

ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Impaired airway epithelial cell wound-healing capacity is associated with airway remodelling following RSV infection in severe preschool wheeze

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Abstract

Background: Respiratory syncytial virus (RSV) causes exacerbations of asthma and preschool wheeze (PSW). However, the anti-viral and repair responses of the bronchial epithelium in children with severe therapy-resistant asthma (STRA) and PSW are poorly understood.

Methods: Children with STRA (age 12 [6-16] years), PSW (age 2 [1-5] years) and non-asthmatic controls (age 7 [2-14] years) underwent bronchoscopy with endobronchial brushings and biopsies. Anti-viral, wound injury responses were quantified in biopsies and primary bronchial epithelial cells (PBECs) in response to RSV, poly(I:C), house dust mite (HDM) or IL-33 using RT-qPCR, Luminex and live cell imaging. Collagen deposition and tissue expression of epithelial growth factor receptor (EGFR), IL-33 and receptor ST2 were investigated in bronchial biopsies.

Results: PBECs from STRA and PSW had increased TLR3 gene expression and increased secretion of anti-viral and pro-inflammatory cytokines (IFN- γ , IL-6 and IL-13) in response to RSV compared to controls. Exposure of PBECs to concomitant TLR3 agonist poly(I:C) and HDM resulted in a significant reduction in epithelial cell proliferation in PSW compared to controls. Wound-healing was also impaired in PSW compared to controls at baseline and following IL-33 stimulation. In addition, tissue EGFR expression was significantly reduced in PSW and correlated with collagen deposition in endobronchial biopsies.

Conclusions: Despite increased anti-viral responses, preschool children with severe wheeze had impaired airway epithelial proliferative responses following damage. This might be connected to the low expression of EGFR in PSW which may affect epithelial function and contribute to asthma pathogenesis.

KEYWORDS

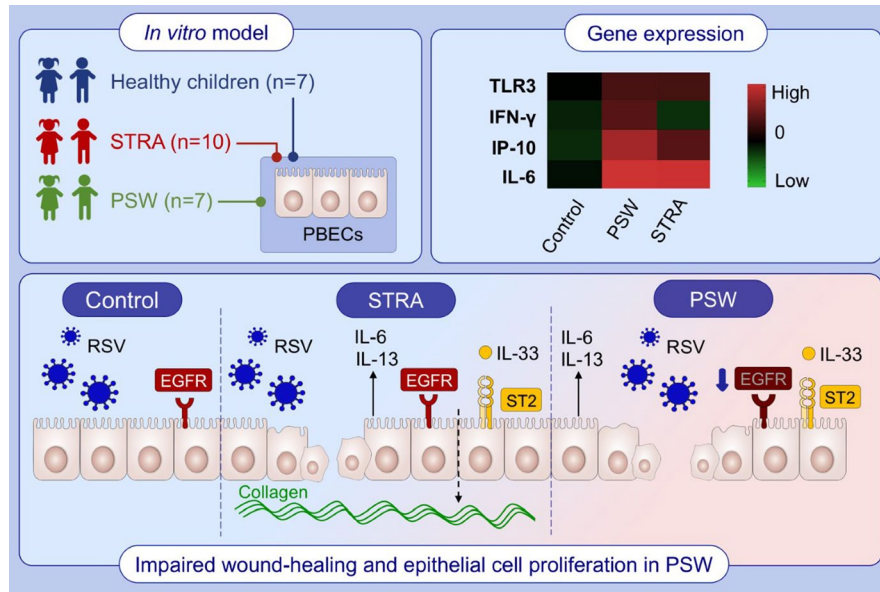
epithelium, paediatric asthma, RSV, severe therapy-resistant asthma, wheeze

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GRAPHICAL ABSTRACT

In response to RSV, PBECs from children with STRA and PSW demonstrate increased TLR3 gene expression and enhanced secretion of pro-inflammatory cytokines compared to controls. IL-33 expression correlates with collagen deposition in children with STRA and PSW. Despite the increased antiviral responses, airway epithelial proliferative responses following barrier damage are impaired in children with PSW.

1 | INTRODUCTION

Wheezing in response to viral infections during infancy and pre-school years is often the first clinical manifestation of asthma development in childhood.¹ However, not all children who wheeze with viral infections will develop asthma.¹ Infection with either human rhinovirus (RV) or respiratory syncytial virus (RSV) is associated with early wheeze and asthma development.²⁻⁵ RSV infection is considered particularly important in more severe early wheeze, resulting in hospitalization.⁶ Early allergic sensitization together with virus-associated wheezing further increases the risk of asthma development.^{7,8}

Most children with asthma have mild or moderate disease and achieve symptom control with maintenance-inhaled steroids.^{9,10} However, a small proportion has persistent symptoms despite maximal dose treatment including long- and short-acting inhaled β_2 -receptor agonists, inhaled corticosteroids (ICS) and/or leukotriene receptor antagonists,¹¹ and after addressing adherence and factors such as allergen and smoke exposure. These children have severe therapy-resistant asthma (STRA).¹² The airway epithelium acts as the first line of defence against harmful environmental insults such as infectious pathogens and allergens, and it has been suggested that abnormal responses to these exposures could lead to asthma pathology.¹³ In support of this, epithelial damage has been reported in both adult and childhood asthma.^{14,15} A functioning re-epithelialization of the airways is required to maintain the integrity of its immune and barrier function.¹⁶ Since RSV replication is restricted to airway epithelial cells and induces potent expression of cytokines and chemokines, the epithelium is postulated to be a primary initiator of pulmonary inflammation during RSV infection. Many prospective long-term follow-up

studies have shown that RSV-induced bronchiolitis is associated with later development of asthma, especially in those with severe disease.^{3,17,18} Studies have also reported an association between RSV-induced bronchiolitis and subsequent allergic sensitization.^{2,19-21} RSV infections have shown to reduce IFN expression in peripheral blood mononuclear cells as well as in T-cell populations and have been correlated with subsequent asthma.²²⁻²⁵ A recent study studies showed that RSV-induced EGFR activation suppresses IFN production from epithelial cells and increases RSV infection in infants.²⁶

The epithelial cytokine IL-33 has been shown to increase during viral-induced asthma exacerbations in adults with mild to moderate disease.^{27,28} However, very little is known about airway epithelial function in severe preschool wheeze patients despite the fact that wheezing with viruses is particularly common in preschool children and there are currently few effective treatment strategies for viral wheeze.²⁹ We have previously reported that STRA children had increased submucosal expression of IL-33 which related to reticular basement membrane thickness.³⁰ We and others have also reported increased sub-epithelial reticular basement membrane thickness in severe preschool wheezers,^{31,32} but the relationship between epithelial function and remodelling has not been investigated.

We hypothesized that children with severe preschool wheeze (PSW) and STRA would have impaired epithelial inflammatory and repair responses to RSV infection compared to controls, and this would be influenced further by exposure to the aeroallergen house dust mite (HDM) or IL-33. We investigated this by assessing the functional properties, expression of anti-viral and pro-inflammatory responses following exposure to IL-33 and RSV, alone or in the presence of HDM, in PBECs from preschool children with severe,

TABLE 1 Demographics of control, wheeze and STRA patients undergoing bronchoscopy

	Non-asthmatic controls ^a (n = 7)	Pre-school wheeze (n = 7)	STRA (n = 10)	P-value
Atopy	2/7 (29%)	0/7 (0%)	9/10 (90%)	.08
Male: Female	3:4	3:4	6:4	
Age, years	7 (2-14)	2 (1-5)	12 (6-16)	.01
Weight (Kg)	36 (28-48)	12.4 (8.7-25)	37 (21-88)	.0009
Height (cm)	146 (137-156)	91 (73-126)	142 (103-186)	.002
Intubation for asthma	—	3	2	
Total IgE(IU/ml)	86 (9-180)	18 (1-42)	505 (29-4847)	.003
Sum of inhalant sIgE (IU/mL)	0 (0-0)	0 (0-0)	4.5 (1-2)	<.0001
Sum of all sIgE (IU/mL)	0 (0-0)	0 (0-0)	1 (1-2)	<.0001
BAL Neutrophils(%) ^b	4.0 (3.7-10.3)	11.0 (3.0-19.0)	6.0 (0.3-21.7)	.9
BAL Eosinophils (%) ^c	1.3 (1.0-4.0)	1.5 (1.0-2.0)	5.0 (0.3-10)	.6
Blood Eosinophils (%)	3.1 (1.5-4.4)	3.9 (0.9-6.1)	6.6 (2.2-15.9)	.2
Blood Neutrophils (%)	49.2 (47.0-55.0)	34.3 (22.2-58.3)	50.0 (29.1-75.8)	.08
ACT score	—	—	15 (11-16)	
Baseline FEV ₁ (litres)	—	—	1.9 (0.8-3.7)	
Baseline % predicted FEV ₁ ^d	—	—	83 (47-112)	
Number FEV ₁ 'normal' (>80% predicted)	—	—	5	
Baseline FVC (litres)	—	—	2.6 (1.0-4.6)	
Baseline % predicted FVC	—	—	109 (75-126)	
Baseline bronchodilator reversibility (%)	—	—	7 (0-49)	
Medications				
Daily dose Inhaled ^e corticosteroid (mcg/day) Budesonide equivalent	0 (0-0)	350 (0-500)	1000 (1000-2000)	
Leukotriene receptor Antagonist	2 of 6 (33%)	3 of 7 (43%)	6 of 10 (60%)	
Systemic corticosteroids				
Daily dose (mg/day)	0	0	10 (0-20)	
Theophylline	0	0	3	

Note: Data presented as median (range).

