Supplemental Data

Maternal diesel particle exposure promotes offspring asthma through NK cell-derived granzyme B

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Supplemental Methods

Mice

The following strains were used in this study: *Ncr1*$^{iCre/+}$ (*B6(Cg)-Ncr1tm1.1(icre)Viv/Orl*), *R26*$^{DTA/DTA}$ (*B6.129P2-Gt(ROSA)26Sortm1(DTA)Lky/J*), *Gzmb*$^{-/-}$ (*B6.129S2-Gzmbtm1Ley/J*) and wild type (all on the C57BL/6 background). Wild type C57BL/6J and *R26*$^{DTA/DTA}$ mice were purchased from Jackson Laboratories. *Ncr1*$^{iCre/+}$ mice were generated and donated by Dr. Eric Vivier (Aix-Marseille Université, Marseille, France; Ref: 17) and sent to us by Dr. Joseph Sun (Memorial Sloan Kettering Cancer Center, New York, NY, USA). *Gzmb*$^{-/-}$ mice were generated and donated by Dr. Timothy J. Ley (Washington University School of Medicine, St. Louis, MO, USA; Supplemental Ref #1) and sent to us by Dr. Xuefang Cao (Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA). Male and female mice were used in this study. All mice were housed and bred under specific pathogen-free conditions at Biological Resource Center at National Jewish Health.

Antibodies

Rat monoclonal anti-mouse CD16/32 (clone 93), Armenian hamster monoclonal anti-mouse CD3ε-FITC (clone 145-2C11), mouse monoclonal anti-human CD3-FITC (clone UCHT1), rat monoclonal anti-mouse CD4-PerCP-Cy5.5 (clone RM4-4), rat monoclonal anti-mouse/human CD11b-FITC (clone M1/70), Armenian hamster monoclonal anti-mouse CD11c-FITC (clone N418), rat monoclonal anti-mouse CD25-PE-Cy7 (clone PC61), rat monoclonal anti-mouse/human CD45R (B220)-FITC (clone RA3-6B2), mouse monoclonal anti-mouse CD45.2-PerCP-Cy5.5 (clone 104), mouse monoclonal anti-mouse CD45.2-APC-Cy7 (clone 104), mouse monoclonal anti-human CD56-PerCP-Cy5.5 (clone HCD56), rat monoclonal anti-mouse CD90.2-PE-Cy7 (clone 30-H12), rat monoclonal anti-mouse CD107a-PE (clone 1D4B), mouse monoclonal anti-human CD107a-PE (clone H4A3), rat IgG2a, κ isotype control (clone RTK2758), Armenian hamster monoclonal anti-human/mouse/rat CD278 (ICOS)-PE-Cy7 (clone C398.4A), rat monoclonal anti-mouse CD326 (Ep-CAM)-PE (clone G8.8), rat monoclonal anti-mouse CD335 (NKp46)-PE (clone 29A1.4), rat monoclonal anti-mouse CD335 (NKp46)-PerCP-Cy5.5 (clone 29A1.4), rat monoclonal anti-mouse F4/80-FITC (clone BM8), mouse

monoclonal anti-human IL-4-PE (clone 8D4-8), rat monoclonal anti-mouse/human IL-5-APC (clone TRFK5), rat monoclonal anti-mouse/human IL-5-BV421 (clone TRFK5), Armenian hamster monoclonal anti-mouse FcεRIα-FITC (clone MAR1), mouse monoclonal anti-mouse/human GATA3-APC (clone 16E10A23), mouse monoclonal anti-mouse/human Granzyme B-Alexa Fluor 647 (clone GB11), rat monoclonal anti-mouse Ly-6G/Ly-6C (Gr-1)-FITC (clone RB6-8C5), rat monoclonal anti-mouse Ly-6C-BV421 (clone HK1.4), Syrian hamster anti-mouse/human KLRG1 (MAFA)-APC-Cy7 (clone 2F1/KLRG1), mouse monoclonal anti-mouse NK1.1-FITC (clone PK136), mouse monoclonal anti-mouse NK1.1-APC (clone PK136), mouse monoclonal anti-mouse NK1.1-PE-Cy7 (clone PK136), mouse monoclonal anti-mouse NK1.1-PerCP-Cy5.5 (clone PK136), rat monoclonal anti-mouse/human RORγt-PE (clone B2D), mouse monoclonal anti-mouse/human T-bet-APC (clone 4B10), Armenian hamster monoclonal anti-mouse TCRβ-FITC (clone H57-597), Armenian hamster monoclonal anti-mouse/rat IL-1β (clone B122), purified Armenian hamster IgG isotype control (clone HTK888), rat monoclonal anti-mouse TSLP (clone 28F12), purified rat IgG2a, κ isotype control (clone RTK2758), rat monoclonal anti-mouse IL-25 (clone 35B), LEAF purified rat IgG1, κ isotype control (clone RTK2071) were from BioLegend. Rat monoclonal anti-mouse CD19-FITC (clone 1D3), Armenian hamster monoclonal anti-mouse/human/rat CD27-PE (clone LG.7F9), rat monoclonal anti-mouse IL-13-PE (clone eBio13A), rat monoclonal anti-mouse EOMES-PE-Cy7 (clone Dan11mag), rat monoclonal anti-mouse IL-25R-PE (clone MUNC33) and rat monoclonal anti-mouse IL-33R (ST2)-APC (clone RMST2-2) were from eBioscience. Rat monoclonal anti-mouse/human CD11b-PE-Cy7 (clone M1/70), rat monoclonal anti-mouse CD127 (IL-7Rα)-PE-Cy7 (clone SB199), rat monoclonal anti-mouse CD127 (IL-7Rα)-BV421 (clone SB199), mouse monoclonal anti-human IFN-γ-PE-Cy7 (clone 4S. B3), rat monoclonal anti-human IL-13-BV421 (clone JES10-5A2) were from BD Biosciences. Rat monoclonal anti-mouse IL-25 (IL-17E)-Alexa Fluor 647 (clone 207702), goat polyclonal anti-mouse IL-33 (Cat# AF3626), normal goat IgG control (Cat# AB-108-C) were from R&D Systems. Rabbit polyclonal anti-mouse/human IL-25 (for immunofluorescent microscopy, Cat# 06-1080) was from Millipore. Anti-rabbit IgG (H+L) antibody-Alexa Fluor 488 (secondary antibody for immunofluorescent microscopy, Cat# A-11008) and rabbit IgG isotype control (Cat# 08-6199) were from

Thermo Fisher. Rabbit anti-Asialo-GM1 serum (Cat# 986-10001) and Normal Rabbit Serum (Cat# 140-06571) were from Wako.

Mouse model of preconceptionally-programmed allergic airway disease (AAD)

Female mice received 6 intranasal applications of DEP (Standard Reference Material/SRM 2975 from National Institute of Standards and Technology) or PBS (Thermo Fisher) under isoflurane (VetOne) anesthesia, at three-day intervals (days -29, -26, -23, -20, -17, and -14 before mating), with the first application occurring at 6 weeks of age. For instillation, DEPs were suspended in PBS at 1 mg/ml and delivered at the dose of 50 μg (50 μl) per mouse. Volume of 50 μl was delivered through three sequential injections of 16.7 μl, 15 min apart. Two weeks after the final administration, females were mated with unexposed males at 8-12 weeks of age. There were no exposures during pregnancy.

In the OVA-based model of AAD, pups were intraperitoneally injected with either 50 μl of the immunizing mixture containing 5 μg of OVA (Sigma-Aldrich) and 1 mg of Imject Alum (0.5 mg aluminum hydroxide and 0.5 mg magnesium hydroxide; Thermo Fisher) in PBS or 50 μl of PBS on postnatal day (PND) 5. On PND 23, 24, and 25, pups were intranasally challenged with either OVA or PBS under isoflurane anesthesia. OVA-immunized pups were challenged with 50 μg of OVA in 15 μl of PBS. Pups that received PBS on PND 5 were challenged with 15 μl of PBS without OVA. Pups were analyzed on PND 28.

In the HDM-based model of AAD, pups received 5 μg of HDM (Stallergenes Greer) in 5 μl of PBS or 5 μl of PBS intranasally on PND 5 under isoflurane anesthesia. Pups received same amount of HDM or PBS on PND 6. On PND 23, 24, and 25, pups were intranasally challenged with either HDM or PBS. HDM-immunized pups were challenged with 30 μg of HDM in 30 μl of PBS. Pups that received PBS on PND 5 were challenged with 30 μl of PBS. Pups were analyzed on PND 28.

Adult mouse model of AAD

On postnatal days (PND) 42 and 49 (i.e. at six and seven weeks of age, respectively) mice were intraperitoneally injected with 100 μl of the immunizing mixture containing 20 μg OVA and 2 mg Imject Alum.

Mice were then intranasally challenged with 50 μg of OVA in 15 μl of PBS on PND 56, 57 and 58. Mice were analyzed on PND 61.

Antibody-mediated depletion of NK cells in vivo

On PND 5, wild type C57BL/6 neonates of DEP-exposed wild type C57BL/6 females were intraperitoneally injected with 15 μl of anti-asialo-GM1 or control rabbit sera (both reagents from Wako) diluted to 50 μl in PBS. On PND 6, pups were immunized with OVA in Imject Alum as described in the parent protocol. Sera administrations were then repeated on PND 22 and PND 25 when reagents were delivered intravenously at 25 μl of anti-asialo-GM1/control sera diluted to 100 μl in PBS. All pups were intranasally challenged with OVA on PND 23, 24 and 25 as in the parent protocol.

Antibody-mediated neutralization of IL-1β, IL-25, IL-33 and TSLP in vivo

On PND 5, wild type C57BL/6 neonates of DEP-exposed wild type C57BL/6 females were intraperitoneally injected with an anti-cytokine antibody or isotype control immunoglobulin [20 μg of anti-IL-1β antibody or isotype control Armenian hamster IgG (both reagents from BioLegend); 20 μg of anti-IL-25 antibody or isotype control rat IgG1 (both reagents from BioLegend); 4 μg of anti-IL-33 antibody or isotype control goat IgG (both reagents from R&D Systems); 20 μg of anti-TSLP antibody or isotype control rat IgG2a, κ (both reagents from BioLegend)], diluted to 50 μl in PBS. On PND 6, pups were immunized with OVA in Imject Alum as in the parent protocol. Antibody/IgG injections were then repeated on PND 22 when reagents were delivered intravenously at the dose of 100 μg (anti-IL-1β, anti-IL-25, and anti-TSLP) or 20 μg (anti-IL-33) diluted to 100 μl in PBS. All pups were intranasally challenged with OVA on PND 23, 24 and 25 as in the parent protocol.

