

## **SUPPLEMENTAL MATERIAL**

### **ET<sub>B</sub> Receptor Deficiency Amplifies Allergic Airway Inflammation and Hyperresponsiveness**

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## Materials and Methods

### Mice

Animal procedures were ethically approved by institutional authorities (Charité - Universitätsmedizin Berlin) and the Local State Office of Health and Social Affairs Berlin (LAGeSo; Berlin, Germany). Experiments were in accordance with the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations for the care and use of laboratory animals, which is equivalent to American ARRIVE. Generation of rescued ET<sub>B</sub>-deficient mice [1] and mice overexpressing human prepro-ET-1 (<sub>pre</sub>ET<sup>tg</sup>) [2] has been described before. ET<sub>B</sub>-deficient mice were rescued from lethal inherited Hirschsprung disease by carrying a dopamine-β-hydroxylase ET<sub>B</sub> transgene [1]. Rescued ET<sub>B</sub><sup>-/-</sup> were on a mixed C57BL/6/129 background, <sub>pre</sub>ET<sup>tg</sup> were on a mixed NMRI/C57BL/6 background. Female transgenic mice and the corresponding wild-type (WT) mice were housed under specific pathogen-free conditions with free access to food and water.

### Lung Histology

Lungs were immersion fixed with buffered formaldehyde solution (4%; pH 7.0; Merck KGaA) for 24 h. Automated paraffin embedding was performed (SLEE medical GmbH, Nieder-Olm, Germany). Lungs were sliced with a microtome (HM 355S, Thermo Fisher Scientific GmbH, Dreieich, Germany) and 5-μm lung sections were mounted onto glass slides. Tissues were stained with hematoxylin and eosin (H&E) or Masson-Goldner trichrome and microscopically studied (Axiophot; Carl Zeiss Microscopy GmbH, Jena, Germany) [3]. The anatomist was blinded to the study groups. Digital images were taken (Color View II camera, CellSens software; Olympus Europa SE Co. KG, Hamburg, Germany) [4]. ET<sub>B</sub> expression was analyzed via immunofluorescence microscopy. Formalin-fixed and paraffin-embedded 5-μm lung sections were deparaffinized and rehydrated. After antigen-retrieval, sections were blocked and incubated with anti-ET<sub>B</sub> (ab117529, Abcam) and anti-SMA (MCA5781GA, Bio-Rad) at 1:200 dilution, followed by secondary anti-rabbit Alexa Fluor 555 (A31572, Invitrogen) and anti-mouse Alexa Fluor mouse 647 (A211235, Invitrogen) at a 1:500 dilution. Stained sections were washed in PBS and mounted with Fluoromount-G™ (ThermoFischer). The increased autofluorescence of lung tissue was utilized to visualize lung parenchyma at 488 nm. Fluorescence was visualized using a Nikon Scanning Confocal A1Rsi+ microscope with a 60x PlanApo Oil immersion objective.

### Isolated perfused and ventilated mouse lung

Mice were anesthetized and lungs isolated as described previously [5–7]. Briefly, following cannulation of pulmonary artery and left atrium, pulmonary perfusion with sterile and carbonated Krebs-Henseleit hydroxyethyl amylopectin buffer (37°C, Serag-Wiessner, Naila, Germany) was initiated in a continuous nonrecirculating manner (1 mL/min) with left atrial pressure set at 2.2 cmH<sub>2</sub>O. Lungs were ventilated (90 breaths/min) in a closed chamber to enable negative pressure ventilation (P<sub>exp</sub> -4.5, P<sub>ins</sub> -9.0 cmH<sub>2</sub>O). Airway resistance (res<sub>aw</sub>) was constantly assessed (Pulmodyn, HSE-Harvard Apparatus, March-Hugstetten, Germany)

[4, 8, 9]. To analyze airway responsiveness, defined doses of the broncho-constrictive stimulus methacholine (MCh), serotonin, thromboxane analog U46619, or ET-1 (all Merck KGaA, Darmstadt, Germany) was added to the perfusate for 0.5 (MCh, serotonin), 3 (U46619) or 10 (ET-1) min each at 8 (MCh, serotonin), 12 (U46619) or 24 (ET-1) min intervals. Alternatively, MCh was nebulized (Pari LL nebulizer, HSE-Harvard Apparatus) in increasing doses for 1 min each at 8 min intervals [7]. Selective ET<sub>A</sub> antagonist BQ-123 (8 µmol/L; Merck KGaA) or solvent (*aqua dest.*) was administered to the perfusate 10 min before ET-1 administration to study ET<sub>A</sub>-mediated airway constriction [4]. The maximal broncho-constrictive response to each dose was shown as factor of basal airway resistance (fold res<sub>aw</sub>) [10]. Data were not included in further analyses if lungs showed signs of atelectasis, hemostasis, or edema.

