Lung-resident eosinophils represent a distinct regulatory eosinophil subset

Supplemental Data

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Supplemental Methods

Reagents and Abs

Lyophilized HDM (*Dermatophagoides farinae*) extracts were from Greer Laboratories. Grade V and grade III OVA, and LPS from E. *coli* O55:B5 were from Sigma. 2.4G2 Fc receptor Abs were produced in house. FITC$^+$ beads (Fluoresbrite ™ plain YG 0.5 μm microsphere) were from Polyscience. Anti(α)-IL-5 neutralizing Abs (clone TRFK5) or control isotype Abs (rat IgG$_1$) were from BD biosciences. Recombinant mouse IL-5 was from Biolegend. Recombinant GM-CSF was provided by Dr. Kris Thielemans (Medical School of the Vrije Universiteit Brussel, Brussel, Belgium).

Mouse studies: Anti-mouse allophycocyanin (APC)-, V450- and V500-conjugated anti-CD45.2 (mouse/IgG2a,κ, clone 104), Alexa fluor 488-, PE-conjugated anti-CD125 (rat/IgG1λ, clone T21), PE-, Alexa fluor 647-conjugated anti-Siglec-F (rat/IgG2a,κ, clone E50-2440), biotinylated anti-CD62L (rat/IgG2a,κ, clone MEL-14), PE-conjugated anti-CD64 mouse/IgG1κ, clone X54-5:7.1), PE-conjugated anti-CD19 (mouse/IgA,κ, clone MB19-1), PE-conjugated anti-NK1.1 (mouse/IgG2a,κ, clone PK136), PE-conjugated anti-CD3 (rat/IgG2b,κ, clone 17A2), PE-conjugated anti-Ly6G (rat/IgG2a,κ, clone 1A8), APC-Cy7-conjugated anti-CD11c (hamster/IgG, clone HL3), PerCp-Cy5.5-conjugated anti-MHCII (rat/IgG2b, clone M5/M6), PE-Cy7-conjugated anti-CD11b (rat/IgG2b,κ, clone M1/70) and FITC-conjugated anti-Ly6C (rat/IgG2c,κ, clone AL-21) were purchased from BD Pharmingen. AlexaFluor 647-conjugated anti-CD193 (CCR3, rat/IgG2a,κ, clone TG14) and streptavidin-conjugated BV421 were from Biolegend. APC-conjugated anti-B220 (rat/IgG2a,κ, clone RA3-6B2), PerCPCy5.5-conjugated anti-CD19 (rat/IgG2a,κ, clone 1D3), PE-conjugated anti-IgA (rat/IgG1,κ, clone 11-44-2), efluor 660-conjugated anti-F4/80 (rat/IgG2a,κ, clone BM8), APC-eFluor780-conjugated anti-CD3 (rat/IgG2b,κ, clone 17A2), biotinylated anti-CD115 (rat/IgG2a,κ, clone AFS98), biotinylated anti-CD80 (mouse/IgG1,κ, clone 16-10A1), biotinylated anti-CD86 (rat/IgG2a,κ, clone GL1), biotinylated anti-CD40 (rat/IgG2a,κ, clone 1C10), PE-conjugated anti-F4/80 (rat/IgG2a,κ, clone BM8), purified anti-CD28 (Gold Syrian Hamster/IgG, clone 37.51) and anti-CD3ε (Armenian Hamster/IgG, clone 145-2C11) were from eBioscience. AlexaFluor 647-conjugated phospho-p44/42 MAPK (Erk1/2)

(rabbit, polyclonal) was from Cell Signaling. APC-conjugated anti-CD101 (recombinant human/IgG1, clone REA301), anti-CD11b microBeads (rat/IgG2b, clone M1.70.15.11.5), APC-conjugated and biotinylated anti-Siglec-F (rat/IgG1, clone ES22-10D8), strepavidin-conjugated beads and the Naive T cell isolation kit were from Miltenyi Biotec. Appropriate isotype control Abs were purchased from the same respective manufacturers.

Human studies: Anti-human APC-conjugated anti-Siglec-8 (mouse/IgG1, clone 7C9), FITC-conjugated anti-CD123 (IL-3R, mouse/IgG1,κ, clone 6H6) were purchased from Sony Biotechnology. PE-conjugated anti-CDw125 (IL-5Rα, mouse/IgG1,κ, clone A14), BV421-conjugated anti-CD45 (mouse/IgG1,κ, clone HI30), BB515-conjugated anti-CD62L (mouse/IgG1,κ, clone SK11), streptavidin-conjugated FITC were purchased from BD Pharmingen. Biotinylated anti-CD101 (mouse/IgG1,κ, clone BB27) was from eBioscience. Appropriate isotype control Abs were purchased from the same respective manufacturers. Anti-CD11b MicroBeads were from Miltenyi Biotec.

Additional reagents are described in the respective sections below.

Mouse cell isolation, staining and flow cytometry

To obtain mouse single-lung-cell suspensions, lungs were perfused with 10 ml of PBS through the right ventricle, cut into small pieces, and digested for 1 hour at 37°C in HBSS containing 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNase I (Roche). Cell suspensions were rinsed with ice-cold PBS containing 10 mM EDTA and passed through a cell strainer (70μm).

To obtain BALF, mouse trachea was catheterized, and the airways were perfused with 2 ml of ice-cold PBS. Blood and perfusion liquid of lung vasculature were collected and mixed with EDTA (final concentration 100 mM), and red blood cells were lysed with RBC lysis buffer (eBioscience).

Small intestinal lamina propria cells were isolated as described previously (1), with slight modifications. Briefly, gut content and Peyer's patches were removed from the small intestine, and the organ was incised longitudinally and washed in ice-cold HBSS containing 2% FBS. To remove epithelial cells, samples were incubated for 45 min in HBSS containing 10% FBS and 2 mM EDTA, followed by enzymatic digestion

for 1 hour at 37°C in RPMI containing 10% FBS, 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNase I (Roche). Cell suspensions were rinsed with ice-cold PBS containing 10 mM EDTA and passed through a cell strainer (70μm). CD11b enrichment was performed by MACS before analysis and cell sorting.

For mouse rEos-like blood cells, cells were obtained from whole blood and Siglec-F enrichment was performed by MACS before cell sorting.

Staining reactions were performed at 4°C after incubation with 2.4G2 Fc receptor Abs to reduce non-specific binding. Cell phenotyping and sorting were performed on a FACSARIA III (BD Biosciences). FSC-W and FSC-A discrimination was used to exclude doublet cells, and ViaProbe (7-AAD) cell viability solution (BD Biosciences) was used to discriminate between dead and living cells. IgA were detected by intracellular staining following intracellular fixation & permeabilization (eBioscience), according to the manufacturer protocol. Results were analyzed with FlowJo software (Tree Star). In experiments aimed at isolating eosinophil subsets, lung cells were first enriched in CD11b-expressing cells by MACS and then sorted based on expression of CD45.2, Siglec-F and CD125 and a high side scatter pattern (SSC^{hi}). Purity of all cell populations was equal or superior to 95% (Supplemental Figure 6).

Quantification of eosinophil numbers

Total lung cell numbers were counted following whole lung digestion by the use of a hemocytometer. The numbers of eosinophils were determined as follows, according to the gating strategy shown in Supplemental Figure 1: Eos number = (total lung cells number) x (% single cells) x (% living cells) x (% $CD45.2^{+}$ cells) x (% Siglec F^{int} $CD125^{int}$ (for rEos) or Siglec $F^{int/hi}CD125^{int}$ (for iEos)) x (% SSC^{hi}) x 10^{-10}.

Eosinophil numbers were also quantified on Congo Red-stained lung sections (Figure 2I, Figure 8B). In each section, numbers of parenchymal and peribronchial eosinophils were counted on a total of 20 fields (magnification 100x) located in parenchymal and peribronchial areas, respectively, and the average number of eosinophils / field was obtained by dividing this number by 20.

Ultrastructural analyses

Pellets of FACS-sorted rEos and iEos from naive and HDM-treated C57BL/6 mice, respectively, were fixed for 60 min at 4 °C in 2.5% glutaraldehyde, in 0.1 M Sorensen's buffer (pH 7.4), acetylated and embedded in epon. Ultrathin sections (600-700 Å) were mounted on nickel grids and stained with uranyl acetate and lead citrate before examination with a Jeol electron microscope (JEM-1400) at 80 kV.

Phagocytic activity

Naive C57BL/6 mice were injected i.t. with 50 µl of 10% (v/v) fluorescent beads. Six hours later, blood cells, cells from the perfusion liquid of the lung vasculature and single lung cell suspensions were obtained. Monocytes and eosinophils from the blood and the perfusion liquid and rEos, AMs and T cells from the lung were analyzed by flow cytometry. Lung rEos, AMs and T cells were FACS-sorted and spread on a slide by cytospin, and intracellular presence of fluorescent beads was assessed by confocal microscopy (Leica SP5).

Lung histology and immunohistochemistry

 Lungs were fixed in 4% formalin, paraffin-embedded and cut in 5 µm thick sections.

For Congo red stainings, sections were immersed in Mayer's hematoxylin (Dako) for 15 sec, rinsed in tap water for 5 min, immersed in Congo Red solution (Sigma) for 30 min and rinsed with absolute ethanol.