IL-25 administration

Ncr1$^{iCre/+}$ females were exposed to DEP and mated with unexposed *R26*$^{DTA/DTA}$ males to generate *Ncr1*$^{iCre/+}$*R26*$^{DTA/+}$ and *R26*$^{DTA/+}$ littermate neonates. On PND 5, neonates were intraperitoneally injected with either OVA/Imject Alum mixed with 0.1 μg of the mouse IL-25 protein (R&D Systems) or OVA/Imject Alum

alone without IL-25. On PND 6, neonates were administrated with either 0.1 μg of mouse IL-25 in 50 μl of PBS or 50 μl of PBS. All pups were intranasally challenged with OVA on PND 23, 24 and 25 as in the parent protocol. Six hours after each intranasal challenge with OVA, pups were intratracheally administered either with 0.5 μg of IL-25 diluted in 30 μl PBS or with PBS alone.

Adoptive transfer of NK cells

NK cells were isolated from donor splenocytes after removal of red blood cells (ACK buffer, Quality Biological) and dead cells (Dead Cell Removal Kit, Miltenyi Biotec), using the NK cell Isolation Kit (Miltenyi Biotec) supplemented by us with biotin-conjugated anti-CD127 (to remove ILCs, clone SB/199 from BioLegend) according to the manufacturer's instructions. Immediately after isolation, $1x10^6$ NK cells (in 50 μl of PBS) were intratracheally injected into aged-matched recipient pups.

Measurement of airway hyperresponsiveness (AHR) to methacholine

Total lung resistance was measured using the FlexiVent apparatus (SCIREQ) as described (13). Anesthetized mice were subjected to tracheotomy. Mouse trachea was then attached through a 19-gauge cannula to the FlexiVent apparatus. A default QuickPrime 3 program was used for measurement of total lung resistance in response to increasing concentrations of methacholine (Sigma-Aldrich; 3.125, 6.25, 12.5, 25, 50, 100, and 200 mg/ml).

Isolation of tissue cells

For isolation of lung cells, lungs were collected from PBS-perfused mice, finely minced and incubated in the digestion buffer [Opti-MEM Reduced Serum Medium (Thermo Scientific) containing 1 mg/ml Collagenase type I (Worthington) and 20 units/ml DNase I (Worthington)] for 30 min at 37℃. Following digestion, cell suspensions were dispersed by gentle pipetting and filtered through a 70 μm cell strainer to eliminate clumps. For isolation of splenocytes, spleens were collected from PBS-perfused mice, finely minced and filtered through

a 70 μm cell strainer. Following centrifugation of filtered lung and spleen cells, red blood cells were lysed using the ACK buffer.

Flow cytometry

To phenotype distinct cell populations by flow cytometry, lung cells or splenocytes were washed with PBS, blocked with anti-CD16/CD32 (clone 93, BioLegend), and then stained with indicated antibodies and the viability dye eFluor506 (eBioscience) in the FACS buffer (PBS supplemented with 2% FBS). For intracellular cytokine staining, single cell suspensions were stimulated for 4 hours with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μg/ml ionomycin in the presence of 10 μg/ml brefeldin A and monensin (2 μM; all from Sigma-Aldrich), washed, blocked with anti-CD16/CD32 and stained for surface markers and viability. Cells were then fixed in 2% paraformaldehyde (Acros Organics) for 20 min followed by permeabilization in the Perm buffer [FACS buffer containing 0.1% saponin (Sigma-Aldrich)] for 15 min. Next, cells were incubated with anti-cytokine antibodies in the presence of anti-CD16/CD32 in the Perm buffer for additional 45 min, and then washed. For transcriptional factor staining, cells were first stained with antibodies for surface markers, then fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer (eBioscience) as instructed by the manufacturer. Samples were run on the BD LSR II flow cytometer. Data was analyzed using the FlowJo version 10.1 software (Tree Star).

NK cell degranulation assay

Single cell suspensions of mouse lungs were blocked with anti-CD16/CD32 and then incubated for 1 hour with IL-2 (10 ng/ml; PeproTech), IL-15 (10 ng/ml; PeproTech), PE-labeled anti-CD107a (clone 1D4B; BioLegend) or PE-labeled isotype control rat IgG2a (clone RTK2758; BioLegend) in RPMI 1640 medium (Thermo Fisher) supplemented with 10% FBS after which brefeldin A (10 μg/ml; Sigma-Aldrich) and monensin (2 μM; Sigma-Aldrich) were added, and cells were incubated for additional 4 hours. Cells were then harvested, stained for surface markers and viability (eFluor506) and analyzed by flow cytometry.

Staining of bronchoalveolar lavage (BAL) cells

The BAL fluid was recovered from mouse lungs and total cell counts were obtained. BAL cells were then deposited on glass slides by cytospin and stained with the Protocol HEMA 3 stain set (Fisher Scientific) to visualize cell morphology. A total of 300 cells were counted per each slide, and percentages of eosinophils, lymphocytes, macrophages, and neutrophils and total BAL cell counts were used to calculate absolute numbers of these subsets in the BAL fluid.

Histology

Lungs were fixed in 4% paraformaldehyde (Acros Organics), embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E) for analysis of inflammation and periodic acid and Schiff (PAS) stain for mucin detection. Images were acquired using a Nikon Eclipse TE2000-U inverted microscope, a 10x objective, a Diagnostics Instruments camera model 4.2 and Spot version 5.0 software. The peribronchial inflammation scoring system includes scores ranging from 0 to 6. A bronchus is scored 0 if there are no inflammatory cells around this bronchus. A score of 1 indicates that less than 50% circumference of a bronchus is surrounded by a thin layer (1-4 cells) of inflammatory cells. A score of 2 indicates that more than 50% circumference of a bronchus is surrounded by a thin layer (1-4 cells) of inflammatory cells. A score of 3 indicates that less than 50% circumference of a bronchus is surrounded by a thick layer (5-8 cells) of inflammatory cells. A score of 4 indicates that more than 50% circumference of a bronchus is surrounded by a thick layer (5-8 cells) of inflammatory cells. A score of 5 indicates that less than 50% circumference of a bronchus is surrounded by an extra thick layer (9+ cells) of inflammatory cells. A score of 6 indicates that more than 50% circumference of a bronchus is surrounded by an extra thick layer (9+ cells) of inflammatory cells. To measure PAS-stained areas of the airway epithelium, ImageJ software was used. For each bronchus, PAS-positive (PAS+) area of the epithelium was divided by the total (PAS+ and PAS-) area of the epithelium.

For statistical analysis of peribronchial inflammation and mucus-positive epithelial areas, multiple mice were evaluated per group. For each mouse, 4-5 randomly selected bronchi were measured; the results were averaged

to obtain a mean value for each mouse. Mean values representing individual mice were then used to calculate a mean value for an experimental group.

Immunofluorescence staining and microscopy

Paraformaldehyde-fixed paraffin-embedded lung tissue was sectioned at 4 μm, deparaffinized and rehydrated with serial passage through changes of xylene and graded ethanol. Tissue sections were blocked for 1 hour with 10% goat serum (Jackson Immunoresearch) in PBS containing 0.05% saponin at room temperature and then incubated overnight with a primary antibody against IL-25 (Millpore) in PBS containing 5% goat serum and 0.05% saponin at 4^0C. Slides were then washed and incubated with Alexa Fluor 488-labeled goat anti-rabbit IgG (Thermo Scientific) in the same buffer for 1 hour at room temperature, counterstained with DAPI (Thermo Scientific) and coverslipped for analysis. Images were acquired using the Nikon Eclipse TE2000-U inverted microscope, equipped with a 10x objective, a mercury lamp (Chiu Technical), excitation and emission filter wheels, and a CoolSnap HQ camera (Roper Scientific-Photometrics). Data acquisition and analysis were performed with the Metamorph version 7.0 software (Molecular Devices). For each mouse, 4-5 randomly selected bronchi were measured; the results were averaged to obtain a mean value for each mouse. Mean values representing individual mice were then used to calculate a mean value for an experimental group.

Preparation of lung homogenates

Mouse lungs were snap-frozen in liquid nitrogen. For cytokine ELISA, lungs were thawed, weighed and homogenized at 100 mg of tissue per milliliter of a homogenization buffer. The buffer contained 0.2x PBS and 1x Protease Inhibitor Cocktail (Sigma-Aldrich). After homogenization, lung samples were subjected to two freeze-thaw cycles, and then centrifuged at 12,000 x g. The supernatants were collected and supplemented with 10X PBS in order to equilibrate their osmolarity to 1x PBS. Supernatants were then assayed for concentration of total protein (Coomassie Plus Assay kit; Thermo Fisher Scientific) and cytokines (see below).

Cytokine ELISAs

Concentrations of IL-1β, IL-5, IL-13, IL-25, IL-33, and TSLP in lung homogenates, BAL fluid supernatants and cell culture supernatants were measured using ELISA kits (Thermo Fisher) according to the manufacturer's instructions. ELISA plates were read using the Emax Microplate Reader and analyzed using the Softmax Pro version 4.3 software (Molecular Devices). Cytokine concentrations in lung homogenates were normalized to total protein concentration.

ELISAs to measure allergen-specific IgE

ELISAs for OVA-specific and HDM-specific IgE were performed using a previously described protocol (14). Flat-bottom microplate strips (Immulon 4 HBX, Thermo Fisher) were coated with 100 μg/ml OVA or 100 μg/ml HDM in the $NaHCO_3$ buffer (35 mM $NaHCO_3$ pH=8.2) at 4°C overnight. The strips were then washed and blocked with the Tris-BSA buffer (100 mM Tris, 1% BSA, pH=9.6) at 37°C for 2 hours. After this step, serum samples were diluted at 1:20 ratio in the Tris-BSA-Tween buffer (100 mM Tris, 1% BSA, 0.05% Tween, pH=7.4) and incubated in the strips overnight at 4°C. Strips were washed and incubated with biotin-conjugated rat anti-mouse IgE antibody (clone R35-118, BD Biosciences) in the Tris-BSA-Tween buffer for 3 hours, followed by wash and incubation with Streptavidin-HRP (BD Biosciences) for 30 minutes at room temperature. The reaction was developed with TMB Substrate Solution (Thermo Scientific) and read at 450 nm.

