Persistent activation of an innate NKT cell-macrophage immune axis that translates respiratory viral infection into chronic lung disease


Supplementary Results

IL-13 dependence of persistent disease traits

Neither pulmonary emphysema nor fibrosis is observed at any time after viral infection (data not shown). The inhibition of airway hyperreactivity was independent of any changes in baseline airway caliber (Supplementary Fig. 1). We found no difference in peak viral load at Day 3-5 PI or the 6 log-fold decrease to trace levels of virus-specific RNA by Day 49 PI in wild-type versus Il13−/− mice. Therefore, the inhibition of chronic lung disease in Il13−/− mice does not appear to depend on any difference in viral replication or clearance. (Supplementary Fig. 1).

IL-13 production by macrophages

For CD4+ T cells and macrophages, the contribution to lung IL-13 mRNA levels was based on an increased number of cells recruited to the lung as well as increased production of IL-13 mRNA per cell (Supplementary Fig. 2). Other minor sources of IL-13 mRNA included NK cells and NKT cells. However, there was no detectable IL-13 mRNA produced by mast cells, basophils, neutrophils, dendritic cells (DCs), B cells, or CD8+ T cells. In some sections, we found a marked accumulation of macrophages in the airway epithelium that was not observed in uninfected control mice (data not shown). The high production of IL-13 per macrophage compared to other cell types (e.g., CD4+ T cells) may be responsible for the detection of CD68+IL-13+ but not CD4+IL-13+ cells in tissue.

To determine whether macrophages are necessary for chronic lung disease, we first studied op/op mice that are macrophage-deficient due to a loss of function mutation in the Csf3 (also known as G-CSF) gene. Since macrophages are required for antiviral defense 15, these mice were infected with a reduced viral inoculum. Compared to wild-type mice inoculated with the same amount of SeV, the op/op mice exhibit markedly decreased levels of IL-13 and Muc5ac gene expression. To preserve the ability to develop an acute immune response to viral infection, we next studied wild-type mice that were treated with clodronate-containing liposomes using a protocol that selectively depletes lung macrophages 15. Liposome treatment was started after clearance of infectious virus to avoid any possible effect on the acute infection. Thus, there was no need to decrease the viral inoculum to preserve survival, and the usual inoculum was administered in these experiments. We found no difference between control and clodronate-liposome treated mice in the levels of IL-13 or Muc5ac mRNA at Day 21 PI, which was consistent with a lack of IL-13 production by macrophages at this early time point. However, macrophage-depleted mice exhibited significantly decreased levels of IL-13 and Muc5ac gene expression at Day 49 PI compared to mice that were treated with empty liposomes. Immunostaining of lung sections indicated that clodronate-treated mice were depleted of IL-13-producing macrophages (data not shown). The loss of IL-13-producing macrophages caused a decrease in IL-13 mRNA and mucous cell metaplasia that was in proportion to the macrophage contribution to total IL-13 mRNA production in the lung at Day 49 PI.

NKT cell requirement for macrophage activation

We investigated whether persistent pressure from another part of the immune system might be necessary to activate macrophages for prolonged periods after viral infection. As noted
in the Introduction, this type of chronic pressure is generally attributed to activation of the adaptive immune response. However, we found that MHC Class II-deficient H2-Ab1−/− mice that lack most CD4+ T cells continued to develop mucous cell metaplasia in concert with increased IL-13 and Muc5ac mRNA levels after viral infection (Supplementary Fig. 3). Similar to wild-type mice, these immunocompromised mice contained only trace levels of viral RNA in the lung by Day 49 PI (Supplementary Fig. 3). Furthermore, we found the same susceptibility to develop chronic airway disease after viral infection in CD4−/− and CD8α−/− T-cell deficient mice (data not shown). To fully avoid interfering with the acute antiviral response, we also achieved T cell depletion using antibody treatment that (like sIL-13Rα2-Fc and clodronate treatment) was not initiated until after clearance of infectious virus. Similar to mice with a genetic T cell deficiency, we found that mice that were antibody-depleted of CD4+ T cells, CD8+ T cells, or both CD4+ and CD8+ T cells also showed the expected increase in the number of IL-13-producing macrophages, induction of IL-13 or Muc5ac gene expression, and mucous cell metaplasia after viral infection (Supplementary Fig. 3 and data not shown). These findings do not exclude a role for T cells in the development of chronic lung disease after viral infection. Indeed, we find that CD4+ T cells are a significant source of IL-13 production at PI Day 21 and 49. The findings with T cell blockade do, however, suggest that other types of immune cells may also influence the chronic disease that develops after viral infection.