^a5 non-asthmatic controls were undergoing clinically indicated bronchoscopies and had agreed to a research bronchoscopy: brushings were collected from 4 and biopsies from 5 subjects. HBECs from 2 non-asthmatic controls were also bought from Lonza. For details, see OR.

^bBAL Neutrophils (<3% is normal).

^cBAL eosinophils (<3% is normal).

^dFEV₁: presented as percentage predicted.

^eICS: inhaled corticosteroids/day. Differences between groups were assessed by Mann-Whitney test or Chi-square test where $P < .05$ is significant.

recurrent wheeze and school-aged children with STRA in comparison to non-asthmatic controls. In addition, we investigated tissue expression collagen, epithelial growth factor receptor (EGFR), IL-33 and ST2 expression in bronchial biopsies from the same patient groups.

2 | MATERIAL AND METHODS

2.1 | Subjects

10 school-aged children with STRA²² (median age 12 [range 6-16] years) and 7 preschool children with severe, recurrent wheeze (median age 2

[range 1-5] years) were recruited from the Royal Brompton Hospital. Children with STRA and PSW underwent a clinically indicated bronchoscopy, and additional research endobronchial brushings and biopsies were taken during the procedure with parental informed consent.⁴ Non-asthmatic control subjects were undergoing clinically indicated bronchoscopy and agreed to research samples being taken at the same time (n = 5). Successful epithelial cell cultures were established from 4 of these, although all 5 had adequate biopsies taken. In addition, normal bronchial epithelial cells from 2 paediatric healthy donors were purchased from Lonza. See Table 1, online repository (OR) for details and Table E1 for information regarding additional medication. The study was approved by the NRES Committee London—Chelsea.

2.2 | Epithelial cell culture and stimulation

Primary bronchial epithelial cells (PBECs) were seeded into collagen-coated tissue culture flasks containing BEGM (Lonza, Walkerville, Md) and used at passage 3 at 80% confluency (except for proliferation) for all experiments. PBECs were stimulated with RSV, with and without concomitant exposure to house dust mite (HDM) (Greer). Proliferation and wound-injury measurements were performed using the TLR3 agonist ds RNA poly(I:C) and recombinant IL-33. For details see OR.

2.3 | Assessment of proliferation and wound repair

The ability of airway epithelial cells to proliferate and repair wounds following exposure to HDM, poly I:C and IL-33 was assessed by image analysis using the IncuCyte live-cell imaging system (Essen Bioscience, Ann Arbor, MI, USA). For proliferation assays, cells were monitored for a total of 60 hours. The density of cells was calculated by IncuCyte S3 Software (Essen Bioscience) and expressed as occupied area (% confluence). For wound-injury assays, PBECs were grown in BEGM until > 80% confluent and cell monolayers were wounded with a single scratch. Cultures were monitored for 48 hours. Results were normalized to each patient's non-stimulated control. For details see OR.

2.4 | RNA extraction and real-time PCR

Total RNA was extracted from epithelial cells using the Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA was synthesized and analysed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was measured using TaqMan primers for specific genes with Taqman Fast Advanced Master Mix (Life technologies) and performed on Vii7 system (Applied Biosystems). For details see OR.

2.5 | Quantification of cytokines

Cell culture supernatant was collected and cytokines were analysed using a customized Milliplex panel (Millipore Corporation, Billerica, Mass) for human cytokines and chemokines (see OR). Soluble ST2 (sST2) was measured using ELISA according to manufacturer's instructions (R&D Systems).

2.6 | Western Blots and densitometry

ST2 protein was detected in protein lysates, and α -Tubulin was used as a loading control and detected using a peroxidase-conjugated anti-rabbit antibody. Membranes were developed with ECL substrate (Pierce) and developed in ECL plus Western blotting detection system

prior to imaging and quantification of band density (intensity per area) on the myECL Imager (Thermo Fisher Scientific). For details see OR.

2.7 | Histopathology

Endobronchial biopsies were fixed in 4% paraformaldehyde processed to paraffin. Consecutive sections were used for immunohistochemical staining for IL-33, ST2 and epidermal growth factor receptor. Antibodies were visualized using DAKO Envision system (Dako) according to manufacturer's directions. Positive staining was quantified manually (number of cells/area of tissue) or as positivity (positive brown pixels divided by all stained pixels) using computerized image analysis on blinded sections using ImageScope (Aperio). For details see OR.

2.8 | Statistical analysis

Sample size was opportunistic since there are no data to inform a power calculation. Nonparametric tests including Mann-Whitney U test and Kruskal-Wallis test with Bonferroni post hoc test were used to detect differences between 2 groups or more than 2 groups, respectively, using GraphPad Prism 6 (GraphPad Software). Correlations were assessed using the Spearman rank correlation test. $P < .05$ was considered significant.

3 | RESULTS

3.1 | PBECs from preschool wheezers and children with STRA had increased secretion of anti-viral, Th1 and Th2 mediators in response to RSV

3.1.1 | Anti-viral response

PBECs from children with PSW and STRA had increased secretion of IFN- γ in response to RSV compared to non-asthmatic controls (NA) (Figure 1A). This was also true for STRA compared to NA when stimulated with HDM + RSV. No difference in TNF- α or CXCL10 secretion was found between the groups (Figure 1B-C). IL-6 was increased in PSW and children with STRA compared to NA when stimulated with RSV alone and RSV + HDM in combination (Figure 1D).

3.1.2 | Type1 and Type2 cytokines

PBECs from children with STRA had increased secretion of neutrophil attractant chemokines IL-8 and GRO in response to RSV alone or in combination with HDM compared to NA and PSW (Figure 1E-F). Children with PSW and STRA had enhanced secretion of the type 2 cytokine IL-13 from PBEC when stimulated with RSV or RSV + HDM in combination (Figure 1G).

3.1.3 | Viral load and replication

No differences in viral load for RSV between non-asthmatic controls, PSW and children with STRA were found, indicating similar viral replication in PBECs between the groups. UV-inactivated virus had impaired viral replication as shown by measurements of viral RNA (Figure 1J). Thus, PBECs from STRA and PSW had increased secretion of anti-viral and pro-inflammatory mediators in response to RSV alone, or in combination with HDM, compared to NA.

3.2 | PBECs from preschool wheezers and children with STRA had an altered gene expression pattern when stimulated with HDM and RSV compared to non-asthmatic controls

Gene expression of 16 genes (TLR3, MDA5, RIGI, IFN- α , IFN- γ , OAS2, IRF3, CXCL10, IL6, CXCL8, ICAM-1, CAR, PCDH1, GCR, EGFR and AREG) related to anti-viral response, pro-inflammatory markers and epithelial growth and repair in epithelial cell samples was evaluated using a fold change heatmap (Figure 2). Red represents increased