Quantitative real-time polymerase chain reaction

Total RNA was isolated from Trizol (Thermo Fisher) lysates using RNeasy Plus Universal Mini Kit or RNeasy Mini Kit (both from Qiagen), and cDNA was synthesized using ImProm-II reverse transcriptase and random primers (all from Promega). cDNA was then amplified using transcript-specific primers, Absolute qPCR SYBR Green mix (Thermo Scientific) and quantified using the ABI Prism 7000 Sequence Detection System (Life Technologies). Primers were designed using the NCBI Primer BLAST tool. Levels of mRNAs were

normalized to levels of 18S ribosomal RNA using the $2^{-\Delta\Delta Ct}$ method. Sequences of transcript-specific primers are shown in Supplemental Table 1.

Mouse primary epithelial cell culture and stimulation

C57BL/6 Mouse Primary Tracheal and Bronchial Epithelial Cells were purchased from Cell Biologics, Inc. and cultured in the Complete Epithelial Cell Medium (Cell Biologics) supplemented with fetal bovine serum, L-glutamine, epidermal growth factor, antibiotic-antimycotic solution and insulin-transferrin-selenium solution (all from Cell Biologics). After cells reached confluence, they were switched to the PneumaCult™-ALI maintenance medium (STEMCELL Technologies) and incubated in this medium under submerged conditions for 48 hours. For stimulation, IL-13 (10 ng/ml, PeproTech), granzyme A (20 nM, MyBioSource), granzyme B (20 nM, Abcam), cathepsin W (20 nM, ProSpec) and an extract of Alternaria alternata (10 μg/ml, Stallergenes Greer) were added. After 24 hours of stimulation with indicated mediators, cell culture supernatants and cell lysates (Trizol) were collected for ELISA and real time PCR, respectively. To measure cell death, cells were gently harvested using the Accutase Cell Detachment Solution (BioLegend), stained with PE-labeled Annexin V and 7-AAD (both reagents from BioLegend), per manufacturer instructions, and analyzed by flow cytometry. In cell death studies, heat shock-treated samples served as positive controls. For heat shock, cells were incubated at 60^0C for 1 minute, followed by 60 min-long incubation at 37^0C. For PAR inhibition studies, epithelial cell layers were treated with SCH79797 (50 nM, Tocris), Vorapaxar (20 μM, Axon Medchem), GB83 (10 μM, Axon Medchem), ENMD-1068 (2 mM, ENZO) or vehicle (DMSO, Sigma-Aldrich). After 2 hours of incubation with inhibitors/DMSO, granzyme B (20 nM) was added and cells were incubated for additional 22 hours. After completion of the culture, epithelial cells were lysed in Trizol for IL-25 mRNA analysis.

Mouse NK cell-epithelial cell co-culture

Mouse splenic NK cells were isolated from wild type, *Gzmb*$^{-/-}$ and littermate control *Gzmb*$^{+/+}$ DEP-OVA pups on PND 28 using an isolation protocol described in the Section "Adoptive Transfer of NK Cells". Purified NK

cells were added to adherent epithelial cell layers at the ratio of five NK cells per one epithelial cell, and cell subsets were co-cultured in the mixed medium (25% PneumaCult™-ALI maintenance medium and 75% RPMI 1640) supplemented with IL-13 (10 ng/ml, PeproTech) for 48 hours. After completion of the culture, epithelial layers were washed with PBS to remove NK cells. Epithelial cells were then harvested using the Accutase Cell Detachment Solution (BioLegend), stained with anti-EpCAM, anti-NK1.1 (to exclude NK cells) and anti-IL-25 antibodies and analyzed by flow cytometry.

Human brushed bronchial epithelial cell culture and stimulation

Human brushed bronchial epithelial cells were obtained from the Human Primary Cell Culture Core at National Jewish Health, Denver, CO. Collection of the bronchial brushing samples was reviewed and approved by the Institutional Review Board at National Jewish Health, Denver, CO. Investigators in this study were blinded from donor identities. The Human Primary Cell Culture Core optimized protocols to expand and generate pure epithelial cell cultures from the brushings (Supplemental Ref #2). These cultures were capable of mucociliary differentiation when grown at the air–liquid-interface (Supplemental Ref #2). All samples used in this study came from healthy non-smokers. Cells were cultured in Completed BEGM™ Bronchial Epithelial Cell Growth Medium (Lonza). After cells reached confluence, they were cultured in PneumaCult™-ALI maintenance medium (STEMCELL Technologies) for 48 hours. For stimulation, human IL-13 (10 ng/ml, PeproTech), human granzyme B (20 nM, Enzo Life Sciences), and an extract of Alternaria alternata (10 μg/ml, Stallergenes Greer) were added. After 24 hours of stimulation, cell lysates were collected for analysis.

Human umbilical cord blood mononuclear cell culture and stimulation

Human umbilical cord blood samples were obtained from the University of Colorado Cord Blood Bank, Aurora, CO. These samples were donated for research because their small volumes precluded future clinical use. Investigators in this study were blinded from donor identities, and the studies were reviewed and approved by the Institutional Review Board at National Jewish Health, Denver, CO. Cord blood samples were processed for mononuclear cell isolation using Histopaque-1077 (Sigma-Aldrich). Mononuclear cells were stimulated with

HDM (50 μg/ml) or DEP (60 μg/ml) or treated with a vehicle (PBS) in RPMI 1640 medium supplemented with 10% FBS for 48 hours. For NK degranulation assay, cells were then incubated for 1 hour with PE-labeled anti-CD107a (clone H4A3; BioLegend) after which brefeldin A (10 μg/ml) and monensin (2 μM) were added and cells were incubated for additional 4 hours. For cytokine assay, cells were incubated with brefeldin A and monensin for 4 hours. Cells were then harvested, stained for viability (eFluor506), extracellular and intracellular markers and analyzed by flow cytometry.

Measurement of protease enzymatic activity

Enzymatic activities of recombinant proteases were determined using spectrophotometric assays. For granzyme A, granzyme B, and cathepsin W activity assays, Z-L-Lys-SBzl (Sigma-Aldrich), Boc-AAD-SBzl (SM Biochemicals, SMSB05) and Z-Phe-Arg-pNA (Enzo Life Sciences) hydrochlorides were used as substrates, respectively (Supplemental Ref #3-5). Substrates were prepared at a concentration of 1 mM. One hundred microliters of a substrate solution were then mixed with 100 μl of a working solution of a protease. To prepare working solutions of proteases, granzyme A and granzyme B were diluted in the 1 mM DTNB buffer, and cathepsin W was diluted in the buffer containing 50 mM sodium acetate, 2 mM EDTA, 10 mM DTT (all from Sigma-Aldrich). Reactions were incubated at 37℃ for 30 min. Substrate hydrolysis was determined at 405 nm as previously described (Supplemental Ref #3-5).

Supplemental References

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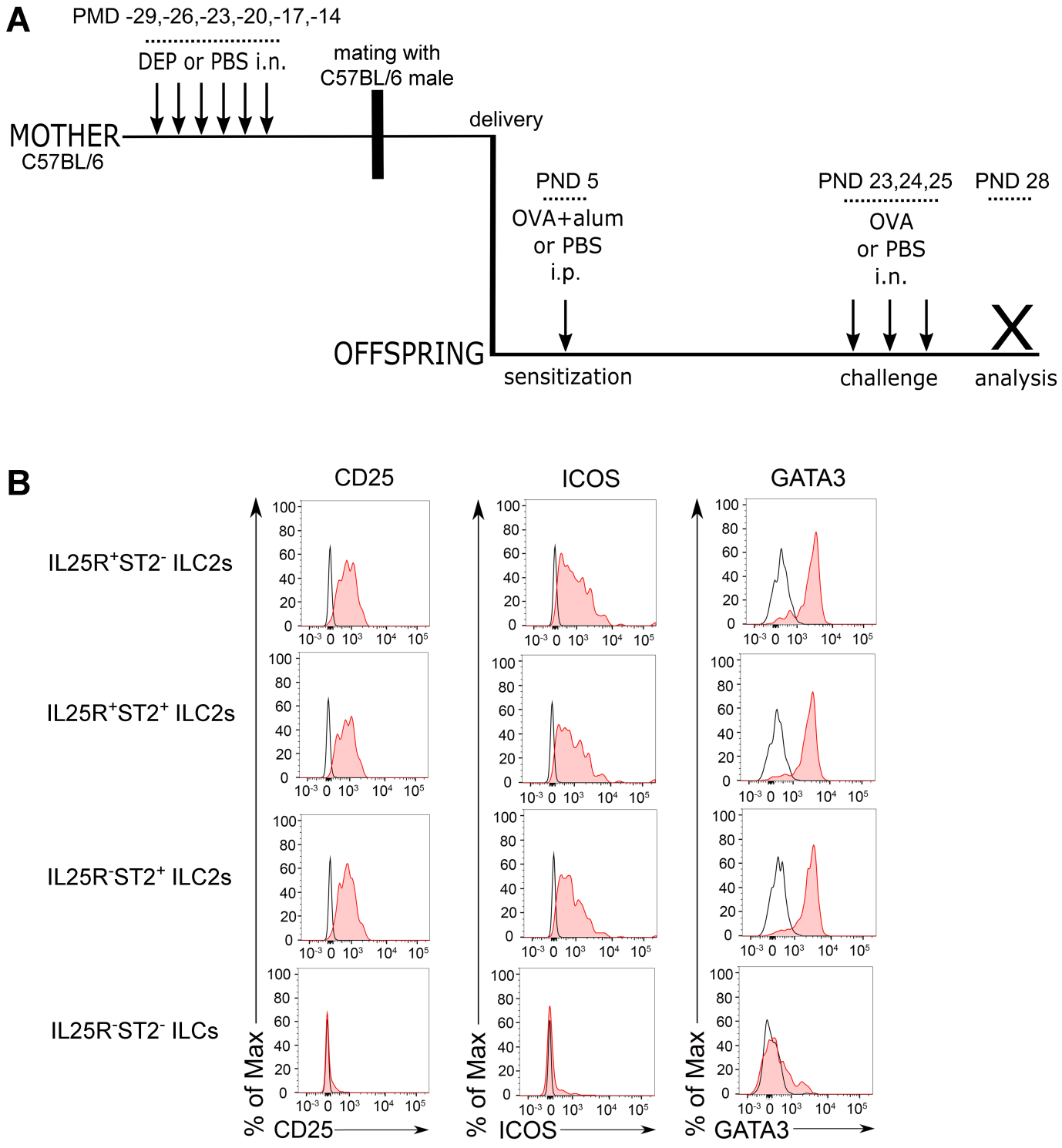
Supplemental Table 1. Primers used in this study.

