remodeling through production of TGF- β ²³.

We found that LIGHT-deficient mice had reduced percentages and absolute numbers of lung macrophages after chronic challenge with either HDM or OVA (**Fig. 3d**). TGF- β is initially complexed with latency-associated peptide (LAP), and LAP expression correlates with TGF- β production in many cell types^{24–26}, including macrophages²⁷.

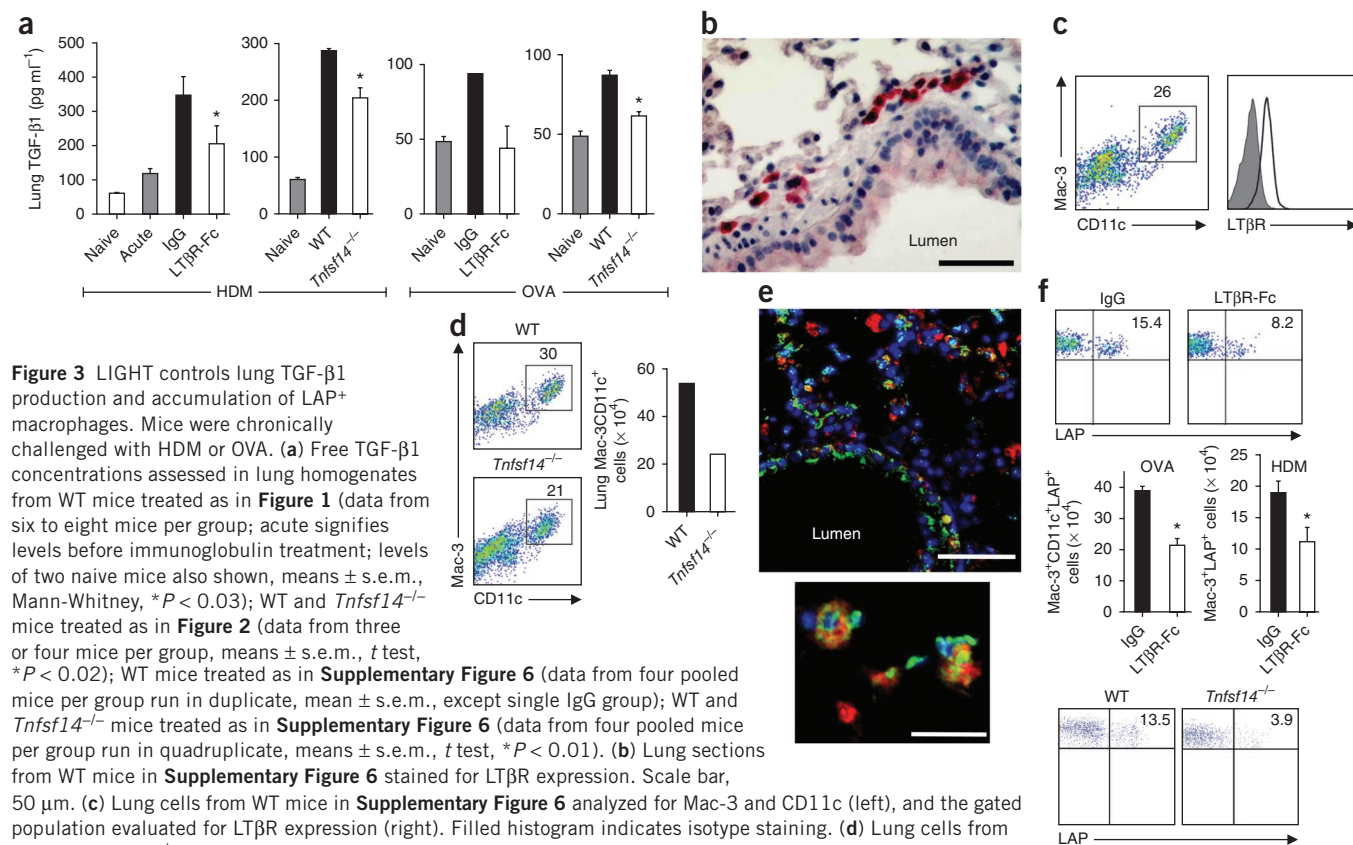
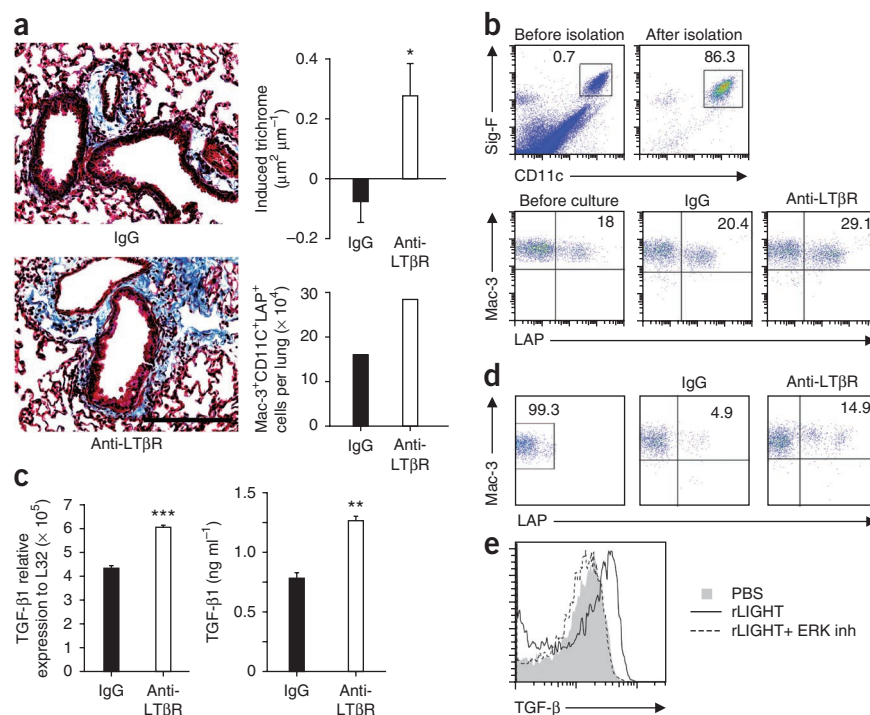


Figure 3 LIGHT controls lung TGF- β 1 production and accumulation of LAP⁺ macrophages. Mice were chronically challenged with HDM or OVA. (a) Free TGF- β 1 concentrations assessed in lung homogenates from WT mice treated as in **Figure 1** (data from six to eight mice per group; acute signifies levels before immunoglobulin treatment; levels of two naive mice also shown, means $\pm$ s.e.m., Mann-Whitney, * $P < 0.03$); WT and *Tnfsf14*^{-/-} mice treated as in **Figure 2** (data from three or four mice per group, means $\pm$ s.e.m., t test, * $P < 0.02$); WT mice treated as in **Supplementary Figure 6** (data from four pooled mice per group run in duplicate, mean $\pm$ s.e.m., except single IgG group); WT and *Tnfsf14*^{-/-} mice treated as in **Supplementary Figure 6** (data from four pooled mice per group run in quadruplicate, means $\pm$ s.e.m., t test, * $P < 0.01$). (b) Lung sections from WT mice in **Supplementary Figure 6** stained for LT β R expression. Scale bar, 50 μ m. (c) Lung cells from WT mice in **Supplementary Figure 6** analyzed for Mac-3 and CD11c (left), and the gated population evaluated for LT β R expression (right). Filled histogram indicates isotype staining. (d) Lung cells from WT and *Tnfsf14*^{-/-} mice in **Supplementary Figure 6** analyzed for Mac-3 and CD11c (top and bottom) and absolute numbers of Mac-3⁺CD11c⁺ cells (right, pooled lung cells from four mice per group). (e) Immunofluorescent staining of lung sections from a representative WT mouse from **Figure 1** stained for Mac-3 (red), LAP (green) and DAPI (blue). Scale bar, 50 μ m. Image zoom also depicted (bottom). Scale bar, 25 μ m. (f) Gated Mac-3⁺CD11c⁺ cells from lungs of mice in **Figure 1** and **Supplementary Figure 6** analyzed for LAP expression (top), enumeration of total LAP⁺ macrophages per lung (middle, $n = 4$ mice per group, means $\pm$ s.e.m., two experiments shown for OVA and one for HDM, * $P < 0.05$, t test) and flow analysis for LAP expression gating on Siglec-F⁺CD11c⁺ macrophages (bottom).