### **Allergic airway inflammation and BAL**

On days 0 and 14, mice were systemically sensitized to ovalbumin (OVA; grade V; Merck KGaA) by intraperitoneal (i.p.) injections of 20 µg OVA dissolved in 10 µL of phosphate-buffered saline (PBS) and 100 µL aluminum hydroxide suspension (1.3%; SERVA Electrophoresis GmbH, Heidelberg, Germany) as described previously [4]. Sham-sensitization was performed via i.p. injections of 10 µL of PBS and 100 µL aluminum hydroxide suspension. On days 28–30, repeated airway exposure to aerosolized OVA (1%; dissolved in PBS; 20 min/day) or PBS (sham-challenge) was performed [7, 11]. Synergistic effects of the respective transgene and allergic airway inflammation were assessed on day 31 (preET<sup>tg</sup>) or 32 (ET<sub>B</sub><sup>-/-</sup>). Bronchoalveolar lavage (BAL) was performed two times in a row. Each time, the right lung was lavaged with 650 µL of ice-cold buffer solution composed of PBS and protease inhibitor cOmplete™ mini (Merck KGaA). Total cell numbers were assessed microscopically as described previously [4, 12, 13]. Total protein (DC protein assay) and cytokines from BAL fluid supernatants were quantified (Bio-Rad; Hercules, California, USA).

### **Humoral immune response**

Plasma levels of murine OVA-specific immunoglobulin (Ig)E and OVA-specific IgG<sub>1</sub> were quantified by ELISA as described [13–15]. For IgG<sub>1</sub> measurements, plates were coated with OVA (Grad V; 5 µg/ml; Merck KGaA, Darmstadt, Germany) and blocked with milk powder (3%; Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Monoclonal anti-OVA (clone OVA-14, mouse IgG<sub>1</sub> isotype; Merck KGaA) served as standard. For detection, biotin-conjugated rat-anti-mouse IgG<sub>1</sub> (clone A85-1; Becton Dickinson GmbH, Heidelberg, Germany) and streptavidin-horseradish-peroxidase (R&D Systems, Inc., Minneapolis, USA) were used. For assessment of OVA-specific IgE plasma levels, plates were coated with rat-anti-mouse IgE-antibody (clone R35-72; Becton Dickinson GmbH) and blocked with bovine serum albumin (3%; Merck KGaA). For detection, biotinylated-OVA and streptavidin-horseradish-peroxidase (R&D Systems, Inc.) were used. Measurements were performed using microtiter plate reader HT-2 and WinRead Anthos Software (both anthos Mikrosysteme GmbH, Friesoythe, Germany). OVA-specific IgE titers were related to pooled standards originated in the

laboratory (lab units/mL) [16]. Total IgE was quantified via ELISA according to the manufacturer's guide.

### **Quantitative real-time reverse transcription PCR**

Lung tissue homogenization, RNA extraction, total RNA reverse transcription (RT), gene expression analyses via quantitative RT-PCR and assessment of relative quantities via comparative  $C_t$  method (relative gene expression set to 1 in sham-treated PBS/PBS WT controls) were performed as described [4, 9, 17]. TaqMan assay IDs (ThermoFischer Scientific, Hennigsdorf, Germany) were Mm01281449\_m1 (vascular endothelial growth factor A, *Vegfa*), Mm00432686\_m1 (colony stimulating factor 1, *Csf1*), Mm00443260\_g1 (tumor necrosis factor alpha, *Tnf*), Mm00432989\_m1 (*ET<sub>B</sub>*). Primer sequences for the housekeeping gene *Gapdh* were TGTGTCCGTCGTGGATCTGA (forward, 5' to 3'), CCTGCTTCACCACTTCTTGA (reverse, 5' to 3'), and CCGCCTGGAGAAACCTGCCAAGTATG (probe, 5'-FAM to 3'-TAMRA) [4, 17].

### **ET-1 lung tissue concentration**

ET-1 was extracted and quantified from tissue as described previously [18]. Briefly, frozen lung tissues were homogenized and centrifuged. ET-1 levels were assessed from supernatants via ELISA (Biomedica, Vienna, Austria).

## Supplemental References

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