

ORIGINAL ARTICLE

Asthma and lower airway disease

ST2 expression and release by the bronchial epithelium is downregulated in asthma

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Abstract

Background: The airway epithelium plays an important role in wound repair, host defense and is involved in the immunopathogenesis of asthma. Genome wide association studies have described associations between ST2/Interleukin (IL)-33 genes in asthma, but its role in bronchial epithelium is unclear.

Methods: ST2 expression was examined in subjects with asthma and healthy controls in bronchial epithelium from biopsies ($n = 27$ versus $n = 9$) and brushings ($n = 34$ versus $n = 20$) by immunohistochemistry and RNA-Seq. In human primary bronchial epithelial cells ST2 mRNA and protein expression were assessed by qPCR, flow cytometry, Western blotting, and immunofluorescence. IL-33 function in epithelial cells was examined by intracellular calcium measurements, wound healing assays, and synthetic activation by gene array and ELISA.

Results: Bronchial epithelial ST2 protein expression was significantly decreased in biopsies in subjects with asthma compared to healthy controls ($P = .039$). *IL1RL1* gene expression in bronchial brushes was not different between health and disease. In vitro primary bronchial epithelial cells expressed ST2 and IL-33 stimulation led to an increase in intracellular calcium, altered gene expression, but had no effect upon wound repair. Epithelial cells released sST2 spontaneously, which was reduced following stimulation with TNF α or poly-IC. Stimulation by TNF α or poly-IC did not affect the total ST2 expression by epithelial cell whereas surface ST2 decreased in response to TNF α , but not poly-IC.

Conclusion: In asthma, bronchial epithelium protein expression of ST2 is decreased. Our in vitro findings suggest that this decrease might be a consequence of the pro-inflammatory environment in asthma or in response to viral infection.

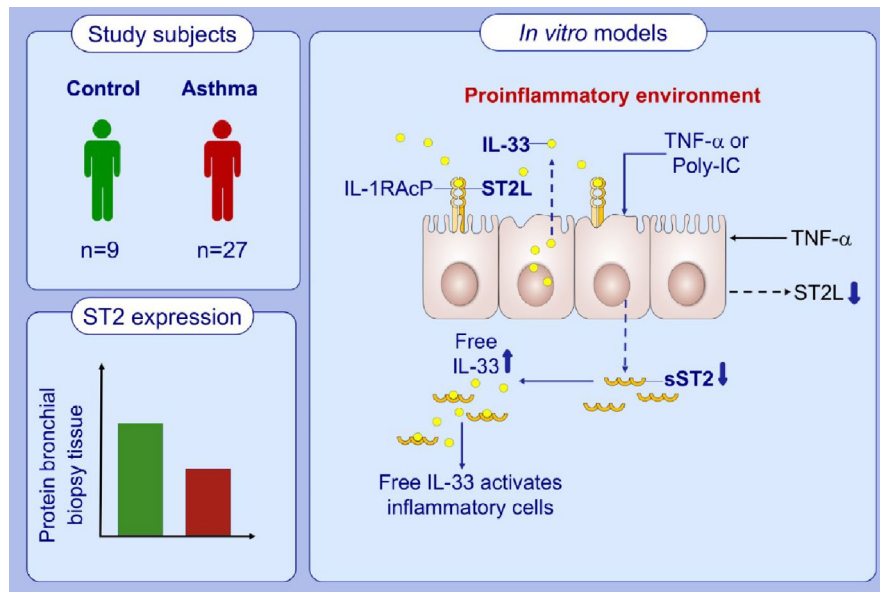
KEYWORDS

epithelial cells, ST2

Abbreviations: ASM, Airway smooth muscle; BEAS-2B, The human virus-transformed bronchial epithelial cell line; DAB, the 3,3'-diaminobenzidine; DAPI, 4',6'-diamidino-2 phenylindole; Eva, Emphysema vs Airway disease; FPKM, fragments per kilobase of transcript per million mapped reads; GINA, Global Initiative for Asthma treatment steps; GMFI, Geometric mean fluorescence intensity; IL-33, Interleukin-33; ILC2, Group 2 innate lymphoid cells; poly-IC, Polyinosinic:polycytidylic acid; qPCR, Quantitative PCR; RNA-Seq, RNA sequencing; r^s , Spearman rank correlation coefficient; sST2, Soluble ST2; ST2L, Transmembrane bound isoform; TBS-T, Tris-buffered saline containing 0.1% Tween-20; χ^2 , Chi-squared.

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GRAPHICAL ABSTRACT

ST2 and soluble ST2 was expressed by primary and BEAS-2B epithelial cells in vitro and ST2 expression was significantly lower in bronchial epithelium in subjects with asthma compared to healthy controls. TNF- α and poly-IC treatment of primary bronchial epithelial cells resulted in down regulation of soluble ST2 whereas only TNF- α contributed to the reduction of ST2 receptor. Downregulation of the release of soluble ST2 could potentially increase free IL-33 leading to amplification of the underlying airway inflammation in asthma.

1 | INTRODUCTION

Asthma is a common chronic disease and a major burden worldwide.¹ The main characteristics of asthma are inflammation, variable airflow obstruction and airway hyper-responsiveness. Asthma is estimated to affect 5%-10% of adults.² Genome wide association studies of asthma have implicated epithelial genes including the *IL1RL1* gene, which encodes ST2, the receptor for IL-33.³⁻⁸

ST2 isoforms are the transcriptional products of the *IL1RL1* gene of which two have been reported to be the most important. The transmembrane bound isoform (ST2L or ST2 receptor) and the truncated soluble form (sST2). The sST2 acts as a decoy receptor by capturing IL-33 and inhibiting its function.^{9,10} IL-33 is predominately expressed in the nucleus of various cells particularly the bronchial epithelium, endothelial cells, fibroblasts, airway smooth muscle (ASM) and mast cells.^{8,11,12} IL-33 functions as a novel alarmin, which is activated by bronchial epithelial cells following injury, airborne allergens, viruses, and air pollutants.¹³ Recent studies have demonstrated the expression of ST2 receptor in epithelial, endothelial, mast cells, and ASM cells.^{12,14,15} The ST2 receptor is a critical component of the Th2 response by activating type 2 innate lymphoid cells (ILC2s) and triggers the production of pro-inflammatory cytokines in allergic and nonallergic asthma.^{16,17}

We hypothesized that in asthma expression of ST2 receptor in the bronchial epithelium is increased, and its function and expression are modulated by the microenvironment. To test our hypothesis, we investigated the expression of ST2 receptor in bronchial biopsies, epithelial brushes in asthma and healthy controls, in vitro human primary bronchial epithelial cells and the BEAS-2B cell line.¹⁸

The effects of the pro-inflammatory cytokine TNF α or the TLR3 agonist polyinosinic:polycytidylic acid (poly-IC) as a viral mimic upon bronchial epithelial ST2 expression.