For Major Basic Protein (MBP) immunostainings, deparaffinized tissues were digested in pepsin (10 min, 37°C) (Dako) and endogenous peroxides were blocked with hydrogen peroxide 3% (Merck). Tissue sections were then immersed in protein block (Dako) for 10 min at room temperature. Mouse lung sections were stained with a rat anti-mouse MBP (dilution 1/500) for 1 hour at room temperature (J.L. Lee Laboratory, Mayo Clinic, Scottsdale, Arizona) followed by a HRP-coupled rabbit anti-rat staining (dilution 1/400) for 30 min at room temperature (Abcam). Human lung sections were stained with a mouse anti-human MBP (clone BMK13, dilution 1/30) for 4 hours at room temperature (ThermoFisher Scientific) followed by a HRP-coupled rabbit anti-mouse staining (dilution 1/500) for 30 min at room temperature (Abcam). Peroxidase activity was

revealed using the 3,3'-diaminobenzidine hydrochloride kit (Dako), and tissues were then counter-colored with Carazzi's hematoxylin.

mRNA isolation and microarray analyses

Singlet living rEos from naive and HDM-treated allergic C57BL/6 mice and iEos from HDM-treated allergic C57BL/6 mice were directly sorted in Trizol® (Life Technologies). The 3 cell populations were sorted in triplicates, yielding a total of 9 samples. mRNA was purified using Direct-zol® kit (Zymo Research) following manufacturer's recommendations. For each cell type, mRNAs were eluted in a total of 175 μl of water. Eluted mRNAs were incubated with DNase for 10 min at room temperature using the RNase-Free DNase Set from Qiagen (Venlo). Finally, mRNAs were purified on RNeasy® Micro columns (Qiagen) and eluted in 50μl water. mRNAs were quantitatively and qualitatively verified using Agilent RNA 6000 Pico Kit run on an Agilent 2100 Bioanalyzer. Concentrations were around 1ng/μl and RNA Integrity Numbers were above 8.

1.5ng RNA was used to generate fragmented and biotin-labeled ssDNA targets using GeneChip® WT Pico Reagent Kit (Affymetrix). The fragmented and labeled targets were hybridized to the arrays following standard Affymetrix protocol, which includes overnight hybridization at 45°C at 60 rpm in an Affymetrix GeneChip Hybridization Oven 645. The arrays were then washed and stained in an Affymetrix GeneChip Fluidics Station 450 (Mouse Gene 2.0 ST arrays).

Microarray raw data was analyzed using R statistical programming language (version 3.2.1) and several Bioconductor packages. The hybridizations were adjusted by using the Robust Multichip Average (RMA) method from the oligo package to acquire summary expression values for each probe set, and the probe sets were subsequently annotated to genes by the mogene20sttranscriptcluster.db package. Probes without gene annotation were removed from the analysis. Differentially expressed genes between two groups were identified using linear models from limma package, with the false discovery rate (FDR) <0.01 and the log 2-fold change >1.

Quantification of IL-5 levels in the lungs of HDM-challenged mice

Perfused lungs were collected from naive and HDM-treated C57BL/6 mice (d 17, see Figure 2A), rinsed in ice-cold PBS and deposited in 1 ml of ice-cold PBS containing Protease Inhibitor Cocktail (Complete, Roche). Weight-matched homogeneous suspensions were obtained by using an Ultra Turrax homogenizer followed by 3 cycles of sonication (1 min each). IL-5 concentration was assessed in the supernatant by ELISA (eBioscience).

In vivo anti-IL-5 treatment

Naive C57BL/6 mice were injected i.p. with 200 µl of 0.5 mg/ml anti-IL-5 Abs or control isotype. Twenty-four hours later, the numbers and the phenotype of blood and lung eosinophils were analyzed. Alternatively, naive C57BL/6 mice were injected i.p. 3 times daily with anti-IL-5 Abs as described above, and the numbers of lung rEos was assessed 24 hours after the last treatment. HDM-sensitized C57BL/6 mice (which received 2 i.n. instillations of HDM [100 µg in 50 µl] on days 0 and 7) were injected i.p. with 100 µg of anti-IL-5 or control isotype on day 21 and received an i.n. instillation of HDM (100 µg in 50 µl) 1 hour later. Three days later, the numbers and the phenotype of blood and lung eosinophils were analyzed.

For assessment of the influence of iEos on HDM-induced Th2 sensitization (Figure 7, A-C), naive BALB/c mice were injected i.p. with 200 µl of 0.5 mg/ml anti-IL-5 Abs or control isotype 1 hour before i.n. instillation of vehicle (saline) or HDM (5 µg) on day 0. Five days later, LN cells were restimulated in vitro with HDM for 3 d, and LN cell proliferation and Th2 cytokine production was quantified as described below. Numbers of lung iEos were also determined by flow cytometry.

Effect of rIL-5 treatment on rEos numbers in vivo and on IL-5-induced signaling and survival in vitro

For assessment of rEos numbers, C57BL/6 naive mice were treated i.p. (50 ng in 100 µl PBS) and i.n. with rIL-5 (50 ng in 50 µl PBS), and numbers of blood rEos-like cells and lung rEos were quantified 1 and 24 hours later by flow cytometry.

For assessment of phospho-ERK activation, total lung cell suspensions from naive and HDM-challenged C57BL/6 mice were cultivated for 3 hours in DMEM containing 10% FBS. Cells were then rinsed, centrifuged and resuspended in DMEM medium. They were pulsed for 5 min with rIL-5 (50 ng/ml) and immediately fixed in paraformaldehyde 4% (15 minutes, room temperature). Control cells were left unpulsed. Extracellular staining for rEos and iEos was performed as described, paraformaldehyde 4% was added for 15 min at room temperature and cells were rinsed, centrifuged and permeabilized by adding ice-cold 100% methanol (30 min, 4°C). Intracellular phopsho-staining was performed according to the manufacturer's instructions.

For assessment or eosinophil survival, $rEos_{ss}$, $rEos_i$ and iEOS were FACS-sorted and cultivated in RPMI medium supplemented with 10% FBS and additives (L-glutamin 8mM, MEAA 0.1 mM, Pyruvate Na 1mM, Penicillin/streptomycin 50UI/ml, β-mercaptoethanol 0.5mM) with or without rIL-5 (50 ng/ml). Cell survival was assessed by flow cytometry before stimulation, and 24 and 48 hours after isolation, by 7-AAD staining.

HDM-induced allergic sensitization and restimulation of LN cells

Lightly isoflurane anesthetized ΔdblGATA or WT control BALB/c mice received an i.n. instillation of vehicle (LPS-free saline) or HDM (5 μg or 100 μg in 50 μl) on day 0. Five days later, MLN cells were collected and cultured in vitro in Click's medium supplemented with 10% FBS, L-glutamin 8mM, modified essential amino acids 0.1 mM, sodium pyruvate 1mM, Penicillin/Streptomycin 50 UI/ml and β-mercaptoethanol 0.5mM, with or without HDM restimulation (30 μg/ml). The proliferation was measured as [^{3}H]thymidine incorporation during the last 16 hours of a 3-d culture. Culture supernatants were assayed for cytokine production (IL-4, IL-5, IL-13) by ELISA (eBioscience).

Assessment of rEos and DC migration

Lightly isoflurane anesthetized BALB/c wild type mice received an i.n. instillation of vehicle (LPS-free saline) or HDM (5 μg in 50 μl) on day 0. Twenty-four, 48 or 72 hours later, the numbers of rEos (identified as SSC^{hi} Siglec-F^{int} $CD125^{int}$ cells) were quantified

by flow cytometry in the MLNs. The numbers of cDCs (identified as CD64^{-}CD19^{-}CD3^{-} NK1.1^{-}Siglec-F^{-}Ly6G^{-}CD45^{+}MHCII^{+}CD11C^{+} cells) in the MLNs 24 hours after HDM injection were used as a positive control.

Anti-CD3/CD28 CD4^{+} T cell stimulation and effect of rEos on T-cell associated Th2 cytokine production

Naive CD4^{+} T cells were purified from spleens of BALB/c mice using the Naive CD4^{+} T cell Isolation Kit (Miltenyi Biotec) (>90% purity, data not shown). Cells (2x 10^5 cells) were then cultured with anti-CD28 Abs (5 µg/ml) in RPMI supplemented with 10% FBS and additives (L-glutamin 8mM, MEAA 0.1 mM, Pyruvate Na 1mM, Penicillin/streptomycin 50UI/ml, β-mercaptoethanol 0.5mM), into 96-well plates pre-coated with anti-CD3 Abs (10 µg/ml). Controls were cultured in uncoated wells without anti-CD28 Abs. To assess their effects of rEos on Th2 cytokine production, FACS-sorted rEos (4x 10^4 cells) from the lungs of naive BALB/c mice were added to the culture. After 96 h, IL-4, IL-5 and IL-13 were measured in the supernatants of cells by ELISA (eBioscience).