Gene name	Forward Primer	Reverse Primer
Mouse *Rn18s*	CGGCGACGACCCATTCGAAC	GAATCGAACCCTGATTCCCCGT
Mouse *Angpt1*	CACACGTGGAGCCGGATTT	CATGGTGGCCGTGTGGTT
Mouse *Angpt2*	ACTACGACGACTCAGTGCAAA	AGCAAGCTGGTTCCAATCTCT
Mouse *Areg*	GCAGCTATTGGCATCGGCATC	TGGCATGCACAGTCCCGTTT
Mouse *Bmp2*	TGGAAGTGGCCCATTTAGAG	TGACGCTTTTCTCGTTTGTG
Mouse *Bpm6*	GCTGCACACTCCTTGAACC	TGAACTCTTTGTGGTGTCGTTG
Mouse *Ctsc*	GTTGGATACTGCCTACGACGA	TGATAGCTGTGTGGCCTCTG
Mouse *Ctsd*	GCACGTCCTTTGACATCCAC	TCCACCTTGATACCTCTTGCC
Mouse *Ctsw*	CTGCCCACCTCTCCTACTTTC	TGTGGGCAAAGATGCTCAGAC
Mouse *Egf*	CAGGAGGTCCGCTAGAGAAATG	ACAGCCATGACTGCACTTTGA
Mouse *Fgf9*	GCAGTCACGGACTTGGATCATT	ACCAGGCCCACTGCTATACT
Mouse *Flt3l*	GTTACTTCAGCCACAGTCCCA	GCTAGGAAGAGGCTCCACAAG
Mouse *Gata3*	CCTACCGGGTTCGGATGTAA	GCAGGCATTGCAAAGGTAGT
Mouse *Gdf15*	GCTGTCCGGATACTCAGTCC	GTCAGGCGCAGGCGTAA
Mouse *Gzma*	ACGATGAGGAACGCCTCTG	GAGTGAGGAACAACCGTGTCT
Mouse *Gzmb*	CTGCTACTGCTGACCTTGTCTC	CTTGCTGGGTCTTCTCCTGTTC
Mouse *Hdgfrp3*	CAGGAGAACGACCGGGATG	AGATAGGGTACTTGTTTGCTGGA
Mouse *Il1b*	GCTGAAAGCTCTCCACCTCA	CGTTGCTTGGTTCTCCTTGT
Mouse *Il5*	GAGGCTTCCTGTCCCTACTC	CCCACGGACAGTTTGATTCTT
Mouse *Il13*	GAGCAACATCACACAAGACCA	GATGTTGGTCAGGGAATCCAG
Mouse *Il17a*	GCCCTCAGACTACCTCAACC	CTTTCCCTCCGCATTGACAC
Mouse *Il25*	GAGCTATGAGTTGGACAGGGA	TTGTGGTAAAGTGGGACGGA
Mouse *Il33*	TCCACGGGATTCTAGGAAGAG	TATGTACTCAGGGAGGCAGGA
Mouse *Ltb*	TACACCAGATCCAGGGGTTC	ACTCATCCAAGCGCCTATGA
Mouse *Negf2*	GTGACTAAGCCCTGCACCTC	ATCTCTTGTCCCTCCCCACT
Mouse *Opn*	CTGGCTGAATTCTGAGGGACTAA	CTTCTGAGATGGGTCAGGCA
Mouse *Osm*	CCCGTCGGGCATAAAGTGG	CCATGCTCAGGATGAGGAGAC
Mouse *Plgf*	GGACGAGCATGGTGATTGTG	TGAGTTGTTCCCAGCAGACAG
Mouse *Tgfb1*	AATGGTGGACCGCAACAAC	CTTCCCGAATGTCTGACGTATT
Mouse *Tnfsf10*	AGGTGGAAGACCTCAGAAAGTG	GAGCACGTGGTTGAGAAATGAA
Mouse *Tnfsf14*	ACGATCTCACCAGGCCAAC	ACATAGTAGTAACCGGGCTCCA
Mouse *Tslp*	TTTGCCCGGAGAACAAGAGA	TGGACTTCTTGTGCCATTTCC
Mouse *Vegfa*	CCTGGCTTTACTGCTGTACCT	TGTCCACCAGGGTCTCAATC
Mouse *Vegfb*	CCCAGCTGACATCATCCATCC	TGTCACTTTCGCGGCTTCC
Mouse *Vegfc*	TGTCTCTGGCGTGTTCCCT	ATCAGCTCATCTACGCTGGAC
Mouse *Xdh*	TGACGAGGACAACGGTAGATG	CTGAAGGCGGTCATACTTGGA
Human *RNA18SN1*	GGCCCTGTAATTGGAATGAGTC	CCAAGATCCAACTACGAGCTT
Human *IL25*	CCCTGGAGATATGAGTTGGAC	GTCTGGTTGTGGTAGAGAAG
Human *IL33*	GTGACGGTGTTGATGGTAAGA	CTCCACAGAGTGTTCCTTGTT
Human *TSLP*	ACCTTCAATCCCACCGCCGGC	GGCAGCCTTAGTTTTCATGGCGA

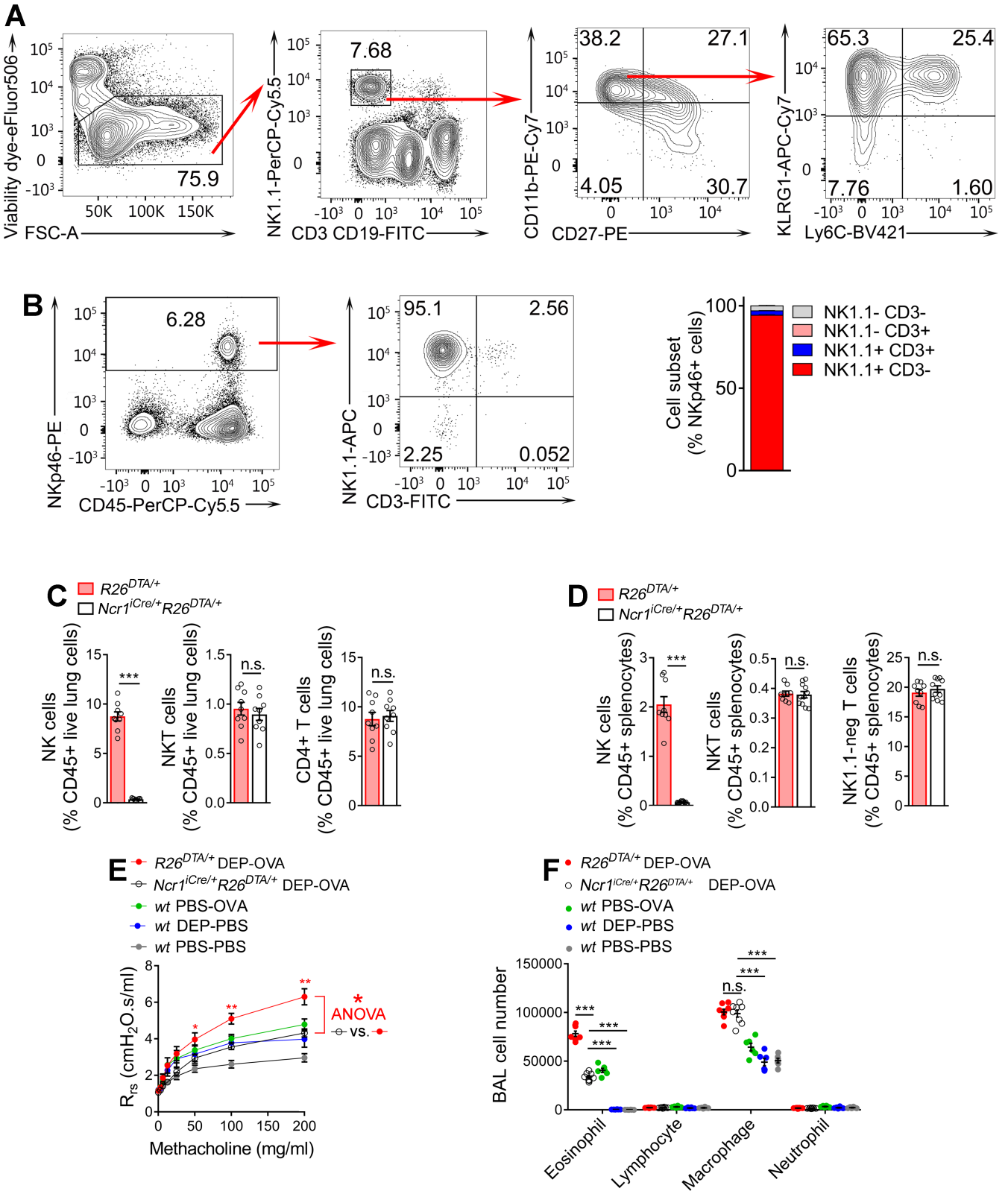
Supplemental figures and figure legends



Supplemental Figure 1: Mouse model of preconceptionally-programmed allergic airway disease (AAD) with OVA as an allergen.