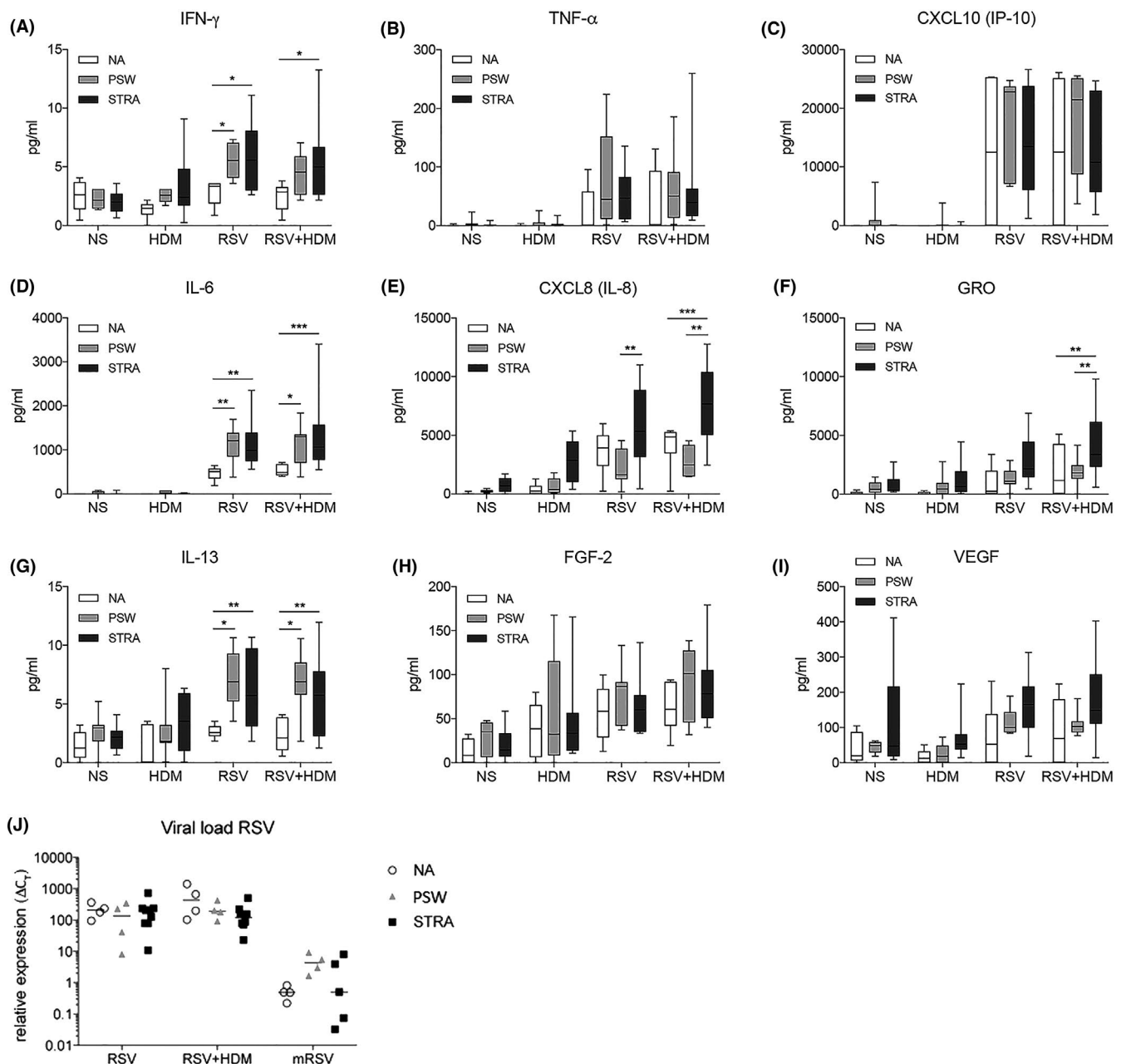


FIGURE 1 Protein secretion in response to RSV infection and HDM stimulation in PBECs from paediatric non-asthmatic controls (NA) ($n = 6$), wheezers ($n = 7$) and children with STRA ($n = 10$) using a custom made Milliplex assay. PBECs were stimulated with media alone, HDM, RSV and HDM and RSV in combination and epithelial cell supernatants analysed at 24 h post-infection: IFN- γ (A), TNF- α (B), CXCL10 (IP-10) (C), IL-6 (D), IL-8 (E), GRO (F), and IL-13 (G), FGF-2 (H), VEGF (I) and viral load (J). Statistical analysis was performed with Kruskal-Wallis test with post hoc test where * $P < .05$, ** $P < 0.01$ and *** $P < 0.001$

expression and green represents decreased expression, and data are presented for non-stimulated cells (Figure 2A) as well as cell cultures stimulated with HDM (Figure 2B), RSV (Figure 2C) and RSV + HDM (Figure 2D). There was a distinct pattern of increased gene expression of anti-viral and proinflammatory mediators in STRA and PSW compared to non-asthmatic controls. Gene expression as individual graphs is shown in Fig E1 in OR.

3.3 | Bronchial epithelial cells from preschool wheezers had impaired proliferation and wound-healing compared to STRA and non-asthmatic controls

Having seen increased anti-viral and pro-inflammatory responses to RSV infection in PBECs, we investigated epithelial function by assessing proliferation and wound healing abilities of PBECs from severe preschool wheezers, children with STRA and paediatric NA in response to poly(I:C) (a viral mimic) and allergen (HDM).

3.3.1 | Analysis per timepoint

Overall, PBECs from children with PSW proliferated very poorly over the selected time period (Figure 3A-D). Exposure of PBEC to concomitant poly(I:C) and HDM at 60 hours resulted in a significant

reduction in proliferation in PSW compared to non-asthmatic controls (Figure 3D). The ability of epithelial cells to heal scratch wounds, measured as percentage of wound closure, was also impaired in PSW compared to controls at baseline (Figure 3E).

3.3.2 | Area under curve

By comparing area under curve between the groups for each stimulus, we found that PSW had significantly lower proliferation than NA and STRA at baseline (media alone) and with all stimuli. PBECs from children with STRA had lower proliferation than NA when stimulated with HDM and poly(I:C) in combination (Figure 3I). By comparing area under curve for wound-healing, we found that PSW had significantly lower wound healing abilities than NA at baseline (media alone) and a tendency when stimulated with HDM ($P = .07$) (Figure 3J).

3.4 | Only children with STRA had increased epithelial and submucosal expression of the IL-33 receptor ST2

We have previously determined that IL-33 expression is increased in STRA patients and is potentially involved in airway remodelling.³⁰ Having found abnormal epithelial wound injury and

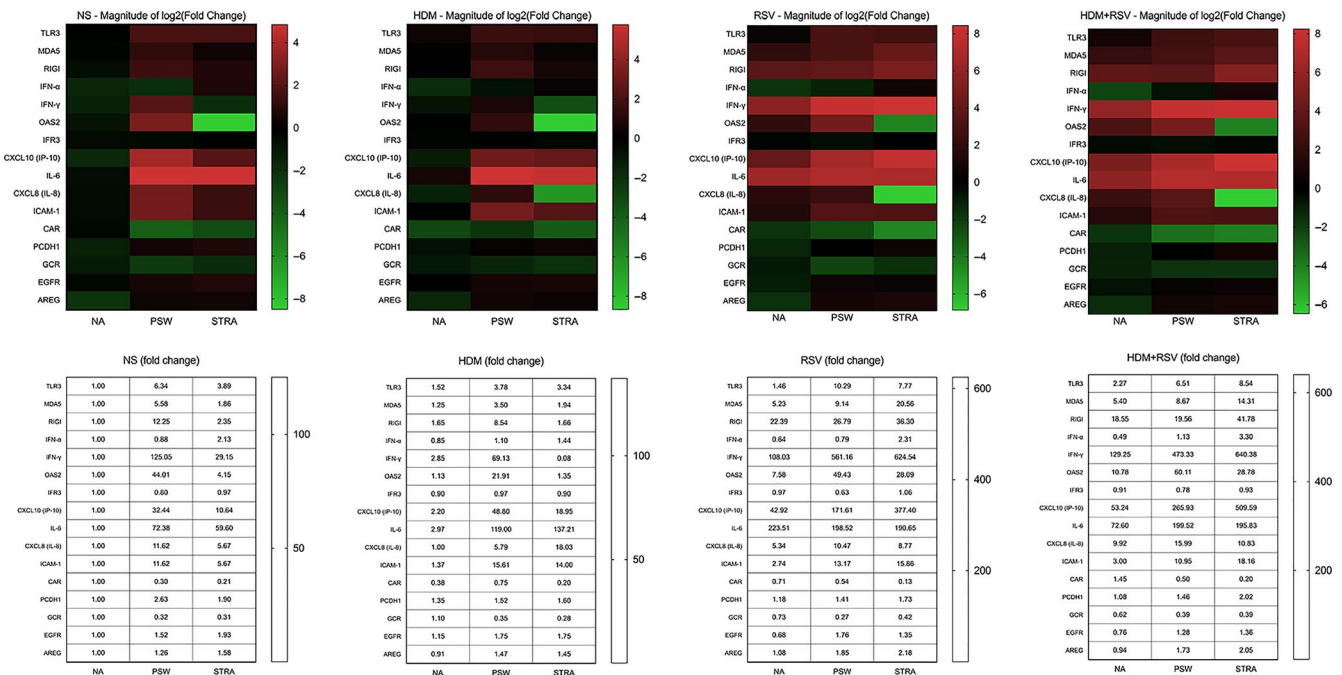


FIGURE 2 Gene expression heatmap of non-stimulated cells (A) as well as in response to HDM stimulation (B), RSV infection (C) or RSV and HDM in combination (D) in PBECs from paediatric non-asthmatic controls (NA) ($n = 6$), wheezers ($n = 7$) and children with STRA ($n = 10$). Epithelial cells were analysed at 8 h post-infection. Data are shown as log₂ fold change values (top) and tables with fold change values (bottom) for TLR3, MDA5, RIGI, IFN-α, IFN-γ, OAS2, IRF3, CXCL10 (IP-10), IL-6, CXCL8 (IL-8), ICAM-1, CAR, PCDH1, GCR, EGFR and AREG. Red and green represent greater and lower expression in compared to non-stimulated non-asthmatic controls, respectively

elevated Th2 responses following RSV infection, we determined the expression of IL-33 and its receptor ST2 in cultured PBECs in response to RSV and HDM. We confirmed in this new patient cohort an increase in IL-33 positive cells in the submucosa in STRA biopsies compared to controls (Figure 4A-C), but no difference in epithelial IL-33 expression between the groups (Figure 4D). Tissue expression of ST2 was increased in STRA compared to controls in both the submucosa and epithelium (Figure 4E-H). However, neither IL-33 nor ST2 was differentially expressed in biopsies from

PSW compared to controls. Gene expression of ST2 was similar between the groups at baseline and following stimulation with RSV (Figure 4I). Epithelial ST2 protein levels were increased in both PSW and STRA compared to controls when stimulated with RSV + HDM (Figure 4J).

Epithelial expression of the IL-33 receptor ST2 was only increased in children with STRA in bronchial biopsies, although expression was increased in both STRA and PSW PBECs stimulated with RSV and HDM.

