Online Repository Materials

Materials and Methods

Animals

C57Bl/6 CD45.1, C57Bl/6 CD45.2, Balb/C, *Rag1*$^{-/-}$ mice were obtained from Jackson Labs. *Rag2*$^{-/-}\gamma c^{-/-}$ mice were kindly provided by Dr. Roberta Pelanda (National Jewish Health). *ERK1*$^{-/-}$ mice were a generous gift from Drs. Gilles Pagés and Jacques Pouysségur**,** University of Nice, France as described previously (1).

Mucosal sensitization of mice

Allergens used include extracts of dust mite (*Dermatophagies farinae*), ragweed (*Ambrosia artemislifolia*), and *Aspergillus fumigatus* (Greer Laboratories). Intranasal allergens (**D**ust mite 5μg, **R**agweed 15 μg, ***A****spergillus* 5 μg; **DRA**) or aspergillus (5μg) were delivered in 15μL quantities in saline.

Acute asthma was produced by immunization twice, one week apart, of Balb/C mice 8-12 weeks of age with *Aspergillus* species (5 μg) in adjuvant (1:1 vol/vol). Adjuvant was aluminum and magnesium hydroxide (Pierce). Asthma was initiated by three consecutive intranasal exposures to *Aspergillus* species (5μg in 15μL saline) and asthma was evaluated 72 hours after the final exposure.

Chronic asthma was produced by intranasal delivery of the DRA mixture twice a week for six consecutive weeks in female mice (C57Bl/6 CD45.2 or Balb/C as appropriate) 8-12 weeks of age. For characterization of asthma chronicity experiments mice were analyzed at the indicated

time points (1, 2, or 6 months after cessation of allergen exposure). A timeline of manipulations and interventions for the chronic asthma protocol with irradiation is shown in figure 2a. For irradiation experiments mice were rested for three weeks after completion of allergen delivery and 2 doses of 600 RADS were delivered three hours apart. Flow cytometric analysis indicates that 6 weeks after irradiation less than 4% of $CD45.2^+$ cells from recipient mice were still present. $5x10^6$ bone marrow cells from C57Bl/6 CD45.1, or Balb/C *Rag1*$^{-/-}$, or Balb/C *Rag2*$^{-/-}\gamma c^{-/-}$ as indicated in the experimental design were transferred i.v. and mice were rested an additional 6 weeks before analyses.

Antibody, pharmacological and genetic interventions

For IL33 blockade experiments i.p. anti-IL33 (15μg/ 200μL injection in saline; R&D) was delivered 3, 5, and 7 days prior to analysis in week 15 (6 weeks after irradiation). All non-irradiated control asthmatic mice were rested an equivalent amount of time before analyses. For CD3 and IL13 blockade hamster anti-mouse CD3ε (clone 145 2C11, e-Bioscience, 200 μg/dose, ref. 2) and anti-IL13 (50μg/dose, Calbiochem) antibodies, hamster IgG or rat IgG were administered i.p. on three consecutive days in week 10 and outcomes examined 3 days later.

Adoptive transfer of CD4 T cells and ILC2

Spleen CD4 T cells were negatively selected by antibody-coated magnetic beads (Miltenyi, Inc). and transferred i.v. (4×10^6 cells) to naïve mice. ILC2 ($Lin^-CD45^+CD25^+$) cells were sorted on MoFlow XDP (Beckman Coulter) and 24 hours after sorting delivered i.v. (2×10^5 cells) to naïve mice. Intranasal DRA (5, 15, 5 μg/ 15μL dose in saline) was performed on three consecutive

days following i.v. transfer of CD4 T cells. CD4 T cell recipient mice were studied on day 6 and 21. ILC recipient mice were analyzed on day 21.

Airway hyperreactivity measurement

Measurement methodologies have been explained in depth elsewhere (17). Briefly, mice were anesthetized with ketamine (180 mg/kg), xylazine (9 mg/kg), and acepromazine (4 mg/kg). After loss of foot-pad pinch reflex, a tracheotomy was performed and the mouse was attached via an 18 gauge cannula to a small-animal ventilator with a computer-controlled piston (Flexivent; Scireq). Mice were ventilated at a frequency of 90 breaths/ min with a tidal volume of 20 mL/kg during nebulization and otherwise with a frequency of 150 breaths/ min with a tidal volume of 20 mL/kg while breathing against an artificial positive end-expiratory pressure of 2.5 to 3 cm H_2O. Lungs were inflated to total lung capacity twice to standardize volume history. Resistance measurements were then taken to establish baselines for total lung resistance and at each methacholine dose. Group averages were expressed as fold increases in baseline resistance (mean +/ - SEM).

Histology and Immunofluorescence Staining

Paraffin embedded lungs were sectioned and stained with hematoxylin and eosin (H&E) for morphometric analysis, or for mucin staining, or toluidine blue for mast cell staining. Sections for immunofluorescence staining were permeabilized with 0.01% saponin in PBS, blocked with 10% goat serum, and stained with a primary antibody against human IL33 and visualized with Alexa-488 conjugated secondary antibody as described previously (3) and counterstained with DAPI for nuclear staining.