Figure 4 LT β R stimulation promotes fibrosis and TGF- β production by lung macrophages. (a) Lung sections stained with Masson's trichrome (left) and extent of induced peribronchial fibrosis (top right; 44–68 airways per group IgG and anti-LT β R, means $\pm$ s.e.m., Mann-Whitney test, $*P < 0.05$). Scale bar, 100 μ m. Mac-3 $^{+}$ CD11c $^{+}$ LAP $^{+}$ cells per lung were enumerated (bottom right, pooled from six mice per group). WT mice were immunized and acutely challenged with OVA over 28 d and then injected with LT β R agonist antibody (anti-LT β R) or rat IgG every 3–4 d for 2 weeks. (b,c) Analysis of Siglec-F $^{+}$ CD11c $^{+}$ lung macrophages (b, top) from naive mice after stimulation with rat IgG or anti-LT β R and analyzed for surface LAP expression after 2 d (b, bottom), TGF- β 1 mRNA (c, left, $***P < 0.0005$) or TGF- β 1 protein after HDM was added in the last 8 h of culture (c, right, $**P < 0.005$). Results are triplicates from each group. (d) Flow cytometry analysis of LAP-Siglec-F $^{+}$ CD11c $^{+}$ Mac-3 $^{+}$ lung macrophages sorted (left) and stimulated with rat IgG or anti-LT β R and analyzed for LAP expression (right). (e) Flow cytometry analysis of intracellular TGF- β in purified lung macrophages stimulated with recombinant LIGHT, in the presence or absence of ERK inhibitor. Data are representative of at least two experiments.



Immunofluorescent staining of lung sections from chronically challenged mice revealed that LAP was expressed predominantly on Mac-3 $^{+}$ cells, in addition to airway epithelium, further suggesting that macrophages are a major source of LAP and TGF- β in the remodeled lung (Fig. 3e). Notably, treatment of chronically challenged WT mice with LT β R-Fc led to a strong reduction in the percentages and total numbers of LAP $^{+}$ macrophages (Fig. 3f), and LIGHT-deficient mice also had a lower proportion of these cells after challenge (Fig. 3f). This implies that LIGHT regulates macrophage accumulation in the lung and directly or indirectly regulates the number of TGF- β -producing cells.

LT β R signaling induces fibrosis and macrophage TGF- β

As LT β R was expressed on lung macrophages, we tested whether LT β R signaling could induce remodeling. We sensitized and challenged WT mice with OVA, followed by injection of agonist antibody to LT β R¹³ given two times per week for 2 weeks, but without further antigen challenge. Mice receiving the agonist antibody showed a marked induction of fibrosis above the level found in acutely challenged mice (Fig. 4a). Furthermore, there was a doubling in the number of LAP $^{+}$ lung macrophages in the agonist group (Fig. 4a).

We then purified naive lung macrophages and cultured them with agonist antibody to LT β R. Macrophage LAP expression and TGF- β 1 mRNA levels increased after stimulation of the LT β R (Fig. 4b,c). The concentration of free TGF- β 1 in the culture supernatant did not increase (data not shown), but when HDM was added, LT β R stimulation significantly increased release of TGF- β 1 (Fig. 4c). This is probably related to a requirement for

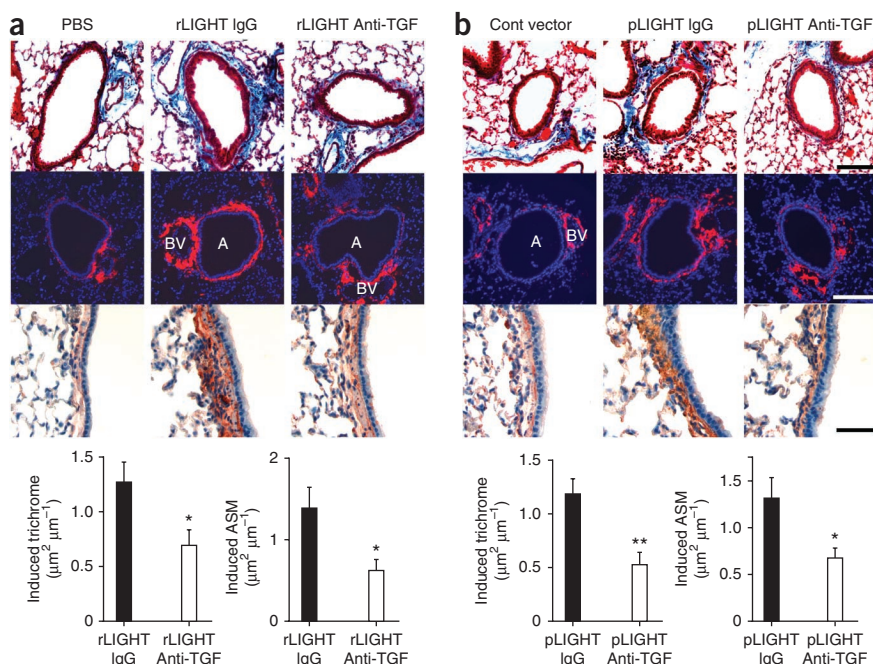
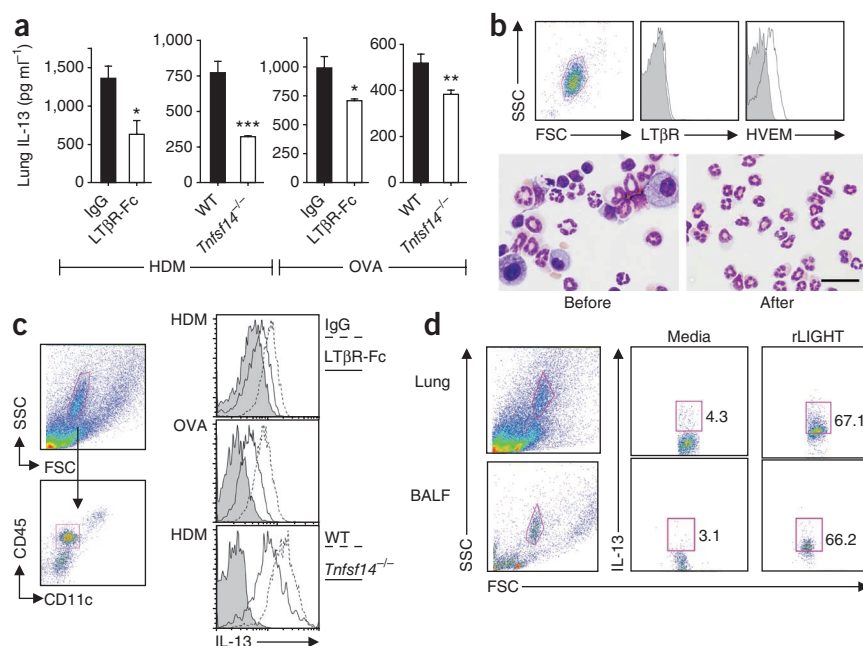


Figure 5 LIGHT-induced airway remodeling is in part dependent on TGF- β . (a,b) Lung sections were stained for trichrome (top row, scale bar, 100 μ m), collagen-1 (second row, scale bar, 100 μ m), α -smooth muscle actin (third row, scale bar, 50 μ m) and scored for fibrosis and smooth muscle area (bottom row, 40 airways per group, means $\pm$ s.e.m., Mann-Whitney, $*P < 0.05$, $**P < 0.005$). WT mice primed with HDM over 3 weeks were treated with intranasal rLIGHT or PBS given eight times over 2 weeks (a) or pCDNA3 mouse LIGHT plasmid or control plasmid given four times over 2 weeks (b). Antibody to TGF- β (anti-TGF) or isotype control IgG was also injected as indicated. Induced reflects levels above those detected in lungs of mice receiving PBS (a) or control plasmid (b). A, airway; BV, blood vessel.

Figure 6 LIGHT promotes IL-13 production by lung eosinophils. (a) IL-13 content in mice primed and challenged with HDM or OVA. Lung homogenates from mice treated as in **Figures 1** and **2** and **Supplementary Figure 6** were analyzed (five to seven mice per group from **Figure 1**, three or four mice per group from **Figure 2** and triplicates of pooled samples from four to seven mice per group from **Supplementary Figure 6**, means $\pm$ s.e.m., *t* test, **P* < 0.05, ***P* < 0.01, ****P* < 0.005).

(b) Flow cytometry analysis of sorted granulocytes (>95% eosinophils by cytoplasm, bottom; scale bar, 50 μ m) from WT mice after acute intranasal OVA challenge for LT β R (top middle) and HVEM expression (top right). Isotype control in gray. (c) Flow cytometry analysis of CD45⁺CD11c⁺ granulocyte-gated lung eosinophils (top and bottom left) from mice in **Supplementary Figure 6** for intracellular IL-13 directly *ex vivo* (middle right). Siglec-F⁺CD11c⁺ eosinophils from mice in **Figure 1** (top right) and **Figure 2** (bottom right) were stained for IL-13 expression. (d) Flow cytometry analysis of BALF (bottom left) and lung cells (top left) and intracellular IL-13 (middle and right) was analyzed in cells gated on forward and side scatter (left, >95% eosinophils). Cells from WT mice immunized and challenged with OVA over 8 days were cultured for 48 h with rLIGHT or medium added during the last 24 h. Data are representative of two independent experiments.



an inflammatory signal for proteolytic release of active TGF- β 1 from LAP. Additionally, stimulation of LT β R on sorted LAP⁺ macrophages resulted in direct upregulation of surface LAP (**Fig. 4d**). We also cultured purified lung macrophages with soluble recombinant LIGHT (rLIGHT) (**Fig. 4e**) in the presence or absence of an inhibitor of extracellular signal-regulated kinase (ERK; U0126), a kinase previously shown to regulate TGF- β production from macrophages²⁷. LIGHT stimulation increased expression of intracellular TGF- β , and ERK inhibition completely abrogated TGF- β induction (**Fig. 4e**). Together, these results suggest that one mechanism by which LIGHT can promote fibrosis is through direct stimulation of LT β R on lung macrophages, resulting in both increased accumulation of these cells in the lung and increased TGF- β expression.

