ET_B receptor deficiency amplifies allergic airway inflammation and hyperresponsiveness

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Shareable abstract (@ERSpublications)
Endothelin-1 is crucial in asthma, interacting with ET_A and ET_B receptors. This study shows that ET_B receptors protect against allergen sensitisation, airway inflammation and hyperresponsiveness in mice, offering new therapeutic strategies for asthma. https://bit.ly/4kHNWQr

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Abstract

Background Endothelin-1 (ET-1) is a proinflammatory mediator that plays a crucial role in regulating airway tone by activating G protein-coupled endothelin receptors A (ET_A) and B (ET_B). The endothelin system has been linked to asthma, but the impact of ET_B receptor deficiency on allergic airway inflammation remains uncharted. This study explores how the endothelin system influences allergic airway inflammation and hyperresponsiveness.

Methods We used rescued ET_B receptor-deficient ($ET_B^{-/-}$) mice to obviate lethal inherited Hirschsprung disease, prepro-ET-1 overexpressing ($_{pre}ET^{tg}$), and wild-type (WT) mice. Basal airway resistance and responsiveness to broncho-constrictive stimuli were assessed in isolated, perfused and ventilated lungs of naïve mice. Additionally, we analysed the humoral immune response and airway hyperresponsiveness following induction of type 2 airway inflammation induced by systemic ovalbumin (OVA) sensitisation and repeated airway challenge with aerosolised OVA.

Results Naïve $ET_B^{-/-}$ mice exhibited significantly heightened airway responsiveness compared to naïve WT mice. After OVA sensitisation and challenge, $ET_B^{-/-}$ mice displayed increased OVA-specific immunoglobulin E levels, intensified allergic airway inflammation and hyperresponsiveness compared to WT mice. Conversely, $_{pre}ET^{tg}$ mice displayed reduced immunoglobulin E levels, airway inflammation and hyperresponsiveness.

Conclusion Our findings suggest ET_B receptors have a protective role in asthma-associated allergic airway inflammation and hyperresponsiveness. The increased asthma phenotype in sensitised and challenged $ET_B^{-/-}$ mice is attributed to ET_B-specific immunomodulatory mechanisms, rather than to elevated levels of ET-1

resulting from impaired ET_B-mediated ET-1 clearance. This conclusion is supported by the diminished asthma-phenotype observed in sensitised and challenged $_{pre}ET^{tg}$ mice. Therefore, adjusting endothelin signalling could offer a promising approach to managing asthma.

Introduction

Worldwide, >260 million people suffer from asthma [1]. Asthma is a chronic airway disease leading to episodes of cough, shortness of breath, wheezing and chest tightness [2]. Type 2 inflammation is believed to play a fundamental role in the pathogenesis of asthma [3]. Of note, comparable gene expression signatures were detected in T-helper (Th)2 cells from type 2-high asthma patients as well as from a murine model of allergic asthma [3, 4]. Interestingly, asthma is associated with a polymorphism of endothelin-1 (ET-1) [5], and airway obstruction in asthma patients is associated with a polymorphism of the endothelin B receptor (ET_B) [6].

Endothelial cells generate and degrade ET-1, which plays a crucial role in regulating vascular and airway tone [7, 8]. The bioactive mediator ET-1 is synthesised *via* enzymatic cleavage of its precursors prepro-ET-1 and big-ET-1 [7]. ET-1 exerts its function *via* two main receptors, ET_A and ET_B, both G protein-coupled receptors found on various cell types, including smooth muscle and endothelial cells [7]. Pulmonary ET-1 levels are increased in eosinophilic airway inflammation, and the endothelin system is believed to contribute to asthma-associated airway inflammation and remodelling [7, 9]. Thus, the endothelin system has been repeatedly proposed as a potential therapeutic target in asthma.

Given the functional role of ET_B in the airways and the ET_B polymorphism in asthma patients, it is pivotal to understand how impaired ET_B receptor signalling may influence its dual role in regulating airway tone and inflammation. We have found murine ET_B deficiency to increase pulmonary eosinophil influx due to ovalbumin (OVA) sensitisation and challenge, significantly elevate IL-12p40 bronchoalveolar lavage fluid (BALF) levels and to enhance pulmonary ET-1 expression, thereby supporting the concept of a protective, anti-inflammatory role of the ET_B receptor in pulmonary allergic disease [10]. In our previous study, we focused specifically on ET_B-mediated pulmonary vascular changes associated with pulmonary arterial hypertension (PAH) [10]. The work presented here highlights the impact of ET_B deficiency on the humoral immune response and on airway hyperresponsiveness in the context of asthma-associated allergic lung disease.

Both rescued $ET_B^{-/-}$ mice and prepro-ET-1 overexpressing mice ($_{pre}ET^{tg}$) were studied to differentiate ET_B-specific effects from those of ET-1. Since age is an important determinant of cardiopulmonary pathologies in $ET_B^{-/-}$ and $_{pre}ET^{tg}$ mice [10], young, matured and aged mice were analysed. It was previously established that ET_B deficiency elevates ET-1 levels by impairing ET-1 clearance from the circulation [8]. Consequently, the observed effects might be attributed to increased ET-1 levels rather than ET_B deficiency itself. However, this possibility was ruled out, as $_{pre}ET^{tg}$ mice displayed the opposite phenotype.

Materials and methods

A detailed description of the methods is provided in the online supplementary material.

Mice

Rescued ET_B-deficient and human prepro-ET-1 overexpressing ($_{pre}ET^{tg}$) mice were studied. ET_B-deficient mice were rescued from lethal inherited Hirschsprung disease by carrying a dopamine-β-hydroxylase ET_B transgene [10].

Lung histology

Lungs were immersion fixed, embedded, sliced, mounted onto glass slides, stained and microscopically studied [10]. ET_B expression was analysed *via* immunofluorescence microscopy.

Isolated perfused and ventilated mouse lung

Isolated lungs were perfused with sterile buffer and ventilated in a closed chamber. Airway resistance (res_{aw}) was constantly assessed and the maximal response to broncho-constrictive stimuli was shown as factor of basal airway resistance (fold res_{aw}) [10].

Allergic airway inflammation

On days 0 and 14, mice were sensitised to OVA by intraperitoneal injections of 20 µg OVA dissolved in 10 µL of phosphate-buffered saline (PBS) and 100 µL aluminum hydroxide suspension [10]. On days 28–30, repeated airway exposure to aerosolised OVA (1%; dissolved in PBS) was performed. On day 31 ($_{pre}ET^{tg}$) or 32 ($ET_B^{-/-}$), bronchoalveolar lavage (BAL) was performed and total cell numbers were assessed [10]. Cytokines from BALF supernatants and immunoglobulins from plasma were quantified.

Quantitative real-time reverse transcription PCR

Gene expression analyses *via* quantitative reverse transcription PCR were performed [10]. Genes of interest were vascular endothelial growth factor A *(Vegfa)*, colony-stimulating factor 1 (*Csf1*), tumour necrosis factor-α (*Tnf*) and ET_B.

Statistical analysis and data presentation

In part, results presented here are from the same dataset published in our previous study [10]. Data were analysed *via* GraphPad Prism 10 (Graph Pad Software Inc., USA). t-test (parametric data) or Mann–Whitney U-test (non-parametric data) was performed for comparison between groups. Unless stated otherwise in the figure legend, dose–response curves were compared using two-way repeated measures ANOVA.

Results

ET_B is expressed in airway smooth muscle cells

Localisation of pulmonary ET_B expression was analysed in murine lungs *via* immunofluorescence. ET_B was expressed in airway and vascular smooth muscle cells, airway epithelial cells and endothelial cells (supplementary Figure S1), in line with the literature [7, 8].