Assessment of IL-10 production by AMs, IMs and eosinophils

AMs (identified as CD45.2^{+}F4/80^{hi}CD11c^{hi} [2]), IMs (identified as CD45.2^{+}F4/80^{int}CD11c^{-} cells after exclusion of granulocytic SSC^{hi} cells [2]) and lung rEOS were sorted by FACS from naive C57BL/6 naive mice and 2x10^5 cells were cultured in RPMI medium supplemented with 10% FBS and additives (L-glutamin 8mM, MEAA 0.1 mM, Pyruvate Na 1mM, Penicillin/streptomycin 50UI/ml, β-mercaptoethanol 0.5mM) with or without LPS (10 ng/ml) in a 96 well plate during 16 hours. Concentrations of IL-10 in culture supernatants were measured by ELISA (eBioscience).

Coculture experiments and Th2 sensitization induced by i.t. administration of OVA_LPS-pulsed BMDCs

To generate BMDCs, bone marrow cells were isolated from naive C57BL/6 mice and were grown for 9 days in GM-CSF-containing medium, as previously described (2). At day 8, the culture medium was replaced with medium devoid of GM-CSF, as GM-CSF

may affect eosinophil functions (3). FACS-sorted rEos, iEos, Int Eos, rEos-like blood cells, IMs or AMs were added to BMDCs cultures at a ratio of 1:1. One hour later, BMDCs were pulsed with 125 μg/ml grade V OVA (i.e. OVA contaminated with low doses of LPS or OVA$_{LPS}$). OVA$_{LPS}$-pulsed BMDCs and unpulsed BMDCs were used as positive and negative controls, respectively. Twelve hours later, cells were collected, washed, and resuspended in PBS. Cell viability was assessed, and BMDC survival was not affected by the coculture with rEos (data not shown). In a first set of experiments, expression of maturation markers (CD40, CD80 and CD86) on CD11c^{hi}MHC-II^{hi} unpulsed BMDCs and OVA$_{LPS}$-pulsed BMDCs cocultured with or without rEos, Int Eos or rEos-like blood cells was evaluated by flow cytometry. In a second set of experiments, $5x10^5$ BMDCs cocultured with FACS-sorted AMs, IMs, rEos or iEos were injected i.t. into anesthetized naive recipients. Ten days after i.t. immunization, mice were challenged with OVA (1% wt/vol in PBS; grade III) aerosol during a daily 45-min challenge on 4 consecutive days. Twenty-four hours after the last challenge, BALF eosinophilia and BLN cell responses were evaluated as previously described (2, 4).

Human eosinophil isolation, staining and flow cytometry

To isolate eosinophils from non-asthmatic lungs, pieces of lung tissue were obtained from the healthy margins of lung carcinoma freshly resected from patients suffering from lung cancer with no clinical history of asthma (see Supplemental Table 2). For each specimen, one part was embedded in paraffin and H&E- and Congo Red-stained lung sections were subjected to histopathological examination for the presence of inflammation and for assessment of the localization of lung eosinophils, respectively. Two out of 10 samples showed abnormal peribronchial infiltration of granulocytes, and was excluded from the analysis. Among the other 8 samples, the resected lung tissue did not exhibit abnormal signs of inflammation, and >90% of eosinophils were found in the parenchyma, but not in the vicinity of the bronchi (data not shown), suggesting that they mostly contained resident eosinophils. The second part of the specimen was dissociated using a gentle MACS dissociator and digested for 1 hour at 37°C in HBSS containing 1 mg/ml collagenase A (Roche) and 0.05 mg/ml DNase I (Roche) to obtain single cell

suspensions. CD11b enrichment was performed by MACS before analysis and cell sorting.

To isolate inflammatory eosinophils from eosinophilic asthmatic patients (see Supplemental Table 3), sputum was induced and processed using the whole expectorate technique, as described in details in (5) and (6), respectively, and single cell suspensions were used. Briefly, after premedication with inhaled salbutamol, sputum was induced by inhalation of NaCl. The whole sputum was collected, weighed and homogenized by adding three volumes of PBS. After centrifugation, supernatant was separated from cell pellet, which was incubated with Sputolysin® reagent 20 min with stirring. Single cell suspensions were then rinsed, filtered, and CD11b enrichment was performed by MACS before analysis and cell sorting.

The number of stainings that were performed on primary lung eosinophils (i.e., anti-CD62L, anti-CD101 and/or anti-IL-3R) was determined according to the number of CD11b$^+$ cells recovered per tissue. Staining reactions were performed at 4°C after incubation with FBS to reduce non-specific binding. Cell phenotyping and sorting were performed on a FACSARIA III (BD Biosciences). FSC-W and FSC-A discrimination was used to exclude doublet cells, and ViaProbe (7-AAD) cell viability solution (BD Biosciences) was used to discriminate between dead and living cells.
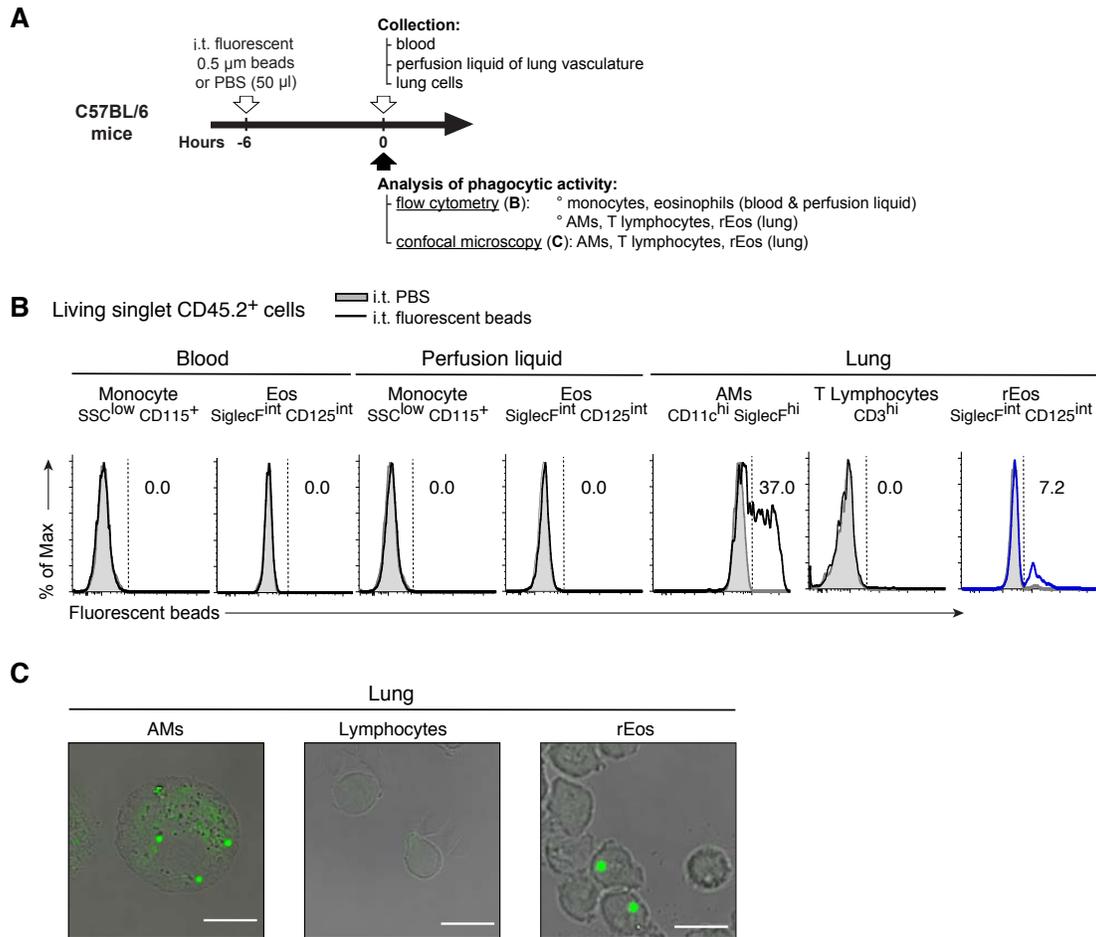
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Supplemental Figures
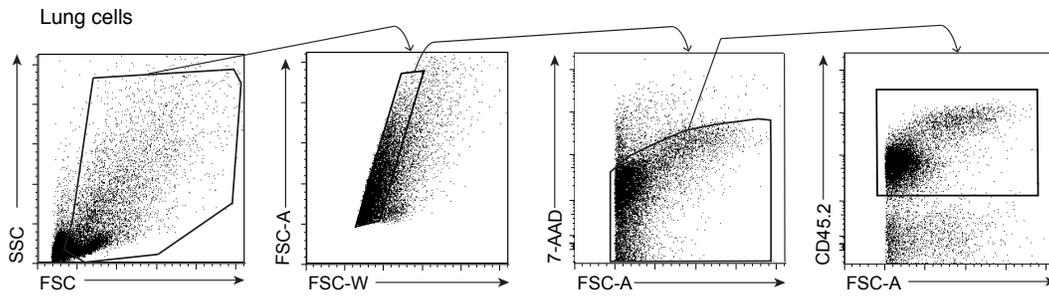
**Supplemental Figure 1. Gating strategy for delineating mouse lung eosinophils.** Whole lungs were extensively flushed through the right ventricle, minced, digested with a collagenase- and DNase-containing solution, and lung cells were collected and stained. Singlet 7-AAD$^-$ CD45.2$^+$ cells were gated and analyzed. FSC, forward scatter; SSC, side scatter.
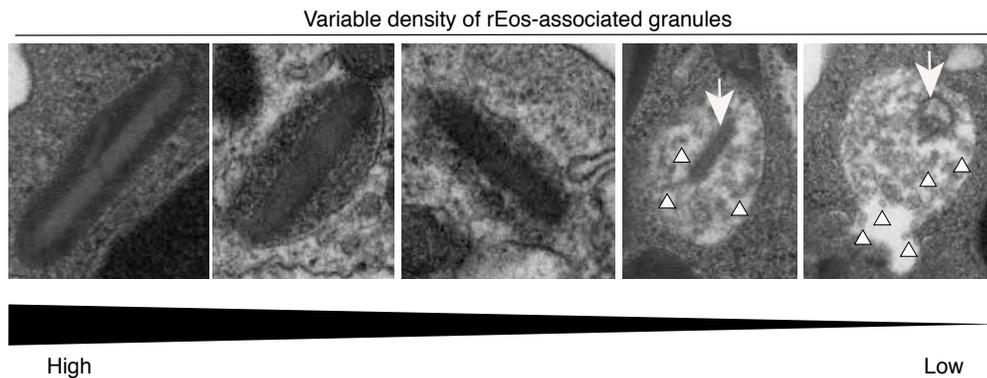
Supplemental Figure 2. Pictures of rEos-associated granules exhibiting variable densities. Steady state lung rEos were sorted from naive C57BL/6 mice (as also shown in Figure 1D), and high magnification transmission electron microscopy pictures of rEos-associated granules are shown. Arrows and arrowheads indicate a central structure most likely corresponding to the central MBP-containing core typical of eosinophil granules and intragranular vesiculotubular structures previously delineated within human eosinophil granules, respectively.