LIGHT directly induces airway remodeling dependent on TGF- β

To test whether LIGHT can directly induce airway fibrosis and smooth muscle hyperplasia *in vivo* by a TGF- β -dependent mechanism, we established acute lung inflammation in WT mice with intranasal HDM given once per week for 3 weeks. This was then followed by intranasal administration of recombinant LIGHT given eight times over the next 2 weeks (in the absence of antigen). Mice that received rLIGHT developed airway fibrosis and smooth muscle hyperplasia compared with those that received PBS, which was reduced by TGF- β blockade (**Fig. 5a**). To confirm this, we challenged mice with the same acute HDM protocol followed by intranasal administration of a plasmid vector encoding mouse LIGHT. Similarly, these mice showed marked fibrosis and smooth muscle hyperplasia, which we did not observe in mice receiving an empty vector (**Fig. 5b**). These features were also suppressed by blocking TGF- β . Thus, LIGHT can induce remodeling in the airway, and this process is partly dependent on TGF- β .

LIGHT regulates lung IL-13

IL-13 is also a pleiotropic regulator of lung remodeling, inducing some activities similarly to TGF- β ⁴. IL-13 is elevated in lung samples

with remodeling from individuals with asthma^{28,29} and can induce epithelial cell proliferation³⁰, myofibroblast proliferation and smooth muscle actin expression³¹. Furthermore, TGF- β and IL-13 can act synergistically to induce features of asthmatic remodeling^{32,33}. The concentration of IL-13 was decreased by 50% in lung homogenates from HDM-challenged, LIGHT-deficient mice, as well as from those treated therapeutically with LT β R-Fc (**Fig. 6a**). We found a smaller, but significant, decrease in OVA-challenged mice that lacked LIGHT or were treated with LT β R-Fc (**Fig. 6a**).

T helper type 2 (T_H2) cells might be a source of IL-13 (ref. 4) and a potential target of LIGHT activity. T cells express HVEM, but not LT β R, and HVEM signals can increase proliferation and cytokine secretion from T cells to augment their response to antigen⁷. However, we did not detect differences in the numbers of T_H2 cells in the airway or lung of Tnfsf14^{-/-} mice or after LT β R-Fc treatment in WT mice (**Supplementary Fig. 9a**). Additionally, CD4⁺ T cell production of IL-13 and IL-5 was unaltered in comparison with controls (**Supplementary Fig. 9b,c**), indicating that LIGHT does not regulate T_H2 cells during chronic repetitive allergen challenges.

Other cell types that might be a source of IL-13 include eosinophils, mast cells and structural cells⁴. Because binding of LIGHT to HVEM increases cytokine production in granulocytes *in vitro*³⁴, we reasoned that HVEM might be expressed on eosinophils. Indeed, sorted BALF eosinophils (>95% of sorted granulocytes) from acute OVA- or HDM-challenged mice expressed HVEM but not LT β R (**Fig. 6b** and data not shown). We then performed intracellular cytokine staining for IL-13 in eosinophils from WT mice after sensitization and chronic challenge with HDM or OVA (**Fig. 6c**). Lung CD45⁺CD11c⁺ granulocytes were predominantly eosinophils and constitutively expressed intracellular IL-13, which suggests these were highly differentiated cells at the end of the chronic antigen challenge (**Fig. 6c**). In contrast, those obtained from mice receiving LT β R-Fc or LIGHT-deficient mice had reduced IL-13 expression (**Fig. 6c**). To directly test the effect of LIGHT, we cultured less differentiated eosinophils isolated from the lung and BALF of antigen-sensitized WT mice that

had received only two intranasal antigen challenges. In this case, the eosinophils did not spontaneously produce IL-13, but IL-13 expression was strongly promoted by stimulation with rLIGHT (Fig. 6d). In contrast, the gated lymphocyte population in these cultures showed minimal induction of IL-13 after LIGHT stimulation (Supplementary Fig. 10). Collectively, these data indicate that LIGHT is required in models of chronic asthma for maximal expression of two of the primary cytokines thought to promote airway remodeling, and that LIGHT-LT β R or LIGHT-HVEM interactions promote proremodeling activities of lung macrophages and lung eosinophils, two of the cell types associated with chronic asthma and remodeling in humans.

DISCUSSION

We show that LIGHT is crucial for airway remodeling and airway hyperresponsiveness in two mouse models of chronic asthma. Notably, our data present a new role for this molecule in lung remodeling and fibrotic disease and suggest LIGHT may be a relevant target for suppressing fibrosis and smooth muscle hyperplasia. Several published reports confirm an association of LIGHT with elements of tissue remodeling. In individuals with scleroderma, a disease that can lead to pulmonary fibrosis, BALF concentrations of LIGHT were higher in those with lung involvement compared to those with only nonpulmonary manifestations³⁵. Moreover, a first report evaluating LIGHT expression in individuals with asthma was recently published¹¹. The authors measured soluble LIGHT in the sputum of a heterogeneous group of 242 people with asthma, including 48 with severe asthma. After analysis of approximately 40 soluble mediators, only LIGHT was found to inversely correlate with lung function, as assessed by baseline forced expiratory volume in one second (that is, higher LIGHT levels were associated with lower baseline forced expiratory volumes, or lung function).

As LIGHT has two receptors, targeting its interaction with LT β R and HVEM may prevent multiple proremodeling activities during the development of chronic asthma. We identified TGF- β as being regulated by LIGHT and found that lung macrophages expressing LT β R are responsive to LIGHT. Of note, rLIGHT has also been found to increase expression of matrix metalloproteinase-9 (MMP-9) in macrophages³⁶. MMP-9 is found in the BALF of people with asthma³⁷, and MMP-9-deficient mice show reduced peribronchial fibrosis after chronic allergen challenge³⁸. Together with our results, these studies suggest that LIGHT-LT β R interactions on lung macrophages might regulate the expression of both TGF- β and MMP-9 during chronic asthma. Although TGF- β is almost universally considered to be involved in lung remodeling⁵, one report in a mouse model of chronic HDM administration found it was dispensable for driving structural changes in the lung³⁹. This may be due to differences in the frequency and periodicity of HDM exposure in that study compared to our and others' studies. Other models with OVA have shown a clear role for TGF- β in airway remodeling^{40,41}, and we found TGF- β expression was reduced by targeting LIGHT in both our HDM and OVA models. This is concordant with the demonstration that direct administration of LIGHT via the airways induced remodeling in a TGF- β -dependent manner.

IL-13 is similarly considered to have a major role in remodeling⁴ and is required for structural changes in the lungs in OVA-, fungal- and HDM-induced animal models^{42–44}. We found LIGHT promotes IL-13 expression in eosinophils and markedly impaired eosinophil IL-13 production was observed after treatment with LT β R-Fc. The importance of eosinophils in human asthmatic

remodeling has been shown after treatment with IL-5-specific antibodies, which reduces eosinophils in the lung and leads to a reduction in airway deposition of extracellular matrix proteins⁴⁵. Eosinophils have also been shown to contribute to airway remodeling in OVA-induced animal models of chronic asthma^{14,46}. One report found that eosinophils were not required for HDM-driven remodeling⁴⁷, but with the caveat that this experimental protocol differed in dose, duration and frequency of challenges compared to that used here. These differences in terms of the molecules required for remodeling may simply reflect variations that might occur in humans and, rather than being contradictory, may provide key insight into subpopulations of people with asthma. Regardless of the ultimate target, or targets, of LIGHT activity, our data show that in various antigen models LIGHT is a primary orchestrator of the remodeling processes.

As well as regulating TGF- β and IL-13, LIGHT may control additional cellular functions that contribute to remodeling. rLIGHT has been shown to enhance proliferation of vascular smooth muscle cells⁴⁸ as well as promote expression of α -smooth muscle actin⁴⁹, actions mediated by HVEM and LT β R, respectively. Bronchial epithelial cells may also be a target. LT β R was found on epithelial cells in human lungs⁵⁰, and we found weak expression of LT β R in lungs of chronically challenged mice, albeit less than on macrophages. Bronchial epithelial cells can undergo EMT driven by TGF- β ^{51,52}, and this might be one source of the increased smooth muscle mass characteristic of airway remodeling. However, we saw induction of fibrosis as well as increased numbers of lung macrophages after injection of an agonist antibody to LT β R without a corresponding increase in peribronchial smooth muscle hyperplasia (data not shown). Notably, we did detect smooth muscle hyperplasia in mice receiving intranasal rLIGHT and LIGHT plasmid, suggesting that HVEM and not LT β R has a role in driving the increase in smooth muscle mass. Future studies are needed to determine whether smooth muscle cells or bronchial epithelial cells are direct targets of LIGHT activity *in vivo*.