2 | METHODS

2.1 | Subjects

Subjects that underwent endobronchial biopsies were recruited from a single center (Glenfield Hospital, Leicester, United Kingdom). Asthma was diagnosed based on the presence of typical symptoms and evidence of variable airflow obstruction or airway hyper-responsiveness and severity determined by the Global Initiative for asthma treatment steps (mild-moderate GINA 1-3, severe GINA 4-5, 19)¹⁹. Healthy controls had no respiratory symptoms and normal lung function. Current smokers were excluded, and all subjects had <10 pack year smoking history. Subjects underwent extensive clinical characterization including sputum induction²⁰ and fiberoptic bronchoscopy as described previously.²¹ The study was approved by the Leicestershire Ethics Committee and all patients gave their written informed consent (08/H0406/189). Bronchial epithelial transcriptome data were available from asthma and healthy control subjects that underwent brushes from previously published studies^{22,23} that were approved by the relevant ethics and review boards Leicestershire, Northamptonshire, and Rutland; approval number 11/EM/0402 and the UK Medicines and Healthcare Products Regulatory Agency. Samples from the Eva (Emphysema vs Airway disease) study approval number 08/H0402/19.

2.2 | Culture cells

Human primary bronchial epithelial cells were obtained from bronchoscopy and grown onto collagen (Advanced Bio Matrix, San Diego, California, USA) coated 12-well plates in bronchial epithelial growth medium (Lonza) supplemented with SingleQuot BulletKit (Lonza), 0.3% Fungizone antimycotic (Thermo Fisher Scientific), and 1% antibiotic-antimycotic (Thermo Fisher Scientific). The primary bronchial epithelial cells were expanded onto collagen-coated T75 cm² flasks and replenished with fresh medium three times a week.²⁴ The human virus-transformed bronchial epithelial cell line (BEAS-2B) was purchased from ATCC.

2.3 | Immunohistochemistry

Sections from glycomethacrylate-embedded bronchial biopsies were stained using rabbit anti-human ST2 antibody (Sigma-Aldrich, 25) and appropriate rabbit isotype control (Immunostep) were applied. The EnVision FLEX kit (Dako) was used for the staining, and all slides were stained on PTLINK/Autostainer Link 48 to assure consistency of staining. The quantitative assessment of ST2 staining in the epithelium was performed using a thresholding technique. The threshold (hue, saturation, and intensity) required to identify high intensity ST2 staining within the epithelium was identified by visually selecting high intensity ST2 stained pixels. The percentage of the epithelium area that was occupied by high intensity ST2 staining was then determined as described previously²⁵ and based on 3,3'-diaminobenzidine (DAB) staining, which was evaluated using the ZEN2 (Zeiss 2012) software.

2.4 | Bronchial brush RNA-Seq and analysis

The RNA extraction, preparation, and sequencing procedure in detail were as described previously.^{22,23} Bronchial brush samples were immediately transferred into RNAprotect and stored at -20°C. Total RNA was eluted in 100 µl of RNase-free water following the manufactures protocol. The extracted RNA from the bronchial brush samples (Qiagen) was tested for quality and concentration with the Bioanalyzer RNA Nanochip (Agilent Technologies, Inc) and cDNA libraries were prepared on Sciclone robot (PerkinElmer Inc) using the RNATRUSEQ protocol (Illumina Inc). Sequencing was performed on the HisSeq 2000 (Illumina) platform (100 bp, paired-end sequencing). Normalized fragments per kilobase of transcript per million mapped reads (FPKM) using the RNA sequencing (RNA-seq) values were calculated to predict the expression levels of the *IL1RL1* gene.²⁶ A cutoff criteria were applied as such that only those samples with a mean FPKM >0 were considered to express the *IL1RL1* gene (or no expression = 0).

2.5 | Flow cytometry and immunofluorescence

Epithelial cells were counted by trypan blue exclusion, and 95%-100% viable cells were re-suspended into PBS-BSA (0.5%, 1×10^6 cells/mL) using gentle cell detachment solution accutase (BioLegend) and cell surface expression was stained with goat polyclonal anti-human ST2-biotinylated antibody which detects ST2/IL-33receptor (12, R&D Systems, Oxfordshire, UK) or its appropriate isotype control Goat IgG biotin (R&D Systems) indirectly labelled with Streptavidin/FITC (Dako). For total ST2 expression, epithelial cells were fixed and permeabilized in 4% paraformaldehyde, 0.1% saponin (Sigma) for 15 minutes on ice and again labelled with ST2 indirectly labelled with Streptavidin/FITC. Expression was assessed in unstimulated or stimulated epithelial cells with human recombinant TNF α (10 ng/mL R&D) or poly-IC (12.5 µg/mL, Invivo Gen, France) for 24 hours by one color flow cytometry (BD FACS Canto, BD, UK) collecting 10 000 events.

For immunofluorescence epithelial cells were cultured on collagen-coated 25-mm glass cover slips in 6 well plates to sub-confluence. Cells were fixed with methanol, blocked with 3% BSA/PBS and labelled with rabbit anti-human ST2 antibody and appropriate rabbit isotype control indirectly labelled with sheep anti-rabbit RPE (AbD serotec, Oxfordshire, UK) and counterstained with 4',6'-diamidino-2 phenylindole (DAPI, Sigma-Aldrich). Images were acquired using a Zeiss Axio Imager Z2 microscope using Zeiss Zen software.

2.6 | Western blotting

Primary bronchial epithelial cells and BEAS-2B cells were seeded at 1.5×10^5 cells in two 60 mm petri dishes and grown to sub-confluence. Whole cell protein extracts were prepared by lysing the cells in Lamli buffer, followed by sonication and boiling for 3 minutes. Cells were normalized for cell number. After denaturation, proteins were separated by SDS-PAGE before wet transfer onto PVDF membrane, which were blocked with 5% skimmed milk in Tris-buffered saline, containing 0.1% Tween-20 (TBS-T). Blocked membranes were, probed with rabbit anti-human ST2 antibody (2 µg/mL) in TBS-T plus 5% milk overnight at 4°C, washed several times with TBS-T and incubated for 1 hour with anti-rabbit peroxidase-conjugated antibody (1:2000). Western blots were visualized using an enhanced chemiluminescence kit according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, United Kingdom). Signals were then visualized using ImageQuant LAS 4000 (GE Healthcare, UK). Total cell lysates of HMC-1 mast cells and human primary ASM cells were also prepared in Laemmli buffer as these were used as positive protein controls on the immunoblots for ST2 expression.¹²

2.7 | Quantitative PCR

Quantitative PCR (qPCR) of ST2L and sST2 messenger RNAs was performed in primary bronchial epithelial cells as described