(A) Mouse model of preconceptionally-programmed AAD with OVA as an allergen. C57BL/6 females were intranasally (i.n.) exposed to DEP or PBS on indicated pre-mating (PMD) days. Offspring were

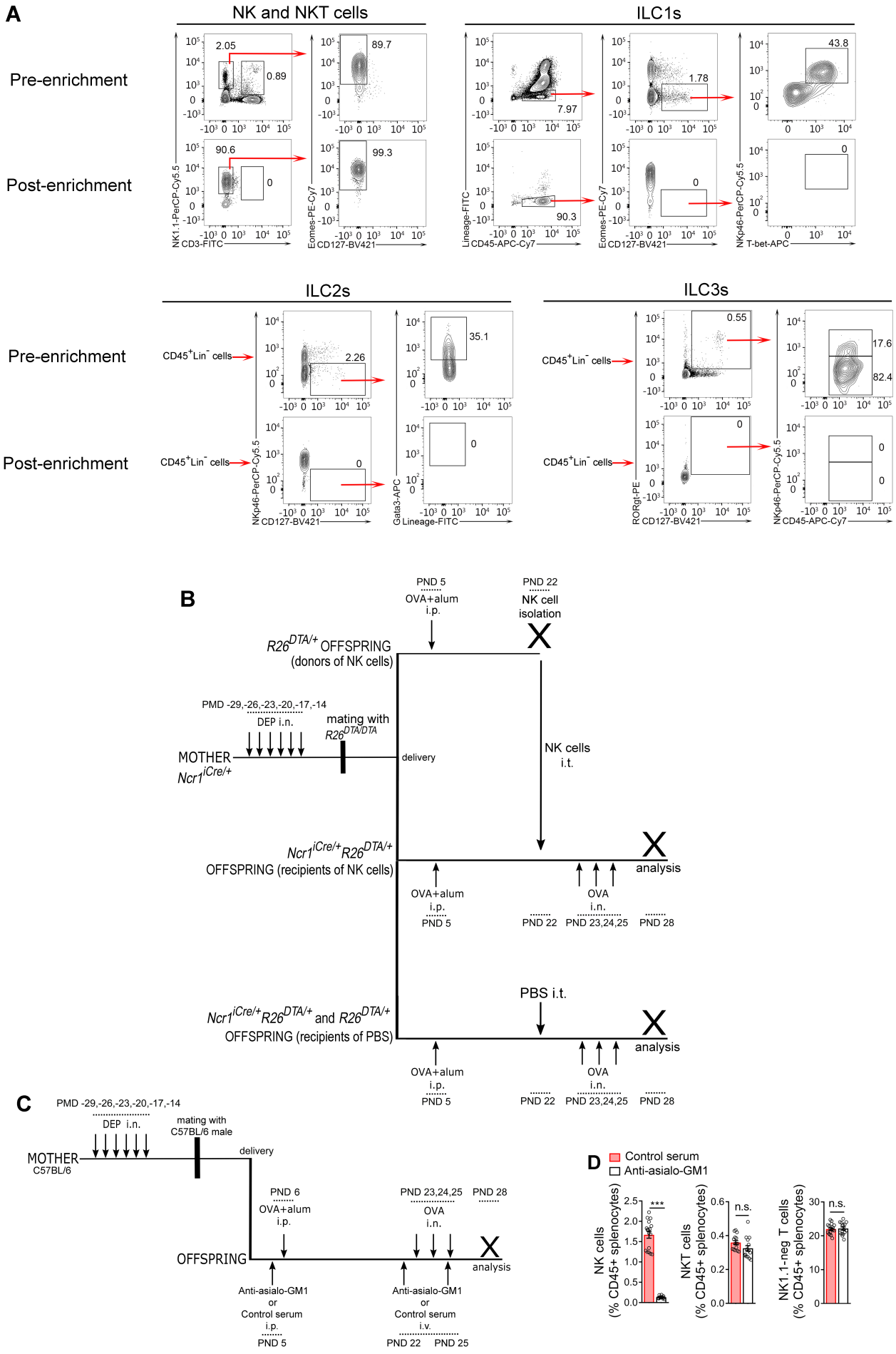
intraperitoneally (i.p.) injected with OVA in alum or PBS, and then, i.n. challenged with OVA or PBS on indicated postnatal days (PND). Pups were analyzed 72 hours after the final i.n. challenge (PND 28). (**B**) Expression of CD25, ICOS and GATA3 by $CD45^+Lin^-CD127^+IL25R^+ST2^-$ cells ($IL25R^+ST2^-$ ILC2s), $CD45^+Lin^-CD127^+IL25R^+ST2^+$ cells ($IL25R^+ST2^+$ ILC2s), $CD45^+Lin^-CD127^+IL25R^-ST2^+$ cells ($IL25R^-ST2^+$ ILC2s) and $CD45^+Lin^-CD127^+IL25R^-ST2^-$ cells ($IL25R^-ST2^-$ ILCs). White histograms show isotype control-stained cells, red histograms show cells stained with an antibody against an ILC2 marker (CD25, ICOS or GATA3). Data are representative of 3 independent experiments.



Supplemental Figure 2: Identification and genetic depletion of NK cells.

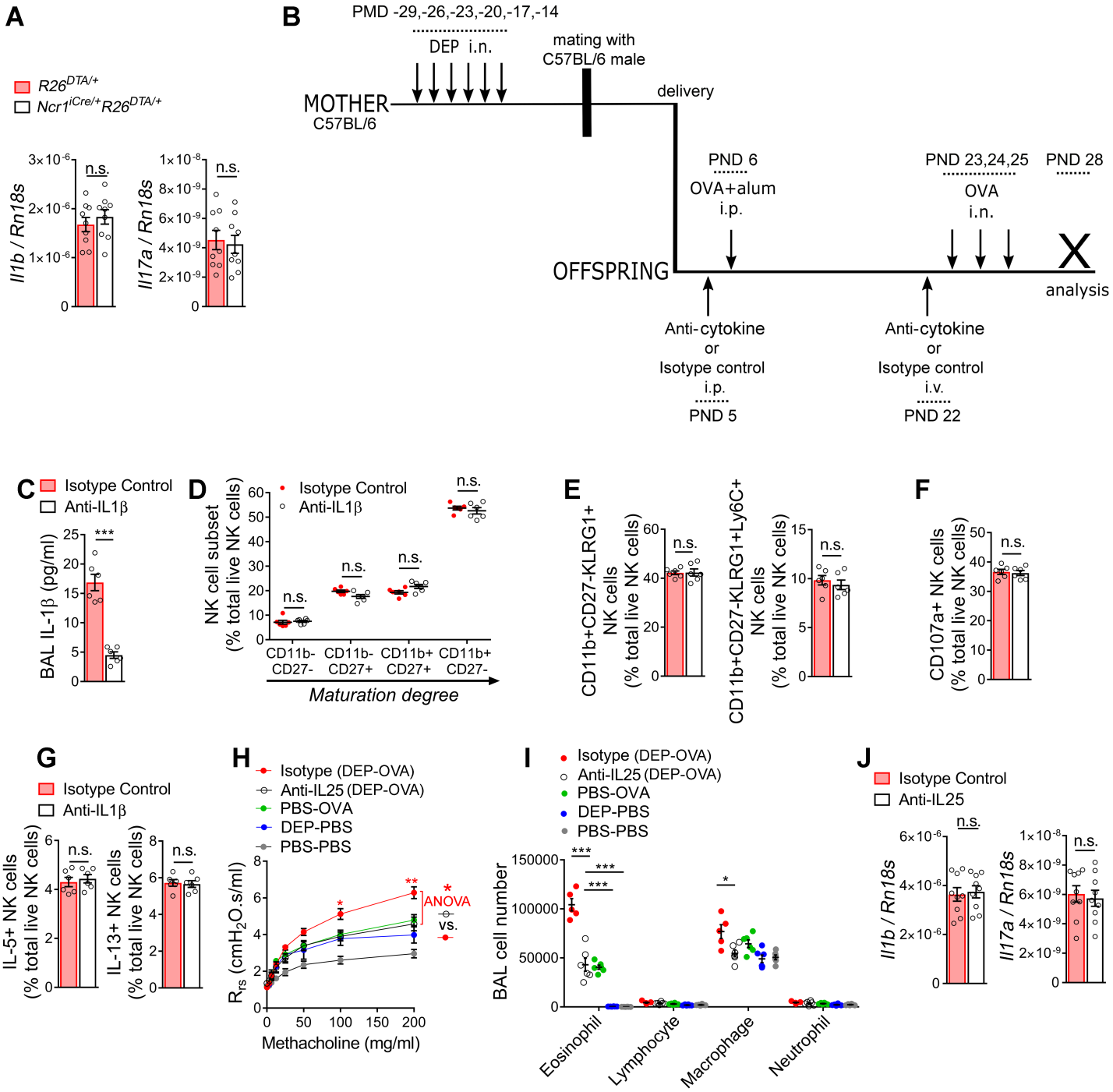
(A) Gating strategy to quantify pulmonary NK cells and their subsets. After exclusion of debris and doublets, single lung cells (singlets) were analyzed for presence of live cells (eFluor506$^-$). Live lung cells were analyzed

for expression of NK1.1 and CD3/CD19. $CD3^-CD19^-NK1.1^+$ cells were analyzed for expression of CD11b and CD27. $CD3^-CD19^-NK1.1^+CD11b^+CD27^-$ cells were analyzed for expression of KLRG1 and Ly6C. **(B**) Left: Gating strategy to identify pulmonary cell subsets that express NKp46. After exclusion of debris, doublets and dead ($eFluor506^+$) cells, live ($eFluor506^-$) lung cells were analyzed for expression of NKp46 vs. CD45. $NKp46^+$ cells were further analyzed for expression of NK1.1 and CD3. Right: The graph summarizes proportions of indicated cell subsets within a population of pulmonary $NKp46^+$ cells. n=4 mice per group. (**C**) Percentages of NK cells ($CD3^-NK1.1^+$), NKT cells ($CD3^+NK1.1^+$), and CD4 T cells ($CD3^+CD4^+$) in live ($eFluor\ 506^-$) $CD45^+$ lung cells from DEP-OVA $Ncr1^{iCre/+}R26^{DTA/+}$ and $R26^{DTA/+}$ littermates. n=9. (**D**) Percentages of NK cells ($CD3^-NK1.1^+$), NKT cells ($CD3^+NK1.1^+$), and NK1.1-negative/neg T cells ($CD3^+NK1.1^-$) in $CD45^+$ splenocytes from DEP-OVA $Ncr1^{iCre/+}R26^{DTA/+}$ and $R26^{DTA/+}$ littermates. n=9. (**E, F**) Total lung resistance to methacholine (E) and leukocyte subset counts in the BAL fluid (F) in DEP-OVA $Ncr1^{iCre/+}R26^{DTA/+}$ pups, DEP-OVA $R26^{DTA/+}$ pups, wild type (wt) PBS-OVA pups, wt DEP-PBS pups and wt PBS-PBS pups. Data are representative of 2 independent experiments. Results are presented as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, by 2-tailed unpaired t test (C, D), 2-way repeated measures ANOVA with Bonferroni post-hoc test (E) and 1-way ANOVA with Tukey's post-hoc test (F). n.s. not significant