Neutralizing LIGHT reduced airway hyperresponsiveness despite no robust effects on the peribronchial immune infiltrates. This is consistent with previous reports that suggest that airway hyperresponsiveness in chronically challenged mice correlates more with remodeling changes than the degree of cellular infiltrates in the lung^{15,51,52}. The precise relationship between airway inflammation, hyperresponsiveness and remodeling in human asthmatics is unclear, but there is evidence that baseline lung function and severity of asthma is related to the smooth muscle mass³. The dichotomy between eosinophil accumulation and fibrosis that we detected is also consistent with previous data that have suggested that airway remodeling may be a distinct process from eosinophilic lung inflammation^{1,2,53,54}. Some studies have additionally suggested that IL-13 might directly contribute to the degree of hyperresponsiveness⁵⁵, and therefore the combination of less fibrosis, smooth muscle hyperplasia and IL-13 after LT β R-Fc treatment or in *Tnfsf14*^{-/-} mice most likely explains the relatively normal lung function we observed.

In summary, we report that the TNF family member LIGHT is essential for airway remodeling in several mouse models of chronic asthma. Blockade of LIGHT, after acute inflammation was established in the lung, substantially reduced airway fibrosis and smooth muscle hyperplasia, two factors associated with a decline in lung function in human asthmatics. This previously unknown role for LIGHT in airway remodeling may lead to strategies targeting this molecule in asthmatic airway remodeling or other diseases involving fibrosis and remodeling.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

T.A.D. and P.S. contributed to animal antigen administration, surgery, data collection, analysis and manuscript writing for all studies; S.F. and J.Y.C. contributed to immunostaining and data analysis; N.K. contributed to remodeling and cytokine data collection and analysis; P.R. contributed to airway hyper-responsiveness testing and analysis; P.S.N. produced plasmids, LT β R-F and antibody to LT β R and contributed to experimental design; H.C. contributed to cytokine data collection; S.S. and K.P. developed mutant mice; B.L.Z. contributed to experimental design; C.F.W. contributed to experimental design and reagent production; D.H.B. contributed to experimental design and remodeling data collection; M.C. contributed to experimental design, data analysis and manuscript writing for all studies.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mouse models of airway remodeling and interventions. Eight- to twelve-week-old C57BL/6 mice (Jackson Laboratories) or *Tnfsf14*^{-/-} mice¹⁶ (bred in house on the BL/6 background) were used. For HDM experiments, mice were given intranasal administrations of *Dermatophagoides pteronyssinus* extracts (Greer Laboratories, 8.42 endotoxin units per mg HDM): 200 µg on day 0 and 100 µg on days 7 and 14 to induce an acute response similar to that previously described¹². To induce a chronic response, 50 µg of HDM was then given twice per week for 4 weeks.

In some experiments, mice challenged acutely with HDM were given 10 µg of intranasal recombinant LIGHT (R&D Systems) or PBS on days 16–18 and on alternating days for five doses. For mouse LIGHT *in vivo* gene transfer experiments, 50 µg of pCDNA3 mouse LIGHT or empty vector was given on days 16, 20, 23 and 27 after HDM challenge. This dose and frequency was based on ranges used in previous reports of successful intranasal plasmid gene transfer in the lung^{56,57}. Full-length mouse *Tnfsf14* was cloned into pCDNA3.1(+) at HindIII and NotI, and endotoxin-free plasmid DNA was purified using the EndoFree Plasmid Mega Kit (Qiagen) following the manufacturer's directions. Antibody to TGF-β (clone 1D11.16.8, Bio X Cell) or isotype control was given in 300-µg doses i.p. every 3–4 d for 2 weeks beginning 1 day after the last HDM challenge.

LTβR-Fc and agonist antibody to LTβR were generated in house as previously described¹³. LTβR-Fc and control human IgG (Bethyl) (100 µg) were given i.p. starting on day 29 for OVA, and day 16 for HDM protocols, and given 24 h before each subsequent intranasal challenge for 4 weeks. LTβR agonist antibody was given on day 30 and every 3–4 d for 2 weeks. For experiments to assess undifferentiated eosinophils, WT mice were injected i.p. with 50 µg of OVA (Sigma) adsorbed to 0.5 mg of alum (Pierce) on day 0, challenged with intranasal OVA on days 7 and 8 and killed on day 9. BALF and lung cells were then cultured with recombinant LIGHT (100 ng ml⁻¹, R&D Systems) for 24 h before intracellular cytokine staining. All experiments were in compliance with the regulations of the La Jolla Institute for Allergy and Immunology Animal Care Committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

Airway hyperresponsiveness. In some experiments, invasive pulmonary function testing was performed using the Flexivent system (Scireq), and airway resistance was analyzed by Scireq Flexivent 5.1 software as previously described⁵⁸.

Airway Inflammation Analysis. We performed bronchoalveolar lavage and processed lung for histology as previously described⁵⁴. Lung eosinophils were detected as the Siglec-F⁺CD11c⁻ population of isolated lung cells¹⁹. BALF neutrophils were detected as the GR-1 granulocyte population. All BALF cell counts were confirmed by manual differential counting of cytospins. Total cell counts were performed using a hemacytometer (Hausser-Scientific).

Lung cytokines. Right lungs were individually placed in 500 µl of RPMI (Gibco) with 0.5% Triton-X and homogenized, followed by centrifugation. In some experiments, lungs were snap frozen in liquid nitrogen before homogenizing as previously described⁵⁹ and analyzed for IL-13 (R&D) and TGF-β (Promega and R&D) 1 by ELISA.

Airway remodeling analysis. The area of peribronchial fibrosis on trichrome-stained sections was evaluated with an image analysis system (Image-Pro Plus; Media Cybernetics)¹⁴. Smooth muscle area was evaluated in the same manner. Quantification of total lung collagen was performed using the Sircoll assay as previously described⁴⁰.

Flow cytometry. Lungs were digested in 2 mg ml⁻¹ collagenase and 1 mg ml⁻¹ DNase (Roche) for 30 min, and lung cells were purified with a 70-µm cell strainer (BD Falcon). Cells were incubated with monoclonal antibody to CD16/CD32 (24G.2) for 10 min to block Fc receptors and then stained for 30 min with various combinations of antibodies to surface markers described in the **Supplementary Methods**. For staining of mouse LIGHT, we first blocked the expression of LTβ with antibody BBF6 to LTβ (kindly provided by J. Browning) followed by LTβR-Fc and phycoerythrin-conjugated antibody to human immunoglobulin (eBioscience) as previously described⁶⁰.

Macrophage isolation and culture conditions. To isolate lung resident macrophages, BALF cells were removed by extensive washing with PBS following digestion of lung tissues. After incubation with mouse Fc-blocking antibody (2.4G2), recovered cells were stained with allophycocyanin-conjugated antibody to CD11c and phycoerythrin-conjugated antibody to Siglec-F followed by anti-PE MicroBeads (Miltenyi Biotec). Siglec-F⁺ cells were enriched on an AutoMACS Pro cell separator (Miltenyi Biotec) following further isolation by a FACSARIA II cell sorter (BD Biosciences) to purify Siglec-F⁺CD11c⁺ autofluorescent lung resident macrophages. Cells were cultured with LTβR-specific agonist antibody (4H8) or rLIGHT as described in the **Supplementary Methods**.

Immunostaining. Immunohistochemical detection of LTβR in paraffin-embedded lung sections was performed as previously described⁵⁸. Immunofluorescent staining for Mac-3, LAP and collagen-1 is described in the **Supplementary Methods**.