The increased population of NKT cells in the lung was detected using either α-galactosylceramide (α-GalCer) analogue-loaded CD1d-tetramer or by staining for NK1.1+CD3+ cells (data not shown). CD4+ NKT cells appeared in greater numbers in the lung at Day 49 PI and produced the same or higher levels of IL-13 mRNA per cell at Day 21 and 49 PI compared to CD4+ NKT cells. In contrast to CD4+ NKT cells, CD4+ NKT cell production of IL-13 mRNA was not accompanied by any increase in IL-4 mRNA at Day 21 or 49 PI (Supplementary Fig. 4). Even for CD4+ NKT cells, the level of IL-4 mRNA production was relatively low at Day 49 PI. This pattern of low IL-4 mRNA production was also found for CD4+ T cells at Day 49 PI, and, consistent with these findings, treatment with an anti-IL-4 mAb caused no change in the development of chronic lung disease after viral infection (Supplementary Fig. 4 and data not shown). Similarly, NKT cell activation was not accompanied by any increase in Ifn-β or Ifn-γ mRNA production by Day 49 PI (Supplementary Fig. 4). The involvement of CD4+ NKT cells in driving chronic inflammation is consistent with our finding that IL-13 or Muc5ac mRNA production persisted despite treatment with CD4-depleting mAb (Supplementary Fig. 3).

In studies of NKT cell-deficient mice, the decreases in IL-13-producing macrophages as well as IL-13 and Muc5ac mRNA levels in the lung was observed at Day 49 PI but was not observed at Day 21 PI. This finding was consistent with the time course for increased macrophage production of IL-13 and with the effects of macrophage depletion. Furthermore, Cd1d1−/− mice at Day 49 PI also had decreased airway hyperreactivity relative to wild-type control mice, and this decrease was independent of any changes in baseline airway caliber (Supplementary Fig. 4). We also observed similar inhibition of virus-induced IL-13 and Muc5ac gene expression in the lungs of Traj18−/− mice. In both strains, lung levels of viral RNA were similar to wild-type mice (Supplementary Fig. 4). Together, these findings indicated that NKT cells (independent of CD1d-dependent actions on NKT cells or APCs) were necessary for chronic lung disease after viral infection.

**Direct NKT cell-macrophage interaction**

We reasoned that NKT cells could act by recruiting macrophages to the lung and by activating macrophage production of IL-13. A role for NKT cell-dependent recruitment of macrophages was substantiated when we found that the usual increase in lung macrophages at Day 49 PI that was found in wild-type mice was absent in Cd1d1−/− mice. In support of a mechanism for NKT cell-dependent recruitment of macrophages, we found that purified lung NKT cells released biologically relevant amounts of macrophage chemokines
(predominantly Ccl3) after stimulation with PMA-ionomycin (Supplementary Fig. 5). Furthermore, lung NKT cells isolated from Day 49 PI produced increased levels of macrophage chemokine mRNA compared to NKT cells from mice without SeV-induced lung disease (Supplementary Fig. 5). The predominant chemokine mRNA produced by NKT cells after viral infection was Ccl3 (consistent with the profile for chemokine production at the protein level), and increased Ccl3 (as well as Ccl2 and Ccl4) mRNA levels were found exclusively in CD4+ rather than CD4− NKT cells (consistent with the increased activity of CD4+ NKT cells in chronic lung disease after viral infection).