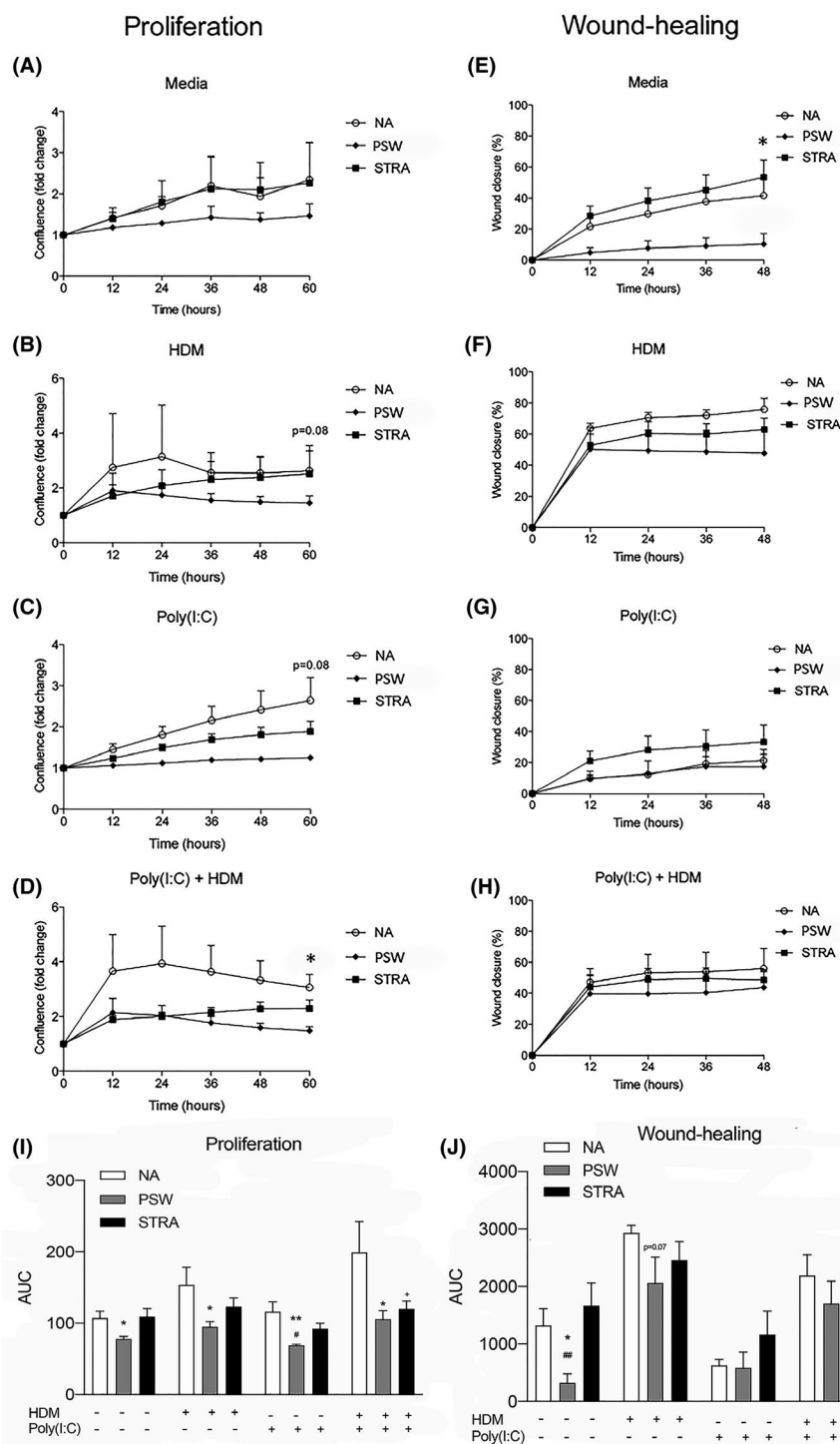


FIGURE 3 Proliferation (A-D) and wound-injury assay (E-H) in PBECs from paediatric non-asthmatic controls (NA) ($n = 6$), PSW ($n = 7$) and children with STRA ($n = 10$). PBECs were stimulated with media alone (A, E), HDM (B, F), viral mimic poly(I:C) (C, G) and HDM and poly(I:C) in combination (D, H). Data as plotted as area under curve (AUC) for proliferation (I) and wound-healing (J). Statistical analysis was performed with Kruskal-Wallis test with Bonferroni post hoc test for each time point. * denotes differences between PSW and media control. # denotes differences between PSW and STRA and + denotes differences between media control and STRA. * $P < .05$ was considered significant

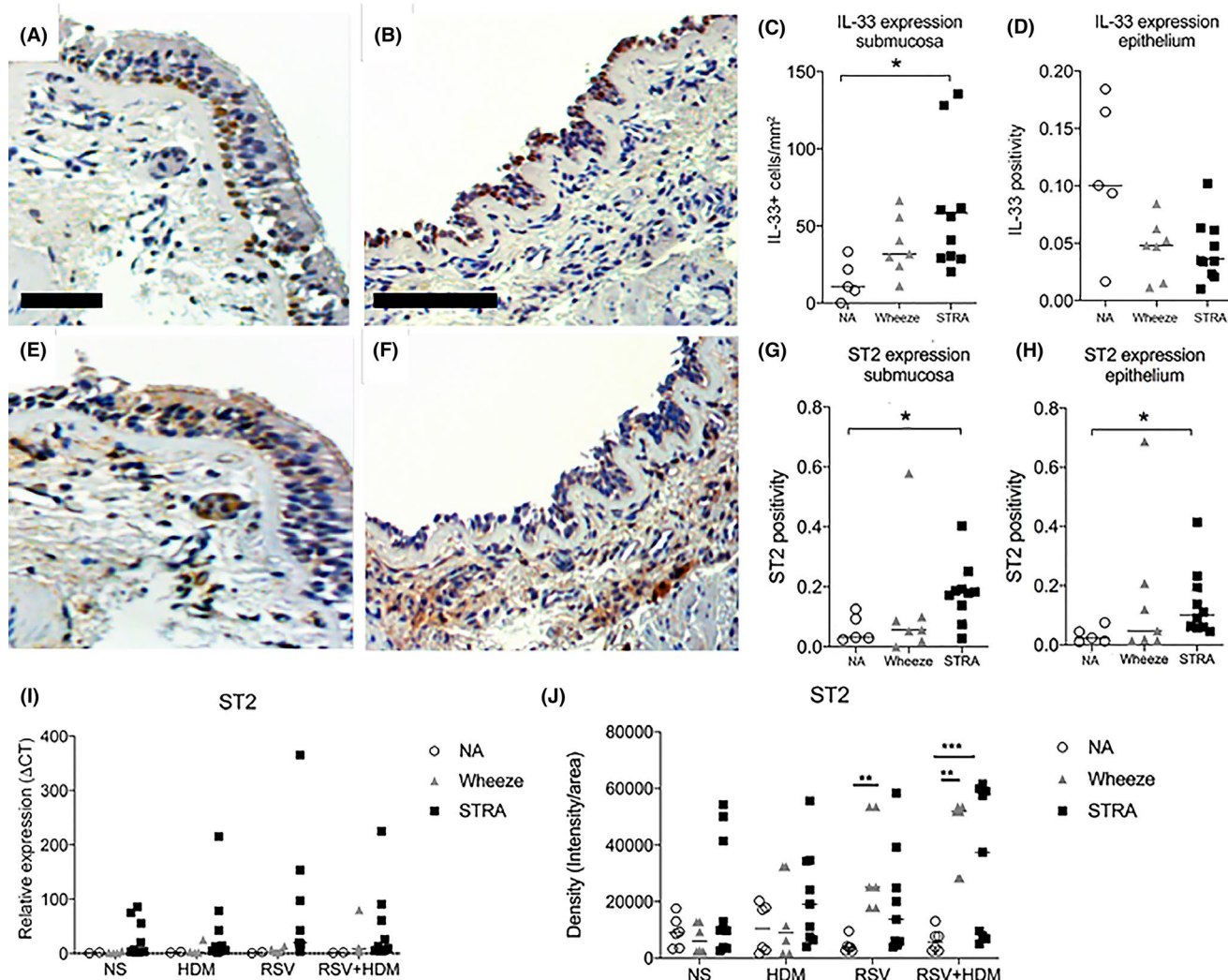


FIGURE 4 Submucosal and epithelial tissue expression of IL-33 (A-D) and ST2 (E-H) in bronchial biopsies from paediatric non-asthmatic controls (NA) ($n = 5$), wheezers ($n = 7$) and children with STRA ($n = 10$). Scale bars: A and E: 50 μm and B and F: 100 μm . One control subject was excluded due to inadequate tissue sample. PBECs were stimulated with media alone, HDM, RSV and HDM and RSV in combination and gene (I) and protein (J) expression of ST2 in response to RSV and HDM in PBECs from paediatric non-asthmatic controls ($n = 6$), wheezers ($n = 6$) and children with STRA ($n = 9$) was measured. Three subjects (1, non-asthmatic control, 1 wheeze and 1 STRA) were excluded due to inadequate RNA/protein concentrations. Statistical analysis was performed with Kruskal-Wallis test with Bonferroni post hoc test where * $P < .05$ and ** $P < .01$

3.5 | IL-33 was associated with both tissue collagen deposition and EGFR expression in preschool wheezers and children with STRA

Since IL-33 has been shown to induce extracellular matrix protein deposition²⁷ and associated with remodelling,³⁰ we investigated the tissue density of collagen in endobronchial biopsies using trichrome staining. The staining displayed positivity (blue colour) for collagen in the submucosal region as well as in the reticular basement membrane.³³ The density of collagen was increased in the submucosa in STRA compared to PSW (Figure 5A-D). To investigate the association between tissue expression of IL-33 and collagen, a correlation analysis was performed, showing a positive correlation between IL-33 and collagen density in both STRA and PSW (Figure 5E) but no significant correlation in non-asthmatic controls (Figure 5F). Since

PBECs from PSW had impaired proliferation and wound-healing, we investigated the tissue expression of the epidermal growth factor receptor (EGFR). The immunohistochemical staining for EGFR displayed a positive signal in the basal layer of the epithelium close to the reticular basement membrane, and staining is in accordance with previous literature.³⁴ Tissue expression of EGFR was lower in PSW compared to both non-asthmatic controls and STRA (Figure 5G-J). As the whole control group were not specifically age-matched to PSW, we compared epithelial EGFR expression in PSW (mean age 2 years) and age-matched non-wheezers (mean age 2.7 years). Epithelial EGFR expression remained significantly reduced in PSW compared to age-matched controls (Figure 5K). We found no significant correlation between EGFR expression and age in the control and PSW groups (see OR, Fig E2A-C). There was a positive correlation between EGFR and collagen density in STRA and PSW but