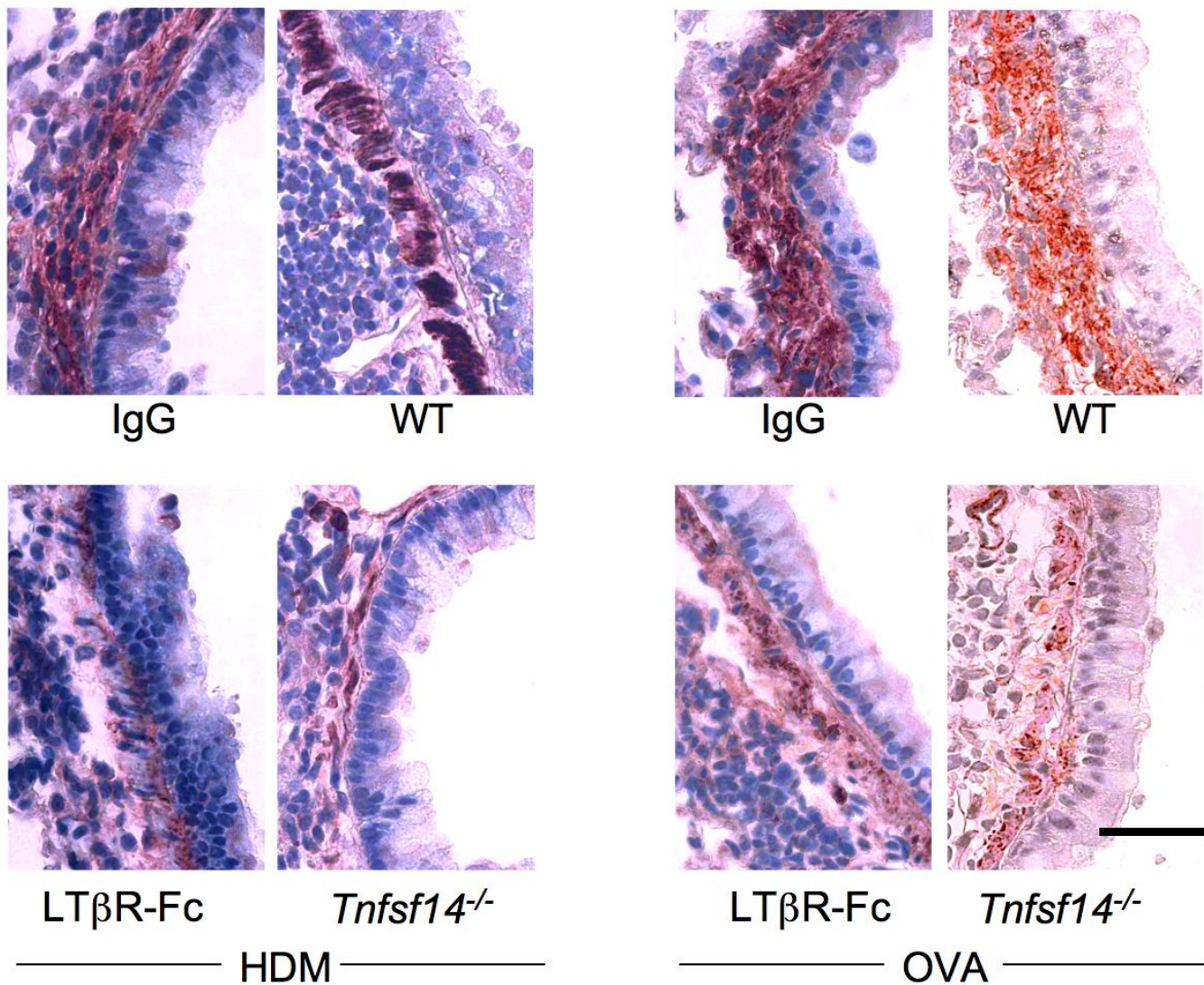
Statistical analyses. Statistical analysis was performed using GraphPad Prism software. A nonparametric *t* test or Mann-Whitney test was used where indicated. A *P* value < 0.05 was considered statistically significant.

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

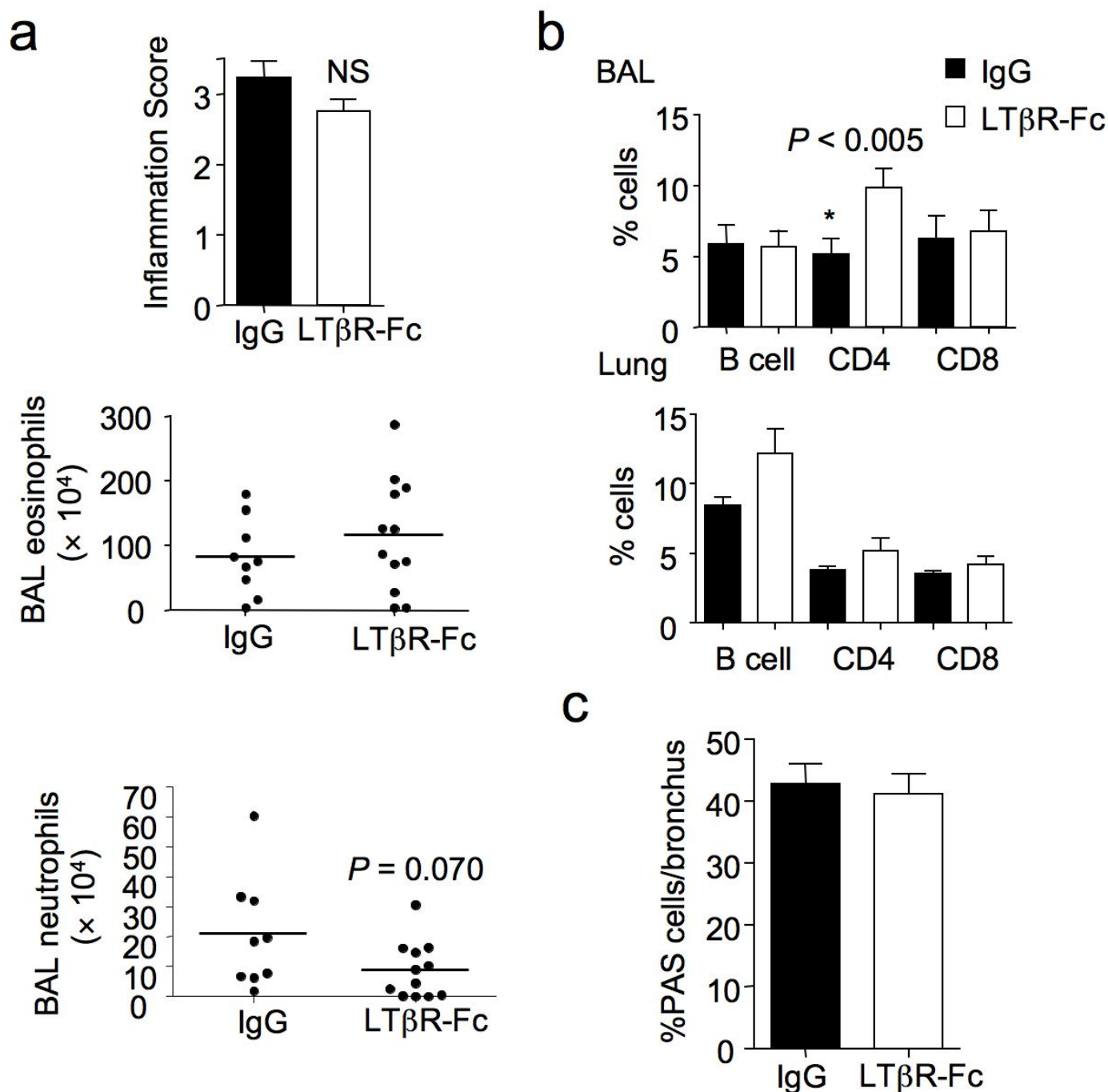
Taylor A. Doherty, Pejman Soroosh, Naseem Khorram, Satoshi Fukuyama, Peter Rosenthal, Jae Youn Cho, Paula S. Norris, Heonsik Choi, Stefanie Scheu, Klaus Pfeffer, Bruce L. Zuraw, Carl F. Ware, David H. Broide, and Michael Croft

Supplementary Fig. 1



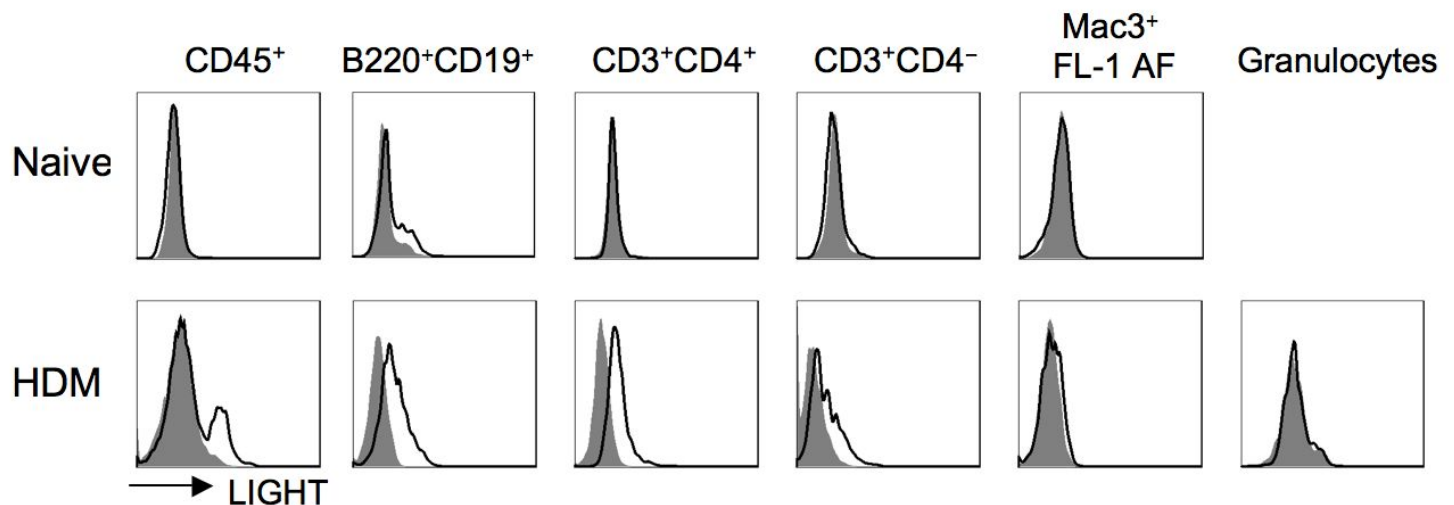
Reduction of peribronchial smooth muscle area in *Tnfsf14*^{-/-} mice and after LT β R-Fc treatment. Mice from Figs. 1,2 and Supplementary Fig. 6 were chronically challenged with HDM (left) or OVA (right) as described in methods. Lung sections were stained for alpha smooth muscle actin. Scale bar, 50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