ET_B deficiency is associated with elevated basal airway resistance and increased airway responsiveness

In isolated perfused and ventilated lungs, basal airway resistance (res_{aw}) was elevated in 2- to 3-month-old and 6-month-old rescued $ET_B^{-/-}$ mice compared to the corresponding wild-type (WT) mice of the same age (figure 1a, b). Furthermore, airway responsiveness (fold res_{aw}) to methacholine (MCh) was highly (5.3-fold) increased in $ET_B^{-/-}$ lungs compared to the corresponding WT mice of the same age (figure 1c, d). In contrast to WT mice (figure 1e), basal airway resistance correlated positively with airway responsiveness to MCh in $ET_B^{-/-}$ mice (figure 1f). This finding is in line with the human situation in asthma patients, who show a negative correlation between baseline forced expiratory volume in 1 s and airway responsiveness to MCh [11].

Airway hyperresponsiveness in naïve $ET_B^{-/-}$ mice is stimulus-independent

In addition to MCh-evoked airway hyperresponsiveness (AHR), airway responsiveness to thromboxane receptor agonist U46619 (figure 2a) and airway responsiveness to ET-1 (figure 2b) were both increased in isolated perfused and ventilated $ET_B^{-/-}$ lungs compared to corresponding WT. To dissect the role of ET_A in ET-1-induced bronchoconstriction, ET_A inhibition *via* BQ-123 diminished ET-1-evoked airway constriction in isolated lungs of WT (figure 2c) and $ET_B^{-/-}$ mice (figure 2d). The attenuating effects of ET_A inhibition on ET-1-mediated airway constriction (figure 2c, d) were, however, stronger in $ET_B^{-/-}$ mice (63%) compared to WT (48%) suggesting a more prominent functional role of the ET_A axis in $ET_B^{-/-}$ mice.

Pulmonary type-2 inflammation exacerbates stimulus-independent AHR in $ET_B^{-/-}$ mice

Next, we analysed the effects of type-2 inflammation as a second hit on airway function in rescued ET_B-deficient mice. In isolated perfused and ventilated murine lungs, ET_B deficiency was associated with elevated basal airway resistance in sham-treated (PBS/PBS=P/P) mice and in ovalbumin-sensitised and ovalbumin-challenged mice (OVA/OVA=O/O) (figure 3a, b). Following OVA sensitisation and OVA airway challenge, airway responsiveness to various bronchoconstrictive stimuli was increased when compared to control mice (PBS/PBS=P/P) (figure 3c–f). $ET_B^{-/-}$ OVA/OVA-treated compared to WT OVA/OVA-treated mice had increased AHR to MCh (figure 3c), to thromboxane receptor agonist U46619 (figure 3d), to serotonin (5-HT) (figure 3e) and ET-1 (figure 3f).

Aggravated allergic airway inflammation and humoral immune response in $ET_B^{-/-}$ mice

Following OVA-sensitisation and OVA-challenge, bronchoalveolar lavage cell numbers were found to be increased in $ET_B^{-/-}$ mice compared to the corresponding WT mice (figure 4a). Representative images of haematoxylin and eosin (H&E) stained lung slides revealed elevated airway inflammation in $ET_B^{-/-}$ OVA/OVA-treated mice compared to WT OVA/OVA-treated mice (figure 4b). Lung oedema formation was restricted to the perivascular space (supplementary figure S2A) without significant differences between both OVA/OVA-treated groups. Other histological signs of alveolar or interstitial oedema were absent in all groups. BALF protein levels were comparable in both groups of OVA/OVA-treated mice (supplementary figure S2B). OVA-specific immunoglobulin E (IgE) was highly increased in OVA/OVA-treated $ET_B^{-/-}$ mice compared to the corresponding WT mice (figure 4c), while OVA-specific IgG_1 levels were not significantly elevated in $ET_B^{-/-}$ OVA/OVA-treated mice compared to WT OVA/OVA-treated mice (figure 4d).