In our co-culture system, we used NKT cells and macrophages from mice without SeV inoculation to achieve low background levels of IL-13 production. We used α-GalCer-analogue-loaded CD1d-tetramer to isolate NKT cells and thereby obtained the same invariant NKT cell population that was targeted in Cd1d1−/− and Traj18−/− mice. The system was also constructed so that NKT cells could be removed after cell-cell interaction to allow for monitoring IL-13 production derived only from adherent macrophages. We found that liver NKT cells were somewhat less effective than lung NKT cells in driving macrophage activation, in that liver NKT cells required α-GalCer ligand to stimulate macrophage production of IL-13 mRNA.

**IL-13R signalling and alternatively activated macrophages**

To determine whether persistent activation of this immune axis was driven by upregulation of cytokines (including IL-13) or cytokine receptors in vivo, we used oligonucleotide microarrays to analyze mRNA isolated from the lungs of mice at Days 21 and 49 PI (Supplementary Fig. 6). Real-time PCR analysis of whole lung samples indicated that Il-13ra1 mRNA was upregulated in wild-type and Il13−/− mice but not in Cd1d1−/− mice, indicating that NKT cells stimulated an increase in Il-13ra1 mRNA. The relatively small increase in Il-13ra1 mRNA after viral infection in whole lung samples suggested that increased expression might be restricted to a subpopulation of lung cells such as macrophages. We therefore analyzed Il-13ra1 mRNA in macrophages that were FACS-purified from whole lung samples. Relative to whole lung samples, we found that there was a much greater increase of Il-13ra1 mRNA in lung macrophages isolated after viral infection, and this increase was also blocked in Cd1d1−/− mice. Similarly, IL-13ra1 was colocalized with the macrophage marker CD68 as well as with IL-13 in lung tissue sections obtained from mice on PI Day 49 by immunostaining (Supplementary Fig. 7). Furthermore, blockade of IL-13 action by sIL-13Ra2-Fc caused a marked decrease in the levels of IL-13-producing macrophages, which, in turn, caused a decrease in Il-13 mRNA levels in the lung.

The microarray gene expression data was re-examined to identify any additional chronic changes in gene expression after viral infection. The mRNAs encoding chitinase-like proteins (Chi3l3/4 and Fizz1), arginase (Arg1), matrix metalloproteinase (Mmp12), and arachidonate 12-lipoxygenase (Alox12e) were significantly up-regulated on Day 49 PI, and these same mRNAs were also upregulated but to a lesser degree on Day 21 PI (Supplementary Fig. 6). Real-time PCR assays for these gene products confirmed the microarray data (Supplementary Fig. 7). Furthermore, these changes were completely blocked in Il13−/− mice, and they were partially inhibited in Cd1d1−/− mice after viral infection (Supplementary Fig. 7, and data not shown). Immunostaining for Chi3l3/4 protein indicated that the increase in the amount of this protein was localized predominantly to lung macrophages and occurred at the same time as the increases in Chi3l3/4 mRNA levels (Supplementary Fig. 7 and data not shown). Moreover, FACS-purified lung macrophages exhibited upregulation of the same markers of alternative activation as whole lung samples at Day 49 PI as well as downregulation of expression in NKT cell-deficient mice (Supplementary Fig. 7).
**Supplementary Figure 1**

Viral induction of IL-13 production causes mucous cell metaplasia. (a) WT mice were inoculated with SeV, and lung Muc5ac and IL-13 mRNA levels were determined at the indicated days PI. (b) WT mice were inoculated with SeV or SeV-UV, treated with control IgG or sIL-13Rα2-Fc from Day 12 to 49 PI, and lung sections were immunostained for Muc5ac. Bar = 20 μm. II-13−/− mice and isotype control IgG showed no signal above background (data not shown). (c) Quantification of Muc5ac+ cells from (b). (d) WT and II13−/− mice were inoculated as in (b) and assessed for pulmonary resistance (Rr) versus concentration of inhaled methacholine. (e) For conditions in (d), corresponding values for baseline Rr. (f) WT and II13−/− mice were inoculated with SeV, and lungs were analyzed for SeV-specific RNA. For (a, d,e,f), (*) indicates a significant increase from Day 0, SeV-UV; for (c), (*) indicates a significant decrease from no treatment control.
Supplementary Figure 2