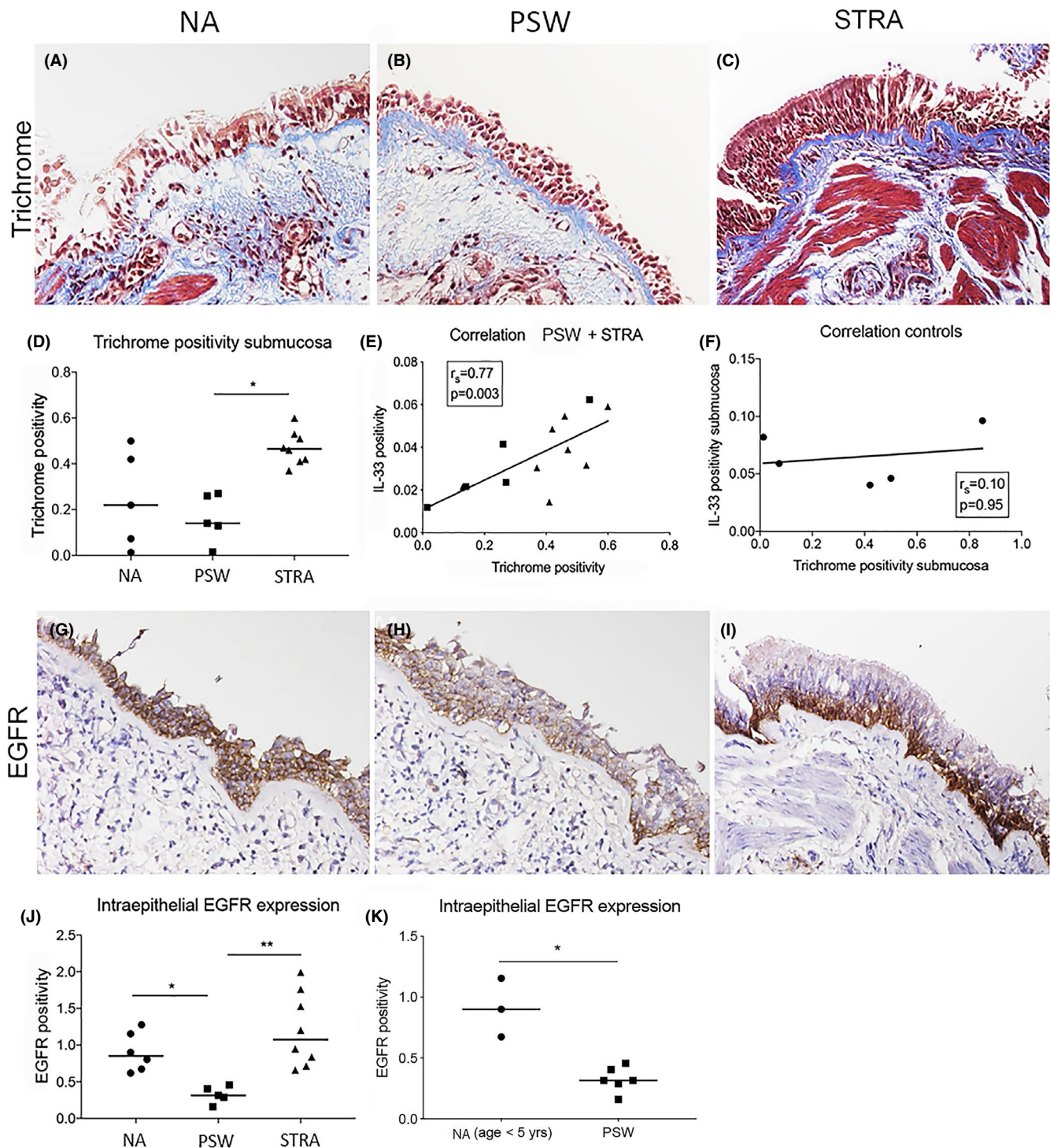


FIGURE 5 Submucosal and epithelial tissue density of collagen (trichrome staining, collagen; blue) (A-D) and correlations between IL-33 and collagen density in (E) wheeze (square) + STRA (triangle) and non-asthmatic controls (F). Epithelial immunohistochemical staining for EGFR (G-I) and quantification (J) in bronchial biopsies from paediatric non-asthmatic controls, wheezers and children with STRA. Since controls and PSW were not age-matched, comparison between non-asthmatic controls < 5 y of age and PSW was analysed separately (K). Paediatric non-asthmatic controls (A,G: n = 5), wheezers (B,H: n = 5) and children with STRA (C, I: n = 8). 5 subjects (2 wheeze and 2 STRA) were excluded due to inadequate tissue sample. Scale bars: A and G: 100 µm. Statistical analysis was performed with Kruskal-Wallis test with Bonferroni post hoc test where * $P < .05$ and ** <0.01 . Correlation analysis was performed using Spearman's rank correlation where significant differences are considered $P < .05$

no significant correlation in non-asthmatic controls (see OR, Fig E2D-E). Children with PSW had reduced epithelial EGFR expression compared to controls, while children with STRA had increased tissue collagen deposition and EGFR expression compared to PSW.

3.6 | IL-33 impaired epithelial wound closing in preschool wheezers compared to controls

In order to investigate cell growth and wound injury in response to IL-33 alone or in combination with poly(I:C), wound closure was measured over 48 hours, using the IncuCyte live-cell imaging system. By comparing area under curve between the groups, we found that PSW had significantly lower proliferation and lower wound healing abilities than NA and STRA when stimulated with IL-33 and IL-33 in combination with poly(I:C) (Figure 6A and B). For the percentage of wound closure for each group, see OR, Fig E3.

4 | DISCUSSION

Bronchial epithelial cells from preschool children with severe recurrent wheeze had reduced proliferative capacity compared to older children with STRA and non-asthmatic controls at baseline and when stimulated with allergen HDM and viral mimic poly(I:C). Furthermore, PSW had reduced epithelial proliferative and wound-healing capacity in response to IL-33. The significantly reduced expression of EGFR in PSW alone suggests an intrinsic defect in the epithelium of children with PSW, and an altered capacity to heal following epithelial injury may lead to reduced epithelial barrier function and exaggerated remodelling.³¹

Repair of a damaged epithelium is a crucial part of restoring airway function to its normal state. It involves migration and spreading of epithelial cells into the damaged region and proliferation of new epithelial cells.^{35,36} In a study using bronchial biopsies, epithelial injury without significant eosinophilic inflammation has been observed in childhood asthma.³⁴ Furthermore, dysregulated repair in

response to mechanical wounding of the epithelium in paediatric asthmatics has been previously described.^{16,37} This indicates an intrinsic reduced ability to respond appropriately to injuries. Our study shows that even younger children with severe preschool wheeze have impaired epithelial proliferation in response to the viral mimic poly(I:C) and allergen HDM. PSW also showed lower proliferation when stimulated with the combination of the two, a condition that experimentally mimics a wheeze attack. We also found that epithelial cells from wheezers had diminished wound-healing capacity at baseline, further indicating an intrinsic impaired ability to repair epithelial damage. Thus, the airway epithelium in severe preschool wheezers appears predisposed to damage by harmful stimuli, such as viral infections or allergens, compared to a healthy epithelium, ultimately leading to the pathological abnormalities including airway remodelling (increased thickness of the reticular basement membrane) that have been described.^{14,31,38} In our model, poly(I:C) alone did not have an effect on wound gap closure in PBECs from PSW. Poly(I:C) lowered wound gap closure in non-asthmatic controls compared to baseline (approximately 20% decrease, $P = .03$). Bronchial epithelial cells from healthy individuals respond to poly(I:C) with for example production of inflammatory mediators and poly(I:C) has been shown to induce cell death and damage in HBECs from normal individuals which might affect their ability to heal wounds³⁹ by affecting proliferation and growth. However, we saw an increased wound gap closure in all groups when treated with HDM compared to media alone. Closing of a wound gap by bronchial epithelial cells is dependent on both proliferation and migration of the cells.⁴⁰ HDM has been found to contain proteases that could cleave cell-to-cell and cell-to-surface binding as well as via protease-activating receptor (PAR)-2 to promote epithelial cell migration.^{41,42}

It has been shown that bronchial epithelial cells from children with STRA elicit ineffective antiviral response to RV infection⁴³. Although RSV gave rise to equal viral load in all groups, PSW and STRA patients responded in a heightened way for some cytokines. We have shown that preschool wheezers and children with STRA had increased gene expression of pattern recognition receptors in response to RSV with and without concomitant stimulation with

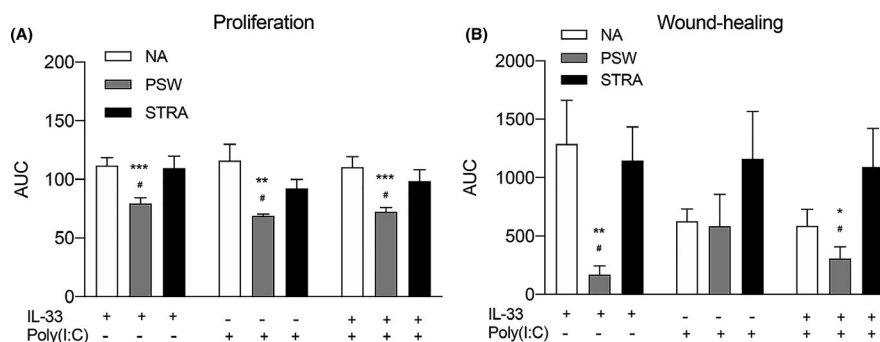


FIGURE 6 Wound-injury assay response at 48h in PBECs from paediatric patients (A) non-asthmatic controls (NA) ($n = 6$), (B) wheezers ($n = 7$) and (C) children with STRA ($n = 10$). PBECs were stimulated with media alone, IL-33, viral mimic poly(I:C) and IL-33 and poly(I:C) in combination. Area under curve (AUC) was calculated for proliferative capacity (A) and wound-healing ability (B). Statistical analysis was performed with Kruskal-Wallis test with Bonferroni post hoc test for each time point. *denotes differences to media control. #denotes differences to STRA. * $P < .05$ was considered significant

HDM compared to controls. This might explain the heightened response for some of the cytokines in PSW and STRA. We used RSV because of epidemiological data that show RSV infection is associated with hospitalization and severe recurrent wheeze, but in future could expand to include RV.^{44,45} However, children are exposed to multiple viruses across the life course; therefore, we also used poly(I:C) as a viral mimic to examine wound injury via live-cell imaging. Poly(I:C) showed an impaired effect on cell proliferation in both STRA and PSW which indicates a widespread change in response to TLR3 agonism that is unlikely to be pathogen specific.