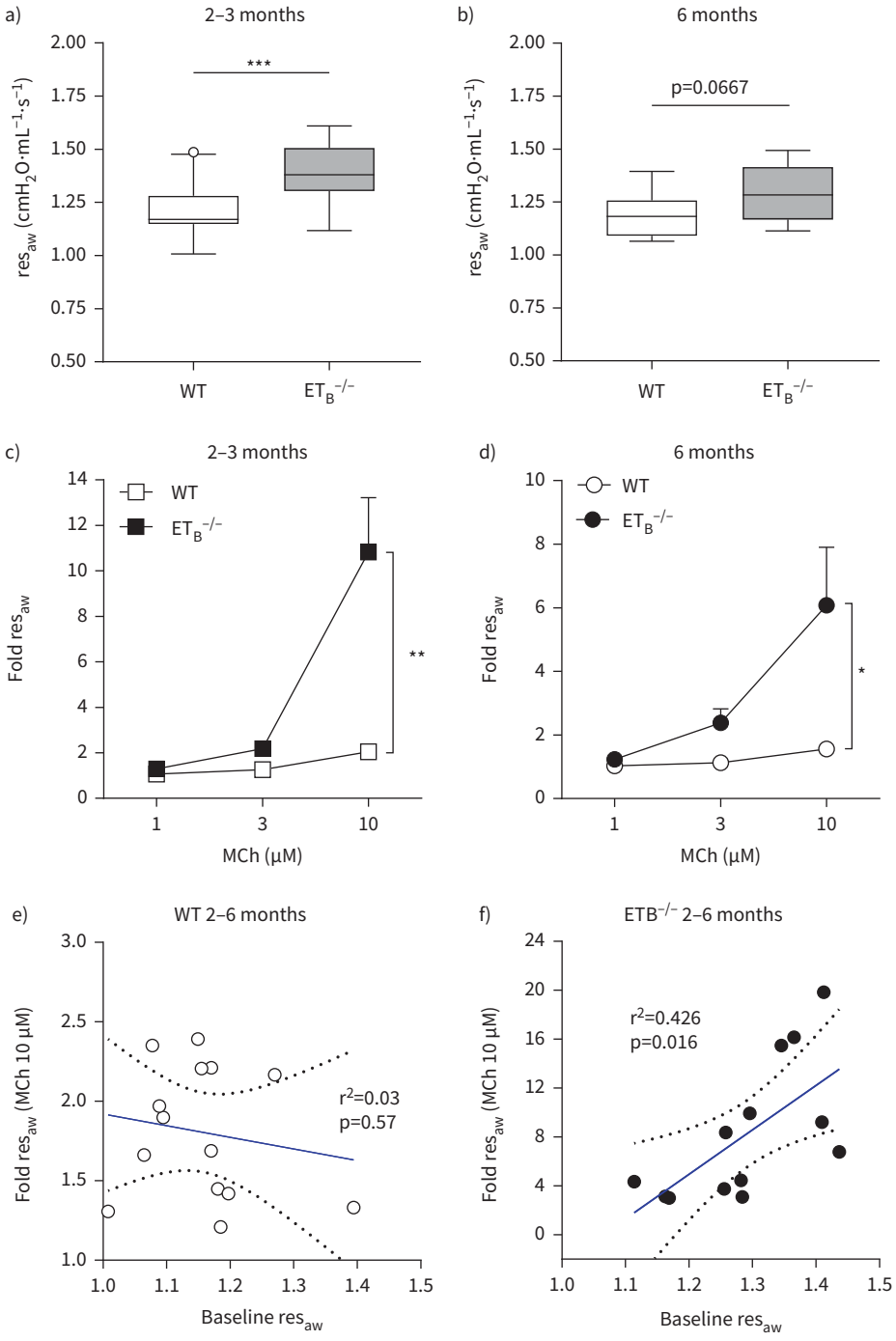

FIGURE 1 Endothelin B receptor (ET_B) deficiency is associated with elevated basal airway resistance and increased airway responsiveness. a,b) Basal airway resistance (res_{aw}) in isolated perfused and ventilated lungs of rescued $ET_B^{-/-}$ mice compared to wild-type (WT) controls at 2 to 3 months and 6 months of age. Data are presented as box plots with median, quartiles and ranges excluding outliers (open circles); analysed by Mann–Whitney U-test n=8–25. c,d) Airway responsiveness (fold res_{aw}) to methacholine (MCh) in $ET_B^{-/-}$ lungs compared to WT controls. Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA. Fold res_{aw} represents the change in res_{aw} in response to MCh application as a factor of baseline res_{aw} (n=6–7). Correlation between basal airway resistance and airway responsiveness to 10 µM MCh in e) WT or f) $ET_B^{-/-}$ mice. In e and f, Pearson correlation was performed and linear regression was calculated; the dotted lines indicate the 95% confidence band (n=13–14). Significant difference between $ET_B^{-/-}$ and corresponding WT controls: *p<0.05, **p<0.01, ***p<0.001.

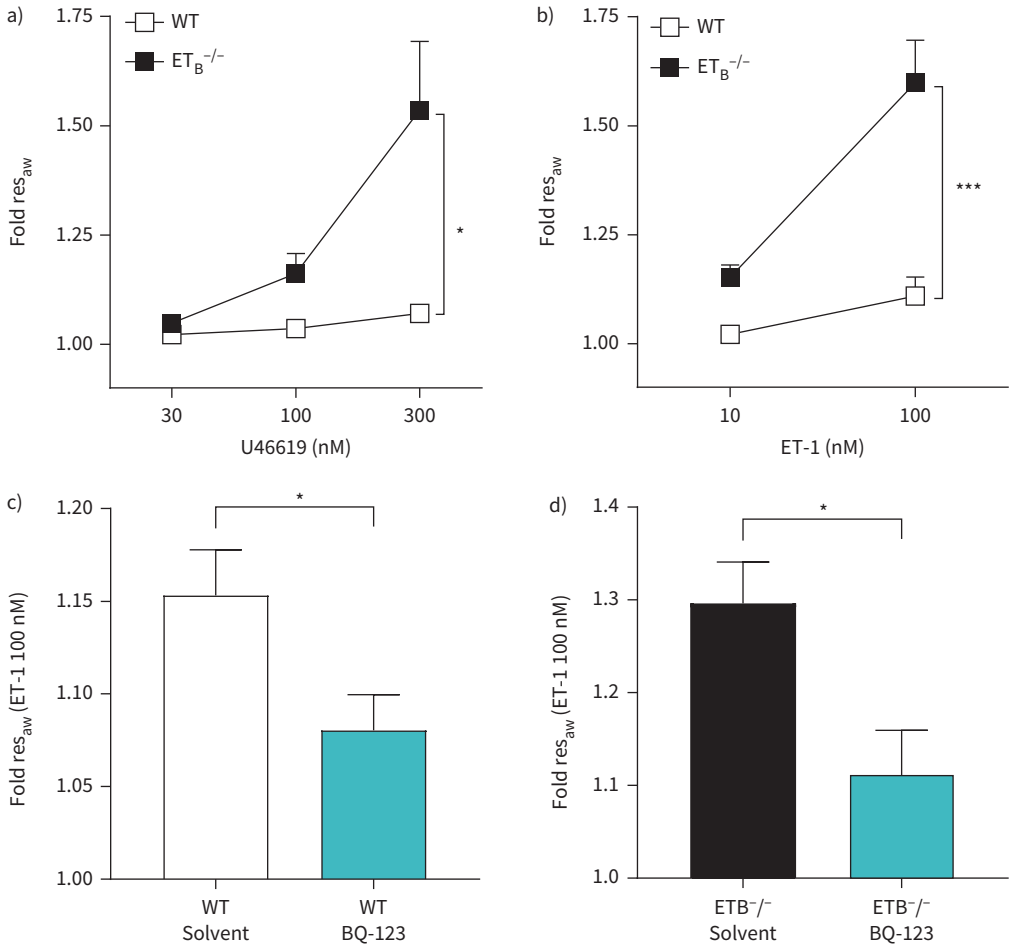

FIGURE 2 Increased airway responsiveness in ET_B receptor-deficient ($ET_B^{-/-}$) mice is stimulus-independent. a, b) Airway responsiveness (fold res_{aw}) to thromboxane receptor agonist U46619 and to endothelin-1 (ET-1) is elevated in rescued $ET_B^{-/-}$ lungs compared to wild-type (WT) controls. Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA (n=7). c, d) Inhibition of the ET_A receptor with BQ-123 reduces ET-1-evoked airway constriction in isolated lungs of both WT and $ET_B^{-/-}$ mice. Data are displayed as bar graphs representing mean±SEM and analysed using an unpaired t-test (parametric data) or Mann–Whitney U-test (non-parametric data) (n=4–6). Significant difference between $ET_B^{-/-}$ *versus* the corresponding WT controls or between BQ-123 *versus* solvent: *p<0.05, ***p<0.001.

Vegfa *mRNA expression is elevated in OVA/OVA-treated* $ET_B^{-/-}$ *mice*

To mechanistically dissect the aggravated allergen-induced humoral immune response, airway inflammation and AHR in rescued $ET_B^{-/-}$ mice, quantitative PCR analyses were performed. Expression of the gene encoding vascular endothelial growth factor (VEGF) is increased in the airways of asthma patients [12], and VEGF is known to propel Th2 immune responses, thereby promoting asthma-associated airway inflammation and AHR [13, 14]. Indeed, pulmonary *Vegfa* mRNA expression was found to be upregulated following induction of allergic airway inflammation (OVA/OVA) compared to sham treatment (PBS/PBS) (figure 5a). In both treatment groups, ET_B deficiency was associated with increased *Vegfa* mRNA expression (figure 5a). Similarly, colony stimulating factor 1 (*Csf1*) mRNA expression was elevated in OVA/OVA-treated $ET_B^{-/-}$ mice compared to the corresponding WT controls (figure 5b). CSF1 expression is known to be upregulated in asthma patients positively correlating with eosinophil inflammation [15] and CSF1 receptor inhibition has been proposed as a novel therapeutic strategy for allergic asthma [16]. In contrast, mRNA levels of tumour necrosis factor-α (*Tnf*) (figure 5c) were unaltered between both OVA/OVA-treated groups. Pulmonary mRNA expression of ET_B was downregulated following OVA/OVA treatment (figure 5d). Similarly, ET_A mRNA expression was reduced in OVA/OVA-treated lungs as previously shown [10]. In both treatment groups, ET_B deficiency was associated with a further reduction in ET_A mRNA expression [10]. Conversely, *ET-1* mRNA expression was found to be elevated following OVA/OVA treatment and highest expression in $ET_B^{-/-}$ lungs [10].