TABLE 1 Clinical characteristics of asthmatic and healthy controls

	Bronchial biopsies			Bronchial samples	brush
	Normal n = 9	Mild-moderate Asthma GINA 1-3 n = 6	Severe Asthma GINA 4-5 n = 21	Normal n = 34	Asthma n = 20 GINA 1-3 (n = 2) GINA 4-5 (n = 18)
Age year, mean ^a	53 (27-65.5)	39 (30-49.25)	53 (42.5-62)	55 (47-60)	52 (44-61)
Gender female/male, n	3/6	3/3	12/9	11/23	9/11
Atopy, n (%)	0 (0)	4 (66.7)	19 (90.5)	0 (0)	17 (85)
ICS (mg/d BDP, equivalent) ^a	0 (0-0)	200 (200-800)	1600 (1600-1800)	0 (0-0)	1600 (1000-1600)
Pre-BD FEV ₁ % predicted ^b	99.8 (17.08)	84.2 (27)	79.36 (26.39)	114.84 (16.46)	77.55 (25.98)
Pre-BD FEV ₁ /FVC ^b	77.4 (3.3)	71.6 (13.16)	64.57 (26.39)	96.31 (5.45)	62.71 (13.14)
% sputum neutrophils ^a	39 (11-48.5)	37 (7.1-75.5)	48.25 (38.56-66.59)	60 (45-81)	45 (31-63)
% sputum eosinophils ^a	1 (0.25-2.25)	1.5 (0.5-7.23)	6.375 (0.81-19.25)	0.37 (0-2)	5.6 (1.4-13)

^aMedian (IQR).^bMean (SD).

previously.¹² Total RNA was purified using Peq Gold total RNA kit (PiqLab, Ltd) followed by DNaseI treatment. RNA quality & quantity were assessed using a TECAN infinite NANO-QUANT plate reader (Tecan Ltd, Reading, UK) and 1 µg of total RNA from each cell culture was reverse transcribed using SuperScript Vilo cDNA synthesis kit (Thermo Fisher Scientific). Amplification of 1 ng of cDNA per reaction in a final volume of 20 µL was performed using the Express SYBR GreenER qPCR SuperMix Universal (Thermo Fisher Scientific) in a Chromo4 Real-Time Detector (Bio-Rad, Herts, UK). After an initial incubation for 2 minutes at 50°C followed by 5 minutes at 95°C, the conditions of amplification were: denaturation at 95°C, annealing at 59°C, extension at 72°C for 36 cycles. 18s rRNA was used as reference gene. The expression data for each transcript was presented relative to the reference gene and determined using the equation 2^{-dCq} , where $dCq = (Cq_{\text{target gene}} - Cq_{18S \text{ RNA}})$, and this expression was arbitrarily multiplied by the factor 10^6 for clearer presentation.

2.8 | Calcium flux

Cells were labelled in parallel with 10 µg/mL fluo-3 and 4 µg/mL Fura Red acetoxymethyl esters (Thermo Fisher Scientific) for 45 minutes at 37°C in Phosphate Saline solution + 2mM Ca²⁺. For determination of cellular response, baseline unstimulated measurements (1 minutes) were followed by addition of recombinant IL-33 (50-200 ng/mL) or calcium ionophore (1.5 µg/mL) as a positive control. The cell flow was halted during this addition, appended and then continued acquiring for a further 3 minutes. Data were collated in a histogram displaying the ratio of fluo-3/Fura Red vs. time. The geometric mean fluorescence intensity (GMFI) of the unstimulated cell population was compared with the stimulated population.¹²

2.9 | Wound repair

Primary bronchial epithelial cells were seeded onto 6 well plates coated with collagen until 80%-90% confluence. Cells were wounded using a sterile 200 µl pipette tip in a predetermined grid pattern.²⁷ Cells were then washed prior to addition of fresh media with or without IL-33 isotype or neutralizing IL-33 (3 µg/mL R&D Systems) as used previously.¹² TGF-β1 (10 ng/mL) was used as a positive control. Wounds were photographed at baseline and after 2, 4, 6, 24, and 48 hours. The percentage of cells that had moved into the wound was analyzed using photoshop CC.

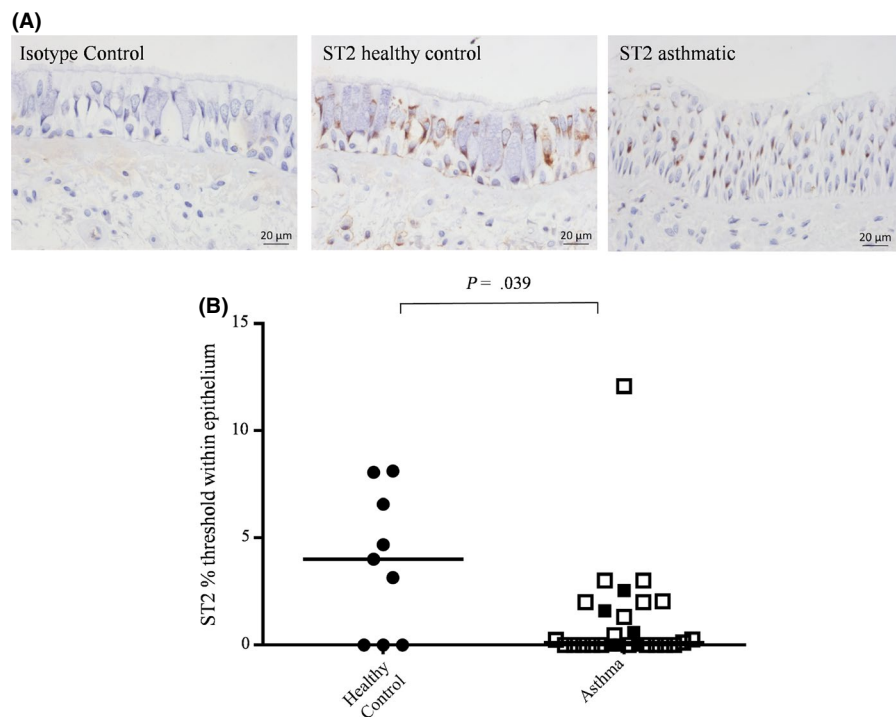
2.10 | Microarray platform

Total RNA purified from primary bronchial epithelial cells treated or untreated with IL-33 (50 ng/mL) for 6 hours followed by DNaseI treatment and RNA integrity was checked on a Bioanalyzer 2100 (Agilent Technologies, Cheshire, UK). RNA was converted to cDNA and then to cRNA using Illumina Total Prep RNA Amplification Kit according to the manufacturer instructions (Ambion, UK). Whole-transcriptome expression profiling was performed on each sample using Illumina HumanHT-12 v4 Expression BeadChips (Illumina, UK according to the manufacturer protocol). The GenomeStudio analytical software was used to assess the raw data quality and determine the average background intensity. Probes with signal intensity below the background were excluded from subsequent analyses.

2.11 | ELISA

The concentrations of sST2 and of chemokines implicated in asthma that demonstrated upregulation in gene expression by

FIGURE 1 ASM and bronchial epithelial IL-33 expression. (A) Representative photomicrographs of normal biopsy specimen illustrating isotype (magnification x400), ST2 staining within the epithelium (magnification x400) and asthmatic biopsy staining (magnification x400). (B) Percentage threshold within the epithelium of ST2 in subjects. Symbol key: ● = Healthy control; ■ = Mild/Moderate asthma; □ = Severe asthma. Horizontal bars represent the median, $P < .05$, unpaired two-tailed nonparametric t-test



primary bronchial epithelial cells following stimulation by IL-33 (CCL2, CCL-5, CCL20, CXCL1, and CXCL10) were measured in the bronchial epithelial cell supernatants by ELISA before and 24-hour stimulation with IL-33 (10 ng/mL and 50 ng/mL). The sensitivity limit of the ELISA assays were as follows: sST2 assay was 31.2 pg/mL, CCL-2 15.6 pg/mL, CCL-5 15.6 pg/mL, CCL20 7.8 pg/mL, CXCL1 31.2 pg/mL, and CXCL10 31.2 pg/mL (R&D Systems).