A

C57BL/6 mice

i.t. fluorescent
0.5 μm beads
or PBS (50 μl)

Collection:
├ blood
├ perfusion liquid of lung vasculature
└ lung cells

Hours -6   0

Analysis of phagocytic activity:
├ flow cytometry (**B**): ° monocytes, eosinophils (blood & perfusion liquid)
│   ° AMs, T lymphocytes, rEos (lung)
└ confocal microscopy (**C**): AMs, T lymphocytes, rEos (lung)

B Living singlet CD45.2$^+$ cells

i.t. PBS
i.t. fluorescent beads

Blood		Perfusion liquid		Lung		
Monocyte SSC^{low} CD115$^+$	Eos SiglecF^{int} CD125^{int}	Monocyte SSC^{low} CD115$^+$	Eos SiglecF^{int} CD125^{int}	AMs CD11c^{hi} SiglecF^{hi}	T Lymphocytes CD3^{hi}	rEos SiglecF^{int} CD125^{int}
0.0	0.0	0.0	0.0	37.0	0.0	7.2

% of Max

Fluorescent beads

C

Lung

AMs	Lymphocytes	rEos

Supplemental Figure 3. Lung rEos have a phagocytic potential and are not located in the lung vasculature. (**A**) Experimental outline. (**B**) Phagocytic activity, quantified by the levels of beads-associated fluorescence (FITC channel) of the indicated cell populations 6 hours after local i.t. instillation of fluorescent beads. Numbers indicate the % of beads$^+$ cells in the subpopulation. (**C**) Representative confocal microscopy pictures of FACS-sorted lung AMs, T lymphocytes and rEos 6 hours after local i.t. instillation of fluorescent beads. Results are representative of 1 mouse from 1 of >4 independent experiments (n=3/group in each experiment), each of them giving comparable results. Scale bar in **C** = 10 μm.
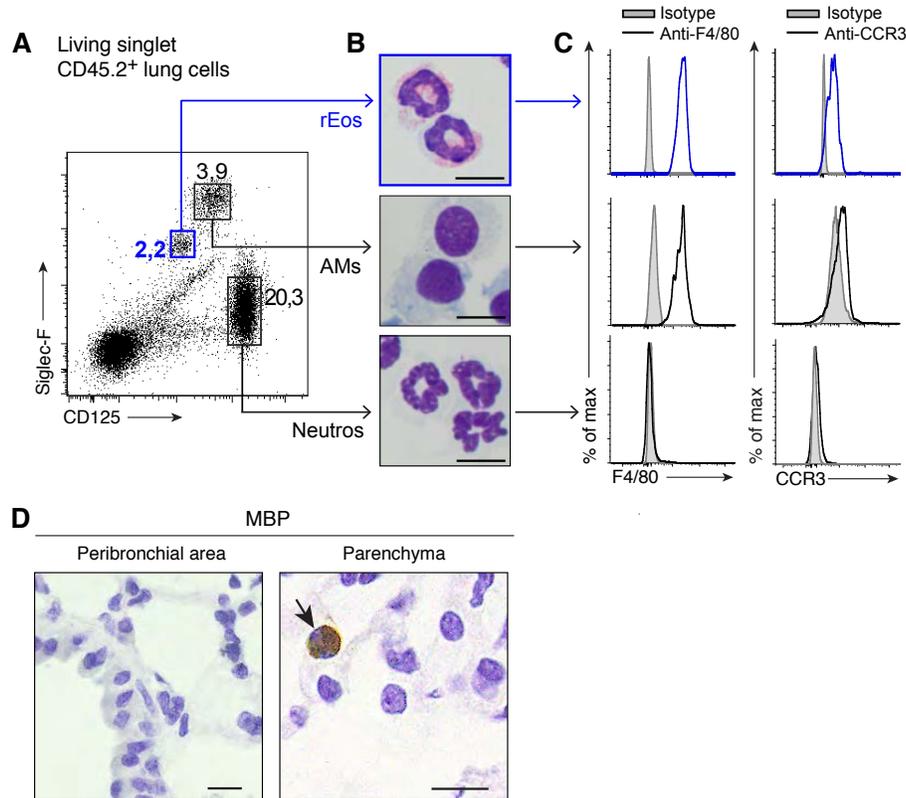
**Supplemental Figure 4. Characterization of rEos in the lung of naive BALB/c mice.** Whole lungs of naive BALB/c mice were analyzed at 6-10 weeks of age. (**A**) Representative flow cytometry dot plot of living singlet CD45.2^{+} lung cells according to Siglec-F and CD125 expression. Numbers indicate % of gated cells in total subpopulation. (**B**) Representative photographs of the gated populations in **A** following cytospin and Hemacolor coloration. (**C**) Representative flow cytometry histograms of F4/80 and CCR3 expression in the populations shown in **B**. (**D**) Representative MBP-stained lung sections. The arrow indicates a MBP-positive eosinophil. Data shown are representative of one of > 8 mice analyzed, each of them giving similar results. Scale bars = 10 μm.
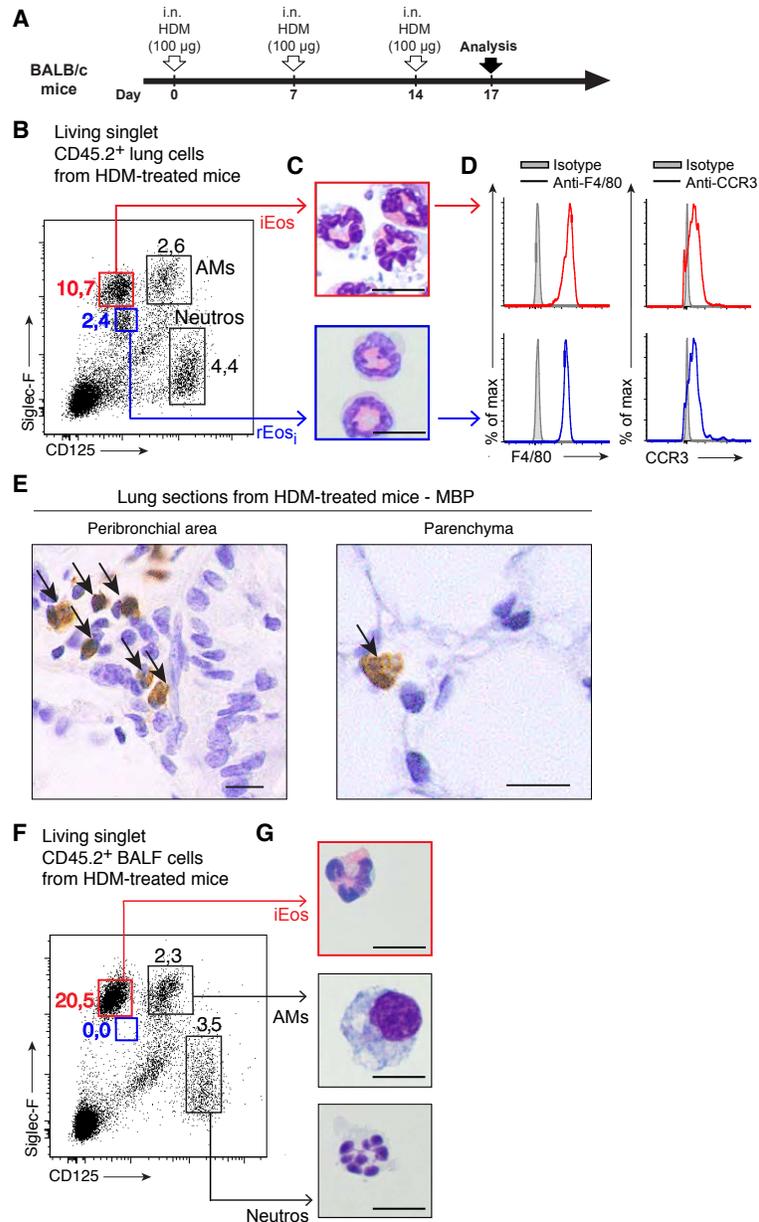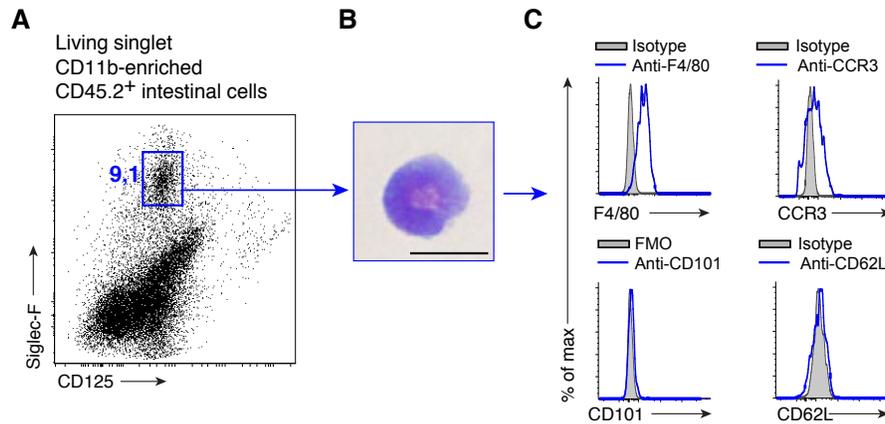