Morphometric measurements

Inflammation was quantified using Metamorph image acquisition and analysis software on H&E stained lung sections at 20x magnification as described previously (17). Airway epithelial hypertrophy and peribronchial smooth muscle hypertrophy were measured as the area of epithelial or smooth muscle per circumference of airway basement membrane. Airway inflammation was measured as the area of inflammatory infiltrates as a percentage of the total field. A minimum of 5 airways per mouse and 5-9 mice per group were quantified.

Images were acquired on a Nikon Eclipse TE2000-U microscope using 20x dry lenses at room temperature through a Diagnostics Instruments camera model #4.2 using Spot software 5.0. H&E sections were mounted using Permount medium. Images were adjusted for brightness and contrast to improve viewing.

Lung digestions

Lungs were perfused with saline and single cell suspensions of lung cells were acquired by mechanical mincing of lungs followed by digestion at 37°C for 45 minutes in RPMI with 10% FBS, 1% penicillin/ streptomyocin and collagenase A (1mg/mL). Cell suspensions were agitated at room temperature for 10 minutes in RPMI with 100U/mL DNAse I prior to filtration through 40μm filters and red blood cell lysis. Single cell suspensions were subsequently fixed in 4% paraformaldehyde for flow cytometric analysis.

Flow cytometric analyses of type 2 ILC and other cell surface markers

Mouse type 2 ILC: For mouse flow cytometric staining all conjugate antibodies were purchased from Biolegend (San Diego, CA) unless otherwise stated. Mouse lung digest cells were fixed with 4% paraformaldehyde, incubated with 10% donkey serum and 1% 2.4G2. ILC cells were stained with PerCp/Cy5.5-labelled CD45.2 (clone 104) and/or PE/Cy7-labeled CD45.1 (clone A20) as appropriate, pacific blue-labeled lineage marker antibodies (CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/Erythroid cells), Alexa Fluor 488-conjugated CD25 (clone PC61), allophycocyanin- labeled IL5 (TRFK5), phycoerythrin- labeled IL13 (eBioscience; clone eBio13A). ILC2 cells were initially characterized with the addition of primary antibody anti-IL33R (ST-2) (R&D, clone AF1004) followed by allophycocyanin-labeled secondary antibody against the primary antibody and allophycocyanin -eFluor780-conjugated IL7Rα (eBioscience clone, eBioRDR5), allophycocyanin-labeled CD117 (c-kit; clone ACK2), phycoerythrin-labeled Ly-6A/E (Sca-1; clone D7), phycoerythrin-Cy7 labeled NK1.1 (clone PK136), and allophycocyanin-labeled FCεR1 (clone MAR-1).

Lung IL33+ cells: IL33 levels and localizations were characterized with primary antibody against IL33 (clone Poly5165) followed by fluorescein isotyhiocyanate- labeled secondary, and primary antibody against proSPC (Millipore, AB3786) followed by allophycocyanin-labeled secondary, or PE-Cy7-labeled CD11b (clone M1/70), or primary antibody against E-cadherin (Santa Cruz, clone H108) followed by pacific blue- labeled secondary, or allophycocyanin-labeled FCεRI (clone MAR-1).

Human BAL type 2 ILC: BAL cells were fixed in 4% paraformaldehyde, and incubated in 10% goat serum. ILC2 cells were stained with pacific blue-labeled lineage marker antibodies (CD3, CD14, CD16, CD19, CD20, CD56), fluorescein isotyhiocyanate- labeled IL7Rα (clone

A019D5). PerCp/Cy5.5- labelled IL13 (clone JES10-5A2), phycoerythrin- labeled IL5 (clone JES1-39D10). IL33R was identified with the addition of primary antibody against IL33R (ST-2) (MBL, clone HB12) followed by allophycocyanin- labeled secondary antibody against the primary antibody. After washing, cells were analyzed by flow cytometry using CyAn ADP cytometer (Beckman-Coulter). Data were analyzed by the software FlowJo version 7.6.5 (Treestar).

T-helper cell proliferation and cytokine production

Splenocytes and mediastinal lymph node cells (2 x 10^6/ml) were cultured for 96 hours on anti-CD3 and anti-CD28 (1μg/mL) coated 48-well plates or stimulated separately with the following allergens—dust mite (10 μg/mL), ragweed (50 μg/mL) and Aspergillus sp. (10 μg/mL) as described previously (3). In one set of cultures cells were labeled with 1μM CFSE (Invitrogen). For measurement of cytokines (IL2 and IL4) monensin was added to another culture set 6 hr before the conclusion. T cells were detected by addition of allophycocyanin-labeled anti-CD4 (clone GK1.5; Biolegend) prior to flow cytometric analysis on CyAn ADP cytometer (Beckman-Coulter) for proliferation and intracellular cytokine expression.

Western blotting

Immediately after Flexivent analysis lung sections were collected in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM Na_3VO_4, 0.1mM PMSF) containing protease and phosphatase inhibitors and homogenized. Lysed samples were subjected to 10% SDS-PAGE, transferred to a PVDF membrane and then immunoblotted with primary antibodies [IL33 (R&D), tubulin (H-235) and β-actin (Santa Cruz)]. After washes the membranes were

incubated with a HRP-conjugated secondary antibody. After additional washing, the membranes were developed with the ECL reagent as previously described (3).