2.12 | Statistical analysis

Statistical analysis was performed using PRISM Version 7 (La Jolla, California). Data are presented as mean ($\pm$ SEM and $\pm$ SD), except cell numbers in bronchial biopsies which are presented as median. Epithelium integrity measurements were calculated to measure the epithelial barrier alteration. Epithelium integrity was assessed by measuring the lengths of the corresponding reticular basement membrane (μ m) of the intact, denuded and damaged (partial) epithelium using microscope drawing tool. The data are presented as the percentage of the total reticular basement membrane length in the section. Correlations were assessed by Spearman rank (r_s) correlation coefficient. Nonparametric data were analyzed using the Mann-Whitney test and the parametric data by using t tests analysis of variances. The Chi-squared (χ^2) test was used to test statistical differences between groups. Differences were considered significant when P values were less than $< .05$. For microarray data, raw fluorescence signal intensities of each Illumina probe were averaged across all samples. Probes with an averaged raw signal intensity across the samples below the confidence detection level determined by the Genome Studio control panel were considered absent. Those

considered present raw signal intensities were base-2 log transformed and transcripts significantly differentially expressed between untreated and IL-33 treated cells were determined using a two-tailed unpaired student's t test in Microsoft Excel. Transcripts were then ranked according to the P value and fold changes. Transcripts with a P value $< .1$ and a fold difference 2 between groups were selected and taken forward.

3 | RESULTS

3.1 | ST2 expression in the bronchial epithelium in asthma

The clinical characteristics of the subjects that provided bronchial biopsies and brush samples are presented in Table 1. A representative photomicrograph of ST2 expression in a bronchial biopsy is shown in Figure 1A for both a healthy and asthmatic subject. The ST2 percentage threshold within the bronchial epithelium was significantly decreased in subjects with asthma compared to healthy controls ($P = .039$, Figure 1B). There was no significant correlation between ST2% thresholding expression in asthma and epithelial percentage integrity, damage or denudation (Figure S1A). There was no correlations between ST2% thresholding within the epithelium of asthma subjects and their clinical characteristic as stated in Table 1 (data not shown).

The *IL1RL1* gene was present in most bronchial brush samples from subjects with asthma and healthy controls and was not different between health and disease (91% normal and 100% asthma, respectively, $P = .17$). Their clinical characteristics are stated in Table 1.

3.2 | ST2 expression by bronchial epithelial cells

Primary bronchial epithelial cells and BEAS-2B cells expressed ST2 as evidenced by flow cytometry (Figure 2A, 2B), immunofluorescence (Figure 2C), and Western blotting (β -actin was used as a loading control, HMC-1 cells and ASM cells were used as positive staining controls for ST2 Figure 2D). There was no difference in ST2 expression between primary bronchial epithelial cells derived from patients with asthma and normal control subjects in all studies (data not shown). Therefore, data from primary bronchial epithelial cells derived from normal and asthmatic subjects were combined throughout. The soluble form of ST2 was spontaneously released from bronchial epithelial cell supernatants and BEAS-2B cells measured by ELISA (Figure 2E). There was significantly more sST2 released by primary bronchial epithelial cells compared to the epithelial BEAS-2B cell line (<0.0001). The *IL1RL1* gene (encodes ST2L the sST2) was found present in 6 of 6 primary bronchial epithelial cell donors and 5 of 6 donors for the *IL-33* gene expression. To further support the presence of ST2L and sST2, mRNA analysis was performed as shown in Figure 2F.

3.3 | Functional responses of primary bronchial epithelial cells

IL-33 triggered Ca^{2+} flux with increased intracellular calcium in primary bronchial epithelial cells as indicated by an increase in the fluo-3/Fura red ratio showing a maximum response at 100 ng/mL (Figure 3 A, B). Primary bronchial epithelial cell wound repair was not upregulated over 2, 4, 6, 24, and 48 hours (Figure S2A) by IL-33. Neutralizing IL-33 with anti-IL-33 (3 $\mu\text{g/mL}$) had no effect on primary bronchial epithelial cell wound repair (Figure 3C).

Following IL-33 stimulation of primary bronchial epithelial cells 39 genes were differentially upregulated (none were downregulated) using a cutoff $P < .1$, and 2-fold increase (Table S1). These included the upregulation of the chemokines CCL2, CCL5, CCL20, CXCL1, CXCL2, CXCL6, and CXCL10, which have been implicated in the pathogenesis of asthma.²⁸⁻³³ Secretion of 6 of these chemokines by primary bronchial epithelial cells was measured following IL-33 stimulation and showed increased secretion for CCL2, CCL20, and CXCL1, but not for CCL5 and CXCL10 (Figure 3D-H).

Stimulation of primary bronchial epithelial cells by the pro-inflammatory cytokine $\text{TNF}\alpha$ or the TLR3 agonist poly-IC significantly reduced sST2 release (Figure 4A). Whereas only $\text{TNF}\alpha$, but not poly-IC significantly reduced the ST2 cell surface receptor expression (Figure 4B). There was a small numerical, but not-significant,

increase in the total ST2 expression detected by flow cytometry in response to $\text{TNF}\alpha$ and poly-IC stimulation (Figure 4B).