Supplemental Figure 5. Characterization of lung rEos and iEos in HDM-challenged BALB/c mice. (**A**) Experimental outline. (**B**) Dot plot of lung leukocytes according to Siglec-F and CD125 expression. Numbers indicate % of gated cells. (**C**) Photographs of the gated populations in **B**. (**D**) Histograms of F4/80 and CCR3 expression in the populations shown in **C**. (**E**) MBP-stained lung sections. Arrows indicate MBP-positive eosinophils. (**F**) Dot plot of BALF leukocytes according to Siglec-F and CD125 expression. Numbers indicate % of gated cells. (**G**) Photographs of the gated populations in **F**. Data are representative of one of >8 mice analyzed, each of them giving similar results. Scale bars = 10 µm.
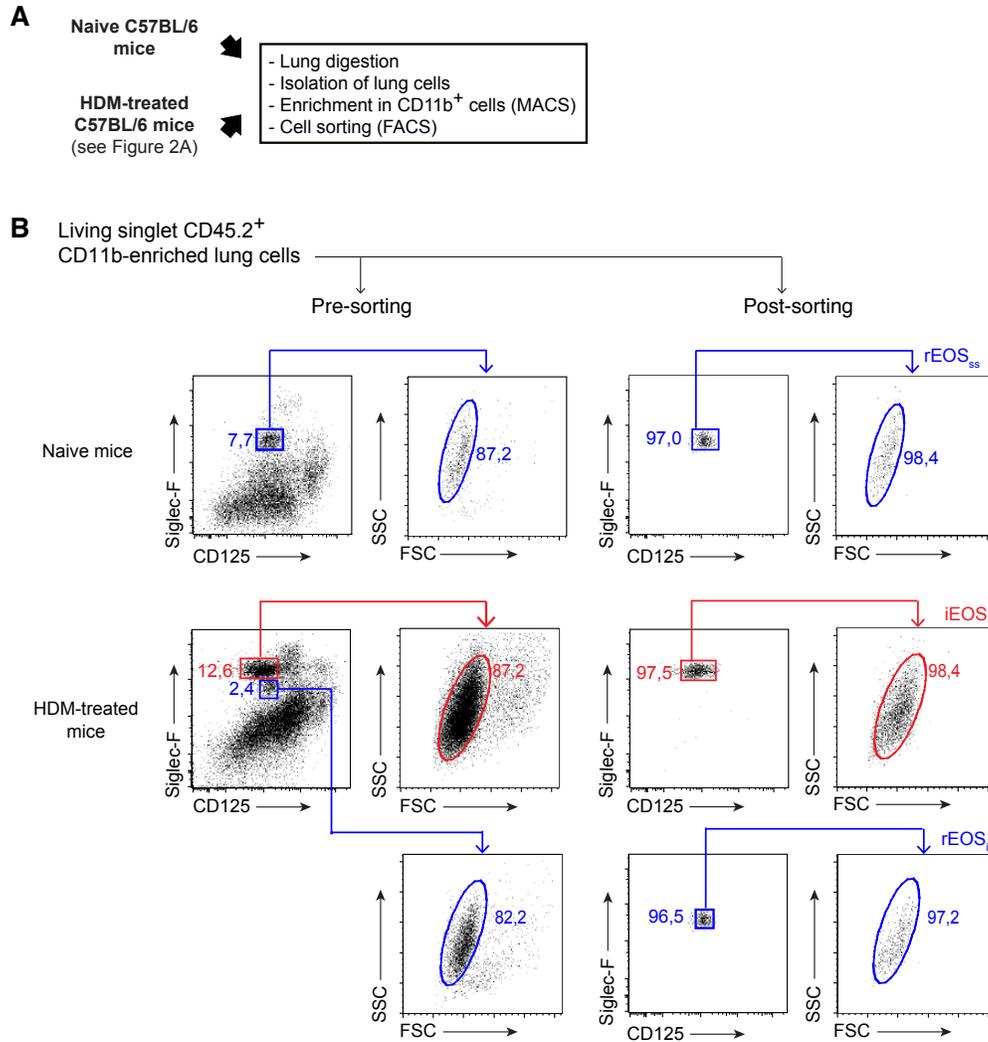
Supplemental Figure 6. Purity of FACS-sorted eosinophil populations. (**A**) Experimental outline. Steady state rEos were collected from naive C57BL/6 mice. iEos and rEos$_i$ were collected from HDM-treated C57BL/6 mice at day 17, i.e. C57BL/6 mice that received 3 i.n. instillations of 100 μg HDM at days 0, 7 and 14. Lung single cell suspensions were obtained and enriched in CD11b-expressing cells by MACS before cell sorting. (**B**) Representative pre-sorting and post-sorting flow cytometry dot plots of living singlet CD45.2$^+$ CD11b-enriched lung cells according to Siglec-F and CD125 expression and SSC pattern before (left) and after (right) sorting of the gated populations. Numbers indicate % of gated cells in total subpopulation. Results are representative of one of three independent experiments, each of them yielding similar cell purities.
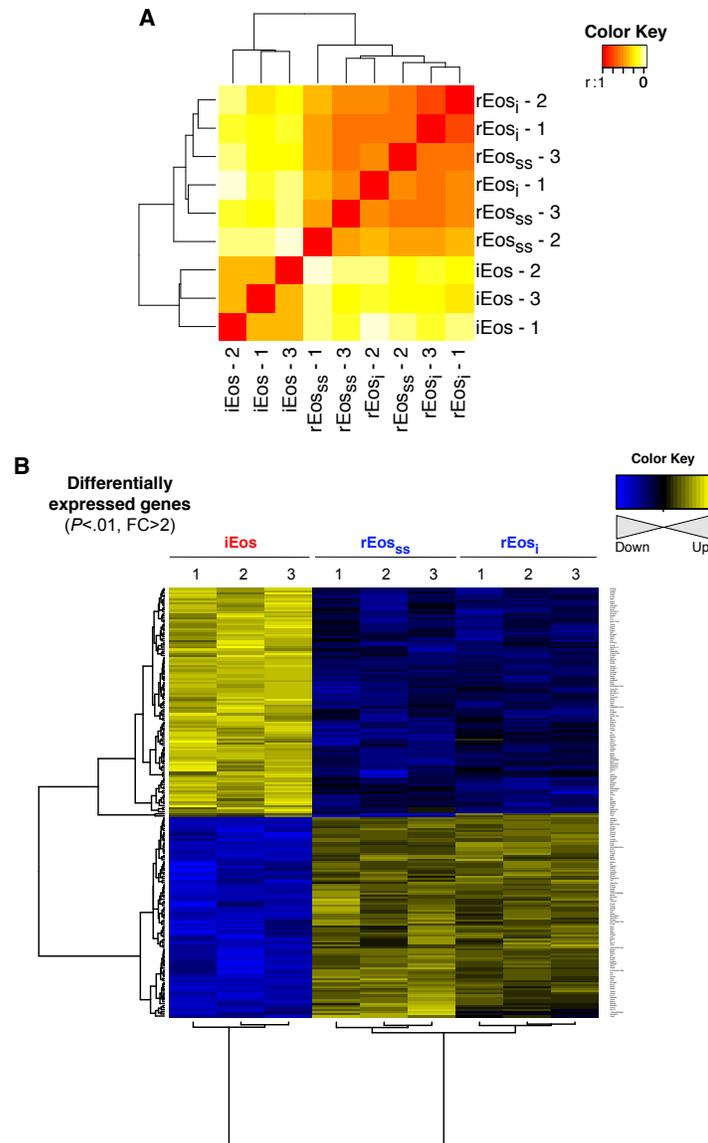
Supplemental Figure 7. Hierarchical clustering and differentially expressed genes between rEos$_{ss}$, rEos$_i$ and iEos. (**A**) Unsupervised hierarchical clustering of biological replicates. The yellow and red colors in cells of the correlation map reflect the low and high correlation respectively between gene expression patterns of two samples. (**B**) Heatmap of hierarchical clustering of 206 differentially expressed genes and 9 samples. All differentially expressed genes between iEos and rEos$_{ss}$, and iEos and rEos$_{i,}$, were collected and clustered with respect to the 9 samples. The colors in the map display the relative expression of genes across different samples. Yellow indicates high expression, blue low expression, and black intermediate expression. FC, fold change.