Supplementary Fig. 2



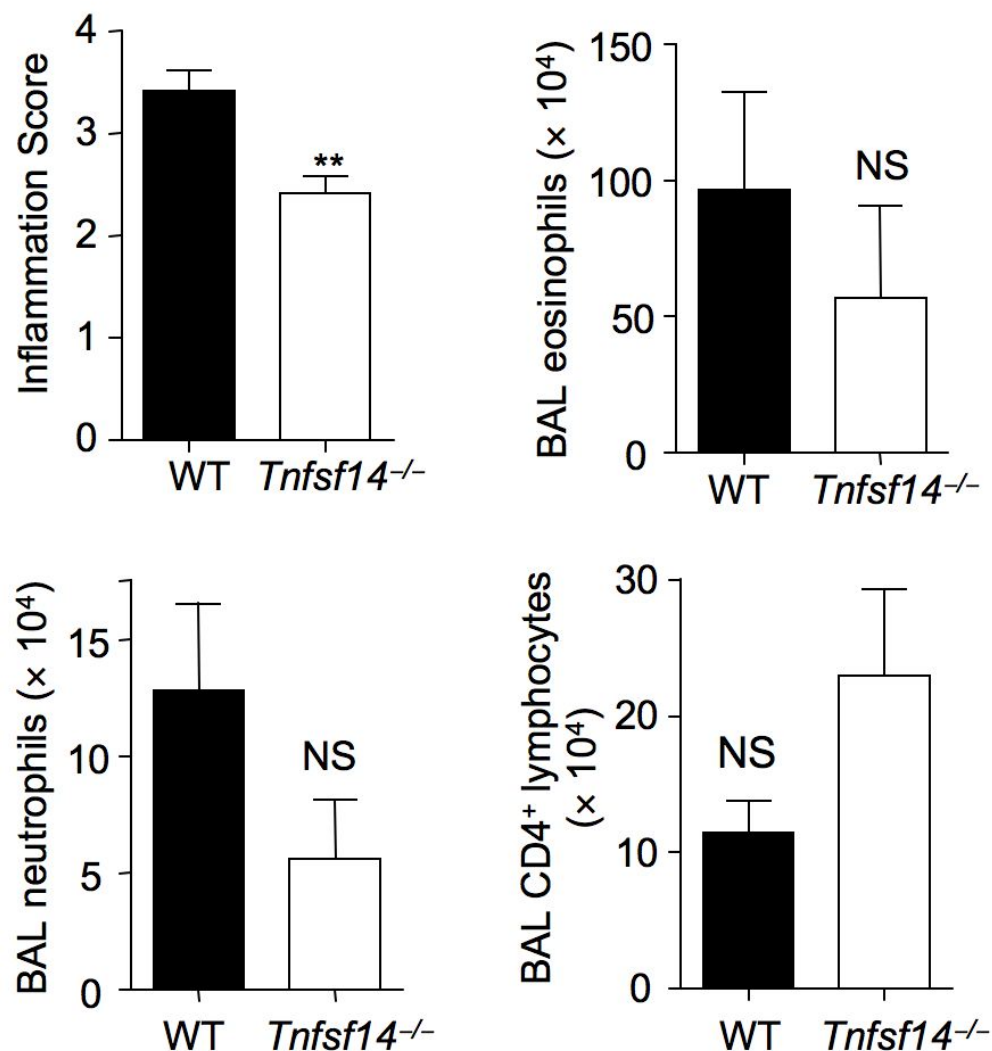
Lung cellular infiltration and mucus production after blockade of LIGHT/LT $\alpha\beta$. WT mice were given three challenges with HDM extract, once per week. LT β R-Fc or IgG was given 24 hours prior to each additional intranasal HDM challenge over the next four weeks (Fig. 1a). **(a)** Peribronchial 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 $\pm$ sem, Mann-Whitney, and/or values from individual mice.

Supplementary Fig. 3



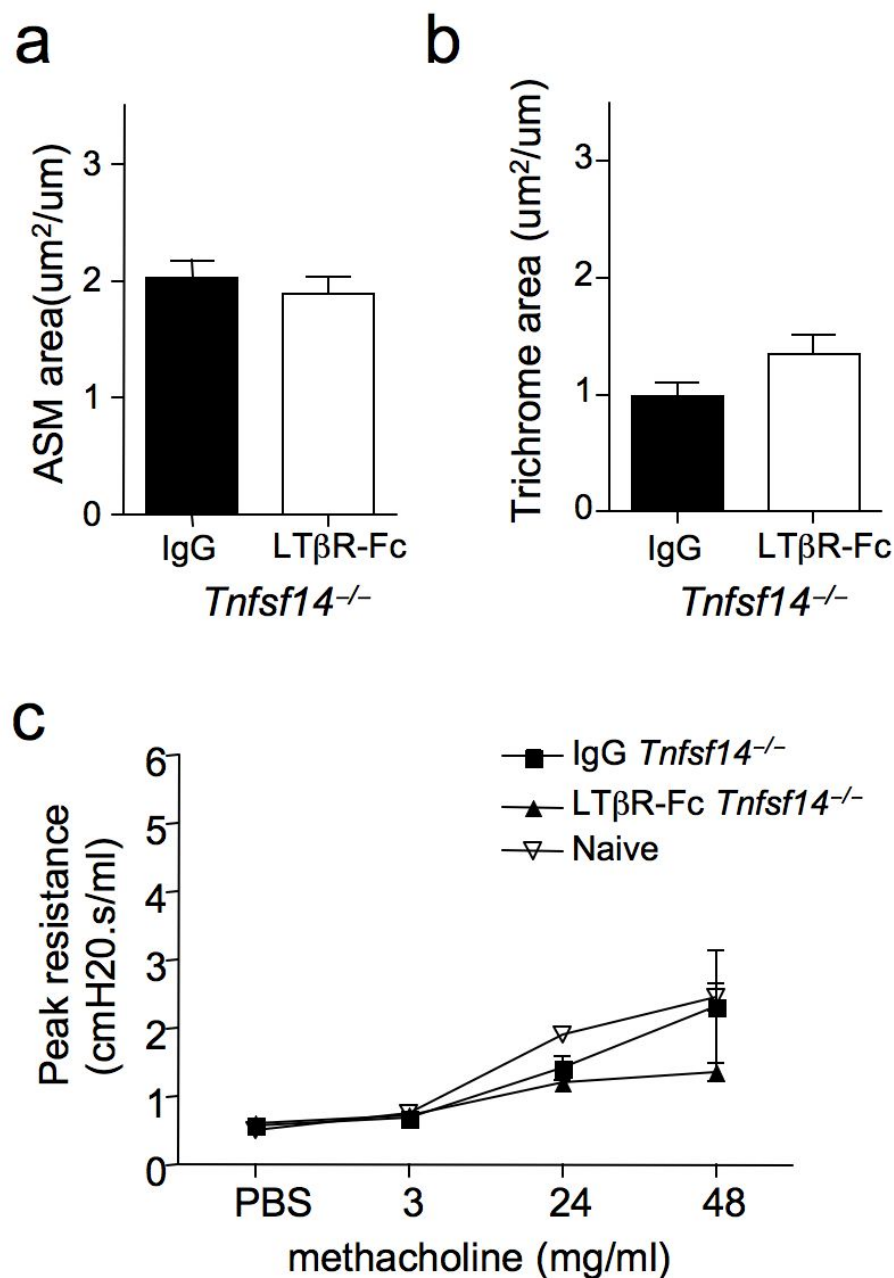
LIGHT expression on lung cells after HDM exposure. WT mice received intranasal HDM once per week for three weeks. One day after the last challenge, lungs cells were stained for CD45, CD4, CD3 (T cells), CD19, B220 (for B cells), and Mac3 with gating on autofluorescent cells (Macrophages). Granulocytes 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



