ONLINE DATA SUPPLEMENT

# Title: IL-5 exposure *in utero* increases lung nerve density and causes airway reactivity in adult offspring

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**Materials and Methods Online Supplement.**

**Animal Models**

Female hemizygous IL5tg or WT mice were housed individually with male WT or (-)Eos mice for breeding (see supplement Figure E1). Genetic outcomes of offspring from 97 litters (n=463 pups) followed Mendelian heritability of the IL-5 transgene: 65.2% IL5tg and 34.8% WT offspring from IL5tg female x IL5tg male and 44.1% IL5tg and 55.9% WT offspring from IL5tg female x WT male. Offspring mice were genotyped by PCR and gel electrophoresis. Briefly, ear tissue was collected from adult mice under 5% isoflurane anesthesia or the distal tail was removed from fetuses. HotSHOT DNA extraction was performed with alkaline lysis reagent (125 μL of 10N NaOH, 20 μL of 0.5M EDTA) followed by neutralization buffer (325 mg Tris-HCl). After DNA extraction, EconoTaq PCR master mix plus green (Lucigen Cat. 30033) was added to 1 μM (-)Eos and 0.1 μM 18s or 0.2 μM IL5tg and 0.2 μM 18s primers (see below) and run through a PCR reaction using a 96 well Veriti Thermal Cycler (Applied Biosystems). After a 5 minute 94°C hot start, PCR denaturation started at 94°C for 30 seconds, followed by annealing step at 53°C for (-)Eos or 56.5°C for IL5tg for 1 minute duration, and extension at 72°C for 1 minute. Both PCR reactions were cycled 30 times, then ran on a 2% agarose gel. (-)Eos Primers:Forward - 5'-AAG TAT GAT GGG GGT GTT TC-3' Reverse - 5'-GAG CGG GTT TTC ATT ATC TAC-3'. IL5tg Primers:Forward - 5'-CAG TGC TTG ACT TTA AAG AGG-3' Reverse - 5'-TGG CAG TGG CCC AGA CAC AGC-3'. 18s Control Primers:Forward - 5'-GTA ACC CGT TGA ACC CCA TT-3' Reverse - 5'-CCA TCC AAT CGG TAG TAG CG-3'. Eosinophils and IL-5 were measured in bronchoalveolar lavage of all offspring to confirm genotyping results. Neither IL-5 nor eosinophils were present in bronchoalveolar lavage of WT offspring from IL5tg mice, confirming genotyping results.

**Allergen Sensitization and Challenge**

Adult offspring mice (F1) were anesthetized with 5% isoflurane and sensitized intranasally with 50 μg of house dust mite (HDM, dissolved in 25 μl PBS, LPS content 83250 EU/vial, Greer Laboratories) on days 0 and 1, followed by challenge with 25 μg HDM (dissolved in 25 μL PBS) daily on days 14 through 17. Controls received intranasal phosphate buffered saline (vehicle). Animals were killed on day 18 for analysis (see supplement Figure E1).

Exposure to inhaled anesthetics can inhibit airway inflammation and suppress bronchoconstriction(1-3), therefore offspring from WT and IL5tg mice were also compared at baseline (ie without prior exposure to isoflurane, vehicle, or HDM).

**Airway Physiology**

Mice were sedated with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.), tracheotomized, and mechanically ventilated via a 21-gauge catheter. The ventilator system consisted of a pneumotachograph to measure airflow (ML141, AD Instruments), a pressure transducer, metering valves (inspiratory time 175 ms, expiratory time 300 ms), two expiratory water columns for positive end-expiratory pressure (2 cm H20) and deep-inhalation (25 cm H20), in-line nebulizer (AeroNeb) and LabChart Pro acquisition software. Mice were ventilated with 100% oxygen at 125 breaths/min with a 0.2 mL tidal volume and paralyzed with succinylcholine (10 mg/kg i.p.) to eliminate respiratory effort. Methacholine and serotonin both elicit consistent, reproducible bronchoconstriction in mice(4). We chose serotonin since it strongly activates vagal sensory nerves(5). Core body temperature was maintained at 36.9°C with a homeothermic

blanket. A 3-lead electrocardiogram monitored heart rate and rhythm and a pulse oximeter recorded oxygen saturation.

Airway resistance was calculated as the difference between peak inspiratory pressure and plateau pressure during an end inspiratory pause, divided by airflow (Resistance = $P_{peak}$-$P_{plateau}$ / flow). Baseline airway resistance was measured in response to 10 μl nebulized PBS vehicle followed by escalating concentrations of serotonin (10 - 1,000 mM). Resistance after each dose of serotonin was expressed as fold change over resistance to aerosolized PBS. For vagotomy experiments, the vagus nerves were isolated bilaterally and cut with scissors immediately before measuring airway responses to distinguish between bronchoconstriction mediated by vagal reflexes versus direct effects of serotonin on smooth muscle. For neurokinin-1 (NK1) experiments, the NK1 antagonist CP99994 (1 μg i.p. dissolved in PBS, Tocris) was given 15 minutes before measuring airway responses to serotonin. For methacholine experiments, the vagus nerves were isolated and cut bilaterally before mice were exposed to escalating concentrations of inhaled methacholine (1 - 100 mM) to test smooth muscle contractility independent of nerve reflexes.