A549 cell culture

A549 cells were cultured in RPMI in 10% fetal bovine serum (HyClone) and 1% penicillin/ streptomycin (Gibco) and maintained at 37°C in a humidified 5% CO_2 incubator. All cytokines were purchased from PeproTech.

In one experiment, cells were treated with medium, IL1β (2ng/mL), IL4 (10ng/mL), IL13 (20ng/mL), IL17 (10ng/mL), IL33 (20ng/mL), TNF (2ng/mL), IFNγ (10 ng/mL) and TNF (2ng/mL) plus IFNγ (10 ng/mL) for 72 hr. Cellular RNA was analyzed for mRNA for IL33 and IL33 receptor. In a second experiment cells were pre-treated for 24 hours with media, IL1β (2ng/mL), IL4 (10ng/mL), IL13 (20ng/mL), IL17 (10ng/mL), TNF (2ng/mL) or IFNγ (10 ng/mL) before stimulation for 72 hours with IL33 (20ng/mL). Cellular RNA was analyzed for mRNA for IL33. In a third experiment, cells were treated for 24 hours with DRA (5 μg/mL of dust mite, 15 μg/mL of ragweed and 5 μg/mL of Aspergillus sp.). Cellular RNA was analyzed for mRNA for IL33. In a fourth experiment cells were treated for 24 hours with IL13 (20ng/mL) and ATP, the supernatant collected and analyzed for IL33 levels by ELISA according to manufacturer's instructions (R&D systems).

RT-PCR

Total RNA was isolated from frozen tissues using Trizol (Invitrogen), or from cells using a kit (Purelink Mini RNA kit, Life Technologies) and cDNA was synthesized using an ImpromII cDNA synthesis kit (Promega) according to manufacturer's instructions as described previously

(3). Gene specific PCR products were amplified using Sybr green (ThermoScientific) and primers outlined in Table 2 using an Applied Biosystems 7000 Sequence Detection System. The levels of target gene expression were normalized to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method.

Microarray and Analysis

We used the "OneArray" microarray service from the Phalanx Biotech (Palo Alto, CA) where 29,922 mouse genome probes and 1880 experimental control probes were used for the array. Each sample was studied in triplicates. The following analyses of the microarray results were performed and provided with the service: 1). Rosetta profile error model calculation; 2). Normalized intensities (excluding flagged and control data) with median scaling; 3). Basic statistic plot and Pearson correlation coefficient; 4). Pairwise ratio calculation; 5). Principal component analysis; and 6). Gene ontology analysis.

Patient Samples

Subjects were recruited from our outpatient clinic at National Jewish Health in Denver, Colorado. Human subject protocols were approved by the Institutional Review Board. We studied bronchoalveolar lavage cells from asthmatic patients and disease controls. Asthma was diagnosed based upon the presence of reversible airway obstruction and/or positive methacholine test (PC_{20}<8 mg/ml) as per the Expert Panel Report 3 (EPR3) criteria. The clinical characteristics of the study subjects are shown in Table 1. Bronchoscopy, BAL and endobronchial biopsy were performed as described previously (4). BAL was processed immediately for flow cytometry. IL33 was analyzed in BAL by ELISA according to manufacturer's directions (R&D).

Statistical analyses

Data are given as mean +/- standard error of means. For comparison of airway hyperreactivity a two-way ANOVA for repeated measures with a Bonferroni *post hoc* test was used. For pairwise comparisons a Student's *t* test was used. Data from human subjects were analyzed by non-parametric tests (Mann-Whitney U test and Kruskal-Wallis test). A P value less than 0.05 was considered significant.

References

1. Goplen N, et al. ERK1 is important for Th2 differentiation and development of experimental asthma. *FASEB J* 2012; 26:1934-1945.
2. Haile S, Lefort J, Joseph D, Gounon P, Huerre M, Vargaftig BB. Mucous-cell metaplasia and inflammatory-cell recruitment are dissociated in allergic mice after antibody- and drug-dependent cell depletion in a murine model of asthma. *Am J Respir Cell Mol Biol*. 1999; 20:891-902.
3. Gorska MM, et al. MK2 controls the level of negative feedback in the NF-kappaB pathway and is essential for vascular permeability and airway inflammation. *J Exp Med* 2007; 204:1637-1652.
4. Good JT, Kolakowski CA, Groshong SD, Murphy JR, Martin RJ. Refractory asthma: importance of bronchoscopy to identify phenotypes and direct therapy. *Chest* 2012; 141:599-606.

Online Tables

Table 1

Clinical characteristics of human subjects

Parameters	Asthma (N=38)	Disease Controls (N=18)
Diagnosis and comorbidities	38 patients with asthma; co-morbidities: 30 patients with allergic rhinitis, 25 with chronic sinusitis, 29 with GERD, 3 with bronchiectasis and 4 with aspiration	7 patients with chronic cough and concurrent allergic rhinitis & GERD, 3 patients with bronchiectasis, 3 patients with MAC infection, 4 with chronic aspiration and 1 with COPD
Age Mean ± SEM (median)	54 ± 4 (56)	56 ± 7 (58)
Sex (F/M)	22/16	11/7
BMI	28 ± 3 (27)	27 ± 3 (26)
FEV1 (%)	69 ± 4 (72)	82 ± 8 (76)
Reversibility	16 ± 3 (12)	5 ± 3 (6)
PC20 for methacholine	2.9 ± 1 (1.58)	Not done
Blood eosinophils	370 ± 80 (200)	116 ± 25 (100)
Medications	ICS+LABA (32) Systemic glucocorticoids (3) ICS (2) Omalizumab (6) Montelukast (8) SABA (38)	ICS + LABA (12) ICS (3) Montelukast (0) Inhaled anticholinergics (3) SABA (18)