Supplemental Figure 8. Phenotypic characterization of small intestinal eosinophils (Int Eos) isolated from naive C57BL/6 mice. CD11b-enriched gut lamina propria cells of naive C57BL/6 mice were analyzed. (**A**) Representative flow cytometry dot plot of living singlet CD11b-enriched CD45.2$^+$ cells according to Siglec-F and CD125 expression. Number indicates % of gated cells in total subpopulation. (**B**) Representative photograph of Int Eos gated in **A** following cytospin and Hemacolor coloration. (**C**) Representative flow cytometry histograms of F4/80, CCR3, CD101 and CD62L expression on Int Eos. Data shown are representative of one of 6 mice analyzed coming from 3 independent experiments, each of them giving similar results. Scale bars = 10 μm.
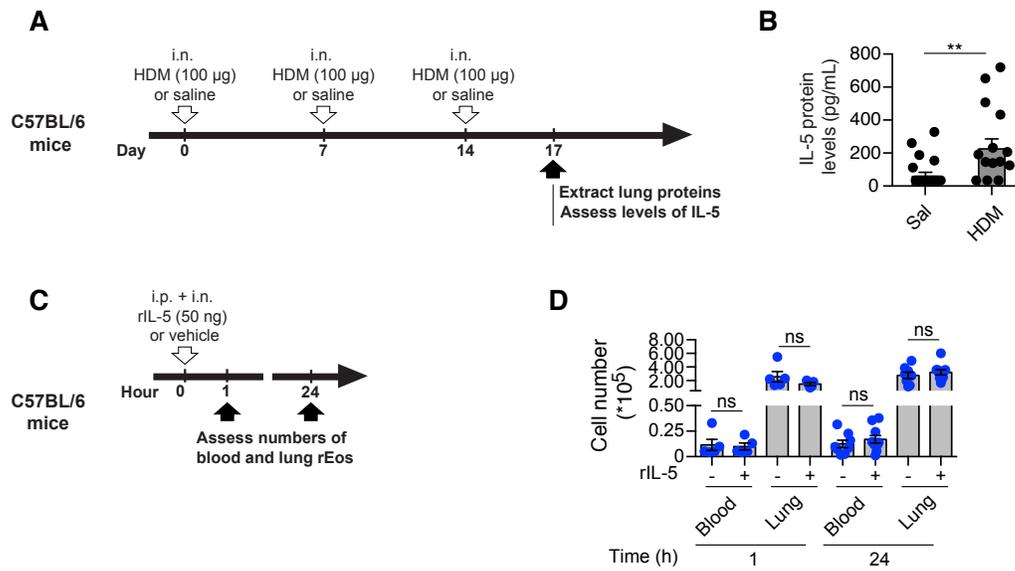
Supplemental Figure 9. Levels of IL-5 in the lungs of HDM-challenged allergic mice and effect of rIL-5 treatment on blood and lung rEos numbers in naive mice. (**A**) Experimental outline for data shown in **B**. (**B**) Concentration of IL-5 was quantified by ELISA on weight-matched lung homogenates from saline- or HDM-treated mice. (**C**) Experimental outline for data shown in **D**. (**D**) Absolute numbers of blood rEos-like cells and lung rEos as assessed by flow cytometry 1 and 24 hours following i.p. and i.n. treatments with 50 ng rIL-5 in 100 µL PBS (i.p.) and 50 ng rIL-5 in 50 µL PBS (i.n.) or PBS alone. Data shown are mean ± SEM, as well as individual values, and are pooled from 2-3 independent experiments (n=5-14/group). *P* values were calculated using (**B**) a Welch *t* test (for comparisons of vehicle *vs.* IL-5-treated groups) and (**D**) a one-way ANOVA followed by Tukey's test for multiple comparisons. **, *P*<0.01; ns, not statistically significant. Sal, saline.
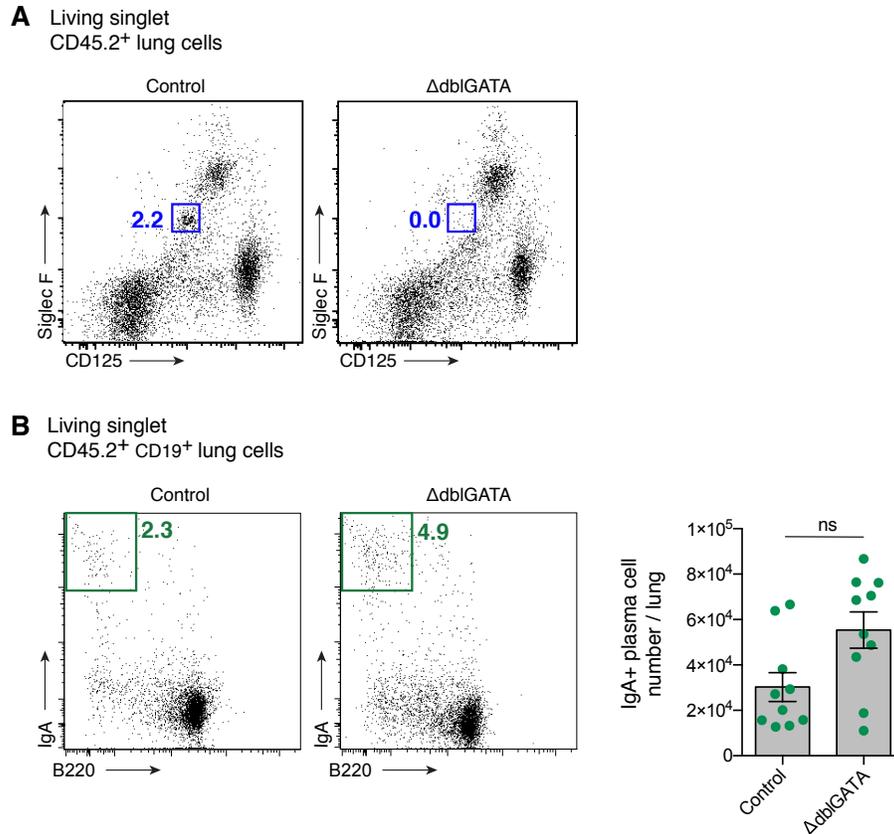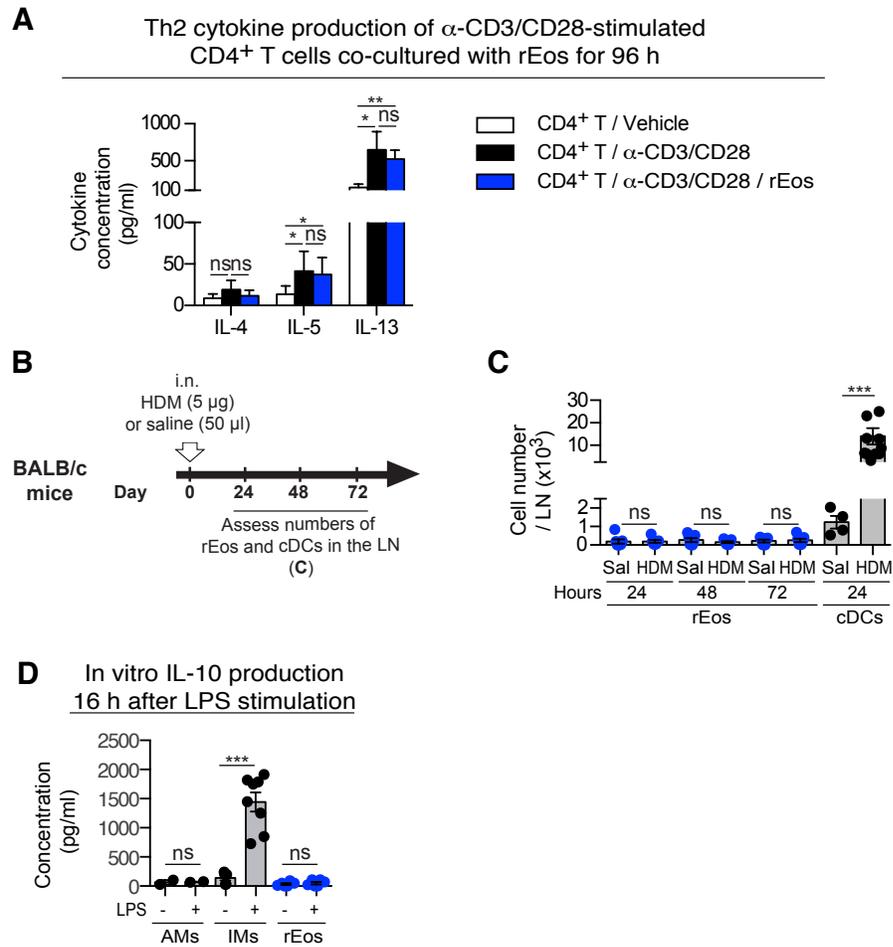
Supplemental Figure 10. Assessment of lung rEos and IgA^{+} plasma cells in ΔdblGATA and control mice. Whole lungs of naive ΔdblGATA and control mice were digested and analyzed. Representative flow cytometry dot plot of (**A**) living singlet CD45.2^{+} lung cells according to Siglec-F and CD125 expression and (**B**) living singlet CD45.2^{+} CD19^{+} lung cells according to IgA and B220 expression. Numbers indicate % of gated cells in total subpopulation. In **B**, absolute numbers of lung IgA^{+} plasma cells in ΔdblGATA and control mice are also shown (n=10/group). Data shown are mean ± SEM, as well as individual values, and are pooled from 3 independent experiments. *P* values were calculated using a two-tailed Student's *t* test. ns, not statistically significant.
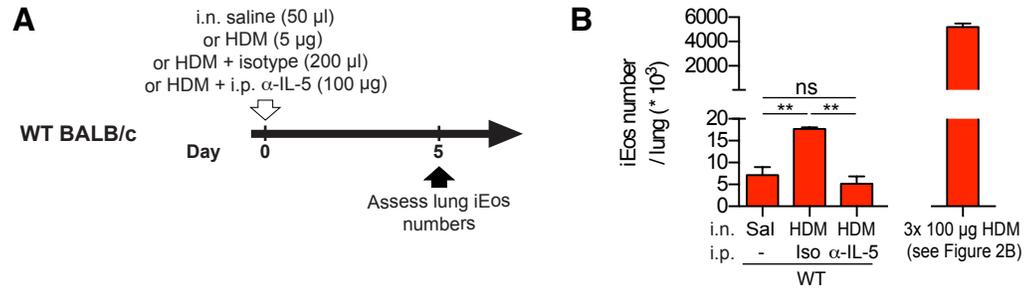