A
NK and NKT cells
ILC1s
Pre-enrichment
Post-enrichment
2.05
0.89
89.7
90.6
0
99.3
NK1.1-PerCP-Cy5.5
CD3-FITC
Eomes-PE-Cy7
CD127-BV421
7.97
1.78
43.8
90.3
0
Lineage-FITC
CD45-APC-Cy7
T-bet-APC
ILC2s
ILC3s
CD45+Lin- cells
2.26
35.1
0.55
17.6
82.4
Gata3-APC
RORgt-PE
B
PND 5
OVA+alum
i.p.
PND 22
NK cell
isolation
R26DTA/+ OFFSPRING
(donors of NK cells)
PMD -29,-26,-23,-20,-17,-14
DEP i.n.
mating with
R26DTA/DTA
MOTHER
Ncr1iCre/+
delivery
NK cells
i.t.
Ncr1iCre/+R26DTA/+
OFFSPRING (recipients of NK cells)
analysis
OVA+alum
i.p.
PND 5
PND 22
OVA
i.n.
PND 23,24,25
PND 28
PBS i.t.
Ncr1iCre/+R26DTA/+ and R26DTA/+
OFFSPRING (recipients of PBS)
C
PMD -29,-26,-23,-20,-17,-14
DEP i.n.
mating with
C57BL/6 male
MOTHER
C57BL/6
delivery
PND 6
OVA+alum
i.p.
PND 23,24,25
PND 28
OVA
i.n.
OFFSPRING
analysis
Anti-asialo-GM1
or
Control serum
i.p.
PND 5
Anti-asialo-GM1
or
Control serum
i.v.
PND 22
PND 25
D
Control serum
Anti-asialo-GM1
NK cells
(% CD45+ splenocytes)
NKT cells
(% CD45+ splenocytes)
NK1.1-neg T cells
(% CD45+ splenocytes)
***
n.s.
n.s.

Supplemental Figure 3: NK cell reconstitution in *Ncr1$^{iCre/+}$R26$^{DTA/+}$* pups and NK cell depletion using anti-asialo-GM1.

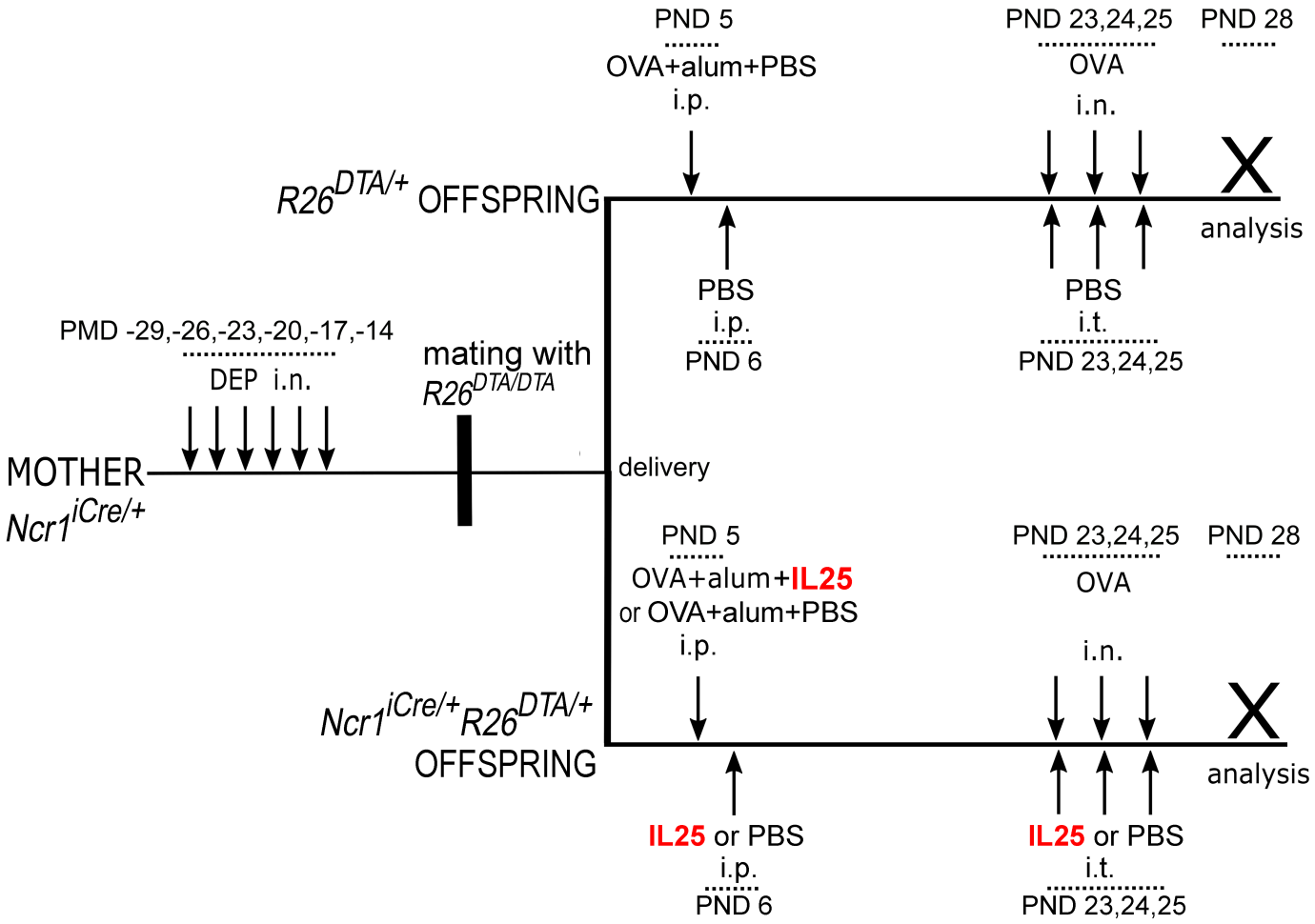
(**A**) Purity check of magnetically-sorted CD127$^-$NK cells. Unfractioned splenocytes (pre-enrichment) and magnetically-sorted live CD127$^-$ NK cells (post-enrichment) were analyzed by flow cytometry. CD127$^-$ NK cells were detected by gating live singlets on NK1.1$^+$CD3$^-$ cells and then on Eomes$^+$CD127$^-$ cells. NKT cells were defined as NK1.1$^+$CD3$^+$ cells. To detect ILCs, live singlets were first analyzed for CD45 and Lineage/Lin markers (CD3, CD19, TCRβ, F4/80, Ly6G, FcεRIα). To detect ILC1s (CD45$^+$Lin$^-$CD127$^+$Eomes$^-$T-bet$^+$NKp46$^+$), CD45$^+$Lin$^-$ cells were gated on CD127$^+$Eomes$^-$ cells and then on T-bet$^+$NKp46$^+$ cells. To detect ILC2s (CD45$^+$Lin$^-$CD127$^+$NKp46$^-$GATA3$^+$), CD45$^+$Lin$^-$ cells were gated on CD127$^+$NKp46$^-$ cells and then on GATA3$^+$ cells. To detect ILC3s (CD45$^+$Lin$^-$CD127$^+$RORγt$^+$) and their NKp46$^+$ subset, CD45$^+$Lin$^-$ cells were gated on CD127$^+$RORγt$^+$ cells and then on NKp46$^+$ cells. (**B**) NK cell reconstitution. *Ncr1$^{iCre/+}$R26$^{DTA/+}$* and *R26$^{DTA/+}$* pups of DEP-exposed *Ncr1$^{iCre/+}$* females and unexposed *R26$^{DTA/DTA}$* males were immunized i.p. with OVA/alum on PND 5. On PND 22, splenic CD127$^-$ NK cells from a sub-group of *R26$^{DTA/+}$* pups were intratracheally (i.t.) transferred into a sub-group of *Ncr1$^{iCre/+}$R26$^{DTA/+}$* littermates. Other pups received PBS. NK cell/PBS recipients were challenged i.n. with OVA on PND 23-25 and analyzed on PND 28. (**C**) Antibody-mediated depletion of NK cells. Pups of DEP-exposed females were immunized with OVA/alum on PND 6 and OVA-challenged on PND 23-25. Pups were injected with anti-asialo-GM1 or control serum on PND 5 (i.p.), PND 22 (i.v.) and PND 26 (i.v.), and analyzed on PND 28. (**D**) Percentages of NK cells (CD3$^-$NK1.1$^+$), NKT cells (CD3$^+$NK1.1$^+$), and NK1.1-negative/neg T cells (CD3$^+$NK1.1$^-$) in CD45$^+$ splenocytes from wt DEP-OVA pups injected indicated sera. n=15. Data are representative of three independent experiments (A) or are pooled from three independent experiments (D). Results are presented as mean ± SEM. *** P <0.001, by 2-tailed unpaired t test.