Abbreviations: BMI: body mass index; COPD: chronic obstructive pulmonary disease; FEV1: forced expiratory volume in 1 second; GERD: gastroesophageal reflux disease; ICS: inhaled corticosteroid; LABA: long-acting bronchodilator; MAC: Mycobacterium avium complex; PC20: provocation concentration inducing a 20% drop in FEV1; SABA: short-acting bronchodilator

Table 2: Nucleotide Sequence of the primers used for real-time PCR

primer name	Sequence 5' --> 3'
mIL4 F	TTGAGAGAGATCATCGGCATTT
mIL4 R	CTCACTCTCTGTGGTGTTCTTC
mIL7 F	CACACTCACGTCCAGATTTAG
mIL7 R	TCCTAGCCTGCCTTAGATC
mIL25 F	CCAGCAAAGAGCAAGAAC
mIL25 R	TTCAAGTCCCTGTCCAAC
mIL33 F	TCCCAACAGAAGACCAAAG
mIL33 R	GATACTGCCAAGCAAGGAT
mTSLP F	CTTCTCAGGAGCCTCTTCA
mTSLP R	AGCCAGGGATAGGATTGA
mST-2 F	GTGACACCTTACAAAACCCG
mST-2 R	TCAAGAACGTCGGGCAGAG
mGAPDH F	ACGGCCGCATCTTCTTGTGCA
mGAPDH R	AATGGCAGCCCTGGTGACCA
hIL33 F	GTGACGGTGTTGATGGTAAGA
hIL33 R	CTCCACAGAGTGTTCCTTGTT
hST-2 F	AACGAGTTACCAATACTTGCTC
hST-R	CAGGCACTATTGCTTCTGGG
h18S F	CTGAGAAACGGCTACCACATC
h18S R	GCCTCGAAAGAGTCCTGTATTG

Legend to Repository Figures

Repository Figure E1. Expression of mucin (red) expression and toluidine positive (dark brown) mast cells in the airways from mice with chronic asthma and saline control.

Repository Figure E2. (**A&B**) Effect of an anti-CD3ε or an isotype control antibody on spleen TCRβ+ T cells (A) and airway inflammation (B). Three doses (200 μg/dose) were administered on alternate days in week 10. N=5, *: P=0.02. (**C-E**) T cell proliferation and cytokine production 6 weeks following irradiation in week 9. Spleen (sp) and mediastinal lymph node (medLN) $CD4^+$ T cells were obtained from three study groups— chronic asthma (CA), chronic asthma + ablation (CA+A), saline control + ablation (S+A). T cells (2×10^6 cells/well) were stimulated with medium, dust mite (10 μg/mL) and anti-CD3 (1 μg/mL) plus anti-CD28 (1 μg/mL) antibodies for 96 hours. Proliferation was assessed by flow cytometric analysis of CFSE dilution. For flow cytometric detection of intracellular IL2 (D) and IL4 (E), cells were treated with monensin 6 hr before conclusion of the culture. 20,000 events (live cells) per well were sampled for flow cytometric analyses. *: $P<0.05$ as compared to medium control, N=4. (**F**) Effect of irradiation and immune ablation on lung inflammation. Representative histologic images of the lung sections from the study mice as in Fig. 2D. Scale bar is 100μm.

Repository Figure E3. Characterization of lung ILC. (A&B) Collagenase-digested single lung cells were gated on live cells via forward scatter/ side scatter and then selected for hematopoietic $CD45^+$ population. The innate lymphoid cells were identified as lineage (CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/Erythroid cells)$^-$ and $CD25^+$ population. This subpopulation was positive for ILC2 markers (C-H) including c-kit, sca-1, intracellular IL-13 and IL-5; and cell surface CRTH2, IL33R and KLRG1 but negative for the NK cell marker NK1.1 or mast cell/basophil marker FCεRI. (I) Total $IL17^+$ lung cells in the chronic asthma and saline control

mice as detected by flow cytometry. Lung ILC (Lin^-CD25^+) cells were negative for IL17. N=3 (J&K) Comparison of ILC between chronic asthma and chronic asthma following immune ablation.

Repository Figure E4. Effect irradiation on ILC. (A&B) Frequency of ILC ($lin^-CD25^+CD45^+$ cells) in the lung digest from mice treated with saline alone (control for chronic asthma) and saline followed by irradiation and transplantation of bone marrow from a naïve mouse. Measurements were done 6 weeks after bone marrow transplantation. (C) Comparison of total lung ILC between the two groups. (D) Comparison of total lung $IL5^+$ cells, and host- and donor-derived $IL5^+$ ILC2. (E) Comparison of total lung $IL13^+$ cells, and host- and donor-derived $IL13^+$ ILC2. N=5 for C-E, n.s: not significant.