Supplemental Figure 11. IL-5-dependent innate recruitment of iEos in the lung of BALB/c mice exposed to i.n. 5 μg HDM. (**A**) Experimental outline for data shown in **B**. Briefly, WT BALB/c mice were sensitized i.n. with 5 μg HDM or injected with saline, and the numbers of lung iEos were quantified 5 days later. HDM-sensitized WT mice also received an i.p. injection of anti(α)-IL-5 or isotype Abs 1 hour before HDM instillation. (**B**) Absolute numbers of lung iEos at day 5. For comparison purposes, numbers of lung iEos during the effector phase of HDM-induced airway allergy (as shown in Figure 2B) are also shown. Data shown are mean + SEM and are representative of 1 of 2 independent experiments, each of them giving similar results (n=4/group). *P* values were calculated using a one-way ANOVA followed by Tukey's test for multiple comparisons. **, *P*<0.01; ns, not statistically significant.
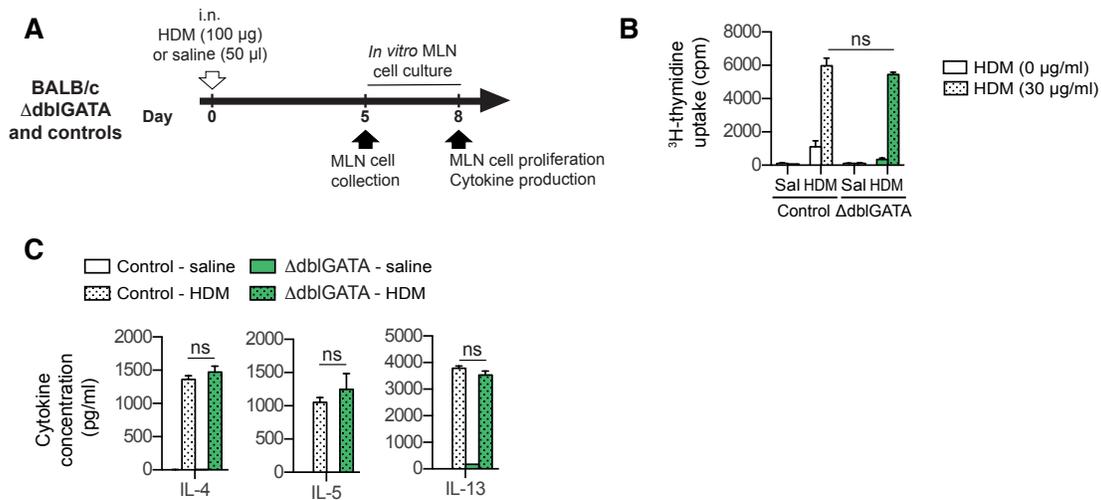
Supplemental Figure 12. Mediastinal lymph node Th2 responses of ΔdblGATA and control mice following i.n. exposure to a high dose of HDM. (**A**) Experimental outline. Eosinophil-deficient ΔdblGATA and control BALB/c mice received an i.n. instillation of a high dose HDM (100 µg). Three days later, MLN cells were cultured with or without HDM for 3 d. (**B**) Proliferation (as quantified by ^{3}H-thymidine incorporation during the last 16 h) of MLN cells. (**C**) Culture supernatants of MLN cell cultures were assayed for IL-4, IL-5 and IL-13 by ELISA. (**B-C**) Data shown are mean ± SEM and are representative of 1 of 2 independent experiments, each of them giving similar results. *P* values were calculated using (**B**) a two-way ANOVA followed by Tukey's test for multiple comparisons and (**C**) a two-tailed Student's *t* test. ns, not statistically significant. cpm, counts per min.
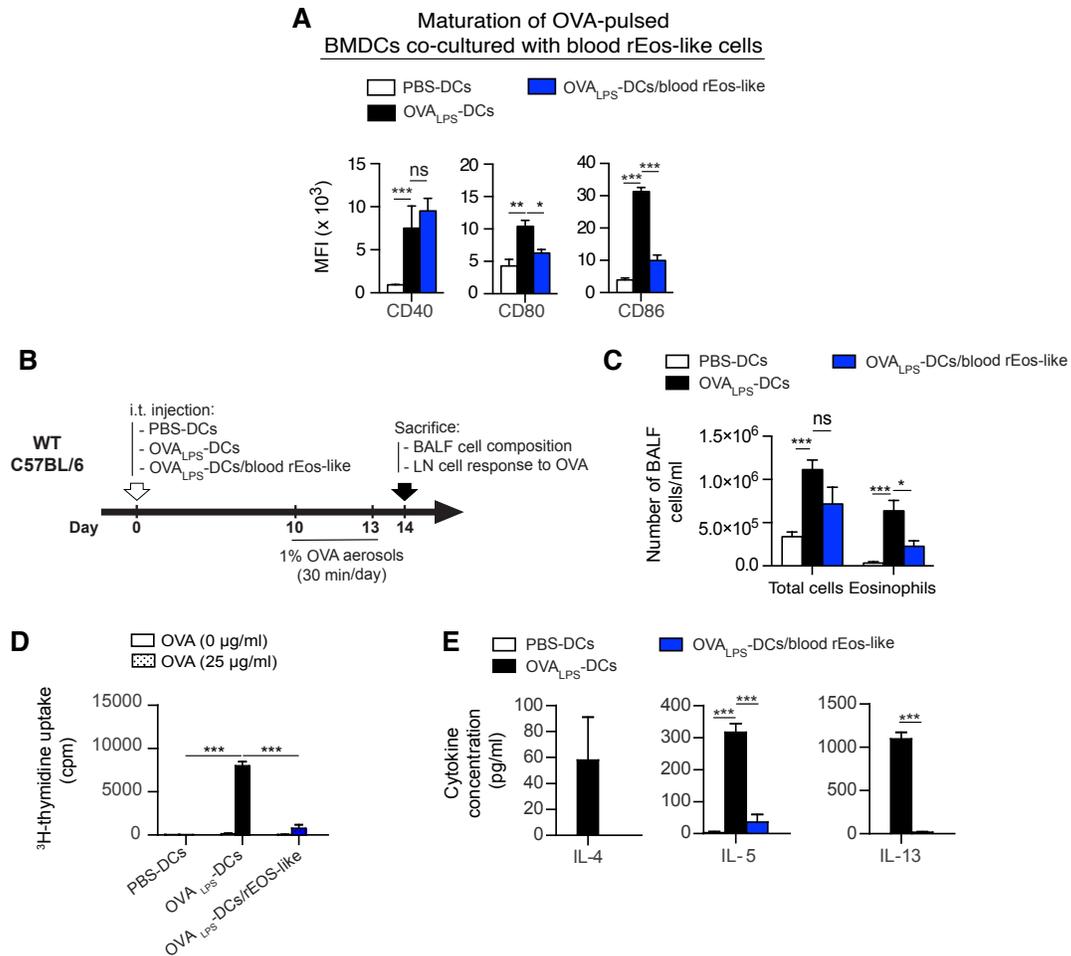
Supplemental Figure 13. Assessment of rEos ability to modulate T cell activation in vitro, to migrate to the LN upon HDM exposure and to produce IL-10 in vitro. (**A**) Th2 cytokine concentrations in culture supernatants of CD4$^+$ T cells isolated from the spleen of BALB/c mice, stimulated with α-CD3/CD28 Abs and cocultured with or without FACS-sorted rEos for 96 hours. (**B**) Experimental outline for **C**. (**C**) Numbers of rEos and cDCs in the draining LN of BALB/c mice were assessed by flow cytometry at the indicated time points after i.n. HDM treatment (5 μg) (n=4-11/group). (**D**) IL-10 concentration in culture supernatants of FACS-sorted AMs, IMs and rEos 16 hours after 10 ng/ml LPS stimulation. Data shown are mean ± SEM and are pooled from 2 (**A,C**) or 3 (**D**) independent experiments. *P* values were calculated using (**A**) a two-way ANOVA followed by Tukey's test for multiple comparisons and (**C,D**) a two-tailed Student's *t* test. *, *P*<0.05; **, *P*<0.01, ***, *P*<0.001; ns, not statistically significant. cDCs, conventional dendritic cells.