**Adult Tissue Collection**

Animals were assigned random numbers at sacrifice to blind study personnel to treatment groups. Airways were lavaged via the tracheal cannula with 500 μl ice-cold PBS three times. Lavage fluid was centrifuged (2000 rpm x 10 min), and supernatants were flash frozen in liquid nitrogen, and stored at -80°C. Cell pellets were resuspended in PBS and total cell counts were determined with a hemacytometer. Cells were cytospun and differential counts were obtained following Wright staining. Airway was filled with 500 μl Zamboni's fixative, excised, and

placed in 5 mL of Zamboni's fixative overnight for quantification of airway nerve architecture (see below). Lavage IL-5, IL-13, GDNF, BDNF, eotaxin, substance P (all from R&D Systems), NGF (Sigma) and NT4 (LSBio) were measured by ELISA.

**Fetal Tissue Collection**

Pregnant WT and IL5tg mice were sedated with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.) at days 18-21 of gestation, abdomen and uterus incised, and a 26-gauge needle inserted into the amniotic sac. After collecting amniotic fluid, the amniotic sac was opened, fetus removed, fetal and maternal blood collected for peripheral smears, and amniotic fluid flash-frozen in liquid nitrogen. The fetal distal tail was removed for genotyping. Blood differential counts were obtained after Wright staining.

**Tissue Optical Clearing, Imaging, and Epithelial Nerve Modeling**

After overnight fixation, airways were dissected free of surrounding tissue. Whole-mount tissues were stained for nerve architecture as previously described (15). In brief, fixed tissues were washed with TBS, blocked overnight (4% normal goat serum, 1% Triton X-100, 5% powered milk in TBS pH 7.4), immunolabeled with antibodies to substance P (to identify epithelial sensory nerves, rat 1:500 BD Pharmigen) or PGP9.5 (a pan-neuronal marker, rabbit 1:250 Millipore) overnight, washed again with TBS, and then placed in secondary antibodies overnight (Alexa goat anti-rat 555 1:1000, Alexa goat anti-rabbit 647 1:1000 Invitrogen). No primary, rat IgG and rabbit IgG controls were run in parallel. Samples were sequentially dehydrated with 50% and 100% ethanol, placed in benzylbenzoate:benzylalcohol (BABB 2:1) for optical clearing, and mounted with BABB in Permount-sealed chambered slides.

Images were obtained with a 780 LSM Zeiss confocal microscope. Three 63x, 1.4N.A. z-stack images extending from the posterior serosal surface through the luminal space were also taken per animal at the bifurcation of main bronchi. For assessment of nerve structure, substance P positive nerves within the epithelial layer were manually traced in 3D using Filaments function of Bitplane Imaris, thus creating 3D models of airway nerves from which the software automatically calculated length and branch points. Each image was treated as a replicate and averaged together for one value per animal. Values represent average nerve length and nerve branch points per 63x image (equivalent to density).

**Nodose-Jugular Ganglia Retrograde Tracing**

Adult offspring mice were anesthetized with 5% isoflurane and administered AlexaFluor 555 tagged wheat germ agglutinin intranasally (ThermoFischer, 10 mg/mL in PBS, 25 μL). Mice were euthanized 16-20 hours later with pentobarbital, vagal ganglia dissected free, and placed in Zamboni's fixative overnight. Ganglia were washed with TBS, mounted with VectaShield, and imaged with an 780 LSM confocal microscope. Wheat-germ agglutinin localized throughout neuron cell bodies, but not the nucleus, allowing for easy identification of individual, single neurons (see figure 4g-h). Each fluorescently labeled neuron within the nodose-jugular ganglia was manually counted in 3-dimensional images (Bitplane Imaris). Nodose-jugular ganglia from mice that did not receive fluorescent wheat-germ agglutinin were used as a negative control. The intensity maximum of figure 2H is decreased 3-fold compared to figure 2G to show tissue outline since background autofluorescence was very low.