We have shown that preschool children with severe wheeze and school-aged children with STRA exhibited an augmented response of both Th1 and Th2 cytokines to RSV with and without concomitant stimulation with HDM. One important feature that distinguished epithelial cells from children with STRA compared to preschool wheeze was the increased production of GRO and IL-8 only in STRA compared to controls. These chemokines are crucial for generating an appropriate neutrophilic response towards pathogens.⁴⁶ We have previously shown that a subpopulation of children with STRA is characterized by an increased density of intra-epithelial neutrophils that correlated to better lung function, better asthma control and lower dose of maintenance inhaled steroids.⁴⁷ Results from the current study support our previous findings that intra-epithelial neutrophils might be beneficial during STRA-associated pathology. To further investigate the connection to inflammatory response, all parameters (proliferation, wound-healing, gene/protein expression and tissue expression of IL-33, ST2, EGFR and collagen) was correlated to levels of eosinophils and neutrophils bronchoalveolar lavage or blood. However, no meaningful strong correlations were found.

Injury to epithelial surfaces triggers release of a variety of pro-inflammatory and Th2-promoting innate cytokines, including IL-25, IL-33 and TSLP.^{13,48} Elevated IL-33 levels correlate with increased asthma severity in children, and expression of IL-33 correlates with airway hyper-responsiveness and remodelling in asthma.^{30,49,50} In the present study, we were able to confirm our previous finding of increased submucosal IL-33⁺ cells in children with STRA,³⁰ but have shown for the first time that submucosal IL-33 expression was not increased in preschool wheeze. Looking specifically at the epithelial compartment, we found no difference in epithelial IL-33 expression between any of the groups, in contrast to previous reports in adults.⁵⁰ Furthermore, we could not detect any differences in IL-33 secretion, protein or mRNA, in our cultured epithelial cells at baseline or in response to RSV. The inability to detect IL-33 may be due to the time point, since IL-33 has been shown to be secreted and rapidly oxidized after stimulation.^{51,52} The airway samples in our study were collected during stable disease, which may also reflect the expression pattern of IL-33 in the epithelium. IL-33 binds to a receptor complex consisting of two transmembrane proteins: IL-1R1 (ST2) and IL-1RAcP. Our previous study of neonatal ST2 knockout mice showed that HDM activation of the IL-33/ST2 pathway is important for the development of airway hyper-responsiveness

with reduced airway remodelling.³⁰ However, to our knowledge ST2 expression in human bronchial epithelium in children with PSW or STRA has not been reported. We detected an increase in ST2 expression in both submucosa and epithelium in STRA, but not PSW, compared to controls. We therefore determined the expression of ST2 in cultured PBECs in response to RSV and HDM and found that protein expression of ST2 increased in both wheezers and STRA compared to controls when stimulated with RSV + HDM. Our results suggest a higher responsiveness in wheezers and STRA during virally induced asthma exacerbations. The gradual increase in ST2 expression, from post-viral stimulation in preschool wheeze, to constantly expressed in STRA might also be an indicator of disease progression and severity.

Little is known about the effect of IL-33 on epithelial wound injury in children. IL-33-mediated activation of ILCs induced production of amphiregulin, a ligand for the epidermal growth factor receptor (EGFR) which promotes collagen production and remodelling,⁵³ and induced proliferation and differentiation in epithelial cells during infection.⁵⁴ In our study, we were able to confirm a correlation between epithelial expression of EGFR and collagen deposition in the submucosa of PSW and STRA children. We also showed a positive association between IL-33 expression and collagen deposition in wheeze and STRA patients. Additionally, PBECs from wheezy children had an impaired wound healing and proliferative capacity in response to IL-33. The different effects of IL-33 in preschool wheezers and STRA compared to healthy controls may explain why wheezing/asthmatic children are more susceptible or have an exaggerated response to virus and allergens.

The wheeze patients had lower EGFR expression than both STRA and controls, which may affect their proliferative and migrative ability.⁵⁵ EGFR has multiple ligands, therefore, investigating the expression and effects of these ligands on bronchial epithelial cell function, and airway remodelling in patients with severe PSW and STRA is an important avenue to pursue for potential novel therapeutic targets, particularly since EGFR inhibitors are already available as anti-cancer therapies. We acknowledge that our control and PSW groups are small and not age-matched and conclusions might be limited. However, it is exceedingly challenging to have young children undergoing clinical bronchoscopy and moreover to obtain parental consent for extra samples to be taken for research. Hence, the number of patients we can recruit is very limited. To compensate for the age mismatch, EGFR tissue expression in PSW (mean age 2 years) was compared to a subgroup of age-matched non-wheezers (mean age 2.7 years) which confirmed that EGFR expression remained significantly lower in wheezers, suggesting a true disease effect. This suggests that duration of disease, since diagnosis of asthma is established at school-age, may affect EGFR expression.

In conclusion, bronchial epithelial cells from children with severe, recurrent wheeze had lower proliferative capacity at baseline and in response to HDM and viral mimic poly(I:C), alone and in combination. The reduced epithelial EGFR expression in PSW, together with lower wound healing response to IL-33 and common allergen

HDM, may lead to a damaged epithelial barrier function in early life in preschool wheezers and contribute to the development of airway remodelling and asthma pathogenesis.

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CONFLICT OF INTEREST

All authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

CA, JI, SS and CL involved in conception and design; CA, JI, PR, JC, PN, SM and LF involved in analysis and data acquisition. CA, JI, PN, AB, SS and CL interpreted the data; CA, JI, AB, SS and CL drafted the manuscript for important intellectual content.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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ONLINE SUPPLEMENT

Impaired airway epithelial cell wound-healing capacity is associated with airway remodelling following RSV infection in severe preschool wheeze

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Running title: Respiratory viruses in paediatric severe therapy resistant asthma

1 MATERIAL AND METHODS

2 Subjects

3 10 school-aged children with STRA (22) (median age 12 [range 6-16] years) and 7 preschool children
4 with severe, recurrent wheeze (median age 2 [range 1-5] years) were recruited from the Royal
5 Brompton Hospital. Children with STRA underwent a clinically indicated bronchoscopy to characterize
6 airway pathology, exclude alternative or associated diagnoses such as upper airway malacia and
7 develop personalised treatment plan. Additional research endobronchial brushings and biopsies were
8 taken during the procedure with parental informed consent (4). All STRA children had previously
9 undergone a detailed assessment to ensure any modifiable factors such as poor adherence or
10 persistent allergen exposure had been addressed prior to the bronchoscopy (23). Non-asthmatic
11 control subjects were undergoing clinically indicated bronchoscopy and agreed to research samples
12 being taken at the same time (n=5). One of these controls had a bronchoscopy with collection of
13 biopsies but no viable brush cells. Normal bronchial epithelial cells from 2 paediatric healthy donors
14 were also purchased from Lonza (no biopsies were sampled from these donors). The study was
15 approved by the NRES Committee London - Chelsea, and informed parental consent and child assent,
16 when appropriate, were obtained. Ethical approval was obtained from National Research Ethics
17 Committee, Royal Brompton Hospital (RBH) Research and Design department (15/LO/1885,
18 08/H0708/3, 15/SC/0569).

19

20 Epithelial cell culture and stimulation

21 Primary bronchial epithelial cells (PBECS) were seeded into collagen coated tissue culture flasks
22 containing BEGM (Lonza, Walkerville, Md) and used at passage 3 at 80% confluency (except for
23 proliferation) for all experiments. PBECS were stimulated with RSV [moi 1] (strain RSV A2, isolated from
24 infant bronchiolitis), with and without concomitant exposure to house dust mite (HDM) (5µg/mL)
25 (Greer, London, UK). Proliferation and wound-healing measurements were performed using the TLR3

agonist ds RNA poly(I:C) (10ug/mL) (Sigma) and recombinant IL-33 (100 ng/mL, BioLegend). Samples for mRNA extraction were harvested after 8h stimulation, while culture supernatants were collected after 8 and 24h and cells for protein analysis in RIPA buffer (Sigma-Aldrich, Gillingham, UK) after 24h. Cell cultures were collected from brushes in BEGM medium at the Royal Brompton Hospital and cultured as previously described (1). At this stage (cell passage 0) 1/10 of the cultures from controls and STRA were unsuccessful and 1/5 of the cultures from PSW were unsuccessful. This was mostly due to infections or more commonly that too few cells were obtained to start with). From passage 1, and up to 3 where the experiments were performed, only one culture (from a non-asthmatic control) was unsuccessful.