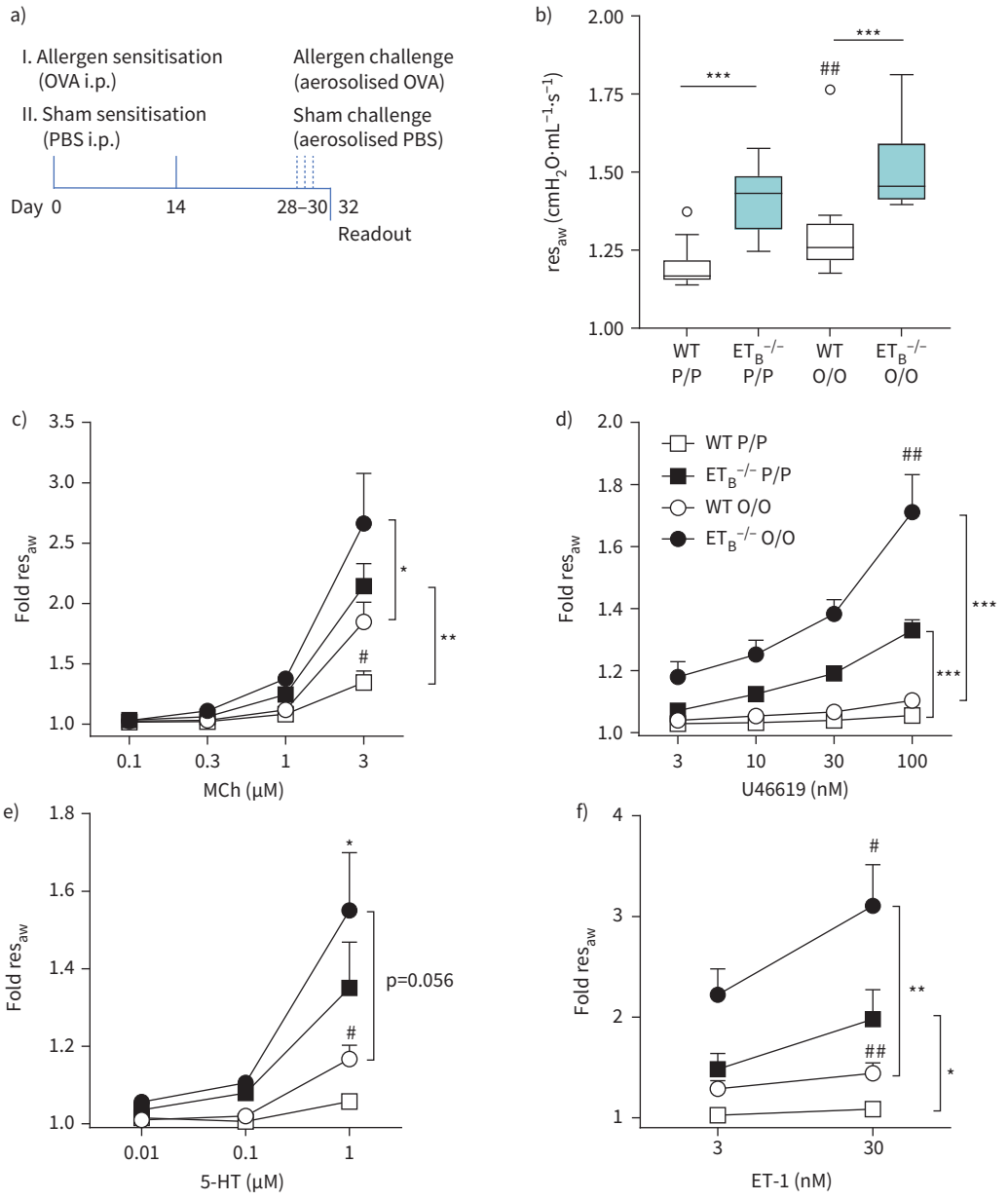

FIGURE 3 Pulmonary type-2 inflammation exacerbates airway hyperresponsiveness (AHR) in ET_B receptor-deficient ($ET_B^{-/-}$) mice. a) Schematic representation of experimental setup. b) Rescued ET_B deficiency is associated with elevated basal airway resistance in both sham-treated (PBS/PBS=P/P) and ovalbumin-sensitised and challenged mice (OVA/OVA=O/O). Data are shown as box plots with median, quartiles and ranges excluding outliers (open circles), analysed by Mann–Whitney U-test (n=12–14). c–f) Airway responsiveness to various bronchoconstrictive stimuli after OVA sensitisation and airway challenge (OVA/OVA=O/O) compared to control mice (PBS/PBS=P/P). ET_B deficiency increases AHR to c) methacholine (MCh), d) to thromboxane receptor agonist U46619, e) to serotonin (5-HT) and to f) endothelin-1 (ET-1). Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA (n=4–8). In e, additional Mann–Whitney U-test was performed comparing values of both groups at the highest dose of 5-HT (*). *: significant difference between $ET_B^{-/-}$ *versus* the corresponding wild-type (WT) controls; #: significant difference between OVA/OVA *versus* the corresponding PBS/PBS group. */#: p<0.05, **/##: p<0.01, ***: p<0.001.

Airway responsiveness is reduced in isolated lungs of prepro-ET-1 overexpressing ($_{pre}ET^{tg}$) mice

To differentiate ET-1- from ET_B-mediated effects, $_{pre}ET^{tg}$ mice were additionally studied. Basal airway resistance in young to matured (2 to 6 months old) and aged (16 to 18 months old) $_{pre}ET^{tg}$ mice was