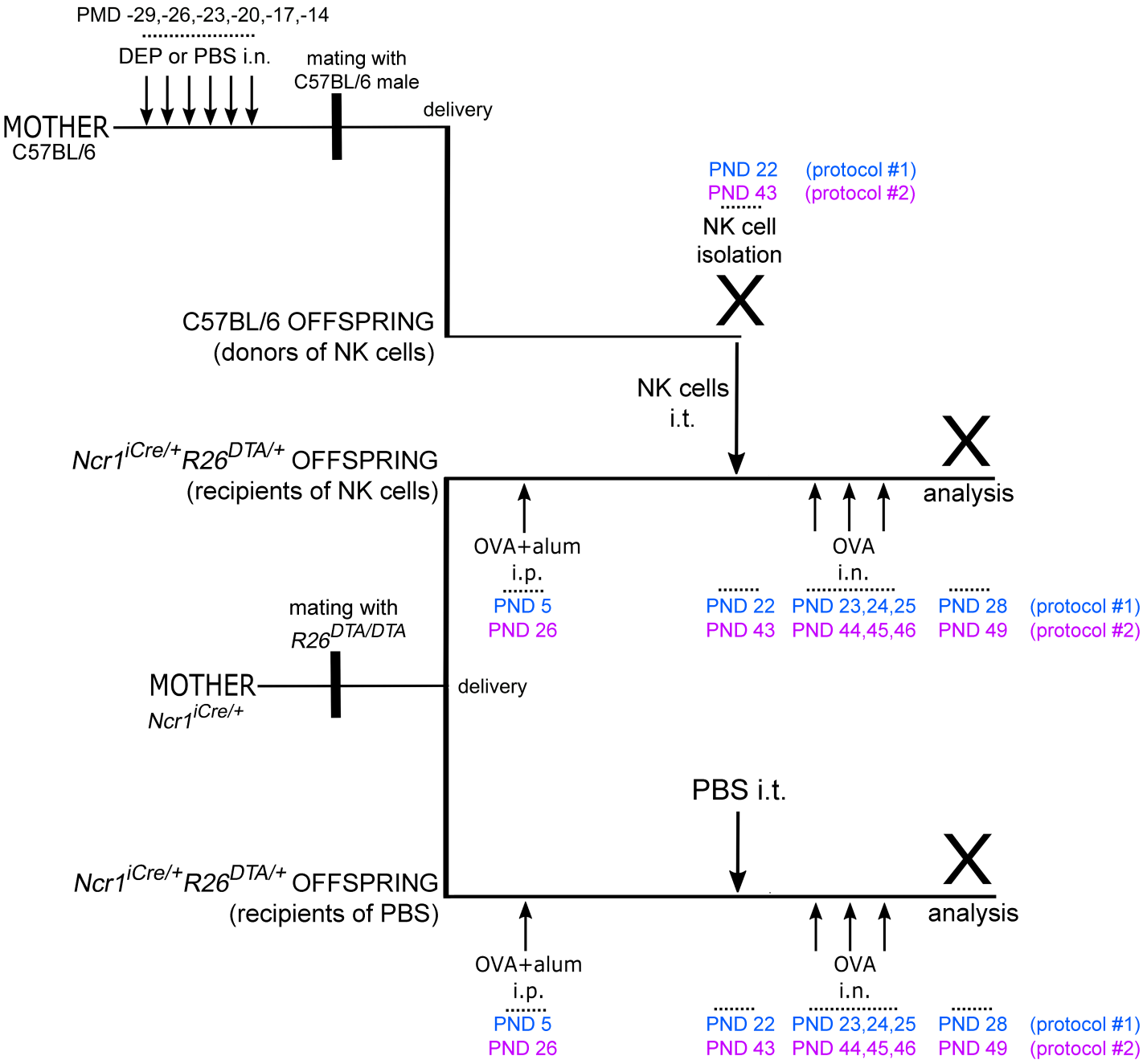
Supplemental Figure 4: Effects of NK cell depletion on cytokines and effects of cytokine depletion.

(A) Levels of *Il1b* and *Il17a* mRNAs in lungs of DEP-OVA *Ncr1*$^{iCre/+}$*R26*$^{DTA/+}$ and *R26*$^{DTA/+}$ littermates. n=9 mice per group. (**B**) Experimental strategy to deplete a cytokine (IL-1β, IL-25, IL-33 or TSLP) in DEP-OVA pups. On PND 5, neonates of DEP-exposed females were i.p. injected with an anti-cytokine antibody or isotype control IgG. On PND 6, pups were immunized with OVA in alum. Antibody/IgG injections were then repeated

on PND 22 when reagents were delivered intravenously (i.v.). All pups were i.n. challenged with OVA on PND 23, 24 and 25, and analyzed on PND 28. (**C-G**) Analysis of DEP-OVA pups receiving an anti-IL-1β antibody or isotype control IgG. (**C**) Concentration of IL-1β in the BAL fluid. n=6. (**D, E**) Percentages of indicated NK subsets in live lung cells. n=6. (**F**) Percentage of $CD107a^+$ (degranulated) NK cells in live lung NK cells. n=6. (**G**) Percentages of $IL\text{-}5^+$ and $IL\text{-}13^+$ NK cells in live lung NK cells. n=6. (**H, I**) Total lung resistance to methacholine (H) and leukocyte subset counts in the BAL fluid (I) in DEP-OVA pups receiving an anti-IL-25 antibody, DEP-OVA pups receiving isotype control IgG, PBS-OVA pups, DEP-PBS pups and PBS-PBS pups. (**J**) Levels of *Il1b* and *Il17a* mRNAs in lungs of DEP-OVA pups receiving an anti-IL-25 antibody or isotype control IgG. n=9. Data are representative of 3 independent experiments. Results are presented as mean ± SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, by 2-tailed unpaired t test (A, C-G, J), 2-way repeated measures ANOVA with Bonferroni post-hoc test (H) and 1-way ANOVA with Tukey's post-hoc test (I). n.s. not significant



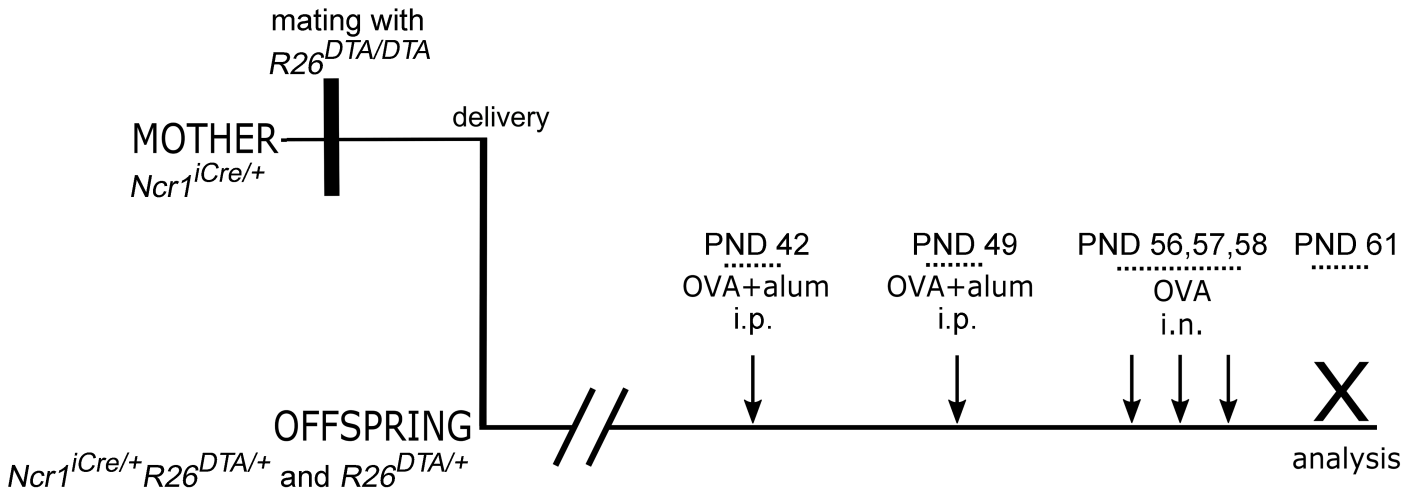
Supplemental Figure 5: Experimental strategy to reconstitute IL-25 in DEP-OVA *Ncr1*$^{iCre/+}$*R26*$^{DTA/+}$ pups. *Ncr1*$^{iCre/+}$*R26*$^{DTA/+}$ and *R26*$^{DTA/+}$ littermates were produced as in Supplemental Figure 3B. On PND 5, pups were i.p. injected with OVA/alum mixed with IL-25 or PBS. IL-25/PBS injections were repeated on PND 6 but this time no OVA/alum was used. All pups were i.n. challenged with OVA on days 23-25. Six hours after each challenge, pups were i.t. administered with IL-25 or PBS. Pups were analyzed on day 28.



Supplemental Figure 6: Experimental strategy to study transmission of predisposition to AAD by NK cells.

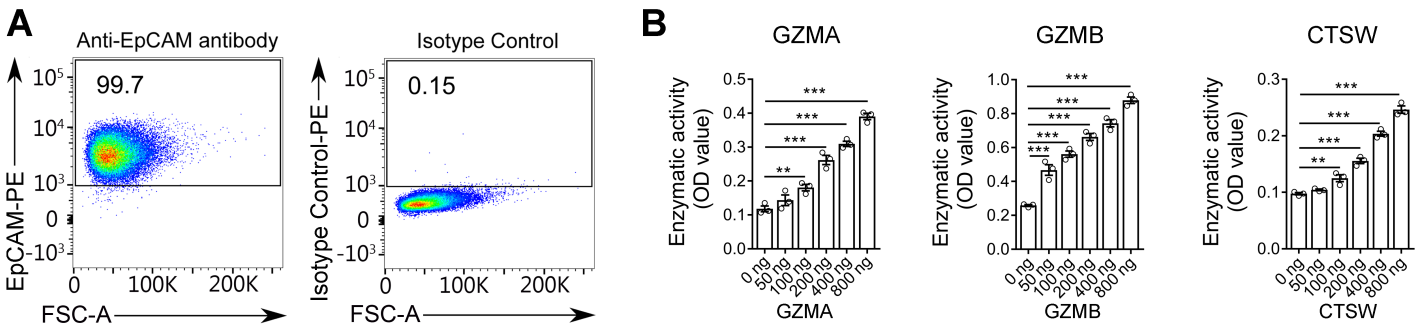
Transfer of NK cells from wild type pups of DEP and PBS exposed mothers into *Ncr1*$^{iCre/+}$*R26*$^{DTA/+}$ pups of unexposed mothers. To generate donor pups, wild type C57BL/6 female mice were exposed to DEP or PBS and mated with unexposed wild type C57BL/6 males. To generate recipient pups, unexposed *Ncr1*$^{iCre/+}$ females were mated with unexposed *R26*$^{DTA/DTA}$ males. Prospective recipient pups were immunized with OVA/alum on

postnatal day (PND) 5 (protocol #1) or PND 26 (protocol #2). Prospective donors were not immunized. Intratracheal (i.t.) transfer of $CD127^-$ NK cells or i.t. injection of PBS took place on PND 22 (protocol #1) or PND 43 (protocol #2), as in Supplemental Figure 3B. Recipients were intranasally (i.n.) challenged with OVA on PND 23-25 (protocol #1) or PND 44-46 (protocol #2) and analyzed on PND 28 (protocol #1) or PND 49 (protocol #2).