(a) WT mice were inoculated with SeV, and lung cell subsets were isolated from mice at Day 21 and 49 PI. Each cell fraction was analyzed for IL-13 mRNA levels. Values represent IL-13 mRNA per cell for each cell fraction. (b) Corresponding number of cells in each IL-13-expressing cell fraction. For (a,b), (*) indicates a significant increase from Day 0 PI. (c) Purified lung macrophages from Day 49 PI were analyzed for IL-13 mRNA at the indicated times after isolation. (*) indicates a significant difference from corresponding SeV-UV control.
Supplementary Figure 3. Effect of T cell depletion on mucous cell metaplasia after viral infection. (a) H2-Ab1−/− mice were inoculated with SeV at 0.5 x 10^5 pfu, and lung sections were immunostained for Muc5ac at Day 0, 21, and 49 PI. Bar = 20 μm. (b) From conditions in (a), quantification of Muc5ac+ cells. (c) For conditions in (a), lungs were analyzed for Il-13 and Muc5ac mRNA and SeV RNA levels. For (b,c), (*) indicates a significant increase from Day 0 PI. (d) WT mice were inoculated SeV or SeV-UV and treated with control IgG, anti-CD4 mAb, and/or anti-CD8a mAb from Day 12 to 49 PI. Lung sections from Day 49 PI were immunostained for Il-13+CD68+ cells, and corresponding lungs were analyzed for Il-13 and Muc5ac mRNA levels. (*) indicates a significant increase from SeV-UV.
Supplementary Figure 4.

Characterization of lung NKT cell function after viral infection. (a) Representative cytograms and gating strategy for analysis of lung NKT cells by flow cytometry using forward and side scatter as well as FITC-labeled anti-CD3e mAb and APC-labeled CD1d tetramer. (b) Using the strategy in (a) plus anti-CD4 mAb, CD4+ and CD4− NKT cells were purified from WT mice at Day 0, 21, and 49 PI, and each subset was analyzed for Ifn-β, Ifn-γ, and IL-4 mRNA level. (c) WT mice were inoculated with SeV or SeV-UV, treated with anti-IL-4 mAb or isotype control Ig from Day 14 to 21 or 49 PI, and lung RNA was analyzed for IL-13 and Muc5ac mRNA levels. (d) WT and Cdlf−/− mice were assessed for airway reactivity at Day 49 PI (n = 5-10 mice per group). (e) Baseline Rγ (cm2 H2O/ml/sec) was analyzed for SeV RNA. (f) SeV RNA levels. (*) indicates a significant increase from Day 0 PI.
Supplementary Figure 5