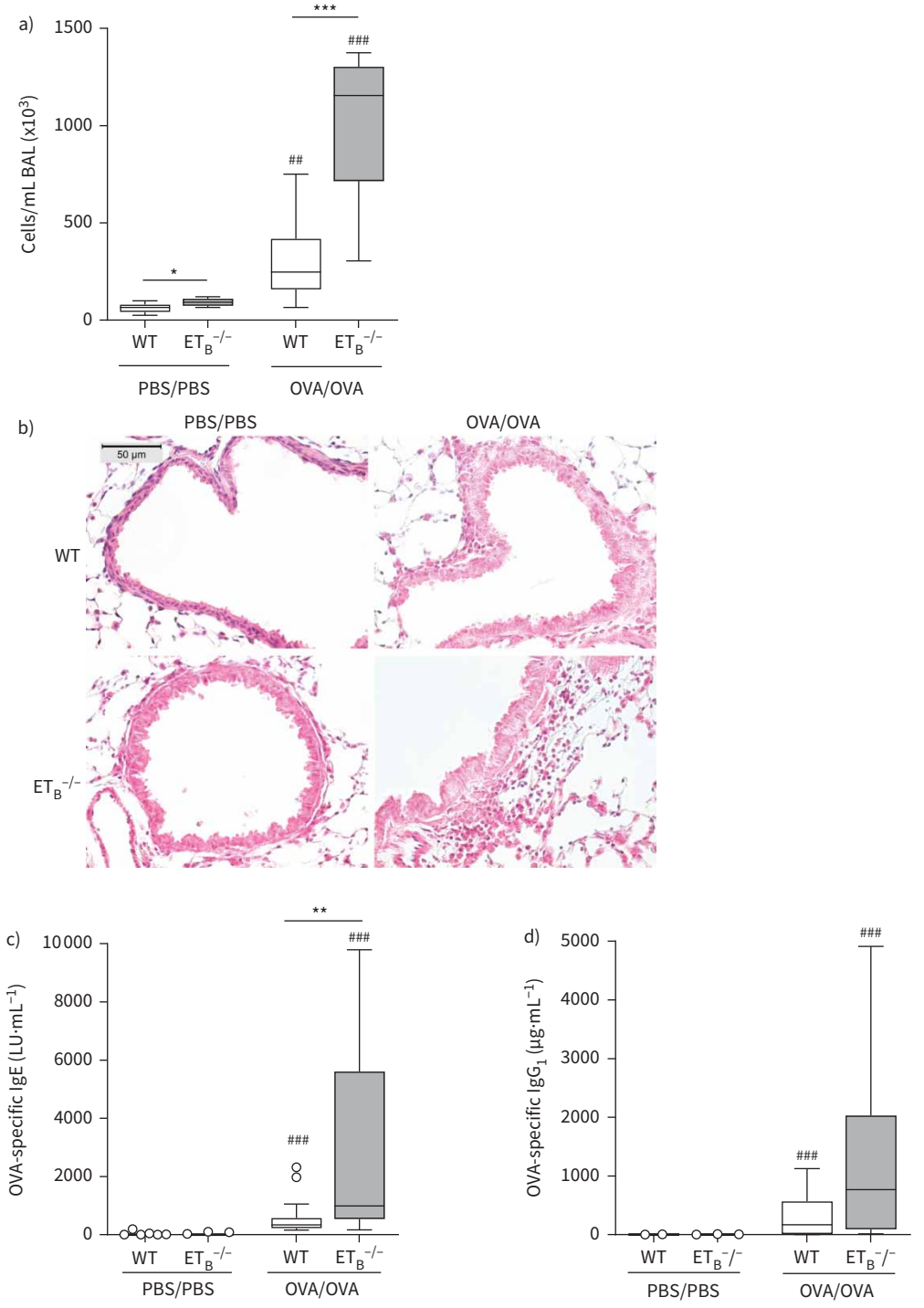

FIGURE 4 Aggravated allergic airway inflammation and humoral immune response in endothelin B receptor (ET_B)-deficient mice. a) Increased bronchoalveolar lavage (BAL) cell numbers in rescued $ET_B^{-/-}$ mice compared to wild-type (WT) controls after OVA-sensitisation and OVA-challenge (OVA/OVA). b) Representative images of haematoxylin and eosin (H&E) stained lung slides showing increased pulmonary immune cell influx in $ET_B^{-/-}$ OVA/OVA-treated mice compared to WT OVA/OVA-treated controls. Scale bar: 50 µm. c) Elevated OVA-specific IgE levels in OVA-sensitised and OVA-challenged (OVA/OVA) $ET_B^{-/-}$ mice compared to WT controls. d) OVA-specific IgG_1 levels. Data represented as box plots depicting median, quartiles and ranges excluding outliers (open circles) analysed by unpaired t-test (parametric data) or Mann–Whitney U-test (non-parametric data) in a, c, d (n=8–24). For b, n=7. *: significant difference between $ET_B^{-/-}$ *versus* corresponding WT controls; #: significant difference between OVA/OVA *versus* corresponding PBS/PBS group. *: p<0.05, **/##: p<0.01, ***/###: p<0.001.

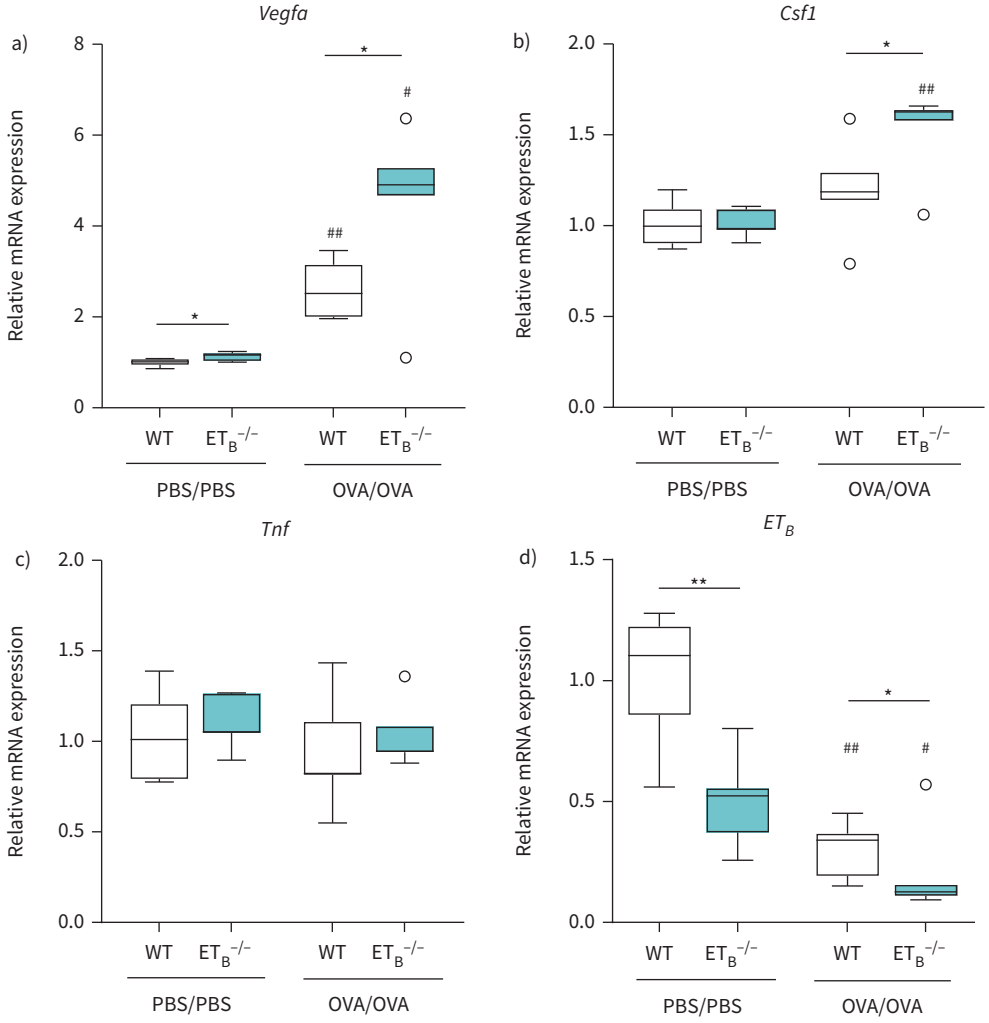

FIGURE 5 Quantitative PCR analysis of lung tissue following induction of type 2 airway inflammation. Quantitative PCR was performed to assess relative mRNA expression in lung tissue after OVA sensitisation and airway challenge (OVA/OVA) or sham treatment (PBS/PBS). The comparative C_t method (relative gene expression set to 1 in sham-treated PBS/PBS wild-type (WT) controls) was used for relative mRNA quantification of a) vascular endothelial growth factor A (*Vegfa*), b) colony stimulating factor 1 (*Csf1*), c) tumour necrosis factor-α (*Tnf*) and d) *ET$_B$*. Data are presented as box plots showing median, quartiles and ranges excluding outliers (open circles), and were analysed using an unpaired t-test (parametric data) or Mann–Whitney U-test (non-parametric data) (n=6–7 per group). *: significant difference between ET_B receptor-deficient ($ET_B^{-/-}$) and the corresponding WT group; #: significant difference between OVA/OVA and the corresponding PBS/PBS group. $^{*/\#}$: p<0.05, $^{**/\#\#}$: p<0.01.

comparable to WT mice of the same age (figure 6a, b). MCh-evoked airway constriction was found to be slightly (not significant) reduced in 2- to 6-month-old $_{pre}ET^{tg}$ mice (figure 6c) and significantly reduced in 16- to 18-month-old $_{pre}ET^{tg}$ mice (figure 6d) compared to the respective WT mice of the same age. Lung ET-1 protein levels were elevated in both transgenic mouse lines (figure 6e). These results suggest that ET_B malfunction rather than elevated ET-1 levels alone trigger airway obstruction in naïve $ET_B^{-/-}$ mice. ET_A and ET_B receptor densities and binding affinities are similar in young and aged $_{pre}ET^{tg}$ and the corresponding WT mice [17]. Thus, physiological ET_B signalling may play a protective role in $_{pre}ET^{tg}$ mice.