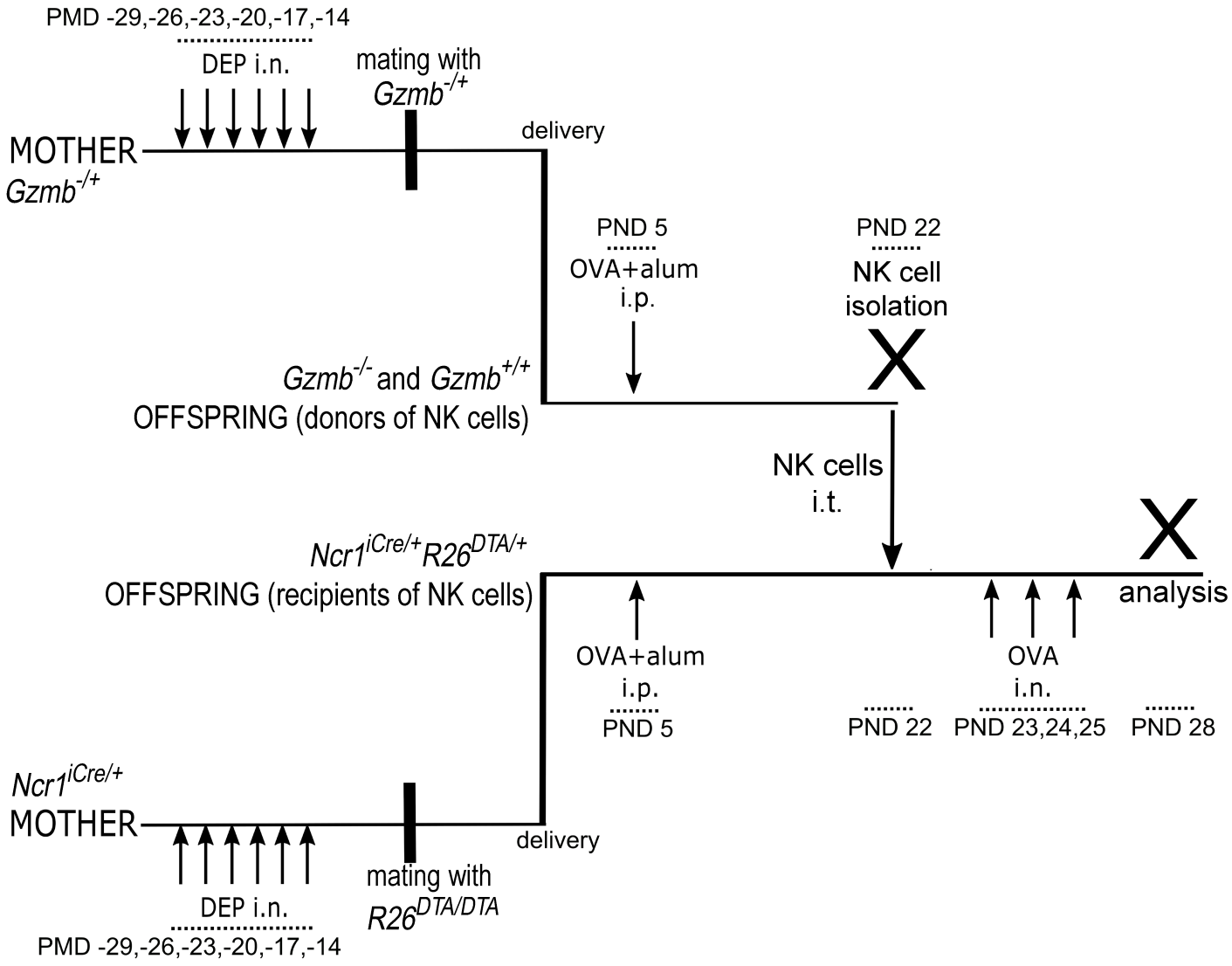
Supplemental Figure 7: Experimental strategy to study roles of NK cells in AAD in normal adult mice.

Unexposed *Ncr1*$^{iCre/+}$ females were mated with unexposed *R26*$^{DTA/DTA}$ males to generate *Ncr1*$^{iCre/+}$*R26*$^{DTA/+}$ and *R26*$^{DTA/+}$ littermates. On postnatal days (PND) 42 and 49 (i.e. at six and seven weeks of age, respectively) littermates were intraperitoneally (i.p.) injected with OVA in alum. Mice were then intranasally (i.n.) challenged with OVA on PND 56, 57 and 58. Mice were analyzed on PND 61.



Supplemental Figure 8: Purity of epithelial cell cultures and enzymatic activities of recombinant granzyme A, granzyme B and cathepsin W.

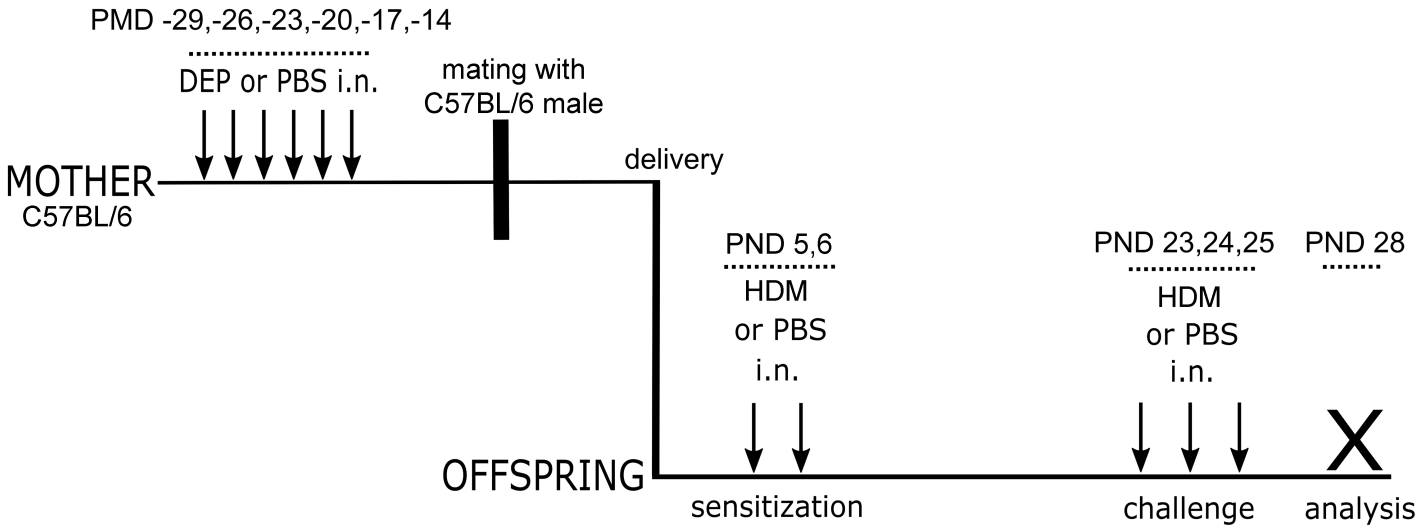
(A) Flow cytometric analysis of C57BL/6 Mouse Primary Tracheal and Bronchial Epithelial Cells (Cell Biologics, Inc.) using an antibody against the epithelial cell marker EpCAM and an isotype control IgG. The experiment indicates that epithelial cells are highly (99.7%) pure. (**B**) Enzymatic activities of recombinant granzyme A, granzyme B, and cathepsin W were measured using specific substrates (Z-L-Lys-SBzl, Boc-AAD-SBzl and Z-Phe-Arg-pNA, respectively) in spectrophotometric assays. Substrates were incubated with a vehicle (0 ng) or increasing amounts of recombinant enzymes in 200 µl of reaction buffer. Substrate hydrolysis was indicated by an increase of optical density (OD) at 405 nm. n=3 wells for each concentration of an enzyme. Results are representative of 2 (A, B) independent experiments. Error bars represent SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, by 1-way ANOVA with Dunnett's post-hoc test



Supplemental Figure 9: Schematic illustration of the NK cell transfer experiment to study importance of NK cell-expressed granzyme B.

To generate donor pups, *Gzmb*$^{+/-}$ female mice were exposed to DEP and mated with unexposed *Gzmb*$^{+/-}$ males. *Gzmb*$^{+/+}$ and *Gzmb*$^{-/-}$ littermate pups were immunized on postnatal day (PND) 5, and euthanized on PND 22 for CD127^{-} NK cell isolation. To generate recipient pups, *Ncr1*$^{iCre/+}$ female mice were exposed to DEP and mated with unexposed *R26*$^{DTA/DTA}$ males. NK cell-deficient *Ncr1*$^{iCre/+}$*R26*$^{DTA/+}$ pups were immunized on PND 5 and then intratracheally transferred with CD127^{-} NK cells from age-matched *Gzmb*$^{+/+}$ or *Gzmb*$^{-/-}$ donor pups on PND

22. After cell transfer, recipients were challenged with OVA on PND 23-25, and analyzed on PND 28. PMD, pre-mating day; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal



Supplemental Figure 10: Mouse model of preconceptionally-programmed AAD with HDM as an allergen.

C57BL/6 females were intranasally (i.n.) exposed to DEP or PBS on indicated pre-mating (PMD) days. Offspring received the house dust mite extract (HDM) intranasally on postnatal days 5 and 6 (sensitization phase) and then on postnatal (PND) days 23, 24 and 25 (challenge phase). Pups were analyzed on PND 28.