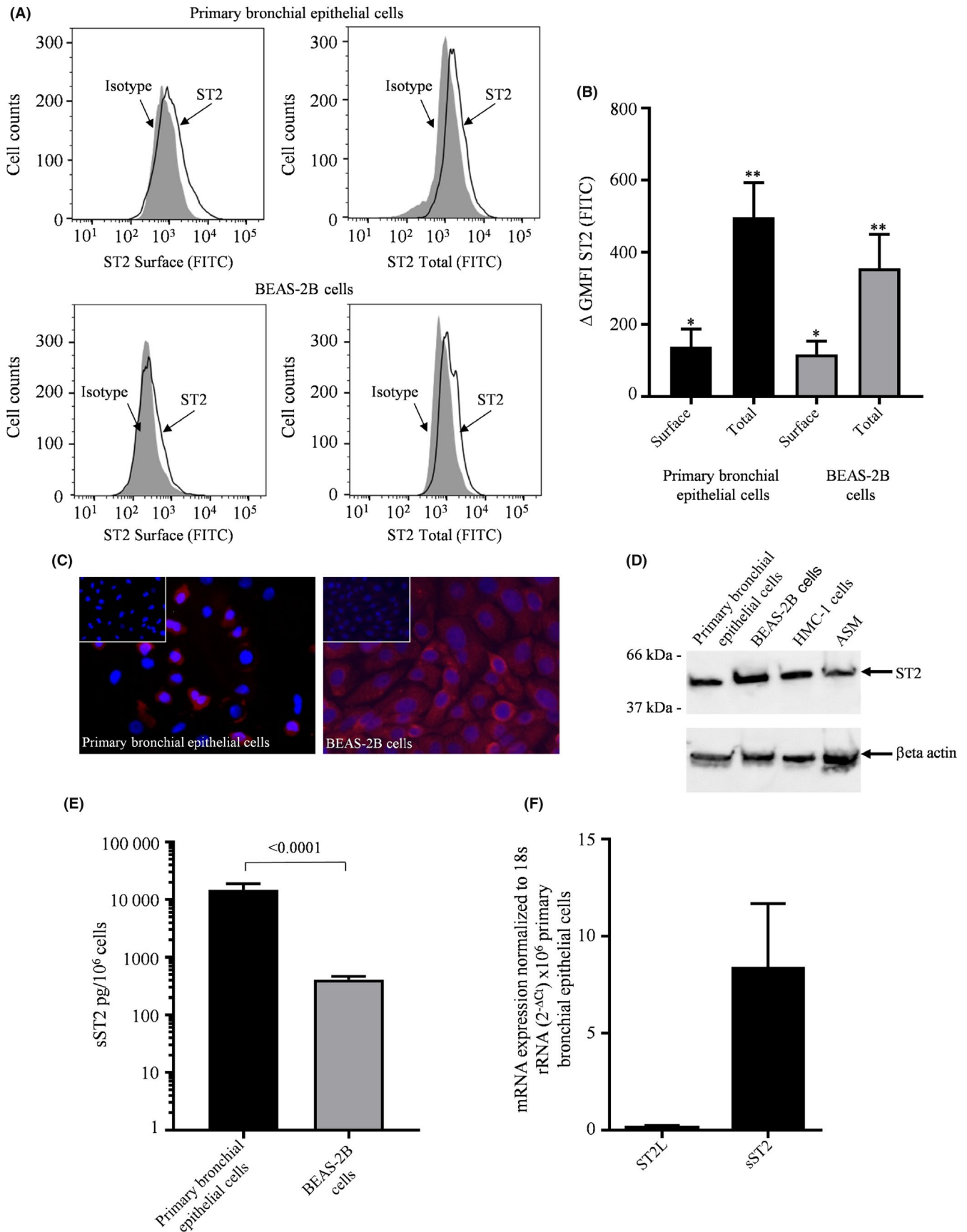
4 | DISCUSSION

In contrast to our hypothesis, we found in asthma that the bronchial epithelium protein expression of ST2 was decreased and the *IL1RL1* gene was expressed in most bronchial brush samples in both health and disease. Our in vitro findings demonstrated that ST2 was functional with increased gene expression and protein release following IL-33 stimulation including important chemokines implicated in asthma. Furthermore, our observations suggest that the decrease in ST2 expression in asthma might be a consequence of the pro-inflammatory environment in asthma or in response to viral infection.

We report here for the first time to our knowledge ST2 expression evident in human bronchial epithelium. Earlier reports have shown ST2 expression in human corneal epithelium tissue³⁴ and nasal epithelium from allergic rhinitis tissue sections.³⁵ We demonstrated that the expression levels of ST2 within the bronchial epithelium were decreased in subjects with asthma compared to healthy control tissue although expression in health and disease was heterogeneous. Our findings from the lower airway samples contrast with the results of recent studies of the upper airways showing that expression of ST2 increased in respiratory diseases such as chronic rhinosinusitis^{36,37} and allergic rhinitis.³⁵ The decreased ST2 expression in asthma we observed was not associated with levels of damaged integrity or denuded epithelium within the biopsies.

This differential ST2 expression between asthma and health in the bronchial epithelium in biopsies was not replicated in unstimulated primary bronchial epithelial cells. However, ST2 expression by primary bronchial epithelial cells was affected by $\text{TNF}\alpha$ (the pro-inflammatory cytokine). We chose to investigate these stimuli as $\text{TNF}\alpha$ has been described as a pro-inflammatory cytokine involved in many aspects of airway pathology in asthma³⁵ and is produced by many cells including mast cells, eosinophils, fibroblasts, epithelial cells, and smooth muscle cells. Studies have shown elevated levels of $\text{TNF}\alpha$ in asthmatic airways of patients with asthma.³⁷⁻³⁹ Poly-IC which is a synthetic mimic of double stranded RNA, agonist for TLR3⁴⁰ a key pathogen-associated molecular pattern receptor for viruses. TLR3 is present on airway epithelium,⁴¹ and studies have shown activation of precursor cells with poly-IC to release IL-33.⁴² Both $\text{TNF}\alpha$ and poly-IC caused downregulation of sST2 release. Surface receptor ST2 expression in primary bronchial epithelial cells was only downregulated by $\text{TNF}\alpha$. These data suggest that $\text{TNF}\alpha$ induced possible

FIGURE 2 ST2 expressed by bronchial epithelial cells. (A) Example fluorescent histograms of ST2 and (B) quantification of both surface and total ST2 (* P value $< .05$, ** P value $< .01$ compared to isotype control $n = 9$) in primary bronchial epithelial cells and BEAS-2B cells. Here, the isotype control was subtracted from the positive ST2 signal. (C) ST2 expression in cells was confirmed by immunofluorescence (nuclei stained blue), ST2 (stained red), isotype control shown as insert, (magnification $\times 400$ $n = 3$). (D) Western-blot analysis of primary bronchial epithelial cells and BEAS-2B cells using a ST2 antibody. Beta-actin was used as a loading control ($n = 3-13$). HMC-1 cells and human ASM cells were used as ST2 protein staining controls. (E) Soluble ST2 release in cell supernatants ($n = 4-17$). (F) Receptor ST2L and sST2 mRNA expression analyzed by qPCR in primary bronchial epithelial cells ($n = 5$). Data are presented as mean $\pm$ SEM. Statistical differences between stimulated vs unstimulated cells were assessed using the paired/unpaired t-test * P value $< .05$, ** P value $< .01$