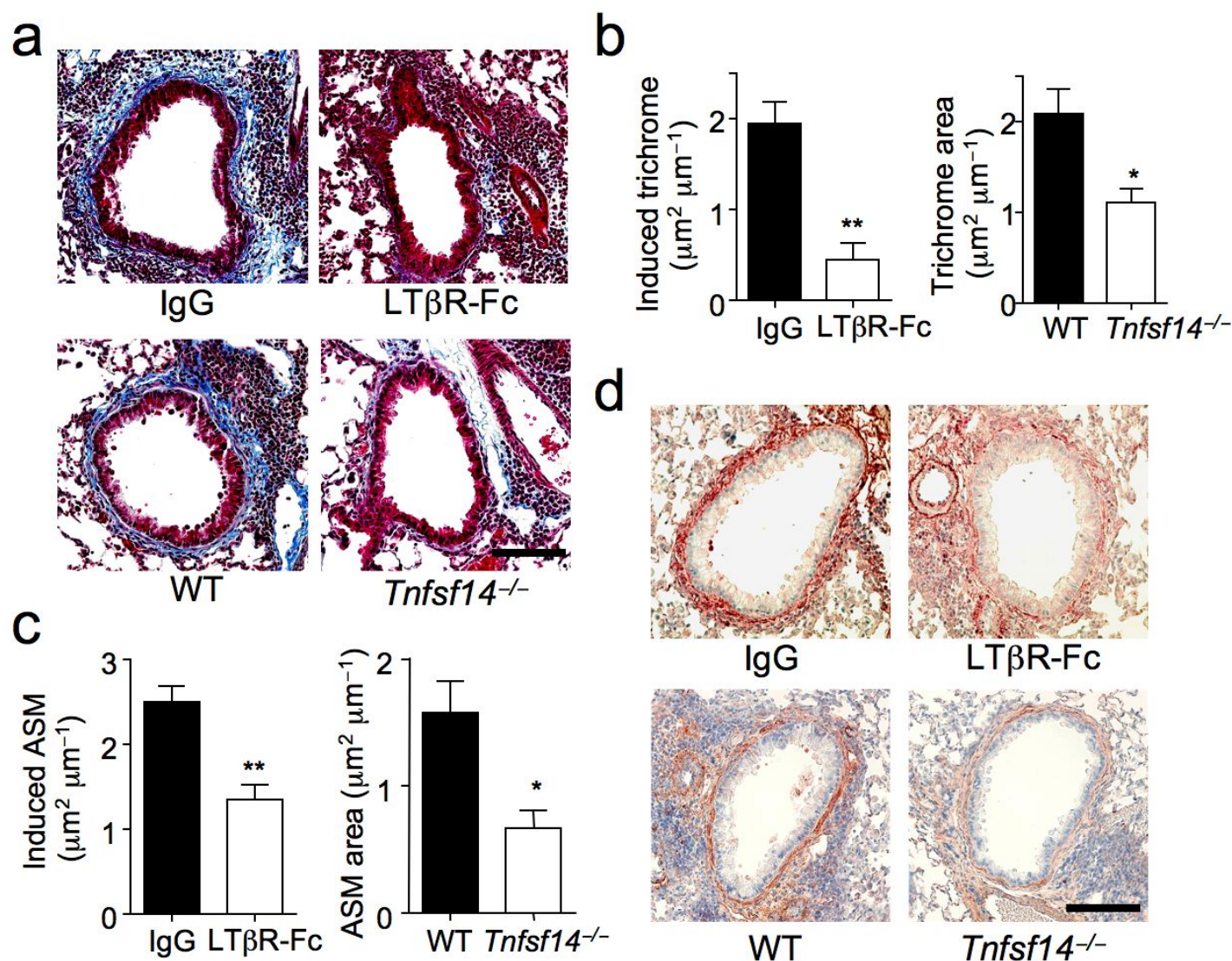
Peribronchial infiltrate, and eosinophil, neutrophil, and CD4⁺ cell numbers in *Tnfsf14*^{-/-} mice after chronic HDM challenge. Lung sections from mice in Fig. 2 were stained with H&E and peribronchial infiltrates scored as described in methods (top left). BAL eosinophils, neutrophils, and CD4⁺ lymphocytes 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 significant difference.

Supplementary Fig. 5



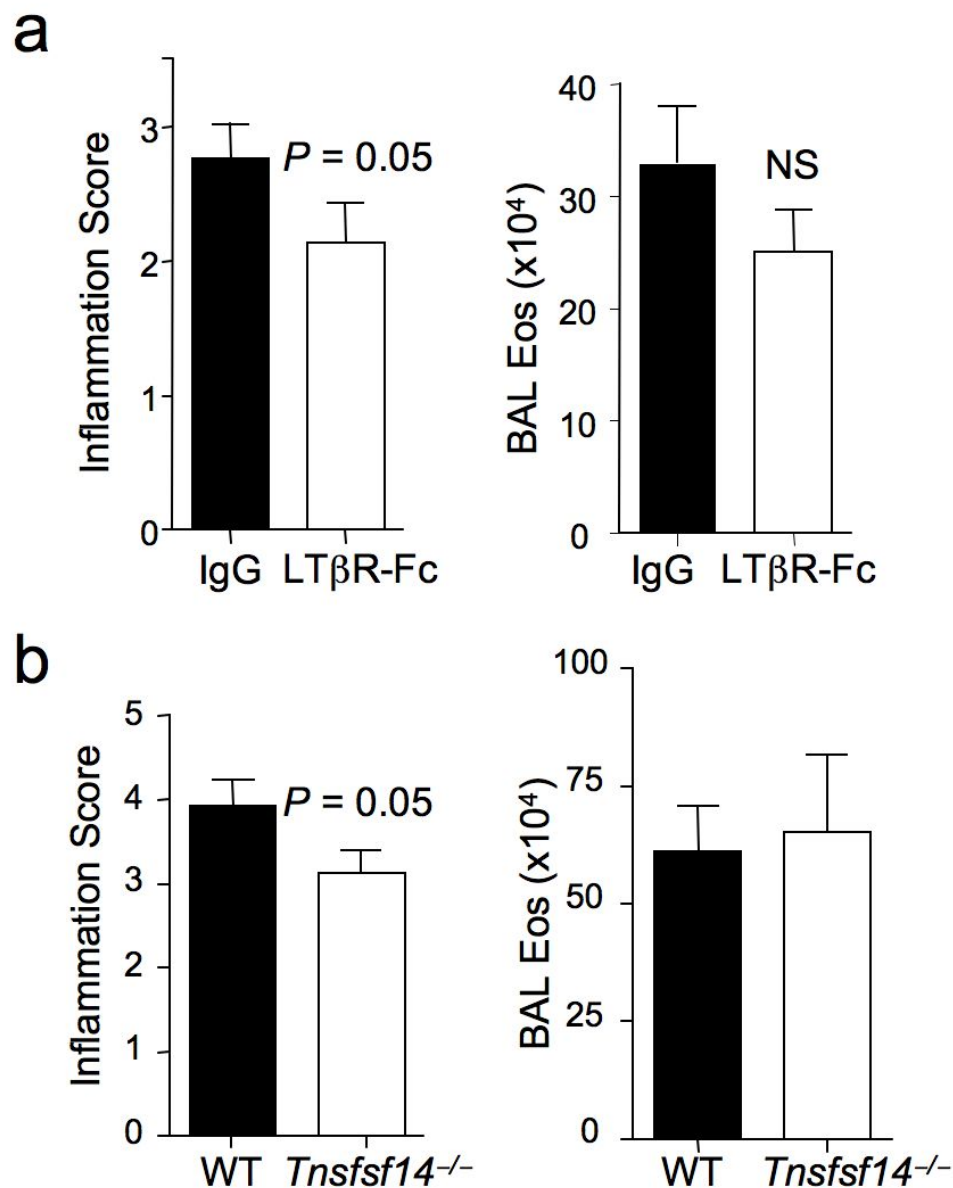
LTβR-Fc treatment in *Tnfsf14*^{-/-} mice does not further suppress remodeling and AHR. *Tnfsf14*^{-/-} mice were immunized and acutely challenged on days 24, 26, and 28 followed by intranasal OVA challenge, twice per week for 4 weeks. Mice then received LTβR-Fc (100ug) or human IgG i.p. beginning on 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



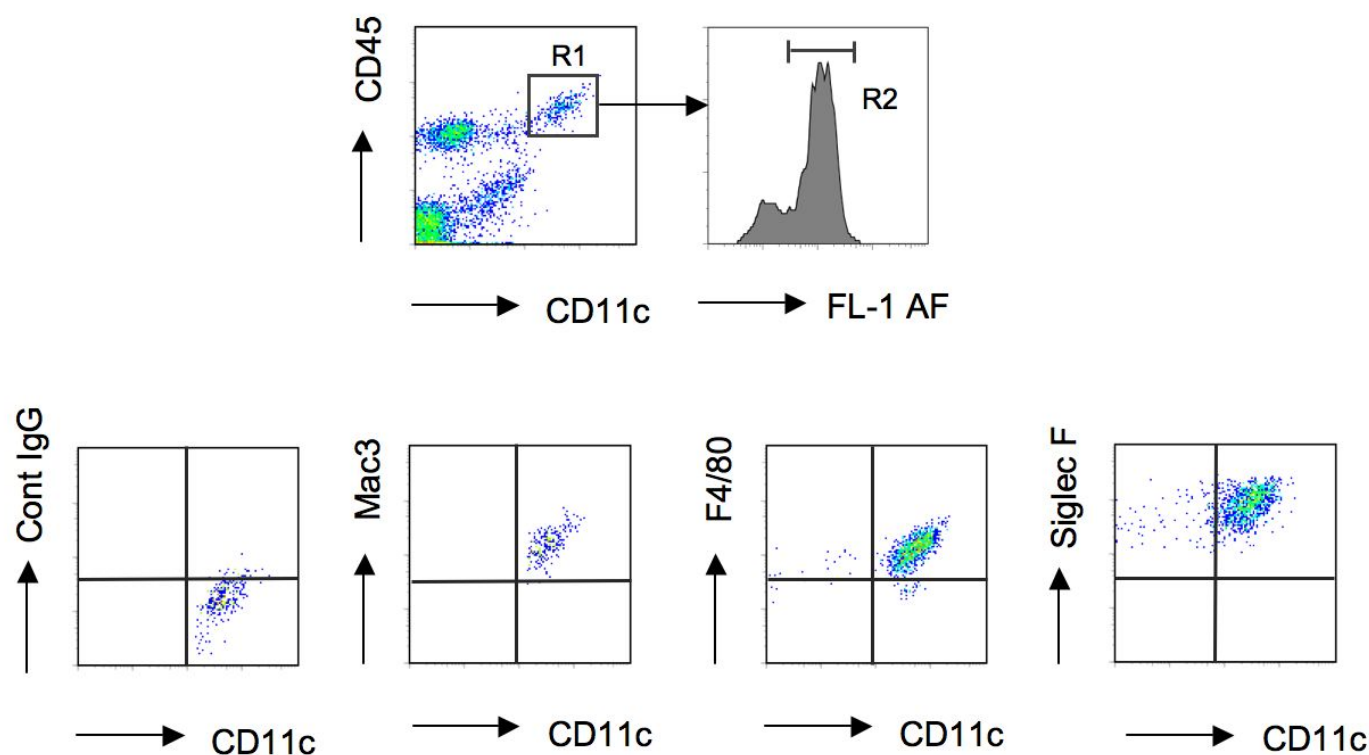
LIGHT is required for airway remodeling induced by chronic exposure to OVA. WT or *Tnfsf14*^{-/-} mice were immunized and acutely challenged on days 24, 26, and 28 followed by chronic intranasal OVA challenge, twice per week for 4 weeks. Some WT mice received LTβR-Fc (100μg) or human IgG i.p. beginning 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