Repository Figure E5. Effect of marrow transplantation on persistence of inflammation. (A&B) Chronic asthma was induced as per Fig. 2A and bone marrow from either *Rag1*$^{-/-}$ or *Rag2*$^{-/-}\gamma c^{-/-}$ was transferred to mice following irradiation. Outcomes were measured 6 weeks after irradiation on week 15. (A) Differential cell counts in BAL fluid. (B) Representative lung sections stained with H&E from the mice represented in A. Scale bar is 100μm. (C) Peribronchial and perivascular inflammation as a percentage of total area. (D&E) Flow cytometric analysis of lung digests from *Rag1*$^{-/-}$ and *Rag2*$^{-/-}\gamma c^{-/-}$ bone marrow recipient mice showing changes in (D) total ILC and (E) $IL13^+$ ILC. * $p<0.05$, ** $p<0.01$. N=5 mice/group.

Repository Figure E6. (A-B) Effect of anti-IL33 on persistence of airway inflammation. (A) Representative lung sections stained with H&E. Scale bar is 100μM. (B) Differential cell counts in BAL fluid. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, N=4. (C) Effect of intranasal an anti-IL33

antibody on airway inflammation. Representative lung sections stained with H&E. Scale bar is 100μM.

Repository Figure E7. (A&B) Effect of adoptive transfer of ILC. $CD45^+Lin^-CD25^+$ cells from the lung digest obtained from chronic asthma model and saline control were adoptively transferred to naïve mice. The presence of host- and donor-derived (A) total ILC and (B) $IL13^+$ ILC2 in the lungs was analyzed on day 21 after the transfer. * $p<0.05$, N=5/group.

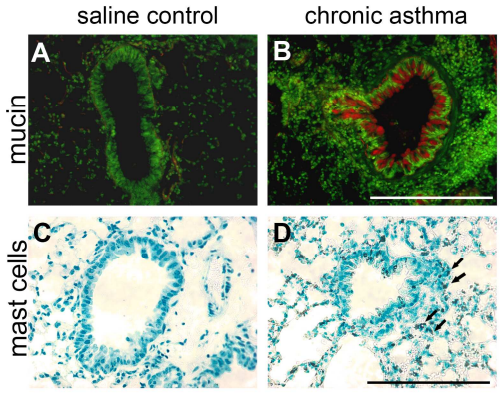
Repository Figure E8. (**A**) Effect of IL13 on IL33 secretion. A549 cells were treated overnight with medium or IL13 (20ng/mL) and ATP (100μM) and the released IL33 was measured by ELISA (*P=0.04, N=6). (**B-E**) Direct effect of IL33 on airway hyperreactivity and lung ILC. C57Bl/6 mice were treated intranasally for 3 consecutive days with 400ng/ dose of IL33. Outcome measures were evaluated 15 days after the final exposure. (B&C) Airway inflammation and its quantification as a percentage of total area. (D&E) Effect on lung ILC. Flow cytometric analysis of lung digests showing (D) total ILC, and (E) total $IL5^+$ lung cells and $IL5^+$ ILC2. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. N=5 mice/ group.

Repository Figure E9. (**A**) Lung digest from the chronic asthma model was stained for IL33 and pro-surfactant protein C (Pro-SPC), CD11b or FcεRI (N=5). (**B**) A549 cells were treated overnight with DRA (dust mite 5 μg/mL, ragweed 15μg/mL, and Aspergillus sp. 5 μg/mL) and IL33 mRNA was quantified by real-time PCR. (*P=0.04, N=5).

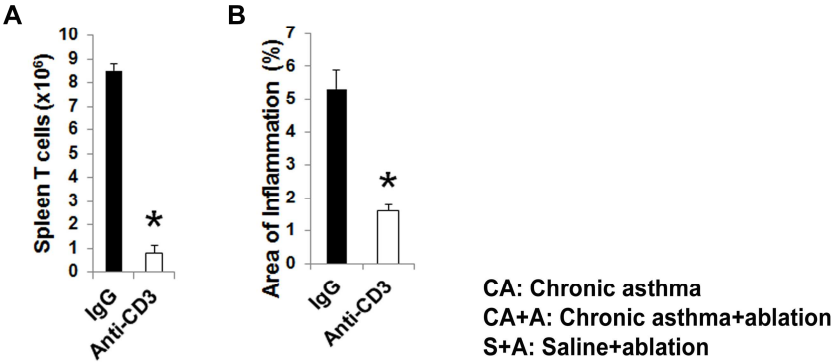
Repository Figure E10. Isolation strategy of type 2 ILC from human bronchoalveolar lavage (BAL). (A) BAL cells were gated for lin (CD3, CD14, CD16, CD19, CD20, CD56)$^-$ and $FceRI^-$ cells. These lin^-FceRI^- cells were stained for IL7Rα and IL33R (ST2). The $IL7R\alpha^+IL33R^+$ cells were identified as type 2 ILC. The majority of these cells were positive for IL5 and IL13. They

were partially positive for CD161 (B). The gating strategy and threshold quadrants were based upon isotype antibody controls (not shown).