**Supplementary Figure 5.** NKT cell production of chemokines for lung macrophages. (a) FACS-purified CD4+ and CD4- NKT cells (2 x 10^5 cells/ml) from mouse lungs at PI Day 49 were cultured in RPMI with 10% FBS and PMA-ionomycin for 1 d at 37 °C, and cell culture media were analyzed for chemokine levels by multiplex bead-based assay. (b) CD4+ and CD4- NKT cells from mouse lungs at PI Day 49 were analyzed for Ccl2, Ccl4, and Ccl5 mRNA by real-time PCR. (*) indicates a significant increase from SeV-UV.
Supplementary Figure 6. Oligonucleotide microarray analysis of lung mRNA after viral infection. Scatter plot depicts normalized gene expression in SeV-UV- versus SeV-inoculated mice at PI Days 21 and 49. Red lines indicate differential expression 2 SD from the mean (dashed line).
Supplementary Figure 7. IL-13R expression and alternative activation of macrophages. (a) Representative photomicrographs from lung sections at PI Day 49 immunostained for CD68, IL-13, and IL-13Rα1 and imaged by laser confocal scanning microscopy. Bar = 50 μm. (b) Lung levels of Chi3l3/4 and Fizz1 mRNA at indicated SeV PI Days. (c) Representative photomicrographs from lung sections at PI Day 49 immunostained for Chi3l3/4. Bar = 50 μm. (d) Lung levels of Arg1 and Mmp12 mRNA in WT, IL-13−/−, and Cd14−/− mice at PI Day 49. (e) Levels of Chi3l3/4, Arg1, Mmp12, Fizz1, and Alox12e mRNA in lung macrophages from WT and Jil1−/− mice at PI Day 49. For (b,d,e), (*) indicates a significant increase from PI Day 0 or SeV-UV control.
Supplementary Figure 8

**Supplementary Figure 8.** Increased IL-13-producing macrophages in humans with asthma. (a) Representative photomicrographs of cytospin samples from BAL fluid of severe asthma patients and normal control subjects that were immunostained for IL-13 and CD68. Arrows indicate a representative IL-13^CD68^ macrophage. Bar = 20 μm. (b) Quantitative analysis of IL-13 and CD68 immunostaining from (a) in asthma patients (n = 3) and non-asthma control subjects (n = 3). (*) indicates a significant increase from value for normal control subjects.
Supplementary Methods

**Mice generation and treatment.** All mice were maintained under pathogen-free conditions for study at 6-8 wk of age as described previously. Sentinel mice and experimental control mice were handled identically to inoculated mice and exhibited no serologic or histologic evidence of exposure to 11 rodent pathogens (including SeV). For IL-4 blockade, anti-IL-4 mAb (clone 11B.11) was administered at 200 μg/kg by intraperitoneal injection at 3-day intervals from Day 12 to 49 after viral inoculation as described previously. For CD4 and CD8 blockade, anti-CD4 mAb (clone YTS 191.1) and anti-CD8 mAb (clone 2.43) were administered on the same schedule as anti-IL-4 mAb and were delivered at levels that inhibit the airway response to allergen.

**Analysis of mRNA and SeV-specific RNA.** For analysis of lung mRNA levels, sequences of the forward and reverse primers and probes were: 5’-GGAGCTGAGCAACATCACACA-3’, 5’-CACACCTCCATTACATGCTGCC-3’, and 5’-CCAGACTCCCTCTGTGCA-3’ for Il-13; 5’-TACCACTCCGCTTCTGCAAGCTGTC-3’, 5’-ATAGTAAACGTGGCCATCAAGGTCTGTTT-3’, and 5’-TATACCCCTTTGAGATCCTCATCTACA-3’ for Mac5ac; and 5’-CATGGAGCTGCAGAGCTCTTTGCGGC-3’, 5’-AAGCTTCTTCAGTGATGAGGACTTGGGAC-3’ and 5’-ATGCCTGGATTCATCGATAAGCTGCACCAT-3’ for Il-4; 5’-CTTGTCAACAGTCTGGCAATTCC-3’, 5’-GTAACACA-TCAGCTGATAGAAGA-3’, and 5’-CTCAAGAAGTACAGTGGG-3’ for Chi31B4; and 5’-ATGAAACAGTGGCCCTCTTGC-3’, 5’-AGCCACACGACACACCACTGAG-3’, and 5’-CCTGCTGGGAGTGACT-3’ for Fizz1. All probes were designed to span an intron and did not react with genomic DNA. Levels of Ifn-β, Ifn-γ, Il-13ra1, Mmp12, Arg1, Alox-12e, Ccl2, Ccl3, Ccl4, Ccl5, and Gapdh mRNA were determined using Taqman gene expression assays (Applied Biosystems). Viral titer was monitored by quantitative real-time PCR for SeV-specific RNA using 5’-CCACCTCTGAGGAGCAGTT (viral nt 68-85) as forward primer, 5’-CCCGCCATCTGTGAA (nt 112-129) as reverse primer, and 5’-CAAGCAAGGTCTG (nt 87-110) as MGB probe. Part of the SeV genome (nt 5-620) that includes leader sequence and part of the nucleocapsid protein (NP) gene was cloned into pGEM-3Zf(-) plasmid, and transcript in genomic orientation was used as RNA standard. All mRNA and SeV-specific RNA levels were normalized to levels of Gapdh mRNA using the TaqMan Rodent GAPDH Control Kit.