Reduced AHR in $_{pre}ET^{tg}$ mice following induction of allergic airway inflammation

Pulmonary type-2 inflammation was induced *via* OVA sensitisation and OVA airway challenge (OVA/OVA=O/O). MCh-evoked AHR was reduced when compared to OVA/OVA-treated WT mice in isolated perfused and ventilated lungs of OVA/OVA-treated $_{pre}ET^{tg}$ mice (figure 7a). BALF total and BALF

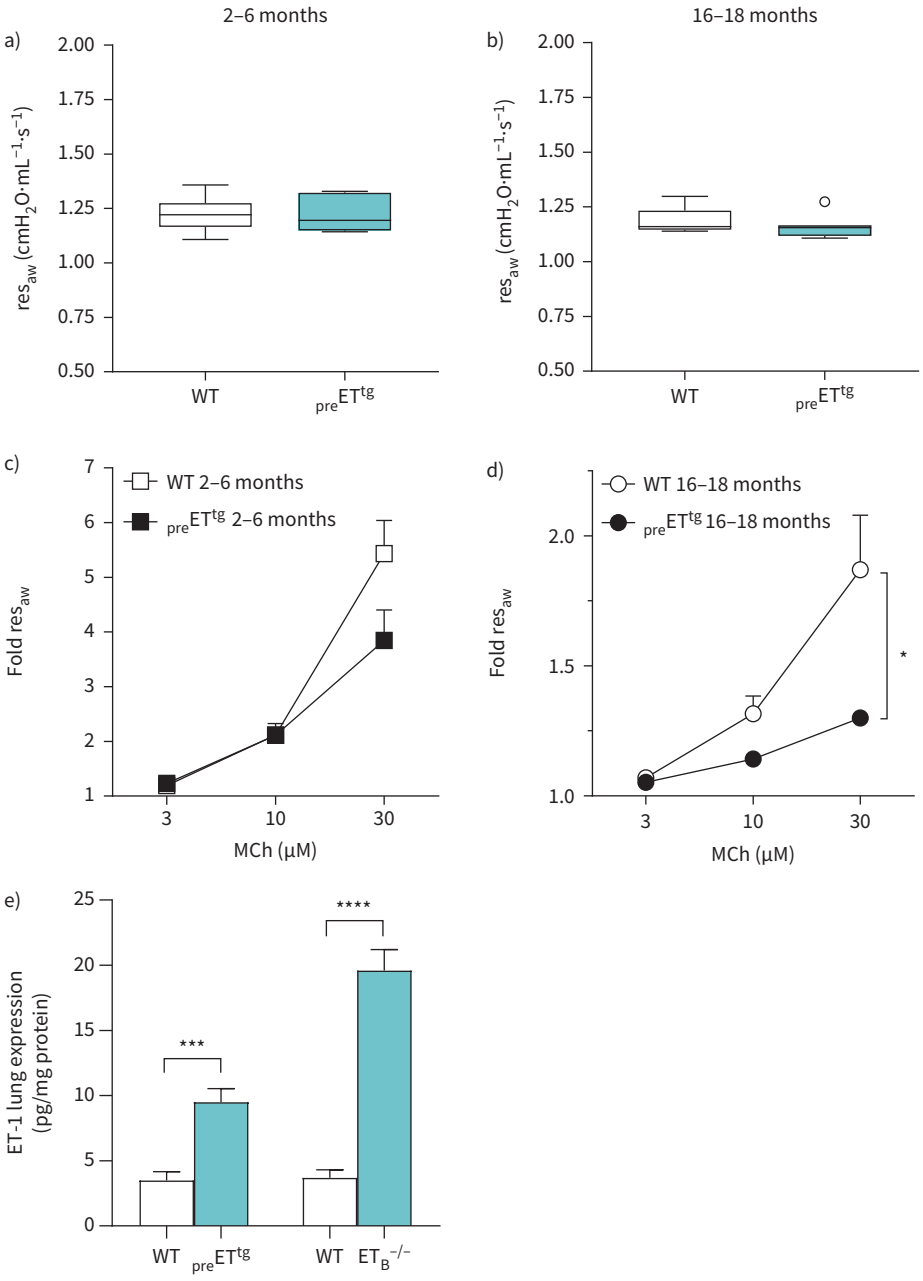

FIGURE 6 Airway responsiveness is reduced in isolated lungs of prepro-ET-1 overexpressing ($_{pre}ET^{tg}$) mice. a,b) Basal airway resistance in $_{pre}ET^{tg}$ and wild-type (WT) mice. Data are presented as box plots showing median, quartiles and ranges excluding outliers (open circles) and analysed using the Mann–Whitney U-test (n=7–11). c,d) Methacholine (MCh)-evoked airway constriction in 2–6-month-old and in 16–18-month-old mice. Data are expressed as mean±SEM and analysed by two-way repeated measures ANOVA (n=7–12). e) Pulmonary ET-1 protein expression in $_{pre}ET^{tg}$, $ET_B^{-/-}$ and the corresponding WT mice. Parametric data are expressed as mean±SEM and analysed by t-test (n=7–10). Significant difference between $_{pre}ET^{tg}$ or $ET_B^{-/-}$ *versus* the corresponding WT controls: *: p<0.05, ***: p<0.001, ****: p<0.0001.

differential cell counts were not significantly altered between both OVA/OVA-treated groups (figure 7b, supplementary figure S3). Of note, however, the median total cell count was 39% lower in OVA/OVA-treated $_{pre}ET^{tg}$ mice compared to OVA/OVA-treated WT mice. In accordance, BALF levels of the chemoattractant CCL5/RANTES were found to be reduced in $_{pre}ET^{tg}$ mice (figure 7c) as well as levels of interleukin (IL)-12p40 in OVA/OVA-treated $_{pre}ET^{tg}$ mice when compared to OVA/OVA-treated WT mice