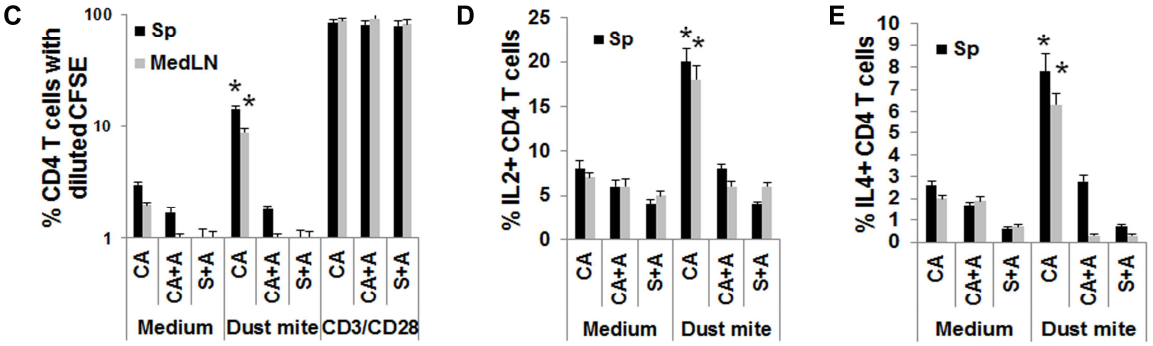
Repository Figure E1

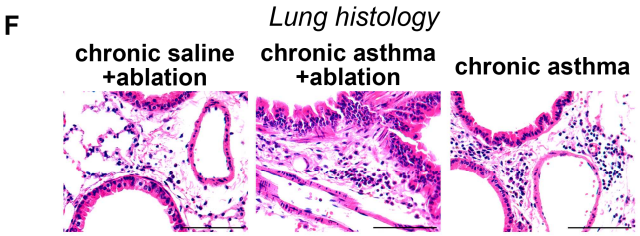


Repository Figure E2



T cell proliferation and cytokine production post irradiation and immune ablation





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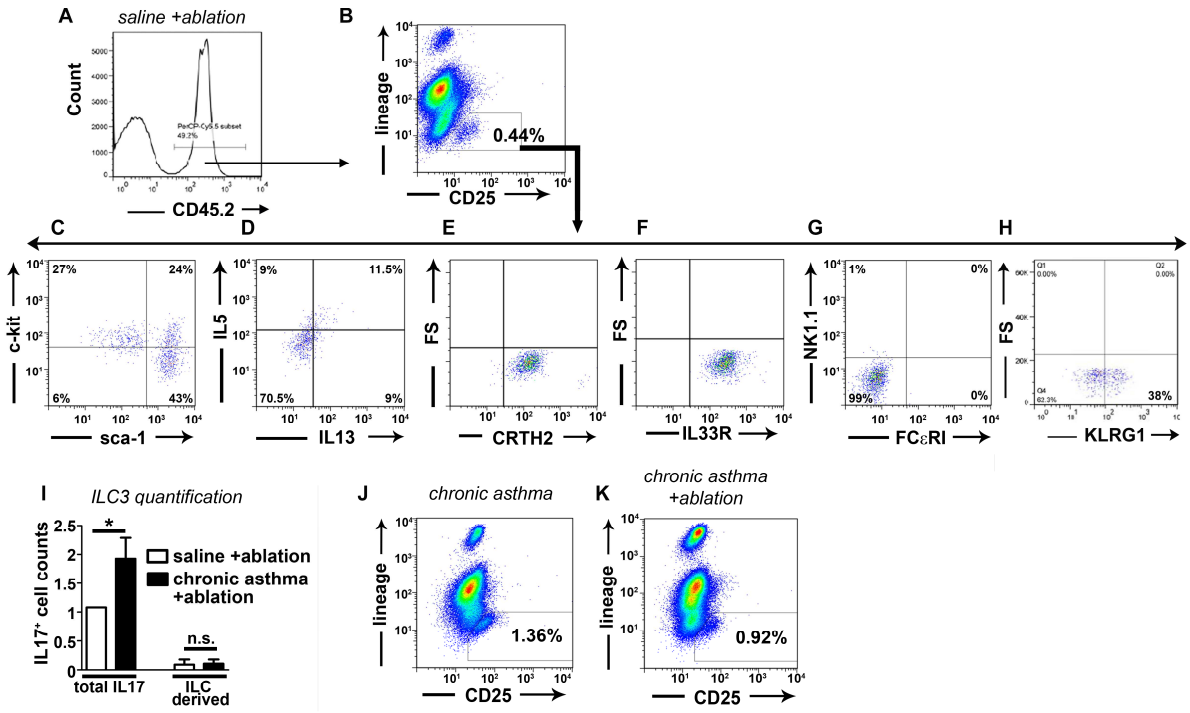
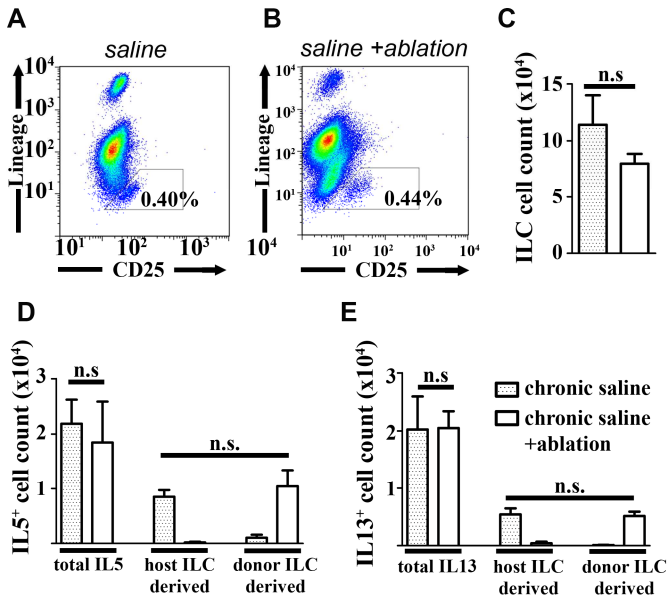