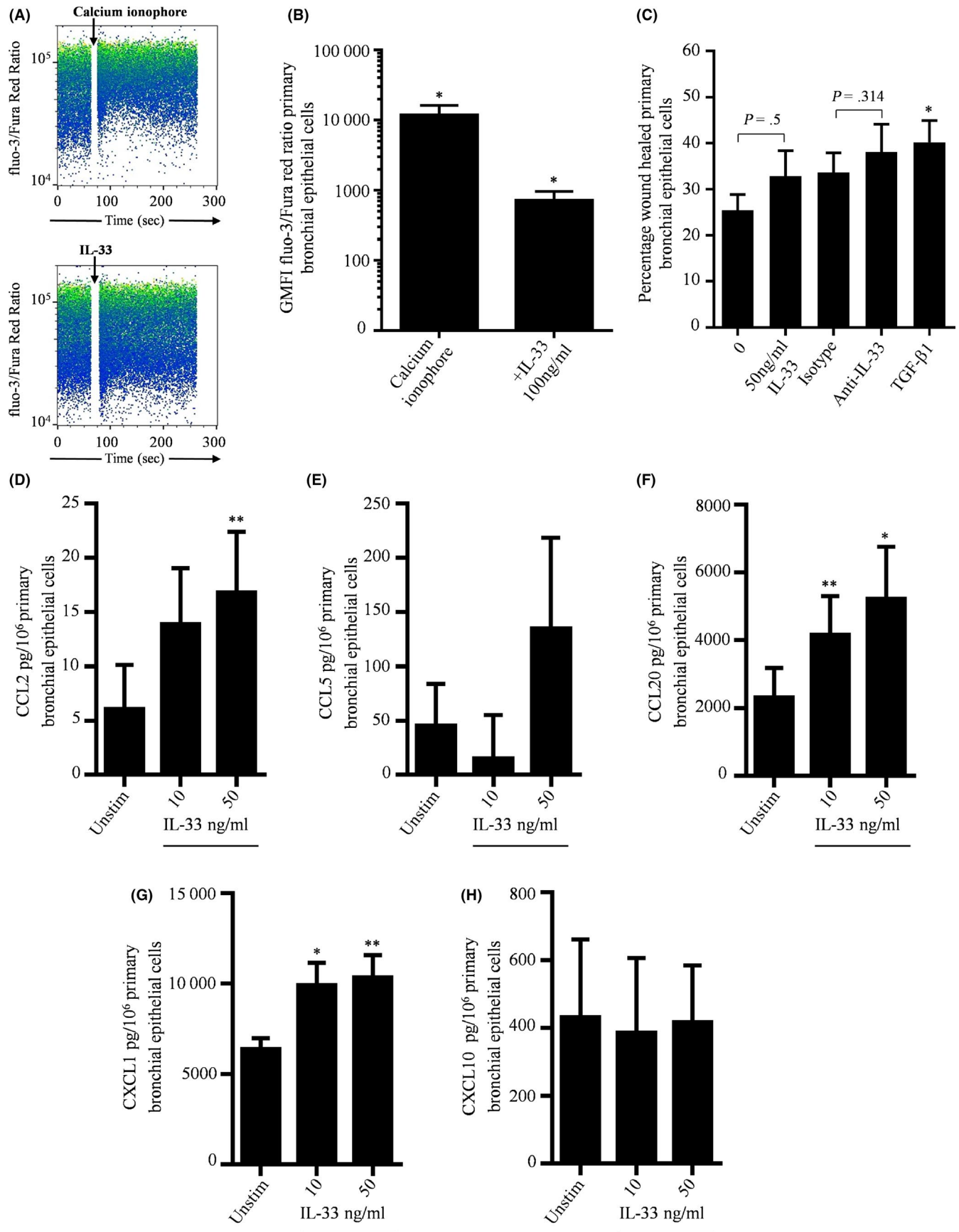


FIGURE 3 Functional responses to ST2 by bronchial epithelial cells. (A) Representative calcium flux display of calcium mobilization in response to calcium ionophore (1.5 $\mu\text{g/mL}$) and IL-33 (100 ng/mL). (B) Calcium flux in primary bronchial epithelial cells in the presence of IL-33 or calcium ionophore ($n = 5$). GMFI equates to total stimulated GMFI minus matched baseline (GMFI * P value < .05 compared to baseline GMFI). (C) Percentage wound repair by primary bronchial epithelial cells $\pm$ IL-33 (50 ng/mL), isotype control, IL-33-neutralizing antibody ($n = 8$), and positive control TGF- β 1 (10 ng/mL, $n = 5$). (D) CCL2, (E) CCL5 (F) CCL20, (G) CXCL1, and (H) CXCL10 protein release in primary bronchial epithelial cell supernatants stimulated with IL-33 for 24 hours ($n = 8$ -17). All data presented as mean $\pm$ SEM. Statistical differences between stimulated vs unstimulated cells were assessed using a paired/unpaired t-test * P value < .05, ** P value < .01

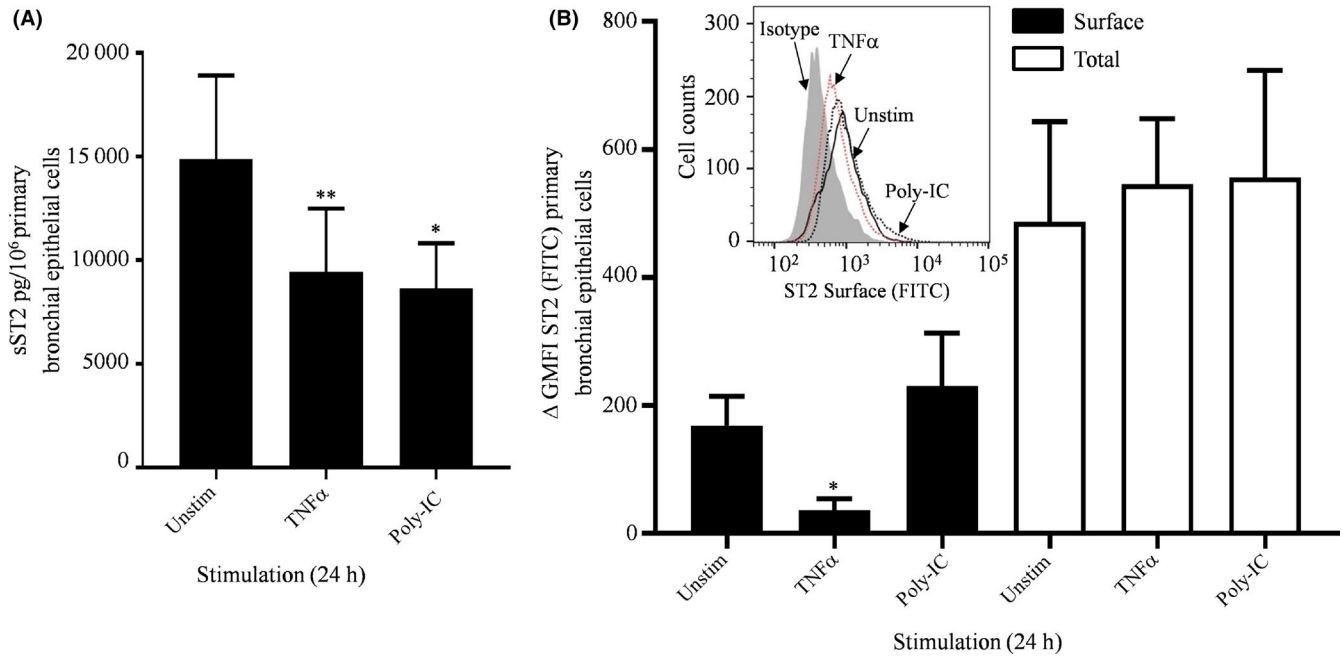


FIGURE 4 Role of inflammatory stimulation and ST2 expression in primary bronchial epithelial cells. (A) Soluble ST2 release in response to TNF α and poly-IC stimulation over 24 hours ($n = 12$ -17), (B) example flow histogram showing down regulation of ST2 surface receptor expression poststimulation with TNF α , no decrease with poly-IC and quantification of receptor in primary bronchial epithelial cells ($n = 6$ -8). All data presented as mean $\pm$ SEM. Statistical differences were assessed using a paired t-test

decreased shedding of ST2 receptor resulting in reduced sST2 release. This could account for the decreased levels of ST2 expression in the asthmatic bronchial submucosa. The downregulation of ST2 expression and possibly the release of sST2 might lead to increased free active IL-33 available resulting in a potential positive feedback loop amplifying the underlying airway inflammation in asthma.