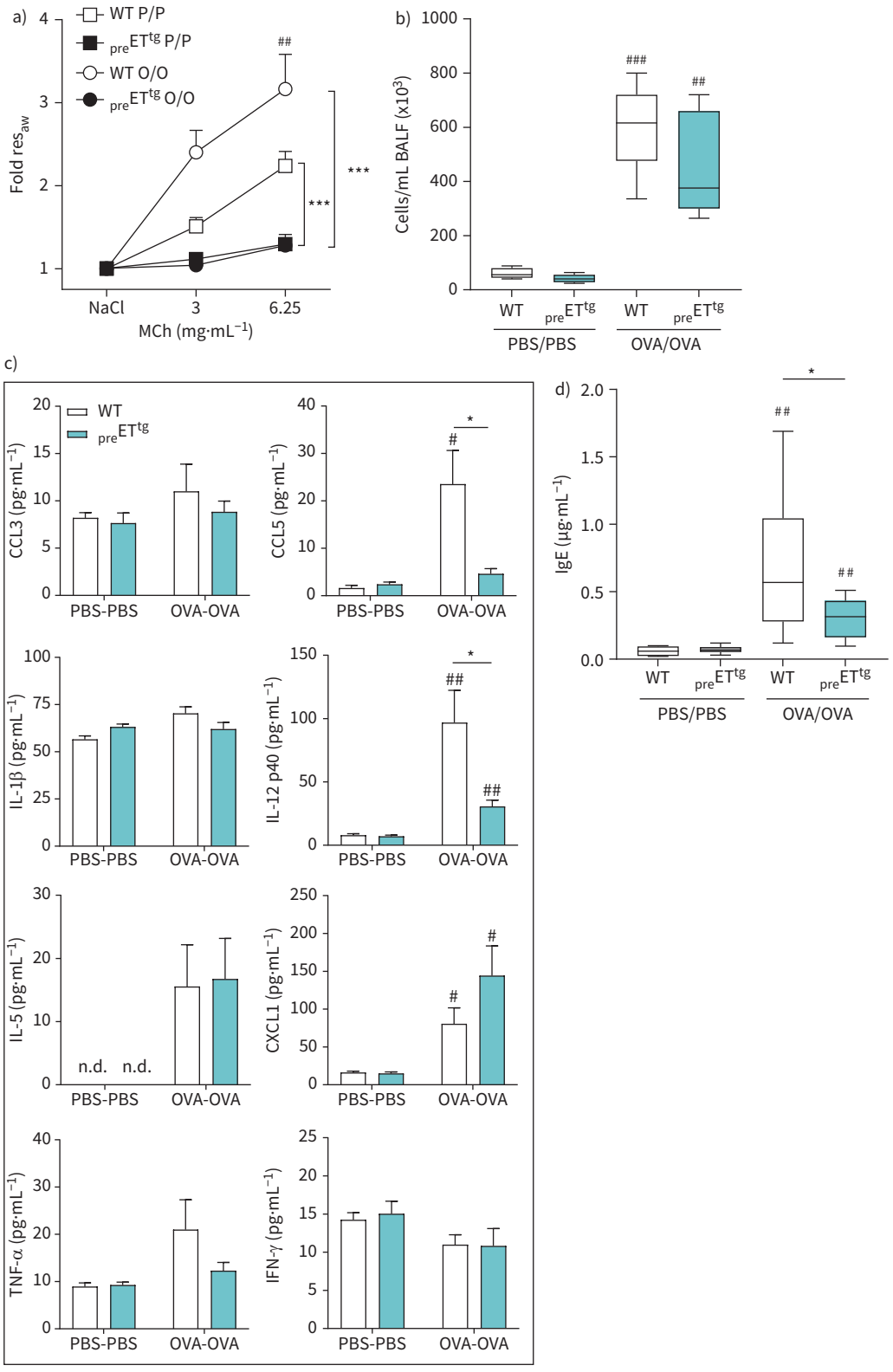

FIGURE 7 Reduced airway hyperresponsiveness (AHR) and inflammation in $_{pre}ET^{tg}$ mice following induction of allergic airway inflammation. a) Methacholine (MCh)-evoked airway responsiveness is increased in isolated, perfused and ventilated lungs of ovalbumin-sensitised and challenged (OVA/OVA=O/O) wild-type (WT) mice compared to PBS/PBS (P/P)-treated WT controls. AHR is reduced in lungs of OVA-sensitised and challenged $_{pre}ET^{tg}$ mice compared to OVA/OVA-treated WT controls. Data are shown as mean±SEM and analyzed by repeated measures analyses (n=5–8). b) Bronchoalveolar lavage fluid (BALF) cell numbers. c) Reduced levels of

CC-chemokine ligand 5 (CCL5/RANTES) and interleukin (IL)-12p40 in BAL fluid supernatant from OVA/OVA-treated $_{pre}ET^{tg}$ mice. d) Reduced IgE levels in OVA/OVA-treated $_{pre}ET^{tg}$ mice compared to OVA/OVA-treated WT controls. Data in b and d are presented as box plots showing median, quartiles and ranges excluding outliers (open circles). Data in c are represented as bar graphs depicting mean±SEM. Data in b–d are analysed using an unpaired t-test (parametric data) or Mann–Whitney U-test (non-parametric data) (n=5–8). CCL3/MIP-1α: CC-chemokine ligand 3; CXCL1/KC: chemokine (C-X-C motif) ligand 1; IFN-γ: interferon-γ; n.d.: not detected; TNF-α: tumour necrosis factor-α. *: significant difference between $_{pre}ET^{tg}$ *versus* the corresponding WT controls; #: significant difference between OVA/OVA *versus* the corresponding PBS/PBS group. */#: p<0.05, ##: p<0.01, ***/###: p<0.001.

(figure 7c). Following OVA/OVA-treatment, $_{pre}ET^{tg}$ mice showed lower IgE levels compared to WT controls (figure 7d). In conclusion, the asthmatic phenotype was attenuated in $_{pre}ET^{tg}$ mice.

Discussion

This study presents novel findings indicating that rescued ET_B receptor deficiency is associated with an asthmatic phenotype, characterised by increased pulmonary inflammation, airway obstruction and aggravated AHR. These observations highlight the critical role of ET_B receptors in maintaining airway homeostasis and in regulating inflammatory responses in asthma. The humoral immune response was found to be significantly elevated in ET_B-deficient mice, indicating a protective role of ET_B receptors in antigen-sensitisation and in modulating allergic airway inflammation and hyperresponsiveness. This finding aligns with our previous work demonstrating that ET_B receptors play a critical role in regulating type 2-mediated immune responses, key factors in the pathogenesis of allergic diseases [10]. Interestingly, ET_B inhibition was shown to improve survival of human dendritic cells and may alter dendritic cell function in a stimulatory manner [18]. Also, sovateltide, a selective ET_B agonist, has been shown to possess anti-inflammatory properties [19].

It is most likely that ET_B-specific immunomodulatory mechanisms, rather than elevated levels of ET-1 resulting from impaired ET_B-mediated ET-1 clearance, drive the asthma phenotype observed in $ET_B^{-/-}$ mice. This conclusion is supported by the observed reduction in circulating IgE, AHR and inflammation in OVA-sensitised and challenged $_{pre}ET^{tg}$ mice.

Consistent with the heightened airway responsiveness in $ET_B^{-/-}$ mice and with the reduced airway responsiveness in $_{pre}ET^{tg}$ mice, mice heterozygous deficient in ET-1 exhibited increased airway responsiveness, potentially due to diminished nitric oxide synthesis [20].

The reduced asthma phenotype in $_{pre}ET^{tg}$ mice is seemingly counterintuitive, as elevated endothelin-1 expression was found in the bronchial epithelium [21], circulation [22], BALF [22] and exhaled breath condensate [23] of asthma patients. Both transgenic mouse lines exhibit elevated levels of lung ET-1. However, ET_B malfunction as a second hit rather than increased ET-1 levels alone seems to be required for induction of asthma-associated pathologies in mice.

In ET_B-deficient mice, lung tissue ET-1 concentrations are markedly elevated due to the absence of functional ET_B receptors, which mediate physiological ET-1 clearance from the circulation and tissues *via* receptor-mediated internalisation and degradation. In the absence of ET_B, ET-1 accumulates not only in plasma but also locally in tissues such as the lung. Interestingly, tissue ET-1 levels in ET_B-deficient mice may exceed those in $_{pre}ET\text{-}1^{tg}$ mice, despite the latter having globally increased ET-1 synthesis. This seeming paradox is explained by the fact that in $_{pre}ET^{tg}$ mice, clearance mechanisms *via* ET_B are still intact and capable of partially counteracting the overproduction, whereas in $ET_B^{-/-}$ mice, even baseline ET-1 production results in disproportionately high tissue accumulation due to impaired clearance. Moreover, local paracrine or autocrine feedback mechanisms may further amplify ET-1 expression in ET_B-deficient tissues, contributing to the observed pronounced elevations [24, 25].