Repository Figure E4

Quantification of ILC



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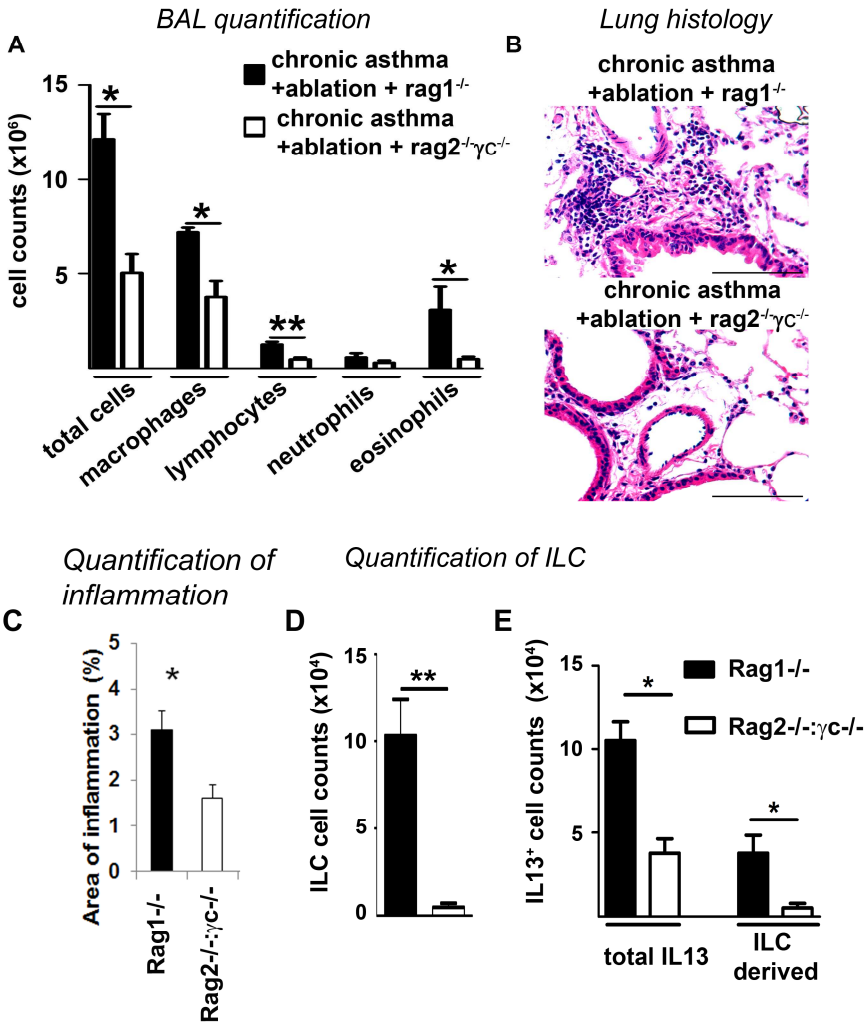