For oligonucleotide microarray analysis, whole lung RNA from PI Days 21 and 49 was used to generate labeled cRNA that was hybridized in triplicate to oligonucleotide microarrays (Affymetrix Mouse Expression Set 430 for PI Day 21, Affymetrix Mouse Genome Array 430 2.0 for PI Day 49) and scanned as described previously. Microarray normalization and statistical analysis was performed using packages from the Bioconductor project executed in the R programming environment. We normalized data using the empirical Bayes version of the GCRMA algorithm as implemented in the GCRMA package. We assessed differential expression (SeV versus SeV-UV) using linear models and empirical Bayes moderated F statistics as implemented in the LIMMA package. We considered differences in gene expression significant if P values were <0.05 after adjustment for multiple testing as described previously, so that false discovery rate was <5%. We performed visualization and plotting using Spotfire DecisionSite for Functional Genomics (Spotfire).

**Cell isolation and culture.** Lung cell suspensions were generated using a protocol modified from one described previously. For the present experiments, PBS was used to flush out the pulmonary and systemic circulations, and BAL was performed to remove any cells in the bronchiolar or alveolar space. Next, 1 ml of digest media was injected intratracheally. Digest medium consisted of DME supplemented with 5% fetal calf serum, 10 mM HEPES, penicillin/streptomycin, 250 U/ml collagenase type I (Worthington Biochemical), 50 U/ml DNase I (Worthington Biochemical), and 0.01% hyaluronidase (Sigma-Aldrich). The lungs were carefully dissected from the trachea, main-stem bronchi, draining lymph nodes, and surrounding tissue, and then were minced and incubated in digest media for 1 h at 37 °C. During the final 15 min of incubation time, EDTA was added to a final concentration of 2 mM. After digestion, the cell mixture was passed through a 40-μm cell strainer to generate single-cell suspensions, and erythrocytes were removed by hypotonic lysis.

For analysis by flow cytometry, samples were blocked with 1 μg anti-mouse CD16/CD32 (BD Pharmingen) per 1 x 10⁷ cells for 15 min, and then stained with mAb (Pharmingen or eBioscience) conjugated to
appropriate fluorophores for 30 min at 4 °C. Labeled rat anti-mouse c-kit, FceRI, GR-1, CD11c, CD19, B220, DX5, NK1.1, CD4, CD8, Mac1, Mac3, Fc RI, and MHC Class II as well as hamster anti-mouse CD3e and isotype control IgGs (rat and Armenian hamster) were from eBioscience or BD Pharmingen. For intracellular staining for CD68, cells were fixed with 2% paraformaldehyde for 15 min at 4 °C and permeabilized with Perm/Wash buffer (BD Biosciences) for 30 min for intracellular staining with rat anti-mouse CD68 mAb (Serotec). Cells were sorted by FACS using a MoFlo high-speed flow cytometer (Dako Cytomation). Cells were collected in either RLT lysis buffer (Qiagen) for RNA isolation or HBSS for further experimentation with viable cells.