We have previously reported increased IL-33 expression in human bronchial ASM and epithelial cells in asthmatic tissue with increased expression in mild/moderate asthma compared to health.¹² IL-33 stimulation increased IL-13 production by human lung mast cells at both the mRNA and protein level and that IL-33 had a direct effect on ASM wound closure and repair in vitro. In contrast, here, we found that IL-33 stimulation did not affect epithelial wound repair. However, IL-33 stimulation led to an increase intracellular calcium and altered gene expression including upregulation of the chemokines CCL2, CCL5, CCL20, CXCL1 and CXCL10 previously implicated in asthma pathogenesis.²⁸⁻³³ Following IL-33 stimulation there was increased secretion by primary bronchial epithelial cells for CCL2, CCL20, and CXCL1, but not for CCL5 and CXCL10 possibly contributing to persistence of airway inflammation in asthma.

One possible criticism of this study is it is likely that ST2 expression by the bronchial epithelium is dynamic, which cannot be captured in a cross-sectional study. Indeed, the expression was heterogeneous and we did not determine the repeatability of its expression in bronchial biopsies. Furthermore, the reduced ST2 expression in subjects with asthma compared to controls was only supported by immunohistochemistry and needs to be validated in future studies. However, with the exception of 1 severe asthma subject, the expression of all the other asthma subjects was lower than the median expression in health. The in vitro findings supported a potential role for the microenvironment in asthma to modulate ST2 expression, but the stimuli were limited to TNF α and a TLR3 mimic whether similar effects are observed with other mediators either alone or in concert warrants further investigation. A further limitation is that the samples for the bronchial biopsies were obtained from the proximal and not lower airways of lung parenchyma limiting the type of cells we could investigate.

In conclusion, our findings showed that in asthma bronchial epithelium protein expression of ST2 is decreased. Our in vitro findings suggest that this decrease might be a consequence of the pro-inflammatory environment in asthma or in response to viral infection.

Future trials are required to validate these findings further. Whether targeting the ST2/IL-33 axis in asthma has clinical benefit and affects inflammation and remodeling of the bronchial submucosa needs to be investigated.

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AUTHOR CONTRIBUTIONS

Dr Brightling guarantees the integrity of the work conducted. Dr Kaur contributed to study design and culture of all ex vivo cell experiments, studied ST2 expression and function, conducted statistical analysis, contributed to the writing of the manuscript, and approved final decision for submission. Dr Chachi contributed to the design, interpretation and analysis for immunohistochemistry. Dr Gomez conducted the qPCR for ST2 and did RNA extraction for gene array analysis. Dr Sylvius ran the gene array assay system and assisted with the statistical interpretation analysis. Dr Singh cultured primary bronchial epithelial cells and stimulation in preparation for ELISA protein expression and acquisition analysis of data. Mohammadali Yavari Ramsheh contributed to analysis and interpretation of RNA-seq data. Ruth Saunders contributed in analysis and interpretation RNA-seq data. Dr Brightling was involved in the recruitment of volunteers and isolation of tissue, supervised all the experimental design, contributed to the writing of the manuscript and approved the final decision for submission. All authors contributed to the review and editing of the manuscript and approved it for submission.