Maturation of OVA-pulsed
BMDCs co-cultured with small intestinal Eos (Int Eos)
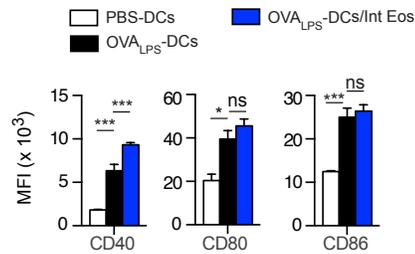
Supplemental Figure 14. Ability of small intestinal eosinophils (Int Eos) to modulate DC maturation in vitro. Expression of the indicated maturation markers on the surface of OVA_{LPS}-pulsed BMDCs cultured alone or cocultured with Int Eos for 12 hours. Data shown are mean + SEM and are pooled from 3 independent experiments with 3 different batches of BMDCs cocultured with primary Int Eos isolated from independent cohorts of mice. *P* values were calculated using a one-way ANOVA followed by Tukey's test for multiple comparisons. *, $P<0.05$; ***, $P<0.001$; ns, not statistically significant. MFI, mean fluorescence intensity.

Supplemental Figure 15. Assessment of immunosuppressive functions of blood rEos-like cells in DC coculture experiments. (**A**) Expression of the indicated maturation markers on the surface of OVA$_{LPS}$-pulsed BMDCs cultured alone or cocultured with blood rEos-like cells for 12 hours. (**B**) Experimental outline for **C-E**. (**C**) Total and eosinophil cell counts of the BALF of the indicated groups. (**D**) Proliferation of LN cells restimulated for 3 days with or without OVA. (**E**) Cytokine concentrations in culture supernatants of LN cell cultures restimulated with OVA. Data shown are mean + SEM and are pooled from 3 independent experiments with 3 different batches of BMDCs-cocultured with primary blood rEos-like cells isolated from independent cohorts of mice. *P* values were calculated using a one-way ANOVA followed by Tukey's test for multiple comparisons. *, *P*<0.05; **, *P*<0.01, ***, *P*<0.001; ns, not statistically significant. cpm, count per minute; MFI, mean fluorescence intensity.

A

B
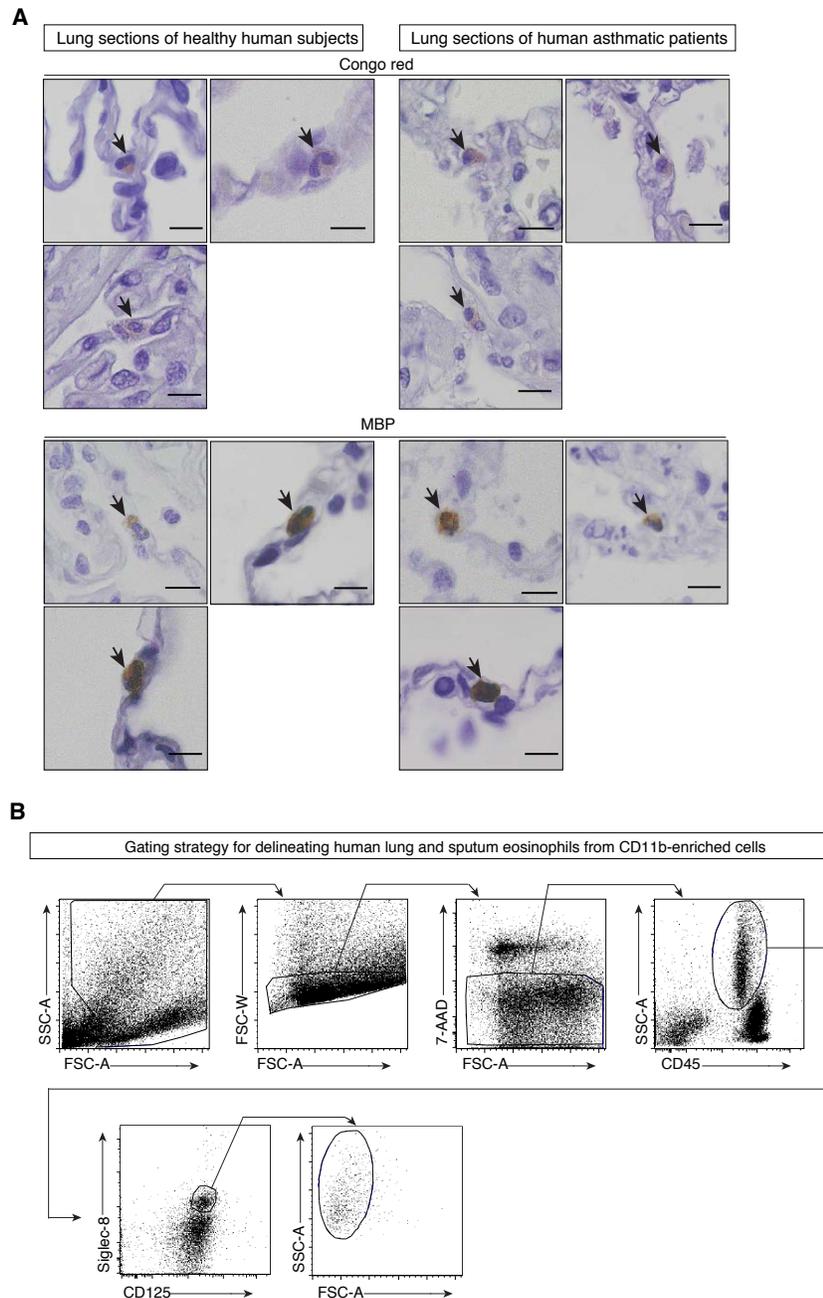
Supplemental Figure 16. Additional pictures of human lung parenchymal eosinophils and gating strategy for delineating human lung and sputum eosinophils. (**A**) Histological pictures of Congo red- and MBP-stained lung parenchymal sections of healthy subjects (left) and asthmatic patients (right). Arrows indicate red and MBP$^+$ eosinophils, respectively. Scale bars: 10 μm. (**B**) Gating strategy for delineating human lung and sputum eosinophils. Singlet 7-AAD$^-$ CD45.2$^+$SSC^{hi} cells were gated and analyzed for Siglec-8, CD125, CD62L, CD101 and IL-3R surface expression (see Figure 8). FSC, forward scatter; SSC, side scatter.

Supplemental Tables

Gender	Age	Disease status	Smoking history
Male	43		Smoker
Male	65		Never smoked
Female	64	Healthy donor	Never smoked
Female	54		Never smoked
Female	58		Smoker
Male	59		Non smoker
Female	39		Smoker
Female	38	Asthmatic	Smoker
Female	61		Smoker
Female	75		Non smoker

Supplemental Table 1. Characteristics of human healthy and asthmatic subjects analyzed in Figure 8, A and B.

Gender	Age	Disease status	Smoking history
Female	62	Lung cancer No clinical history of asthma No histopathological abnormalities found in the lung tissue analyzed	Ex-smoker
Female	58		Ex-smoker
Female	57		Smoker
Female	58		Smoker
Female	65		Ex-smoker
Female	41		Smoker
Male	71		Ex-smoker
Male	62		Ex-smoker

Supplemental Table 2. Characteristics of human lung cancer patients from which originate the normal lung tissue analyzed in Figure 8, C-E. Also see the supplemental methods for additional information regarding the inclusion criteria of the lung tissue.

Gender	Age	Disease status	Smoking history	Inhaled corticosteroids	% eosinophils in sputum
Female	31		Ex-smoker	No	8.4
Female	64		Never smoked	No	16.6
Female	47		Never smoked	No	2.5
Female	70	Asthmatic	Ex-smoker	Yes	6.2
Female	75		Never smoked	No	6.4
Male	63		Never smoked	Yes	23
Male	65		Ex-smoker	Yes	4

Supplemental Table 3. Characteristics of human eosinophilic asthmatic patients from which originate the sputa analyzed in Figure 8, C-E.