## References:


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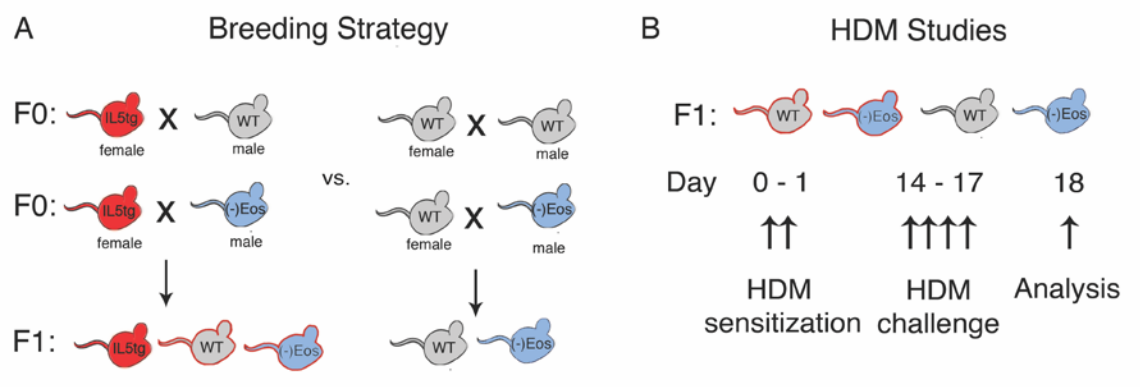
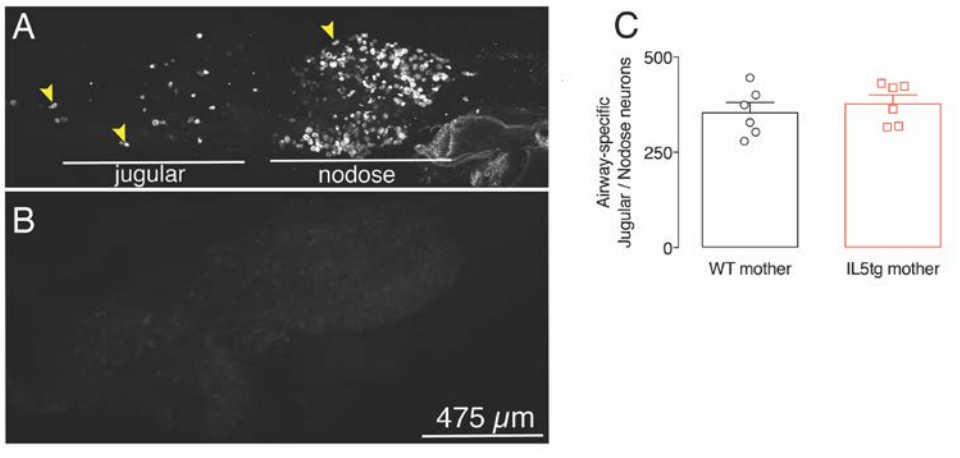
3. Habre W, Petak F, Sly PD, Hantos Z, Morel DR. Protective effects of volatile agents against methacholine-induced bronchoconstriction in rats. *Anesthesiology* 2001; 94: 348-353.
4. Martin TR, Gerard NP, Galli SJ, Drazen JM. Pulmonary responses to bronchoconstrictor agonists in the mouse. *J Appl Physiol (1985)* 1988; 64: 2318-2323.
5. Potenzieri C, Meeker S, Undem BJ. Activation of mouse bronchopulmonary C-fibres by serotonin and allergen-ovalbumin challenge. *J Physiol* 2012; 590: 5449-5459.

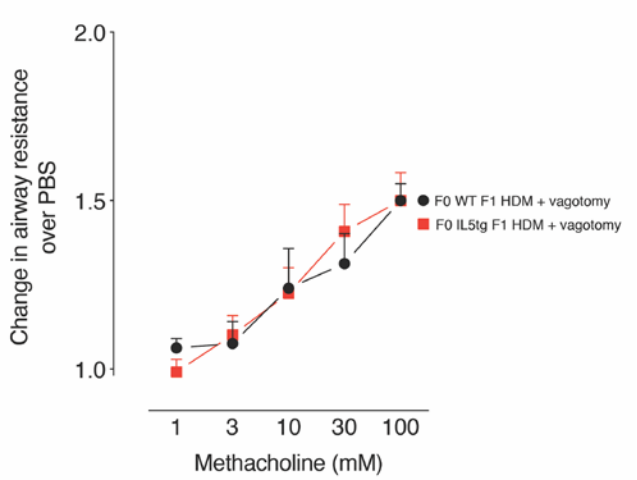
**Supplemental Figures.**




**Supplemental Figure E1**. Breeding strategy and HDM exposure timeline. (A) IL5tg female mice were bred with WT or eosinophil deficient (-Eos) male mice to produce offspring that were IL5tg, WT or eosinophil deficient and had been exposed to maternal IL-5 *in utero*. (B) Adult offspring were sensitized to HDM (50 µg dissolved in PBS, intranasal) or vehicle control (PBS alone, 25µL total volume) followed by intranasal exposure of HDM (25 µg) or vehicle on protocol day 14-17. Animals were euthanized on day 18 for analysis.




**Supplemental Figure E2.** Maternal IL-5 did not change the number of offspring nodose-jugular neurons innervating the lungs. Wheat-germ agglutinin tagged with Alexa Flour 555 was administered intranasally and nodose-jugular ganglia dissected free 16 hours later to identify fluorescent, retrograde labeled neurons (A, yellow arrowheads point to individual neurons). Negative control nodose-jugular ganglia from mouse that did not receive fluorescent wheat-germ agglutinin (B). Intensity maximum of (B) is increased 3-fold compared to (A) to show tissue outline since background autofluorescence is very low. Maternal IL-5 did not change the number of vagal neurons innervating the airway (C).




**Supplemental Figure E3. Airway hyperreactivity was not mediated by increased airway smooth muscle responsiveness.** Methacholine induced bronchoconstriction was not different between vagotomized HDM exposed offspring from WT (●, n=7) and IL5tg (■, n=6) mice.