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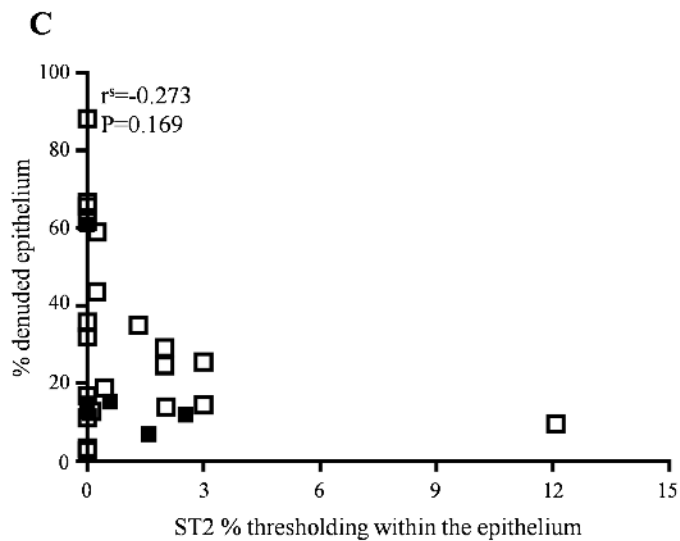
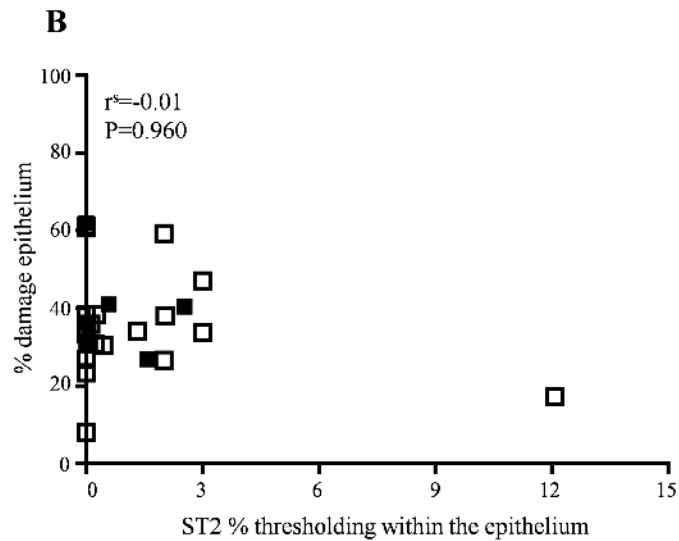
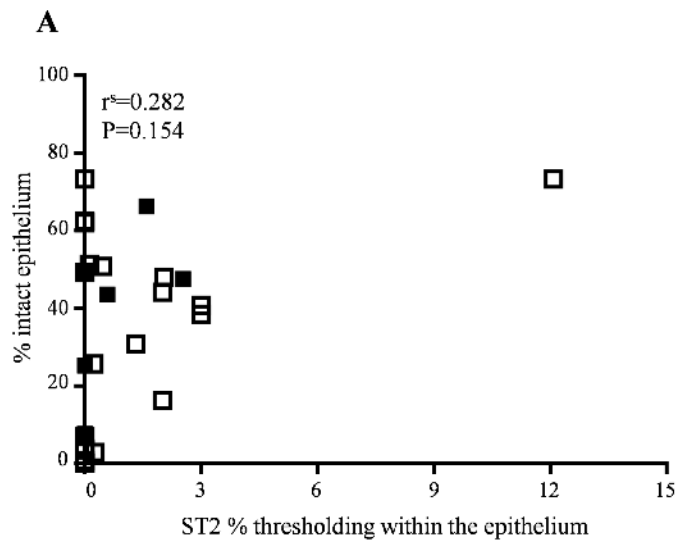
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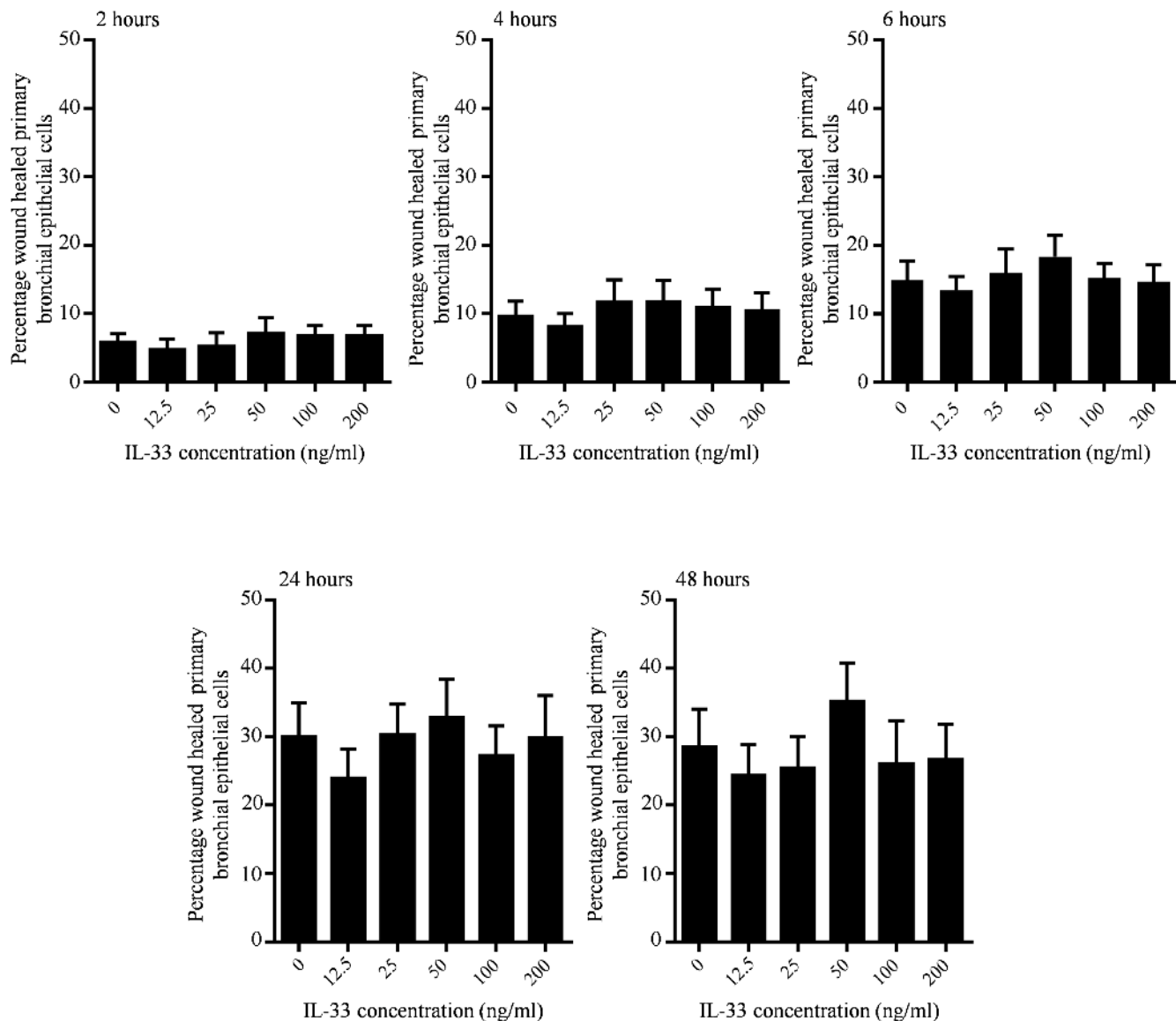
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SUPPORTING INFORMATION

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

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A

1 **Supplementary Table 1: Differentially upregulated genes in IL-33 treated vs untreated**

2 **human primary bronchial epithelial cells**

Probe ID	Gene symbol	P value	Fold change
ILMN_2336781	SOD2	0.021	2.35
ILMN_1680965	LOC728454	0.024	2.87
ILMN_1811364	SGPP2	0.025	2.19
ILMN_2328666	CD83	0.032	4.78
ILMN_1723035	OLR1	0.034	2.29
ILMN_2322768	CSF3	0.037	2.56
ILMN_1812226	ICAM1	0.041	3.49
ILMN_2048043	DEFB4	0.041	3.15
ILMN_1708375	IRF1	0.044	2.14
ILMN_1715603	IL23A	0.047	6.62
ILMN_1791759	CXCL10	0.047	2.98
ILMN_2171384	CXCL5	0.048	10.05
ILMN_1788874	SERPINA3	0.049	4.32
ILMN_1780582	CD83	0.050	3.72
ILMN_2158713	IL1F9	0.051	14.81
ILMN_1728106	TNF	0.051	2.83
ILMN_1727689	TNFAIP2	0.051	3.73
ILMN_1787897	CXCL1	0.055	4.04
ILMN_1752562	CXCL5	0.055	9.95
ILMN_1661861	CSF2	0.056	2.84
ILMN_1792922	SOD2	0.058	2.11
ILMN_1682636	CXCL2	0.062	4.29
ILMN_1805410	C15orf48	0.063	2.34
ILMN_1657234	CCL20	0.063	12.85
ILMN_2211018	SPRR2E	0.066	7.06
ILMN_1720048	CCL2	0.066	2.41
ILMN_2406501	SOD2	0.067	2.37
ILMN_1800091	RARRES1	0.071	2.59
ILMN_1788764	BPGM	0.071	2.23
ILMN_1779234	CXCL6	0.071	3.63
ILMN_2161577	CXCL6	0.071	2.4
ILMN_1672295	ZC3H12A	0.071	2.88
ILMN_1701239	SPRR2E	0.074	4.46
ILMN_2098126	CCL5	0.076	2.07
ILMN_1699651	IL6	0.080	3.1
ILMN_1774287	CFB	0.081	3.42
ILMN_1651838	RND1	0.086	2.06
ILMN_1773546	MRGPRX3	0.087	2.45
ILMN_1788109	IL17C	0.093	5.34

4 Genes with value $p < 0.10$ and more than 2 fold change were deemed to be statistically significant. Paired t
5 test (2 tailed, $n=4$).
6

Supplementary Figure legends:

Supplementary Figure 1. Relationship between ST2 and Epithelial cell integrity

Correlation between percentage threshold within the epithelium of ST2 in asthma vs intact (A), damaged (B) and denuded (C) epithelium. Symbol key: ■ = Mild/Moderate asthma; □ = Severe asthma. Spearman's correlation.

Supplementary Figure 2. Epithelial cell IL-33 mediated wound repair

Percentage wound repair by primary bronchial epithelial cells $\pm$ IL-33 (12.5-200ng/ml) over (A) 2, 4, 6, 24 and 48 hours (n=8). All data presented as mean $\pm$ SEM.