Assessment of proliferation and wound repair

The ability of airway epithelial cells to proliferate and repair wounds following exposure to HDM (5µg/mL), poly I:C (10µg/mL) and IL-33 (100ng/mL) were assessed by image analysis using the IncuCyte live-cell imaging system (Essen Bioscience, Ann Arbor, MI, USA). PBEC cultures were seeded in duplicates in 96 well culture plates at a density of 10 000 cells per well. For proliferation assays, cells were monitored for a total of 60 hours taking 2 images every 3 hours. The density of cells was calculated by identification of cellular confluence through the confluence image mask provided in IncuCyte S3 Software (Essen Bioscience) and expressed as occupied area (% confluence). For wound-healing assays, PBECs were grown in BEGM on 96 well ImageLock tissue culture plates until >80% confluent. Using WoundMaker device (Essen Bioscience), cell monolayers were wounded with a single scratch. Cultures were incubated in EGF free BEGM at 37°C and scratch area was monitored for 48 hours (2 images every 4 hours) using IncuCyte live-cell imaging system (Essen Bioscience), with % wound closure calculated by identification of cellular confluence in the originally created wound area compared to outside the wound. Results were normalised to each patient's non-stimulated control.

RNA extraction and real-time PCR

Total RNA was extracted from epithelial cells using the Qiagen RNeasy Micro Kit (Qiagen, Hilden, Germany). To ensure RNA quality, RIN value and quantity of RNA was determined (Agilent Technologies, Santa Clara, California) and analysed using Agilent 2200 TapeStation System (Agilent Technologies). cDNA was synthesized from 500ng of total RNA and analysed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA) (see OR). Gene expression was measured using TaqMan primers for specific genes. Reactions were carried out in duplicate in 384 well plates (Applied Biosystems). Real time PCR reactions were carried out with Taqman Fast Advanced Master Mix (Life technologies) and performed on Viia7 system (Applied Biosystems). The housekeeping gene GAPDH was used as a reference gene for PCR amplifications.

Quantification of cytokines

Cell culture supernatant was collected and cytokines analysed using a customised Milliplex panel (Millipore Corporation, Billerica, Mass) for human cytokines and chemokines. Soluble ST2 (sST2) was measured using ELISA according to manufacturer's instructions (R&D Systems, Abingdon, United Kingdom).

Western Blots and densitometry

Cell lysates were cleared by means of centrifugation at 10,000 *g* for 10 minutes. Protein was quantified using BCA protein assay kit (Thermo Fisher Scientific) and sample volumes are adjusted to normalise protein. One microgram of protein per well in LDS sample buffer (Invitrogen, Carlsbad, Calif) was loaded onto 4-12% Bis-Tris gels (Bio-Rad Laboratories, Watford, UK) and run at 100V in a Criterion Cell (Bio-Rad Laboratories). Proteins were transferred to the membrane using Pierce Power Blotter (Thermo Fisher Scientific). ST2 primary antibody (1:100, Sigma Aldrich) was added at 1:500 dilution in blocking buffer and incubated overnight at 4°C. α -Tubulin (1:1000, Abcam, Cambridge, United Kingdom) was used as a loading control. Horseradish peroxidase-conjugated anti-rabbit antibody (Cell Signaling Technology, Danvers, Mass, US) was used at 1:1000 dilution, and membranes were

developed with ECL substrate (Pierce, Cheshire, United Kingdom). The membranes were then washed in wash buffer and developed in ECL plus western blotting detection system prior to imaging and quantification of band density (intensity per area) on the myECL Imager (Thermo Fisher Scientific).

Histopathology

Endobronchial biopsies were fixed in formal saline and processed to paraffin. Five-micrometre sections were stained with haematoxylin and eosin and used to assess morphology and consecutive sections were used for immunohistochemical staining for IL-33 (1:100, Nesy; Enzo Life sciences, Exeter, UK), ST2 (1:100, Atlas antibodies/Sigma-Aldrich) and epidermal growth factor receptor (EGFR, 1:100, Dako, Glostrup, Denmark). Binding of primary antibodies were visualised using DAKO Envision system (Dako) according to manufacturer's directions. Sections were counter stained with haematoxylin and mounted in DPX (Sigma-Aldrich). Positive staining was quantified manually and expressed as number of cells/area of tissue if cells were clearly identified as individual objects such as in the case of submucosal IL-33. When positive staining was found in large number of cells or if staining was present in structural cells or matrix, positive staining was quantified as positivity (positive brown pixels divided by all stained pixels) using computerised image analysis on blinded sections using ImageScope (Aperio, Vista, CA). Negative controls (omitting the primary antibody) and isotype controls (Dako) were used for evaluation of antibody specificity. For analysis of collagen deposition one consecutive section was stained with Masson's trichrome and quantified as positivity (positive blue pixels divided by all stained pixels) in blinded sections using computerised image analysis ImageScope (Aperio).

Statistical analysis

Sample size was opportunistic since there are no data to inform a power calculation. Nonparametric tests including Mann-Whitney U test and Kruskal Wallis test with Bonferroni post hoc test were used to detect differences between 2 groups or more than 2 groups, respectively using GraphPad Prism 6

- 1 (GraphPad Software, La Jolla, CA). Correlations were assessed using the Spearman rank correlation
- 2 test. $p < 0.05$ was considered significant.

RESULTS

PBECs from preschool wheezers and children with STRA had increased anti-viral and pro-inflammatory gene expression when infected with RSV

We initially investigated the anti-viral responses to RSV infection with and without HDM stimulation on PBECs by measuring gene expression of pattern recognition receptors, anti-viral and pro-inflammatory cytokines. Data were presented as fold change from non-stimulated non-asthmatic controls.

Pattern recognition receptors: TLR3 expression was significantly higher in PSW compared to controls at baseline (medium alone) (Figure E1A). In RSV infected cells, the expression of TLR3 was higher in PSW and STRA compared to controls. A combination of RSV and HDM, however, resulted in increased expression of TLR3 only in STRA when compared to controls (Figure E1A). Similarly, the expression of melanoma differentiation-associated protein 5 (MDA5) was higher in STRA following exposure to RSV or RSV+HDM compared to controls (Figure E1B). Following exposure to RSV+HDM, cells from STRA exhibited increased gene expression of retinoic-acid inducible gene I (RIGI) in comparison to controls (Figure E1C).

Anti-viral and proinflammatory cytokines: Expression of IFN- α was higher in STRA compared to both controls and PSW only when stimulated with RSV+HDM (Figure E1D). There no differences in IFN- γ between groups at baseline or following stimulation (Figure E1E). CXCL10 was increased only in STRA compared to controls when stimulated with RSV+HDM (Figure E1F). No difference in gene expression of IL-6 and IL-8 was observed between the groups (Figure E1G-H).

EGFR expression does not correlate with age in controls or preschool wheezers

We found that EGFR tissue expression was decreased in PSW compared to non-asthmatic controls and STRA. Since the PSW group are younger than the patients with STRA we wanted to investigate if there are any associations between EGFR tissue expression and age in each of the groups using a spearman rank correlation test. We found no correlation between EGFR and age in controls ($r_s=0.0$, $p>0.99$) and PSW ($r_s=0.08$, $p=0.6$). However, a trend towards a correlation within the STRA group

($r_s=0.51$, $p=0.2$) was found although it did not reach significance which is possible due to low number of patients. There was a positive correlation between EGFR and collagen density in STRA and PSW but no significant correlation in non-asthmatic controls (Fig E2D-E).

IL-33 impaired epithelial wound closing in preschool wheezers compared to controls

In order to investigate cell growth and wound injury in response to IL-33 alone or in combination with poly(I:C), wound closure was measured over 48 hours, using the IncuCyte live-cell imaging system. Neither IL-33 ($p>0.99$) nor poly(I:C) ($p=0.18$) had an effect on wound closure in non-asthmatic controls (Figure E3A). PBECs from PSW had lower capacity to close wounds compared to both non-asthmatic controls and STRA, and this was further impaired following IL-33 stimulation ($p=0.05$) (Figure E3B). No effect was observed with poly(I:C) in preschool wheezers ($p>0.99$). In children with STRA, epithelial wound closure was impaired in the presence of IL-33, either alone ($p=0.01$) or in combination with Poly(I:C) ($p=0.02$) (Figure E3C). Therefore, IL-33 had no effect on epithelial wound-healing in non-asthmatic controls, but wound-healing was significantly impaired in both PSW and STRA compared to controls following IL-33 stimulation.

1 REFERENCES

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5

Table E1. Subject characterization - Additional medications

	Non-asthmatic controls [†] (n=7)	Pre-school wheeze (n=7)	STRA (n=10)
Medications			
Anti-histamines	1	0	8
Pizotifen	0	0	1
Muscarinic receptor antagonist	0	0	1
Antibiotics	0	2	3
EpiPen	0	0	1
Omeprazole	1	1	1
Nasal steroid	0	0	3
Cortisone cream	0	1	1
Vitamin D	0	0	1

[†]5 non-asthmatic controls were undergoing clinically indicated bronchoscopies and had agreed to a research bronchoscopy: brushings were collected from 4 and biopsies from 5 subjects. HBECs from 2 non-asthmatic controls were also bought from Lonza.