Cells were sorted as follows: anti-c-kit positive for mast cells, anti-FcεRIα positive for basophils, anti-GR-1 positive for neutrophils, and anti-CD11c positive for DCs. The B, T, NK, NKT cells, and macrophages were gated for FSC<median>SSC<low> “lymphocytes”. Within this gate, we sorted anti-B220 positive or CD19 positive for B cells (both gave identical results), both anti-CD8 and anti-CD3e positive for CD8+ T cells, anti-CD4 and anti-CD3e positive as CD4+ T cells, anti-DX5 positive and anti-CD3e negative for NK cells, anti-NK1.1 positive and anti-CD3e positive or α-GaICer analogue (PBS57)-loaded CD1d tetramer positive and anti-CD3e positive for NKT cells; anti-Mac-1 positive and intracellular anti-CD68 positive or anti-Mac-1 positive and negative for a pool of FITC-label anti-c-kit, FceRI, GR-1, CD11c, NK1.1, CD3e, and B220 mAbs for macrophages. The latter sort scheme (Mac1-positive, multiple FITC marker-negative macrophages) were >95% positive for intracellular CD68 on subsequent analysis and allowed us to perform further experiments with non-permeabilized live cells. Macrophages were also gated for FSC<median>SSC<low> “macrophages-granulocytes”, but little II-13 mRNA was detected in cells in this gate (<4% of total II-13 mRNA at PI Day 49). Thus, all data for II-13 mRNA is derived from cells found in the FSC<median>SSC<low> gate. Levels of IL-13 and chemokine (KC, CCL2, CCL3, CCL4, and CCL5) proteins were determined by Quantikine, ELISA (R&D Systems) or Bio-Plex bead-based cytokine assay (Bio-Rad), respectively.

**Immunohistochemistry.** For immunostaining with brightfield microscopy, mouse lungs were fixed, embedded in paraffin, cut into sections, dewaxed, rehydrated, and incubated with mouse anti-MUC5AC mAb (clone 45M1; Neomarkers) or control Ab as described previously 15. Tissue sections were counterstained with hematoxylin, dehydrated in graded ethanol, and mounted for viewing in a photomicrography system. For immunofluorescence, paraffinized sections were hydrated and incubated in Antigen Unmasking Solution (Vector Labs) at 90 °C for 10 min for antigen retrieval. Sections were blocked for 1 h at 25 °C in 2% vol/vol teleostean gelatin (Sigma) and 5% goat serum and then overnight at 4 °C with biotinylated goat anti-mouse IL-13 Ab (R&D Systems, 3.6 μg/ml). Staining was visualized with tyramide signal amplification Alexa Fluor 555 (Invitrogen). Peroxidase activity was quenched with 3% H₂O₂ for 5 min at 25 °C. Biotin, biotin receptors, and avidin binding sites were blocked with the Avidin/Biotin Blocking kit (Vector Labs). Sections were also stained with biotinylated rat anti-mouse CD68 mAb (Serotec, 10 μg/ml) followed by avidin HRP (Vector Elite ABC System) and Alexa Fluor 488 (Invitrogen) and with goat anti-mouse IL-13Rα1 IgG (Santa Cruz Biotechnology, 2 μg/ml) followed by biotinylated donkey anti-goat IgG mAb (Jackson Immunoresearch, 13 μg/ml) and Alexa Fluor 647 (Invitrogen). Sections were treated with 3% H₂O₂ for 5 min at 25 °C and the Avidin/Biotin Blocking kit. All antibodies were incubated in PBS with 2% vol/vol teleostean gelatin. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). In some experiments, tissue sections were blocked with 2% teleostean gelatin, incubated with biotinylated anti-MUC5AC mAb and Alexa 555-conjugated streptavidin or anti-CD68 mAb and Alexa 633-conjugated goat anti-rabbit secondary Ab (Molecular Probes), counterstained with Sytox Green (Molecular Probes), and then imaged by confocal microscopy using a Zeiss laser scanning system with LSM-510 software. For immunostaining of Chi3L3/4 (Ym1), rabbit anti-mouse Chi3L3/4 Ab was provided by Shiooko Kimura (NCI, Bethesda, MD) and used as described previously 15.