Both ET-1 receptors ET_A and ET_B are known to mediate airway tone in the human lung and various other species [26–29]. However, due to crosstalk between ET_A and ET_B, their specific function is not easily assessable by selectively inhibiting one or the other [27], as reflected by considerable controversies in the literature. In our study, we cannot exclude that ET_A receptors in $ET_B^{-/-}$ mice partially fulfill functions physiologically regulated by ET_B, either due to mutual interactions [27] or to functional redundancy between both receptors. Similarly, in $_{pre}ET^{tg}$ mice ET-1-mediated effects cannot be clearly attributed to either ET_A and/or ET_B. As such, the data presented here should be interpreted with caution with respect to the definitive role of specific receptor subtypes. Adding to complexity, interspecies variations in the localisation

and density of ET-1 receptors in the airways have been reported [30, 31]. Additional investigations are needed to determine the potential benefit of targeting ET_B receptor, either alone or in combination with ET_A, in allergen sensitisation, allergic airway inflammation and airway hyperresponsiveness.

Our findings indicate that AHR in ET_B-deficient mice is nonspecific, as it manifests in response to various clinically relevant broncho-constrictive stimuli, similar to the functional alterations observed in asthma patients. Remarkably, AHR in asthma patients comprises the here detected increased airway responsiveness to ET-1 [32]. Airway responsiveness is reduced in aged mice [33]. Accordingly, the maximal airway response in 6-month-old $ET_B^{-/-}$ mice was lower relative to 2- to 3-month-old $ET_B^{-/-}$ mice. Similarly, 16- to 18-month-old $_{pre}ET^{tg}$ and WT mice had the lowest airway responsiveness in line with previously reported age-dependent effects [33]. However, since different genetic backgrounds were used in our study, results cannot be compared directly between the two mouse lines. Specifically, different genetic backgrounds can be associated with altered type 2-induced AHR, pulmonary composition of inflammatory cells and cytokines, and allergen-specific immunoglobulins [34].

Eosinophil influx into the alveolar space was highly elevated in OVA-sensitised and challenged $ET_B^{-/-}$ mice, as shown in our previous study [10]. It has been previously reported that pharmacological ET_B inhibition did not affect murine allergic airway inflammation [30, 35]. However, the ET_B inhibitor application was performed exclusively in the context of OVA airway exposure long after OVA sensitisation in the respective studies, whereas the effects of ET_B inhibition on allergen sensitisation were not investigated. As our findings suggest a significant immunomodulatory role of ET_B in allergen sensitisation, as indicated by the markedly elevated allergen-specific IgE levels in OVA/OVA-treated $ET_B^{-/-}$ compared to WT mice, future studies should explore the effects of ET_B receptor modulation in the context of allergen sensitisation in greater depth. A deeper understanding of ET_B-mediated signalling in allergen-induced type I sensitisation could also pave the way for the development of optimised adjuvants in allergen-specific immunotherapy, ultimately enhancing its efficacy.

IL-12p40, an IL-12 subunit, promotes dendritic cell migration and macrophage chemotaxis [36]. In asthmatic patients, airway epithelial inflammation is characterised by an increased airway expression of IL-12p40 and by elevated IL-12p40 BALF levels [37]. IL-12p40 BALF levels were further shown to correlate positively with BALF macrophage counts [37]. Intriguingly, we have previously observed IL-12p40 levels and macrophage counts to be significantly higher in BALF from ET_B-deficient mice compared to WT controls following the induction of pulmonary type 2 inflammation *via* OVA sensitisation and challenge, while type 2 cytokines IL-4, IL-5 and IL-13 were not significantly altered in OVA/OVA-treated $ET_B^{-/-}$ mice [10].

The increase of IL-12p40 levels points towards a dysregulation in cytokine production due to the absence of ET_B [10]. Remarkably, levels of IL-12p40 were observed to be reduced in OVA/OVA-treated $_{pre}ET^{tg}$ mice in the present study. This may be relevant for the induction of the asthmatic phenotype, highlighting the influence of the genetic background of the endothelin system on immune regulation and airway responsiveness in asthma pathology. Therefore, the proposed relevance of endothelin-1 and ET_B polymorphisms for asthma [5, 6] needs to be reevaluated in larger study cohorts. Interestingly, IL-12 levels were found to be increased in PAH patients following the initiation of dual (ET_A/ET_B) endothelin receptor antagonist treatment [38], further highlighting the immunomodulatory role mediated by endothelin receptors.

Although the role of the endothelin system in asthma is established, our study further uncovers the immunomodulatory actions of ET_B in allergic airway inflammation. However, the exact underlying mechanism of ET_B-mediated immune homeostasis remains elusive. Two potential players involved in ET_B-mediated immune signalling are *Vegfa* and *Csf1*, as they were shown to be upregulated in OVA-sensitised and challenged $ET_B^{-/-}$ mice.

VEGF airway expression was shown to be upregulated in asthma patients [12, 39] and VEGF is believed to promote allergic airway inflammation, hyperresponsiveness and remodelling *via* dendritic cell activation and augmented sensitisation [13, 14, 40]. In addition, VEGF-A has been shown to increase chemotaxis of macrophages and granulocytes [40]. In mice, VEGF overexpression led to an asthma-like phenotype with eosinophil airway inflammation, remodelling and AHR [13]. Moreover, airway allergen exposure in VEGF overexpressing mice led to a highly increased allergen-specific humoral immune response with subsequent elevated allergic airway inflammation and AHR compared to the WT controls [13]. In turn, anti-VEGF monoclonal antibody treatment in a murine model of allergen-induced asthma was demonstrated to diminish the humoral immune response, type 2-associated airway inflammation and AHR seemingly *via* VEGF-A neutralisation [40, 41]. VEGF has been repeatedly proposed as a target for asthma treatment [13,

40, 41]. CSF1 and its receptor CSF1R are also believed to play an important role in asthma pathogenesis *via* promoting activation and migration of dendritic cells as well as eosinophil inflammation [15, 16, 42, 43]. Generation of allergen-specific IgE in allergen-sensitised and challenged mice as well as subsequent allergic lung inflammation and AHR critically depend on CSF1 [16, 43]. Therefore, the upregulation of the genes encoding VEGF-A and CSF1 in the lungs of OVA/OVA-treated $ET_B^{-/-}$ mice, as demonstrated in our study, may partially account for the heightened humoral immune response, intensified airway inflammation and increased airway hyperresponsiveness observed in these mice compared to WT controls. Additional analyses targeting VEGF or CSF1 in $ET_B^{-/-}$ mice are needed to confirm their underlying roles.

Conclusion

The findings of this study underscore the significant role of ET_B receptors in the pathophysiology of asthma, particularly highlighting their impact on allergen sensitisation, airway inflammation, airway obstruction and hyperresponsiveness. The observed asthmatic phenotype in ET_B-deficient mice suggests that ET_B receptors are crucial for maintaining airway homeostasis and modulating immune responses. Furthermore, the dysregulation of cytokine and growth factor production, particularly CSF1, IL-12p40 and VEGF-A, points to an important mediating role of endothelin signalling in orchestrating type I sensitisation and airway inflammation. As we advance our understanding of the endothelin system in airway function and immune signalling, it becomes evident that targeted therapies aimed at modulating the endothelin system could offer new avenues for treating asthma and related inflammatory diseases. Future research should focus on elucidating the precise mechanisms by which endothelin-1 receptors mediate allergen sensitisation and airway inflammation, and exploring potential therapeutic strategies that leverage this knowledge.

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