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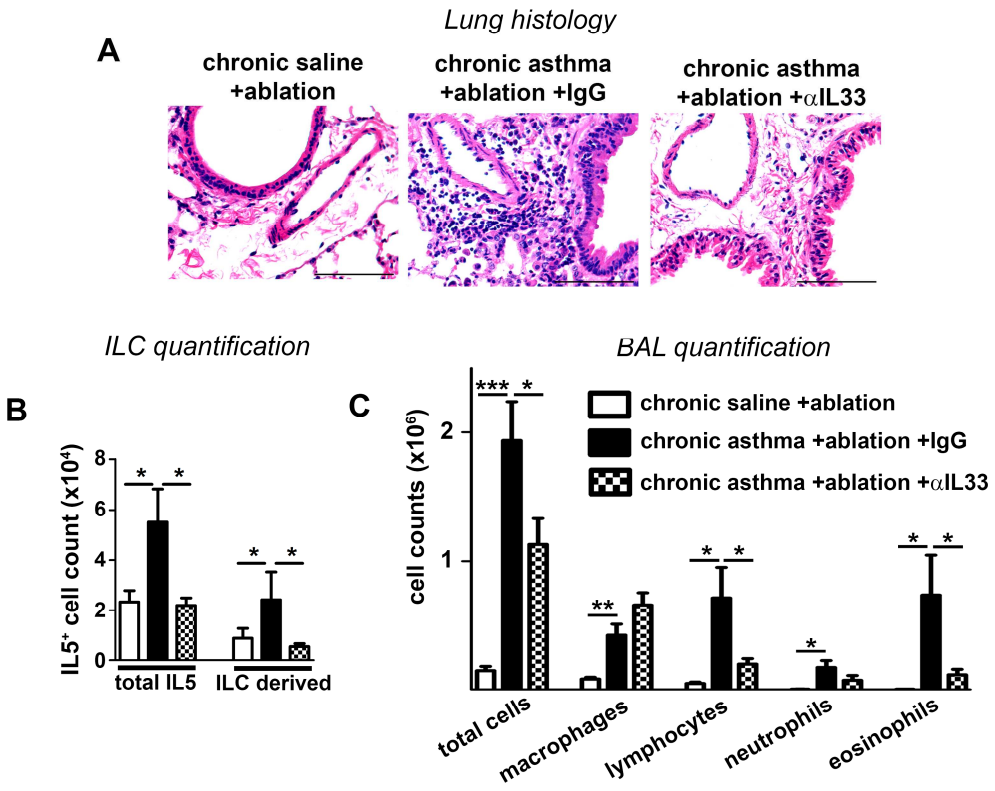
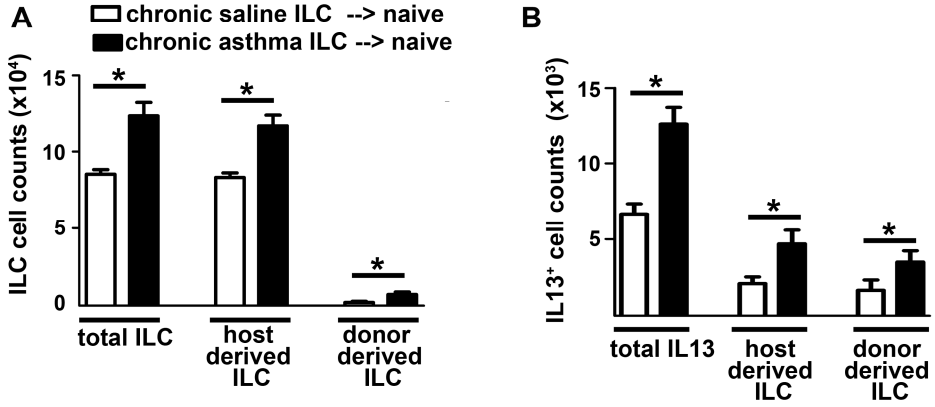


Repository Figure E7

Effect of adoptive transfer of ILC

Quantification of ILC (h-i)



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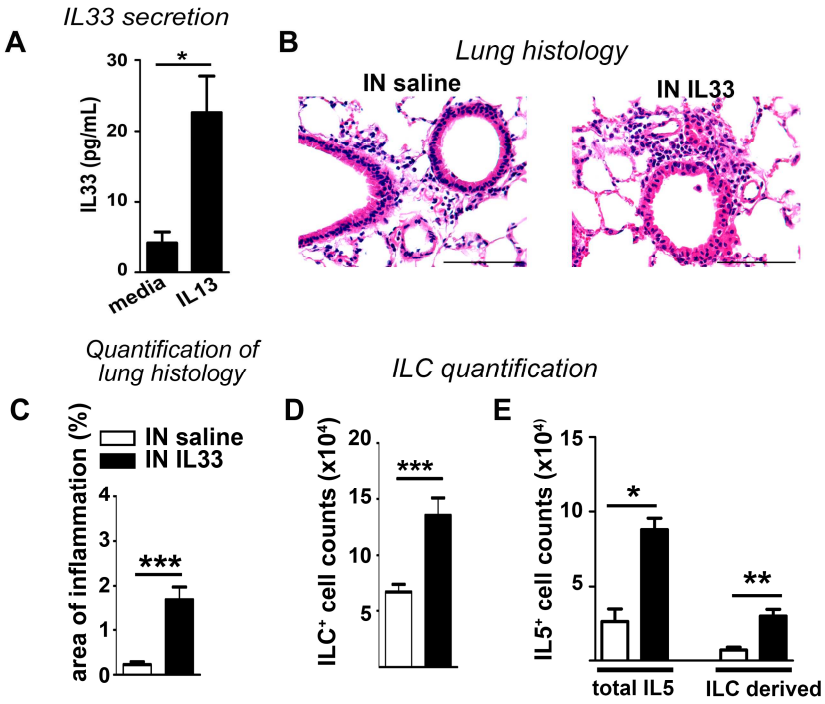

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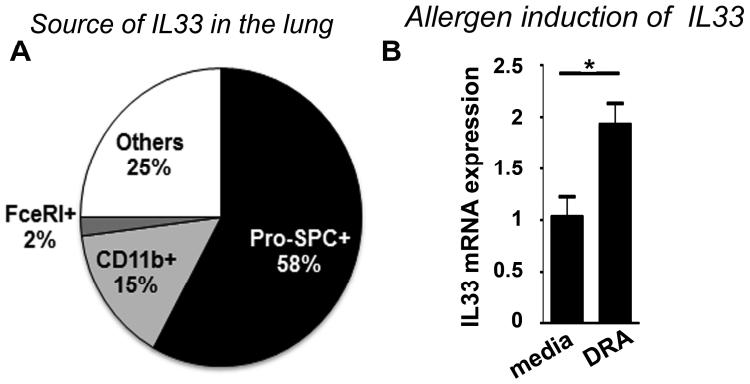

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