**Airway reactivity measurements.** In screening experiments, airway reactivity to nebulized methacholine was determined in unrestrained mice using a whole-body plethysmograph and BioSystem XA Version 2.7.9 software (Buxco Electronic) to derive values for enhanced pause (Penh) as described previously 2,15. Mice were placed in the plethysmograph for a 5-min acclimatization interval, followed by 3-min acquisition intervals before (baseline Penh) and after a 48-sec exposure to nebulized vehicle (PBS) or doubling concentrations of methacholine (5-80 mg/ml) delivered from a Collison 6 jet nebulizer (BGI). In validation experiments, airway reactivity was also determined by measurements of total lung resistance (Rl) and dynamic compliance (Cdyn)
as described previously. In this case, mice were anesthetized with pentobarbital (100 mg/kg ip), ventilated through a tracheostomy with a Harvard Apparatus Model 687 at 6-10 ml/kg and positive end-expiratory pressure of 2-4 cm H₂O, and monitored for intrapleural pressure using an oroesophageal tube. Methacholine was delivered at 3-min intervals using an in-line nebulizer (Aerogen Laboratory; 2.4-4 μm particle size). Between deliveries, respiratory flow signal was obtained with a pneumotach (SenSym SCXL004; Buxco Electronics) and was integrated to calculate lung volume. Intraesophageal and airway pressure were measured via pressure transducers (Validyne DP45; Buxco Electronics) directly connected to the respective catheters.

**Human subject samples.** Patients with severe asthma as well as healthy control subjects were recruited, characterized, and subjected to bronchoalveolar lavage (BAL) as described previously. Severe asthma patients met the American Thoracic Society workshop criteria for refractory severe asthma if they had one or both of two major criteria and two of the minor criteria. The major criteria include: treatment with continuous or near continuous oral glucocorticoids or treatment with high-dose inhaled glucocorticoids. The minor criteria include: daily treatment with a long-term controller medication in addition to inhaled glucocorticoids, asthma symptoms requiring short-acting β-agonist use on a daily or near-daily basis, persistent airflow obstruction, one or more urgent care visits for asthma per year, three or more oral glucocorticoid bursts per year, prompt deterioration with <25% decrease in oral or inhaled glucocorticoid dose, or near fatal asthma event in the past. For the group of severe asthma patients, forced expiratory volume in 1 sec (FEV1) was 63 ± 14% predicted, and for normal controls, FEV1 was 86 ± 4% predicted. For asthma and control subjects, there was no history of endotracheal intubation within the past 5 yr, respiratory tract infection within the past 3 months, or significant cardiac or neurologic disease. Cytospin samples from BAL fluid were immunostained with mouse anti-human CD68 mAb (2 μg/ml) for 18 h that was visualized with anti-mouse HRP mAb (Invitrogen, 1 μg/ml) followed by tyramide signal amplification with Alexa Fluor 488 (Invitrogen), quenched with HCl, and then stained with biotinylated mouse anti-human IL-13 mAb (4 μg/ml) for 18 h that was visualized with avidin HRP (Vector Elite ABC System) and Alexa Fluor 594 (Invitrogen).

For COPD patients, real-time PCR was performed in the same manner as described above for mouse lung. Sequences of forward and reverse primers and probes were: AGGCCAGCTACGGGCGCGG-GCCAGACAT, 5'-TTCCCCCTACTACCCGAGTGCGCCAGCA, and 5'-TGGACACCTGTT-TTTAGCACAGGAT for MUC5AC, 5'-GAGAACCAGAGATGCTGAGGAGATTCT, 5'-ACCTCAGATTCTTGAGCGCA, and 5'-AAGGTCCTGAGGTGGCAGTTCGTTCTTACCTTGTTGACCA, and 5'-AGGTTCGAGTTCAACCGATTGGTGATTTGATG for GAPDH. All target mRNA levels were normalized to GAPDH mRNA levels.