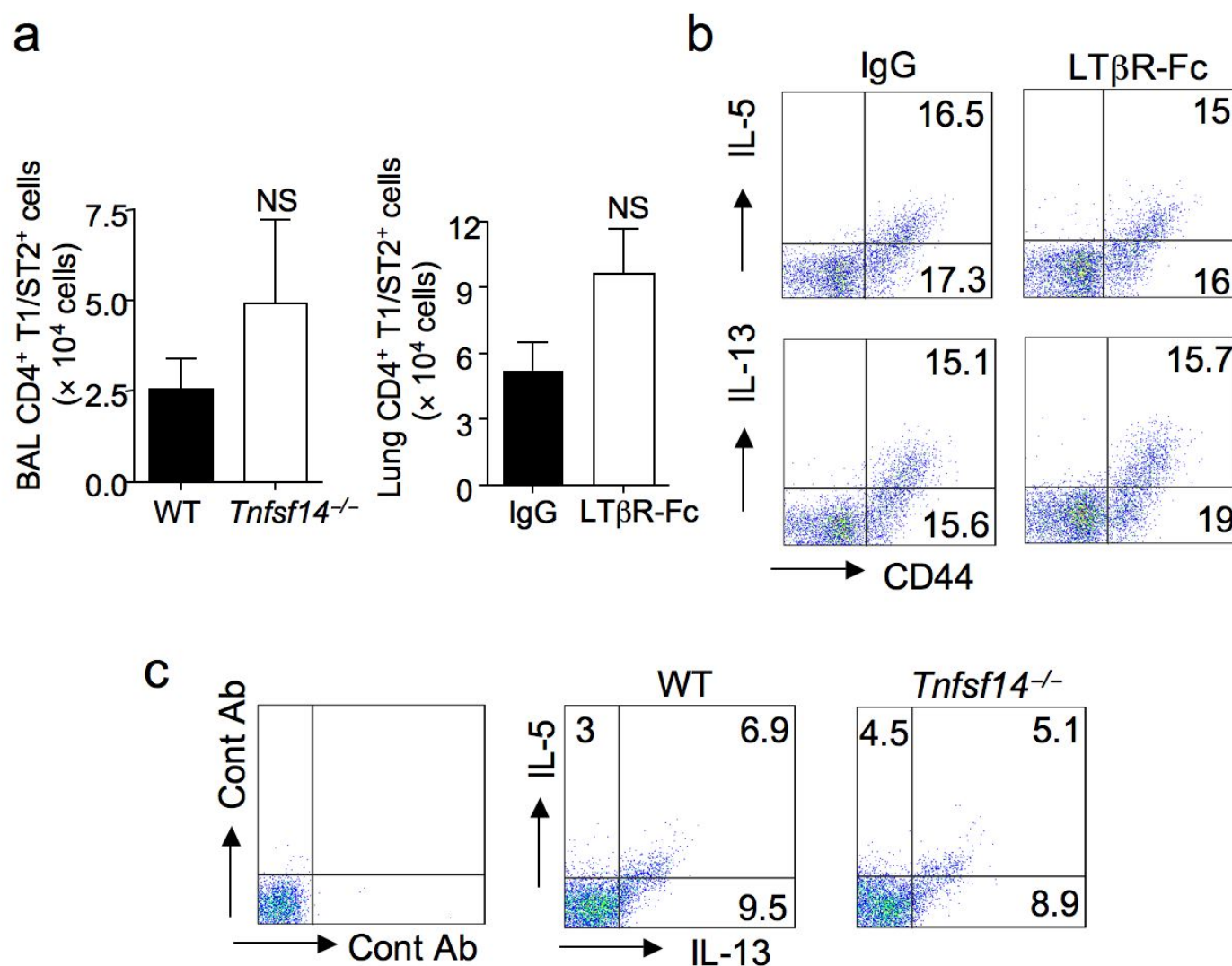
Peribronchial infiltrates and BAL eosinophil numbers after chronic OVA challenge. Lung sections from mice in Fig. 3 were stained with H&E and scored for peribronchial infiltrate (left). BAL eosinophils were enumerated (right). **(a)** WT mice receiving IgG or LT β R-Fc, 14 mice per group for BAL eosinophils, 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 $\pm$ sem, Mann-Whitney test.

Supplementary Fig. 8



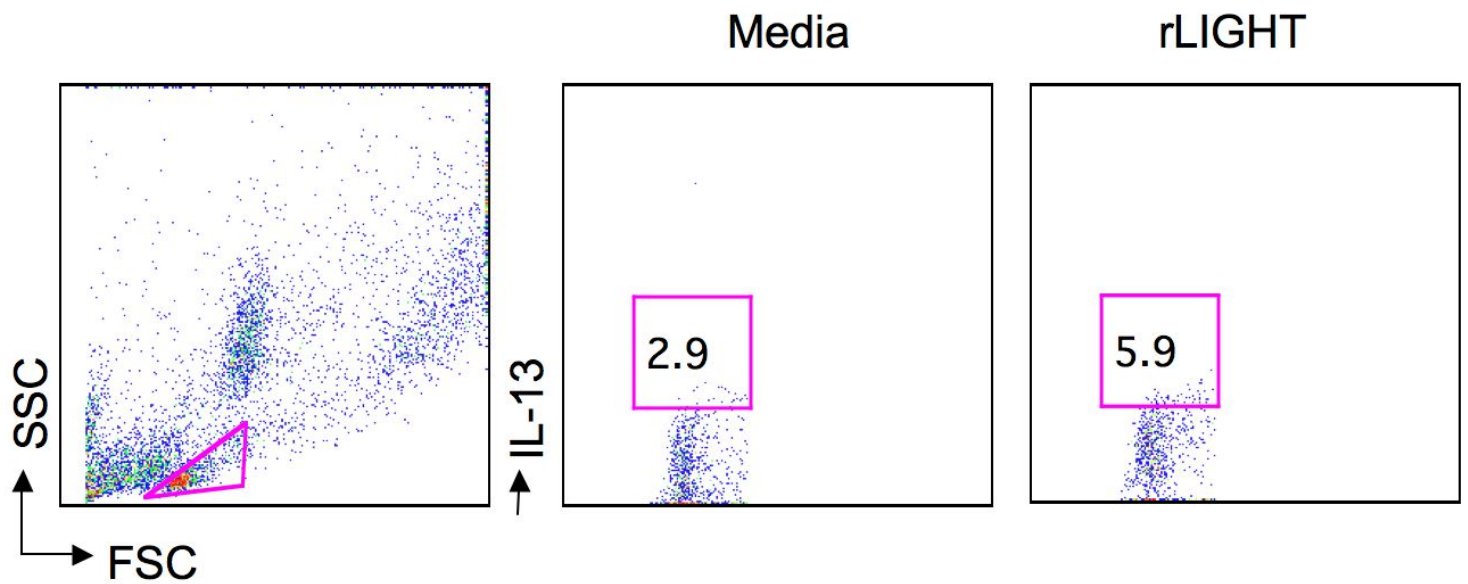
Characterization of lung macrophages and dendritic cells (DC) by flow cytometry. Single-cell suspensions were prepared from collagenase/DNase digested lungs, and stained with CD45 for hematopoietic cells and CD11c. Two peaks of autofluorescence (AF) can be distinguished within CD45⁺ CD11c⁺/hi cells, 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/SSC 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 μ g of OVA (Sigma) adsorbed to 0.5 mg of Alum (Pierce). Intranasal challenges of 20 μ g of OVA in 20 μ l 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, cannulated 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- β 1 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 Allophycocyanin (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 μ g/ml control Rat IgG or agonistic antibody to LT β R antibody (4H8) for 24 or 48 hrs. In order to activate latent TGF- β , HDM extract (10 μ g/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 microbeads 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 70 μ m filter (BD falcon) and cultured (0.1 million per well) in 96 well plate U bottom in the presence of HDM (10 μ g/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 μ g 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 LT β R immunohistochemistry, a primary mAb directed against LT β R (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 Amplification 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).