1 LEGENDS

2 **Figure E1.** Gene expression in response to RSV and HDM in PBECs from paediatric non-asthmatic
3 controls (NA) (n=6), wheezers (n=6) and children with STRA (n=9). PBECs were stimulated with media
4 alone, HDM, RSV and HDM and RSV in combination. Gene expression at 8 hours post infection for:
5 TLR3 (A), MDA5 (B), RIGI (C), IFN- α (D), IFN- λ (E), CXCL10 (IP-10) (F), IL-6 (G), and IL-8 (H). Data were
6 presented as fold change from the average value of each gene for non-stimulated non-asthmatic
7 control. Statistical analysis was performed with Kruskal-Wallis test with post hoc test where *P<0.05,
8 **<0.01 and ***<0.001. Two subjects (1 wheeze and 1 STRA) were excluded due to inadequate RNA
9 concentrations.

10

11 **Figure E2.** Immunohistochemical staining for EGFR in bronchial biopsies was correlated to age in non-
12 asthmatic controls (A), wheezers (B) and children with STRA (C) and to Trichrome positivity in
13 PSW+STRA (D) and controls (E). Correlation analysis was performed using Spearman's rank correlation
14 where significant differences are considered p<0.05.

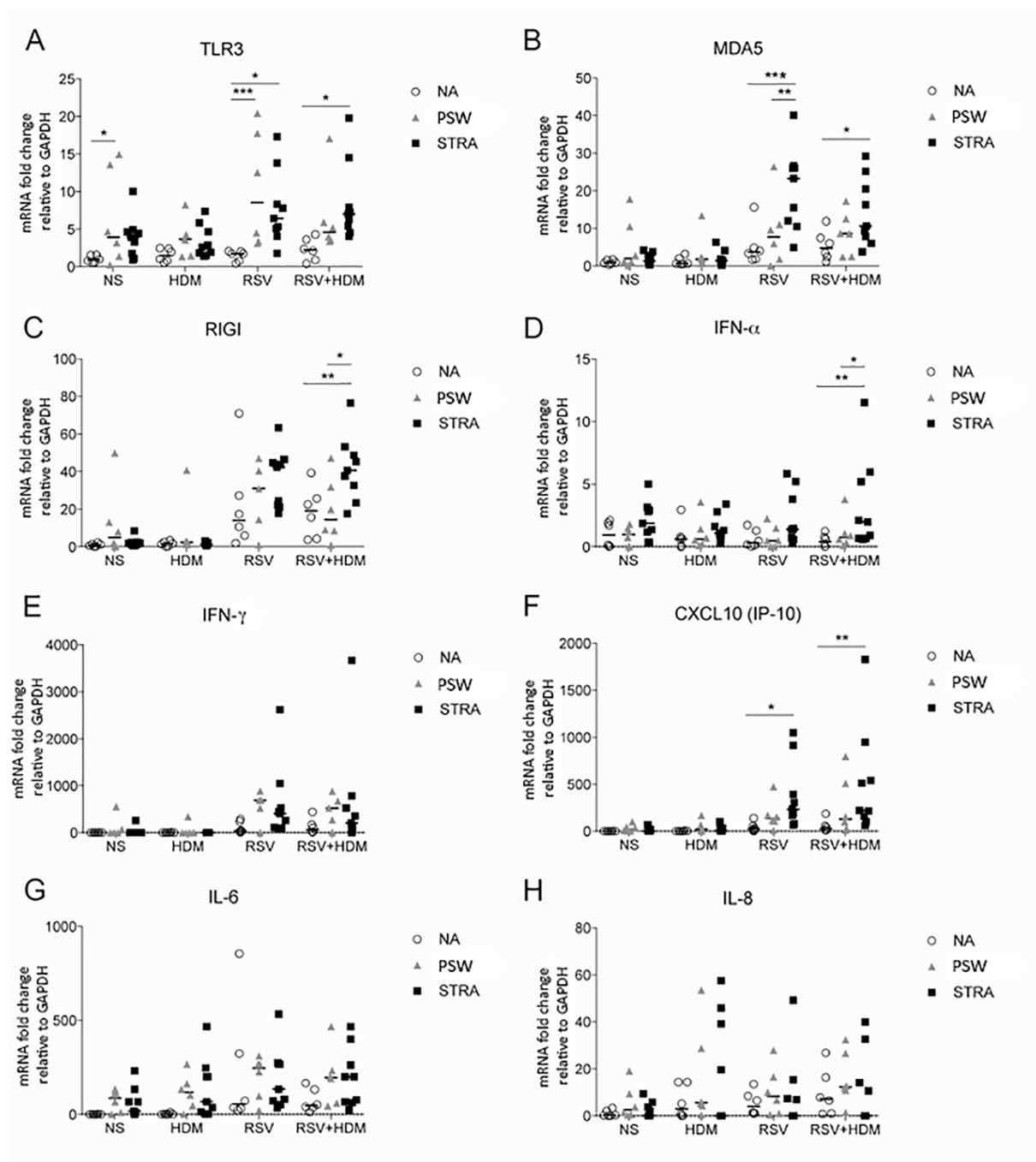
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16 **Figure E3.** Wound-injury assay response at 48h in PBECs from paediatric (A) non-asthmatic controls
17 (NA) (n=6), (B) wheezers (n=7) and (C) children with STRA (n=10). PBECs were stimulated with media
18 alone, IL-33, viral mimic poly(I:C) and IL-33 and poly(I:C) in combination. Statistical analysis was
19 performed with Kruskal-Wallis test with Bonferroni post hoc test for each time point. * denotes
20 differences to media control. p<0.05 was considered significant.

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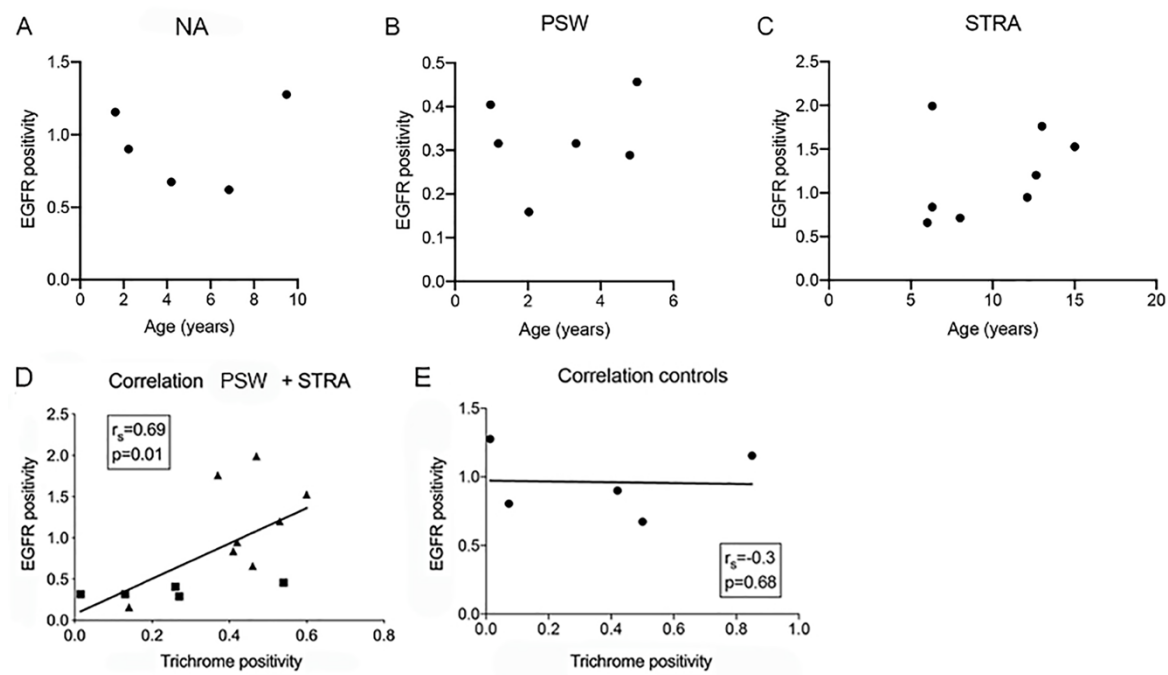
1 Figure E1.

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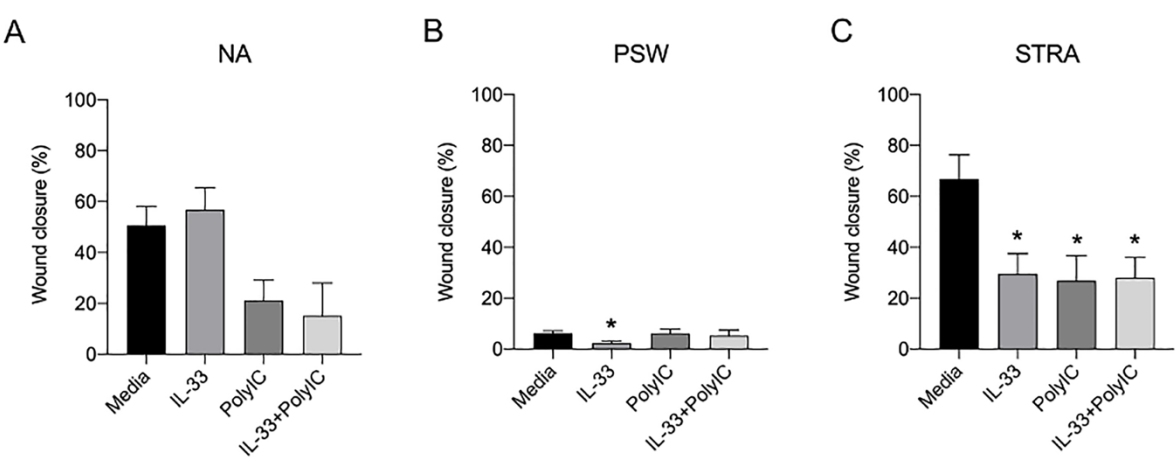
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1 Figure E2.



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1 Figure E3.



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