Supplementary Material

Additional studies

The TIP study used microarray data to investigate gene expression changes in bronchial brushings of current smokers (n=63) (1). The study collected samples 24 hours after 3 hours of smoking activity and 24 hours after non-smoking activity.

The bulk RNA-sequencing *in-vitro* study used primary bronchial epithelial cells that were isolated from tumour-free resected lung tissue and exposed to whole cigarette smoke or air (2). RNA-sequencing was performed on samples collected at 1h, 4h and 24h after smoke or air exposure.

The COPD small airway were collected from the bronchial brushings of healthy current smokers (n=18), healthy never smokers (n=18) and COPD current smokers (n=18) (3). Samples were then processed using Affymetrix Human Genome U133 Plus 2.0 Array to obtain microarray data.

Expression quantitative trait loci (eQTL)

To investigate whether an association between genetics and *IL-33* expression exists, we performed an expression quantitative trait loci (eQTL) analysis (4), focusing on one of the top asthma-SNPs (rs992969) previously shown to influence *IL-33* expression (5-9). This analysis was conducted on the CRUKPAP study, correcting for age and gender.

Cellular deconvolution

Cellular deconvolution of bulk RNA-seq data was performed to estimate the proportions of different cell types from the gene expression for all bulk RNA-Seq datasets. This analysis was performed as previously described (10). Briefly, AutoGeneS software was used to select and filter 400 genes from highly variable ones. The selection was based on minimized correlation

and maximized distance between clusters in which genes with the most stable results across cohorts were selected and used to infer major cell type proportions. RNA-seq signatures of club and goblet cells were combined as secretory cells due to their similar gene expression profiles. The RNA-seq data were subsequently normalized to counts per million (CPM), and highly variable (HV) genes (N=5,000) were selected. Bulk deconvolution on all samples was then conducted using the CIBERSORT support vector regression (SVR) method (11). Afterwards, the level of *IL-33* expression was associated with the predicted cell proportions in each dataset. Correlation between cell type and *IL-33* expression was tested using the Spearman correlation test.

Pathway Analysis

An IL-33 activation signature was compared to the RNA-seq datasets (STOP, GLUCOLD, COPD microarray, NORM, CRUKPAP). The IL-33 activation signature was derived from in vitro IL-33-activated human mast cells, basophils, type 2 lymphoid cells and human umbilical vein endothelial cells (HUVEC) (12). Gene set variation analysis (GSVA) was performed for the IL-33 signature using the GSVA package (version 1.38.2) in R (13). The Mann-Whitney test was used to assess statistical significance.

IL1RL1 Splicing

The reads corresponding to common region between the soluble and membrane bound IL1RL1 and the reads unique to only the membrane bound IL1RL1 were investigated separately for two dataset where the Raw RNA-Seq data was available. This method has been previous published for other genes (14).

Immunohistochemical staining

Immunohistochemical staining for IL-33 was performed on paraffin embedded peripheral lung tissue derived from 10 patients. 3 µm thick sections were cut and deparaffinized and rehydrated; followed by antigen retrieval with citrate buffer (10 mM, pH6). Endogenous peroxidase, avidin and biotin were blocked using 0.3% hydrogen peroxidase (H_2O_2) and Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories, Canada). Blocking was followed by incubation with the primary antibody (monoclonal mouse, anti-human, ALX-804-840-C100, 1:800), diluted in a 1% BSA-PBS, for one hour at room temperature. The sections were washed with PBS and incubated with a biotinylated Rabbit Anti-Mouse (E0413, Dako, Denmark) secondary antibody diluted in 1% human serum + 1% BSA-PBS, after which the sections were washed with PBS and incubated with Horseradish peroxidase (HRP)-conjugated Streptavidin (P0397, Dako, Denmark) diluted in 1% human serum + 1% BSA-PBS. Positive staining was visualized using 15 min incubation with Vector® NovaRED® Substrate (SK-4800, Vector Laboratories, Canada). Sections were counterstained with hematoxylin and scanned at magnification 40x using the Hamamatsu NanoZoomer 2.0HT digital slide scanner (Hamamatsu Photonic K.K., Japan). The R&D anti-IL33 antibody (AF3625) was also used for immunohistology using the protocol previously described (32) at a dilution of 1:400.

Immunofluorescent double staining for IL-33 & p40

Immunohistochemical staining for IL-33 was performed in lung tissues derived from two studies, the first consisted of current smokers (n=9), ex/never smokers (n=26) and ex-smoking COPD patients (n=25) and a second study containing COPD current smokers (n=9), COPD ex-smokers (n=7) and ex-smoking non-COPD patients (n=7). The lung tissues were embedded in paraffin and cut into 3 µm thick slices. These slices were deparaffinized and rehydrated; followed by antigen retrieval with Tris/HCL buffer (0,1 M, pH = 9). Slides were blocked using 5% BSA/PBS and Avidin/Biotin Blocking Kit (SP-2001, Vector Laboratories, Canada).

Blocking was followed by incubation with the primary p40 antibody (polyclonal rabbit, anti-human, ab 167612, 1:100, abcam, UK), diluted in a 1% BSA/PBS, for one hour at room temperature. Secondary antibody incubation for p40 used polyclonal Donkey Anti-Rabbit immunoglobulins (A31573, 1:100, Alexa FluorTM, U.S.A) at room temperature, diluted in 1% human serum + 1% BSA/PBS. Next, slides were incubated for one hour at room temperature with the primary IL-33 antibody (monoclonal mouse, anti-human, ALX-804-840-C100, 1:800, ENZO Life Sciences, U.S.A.), diluted in 1% BSA/PBS. Slides were incubated with biotin labeled polyclonal rabbit anti-mouse (E0413, 1:100, Dako, Denmark), diluted in 1% human serum + 1% BSA/PBS, before incubation with Streptavidin (Alexa Fluortm 555 conjugate, S21381, 1:300, Alexa FluorTM, U.S.A.) diluted in diluted in 1% human serum + 1% BSA/PBS. Autofluorescence was blocked for 3 minutes using Vector® TrueVIEW® Autofluorescence Quenching Kit (SP-8400, Vector Laboratories, Canada) before cell nuclei were stained through incubation with DAPI (D9542, 1:1000, Sigma-Aldrich, U.S.A.) for 15 min at RT. VECTASHIELD Vibrance® Antifade Mounting Medium, provided with the TrueVIEW® kit, and coverslips were applied on the slides. At last slides were scanned at 20x magnification using SLIDEVIEW VS200 (Olympus Corporation, Japan).

Analysis of fluorescence double staining

Scans were imported in QuPath software, after which airways were cut out for analysis. Next, cell detection was performed in the airways using Stardist as a detection model for cell nuclei. In R software V.4.0.0 (Boston, Massachusetts, USA), cytoplasmic intensity values for IL-33 and p40 were used as a way of background correction, because both IL-33 and p40 are specifically expressed in nuclei and not cytoplasm. After background correction, per airway the cells were arranged and plotted from lowest to highest value in a histogram and a linear model was created to fit the curve of the plot. Once staining intensity in the cells increased exponentially, and cells thus did not match the linear model, they were marked as positive for the protein stained. Using this method the total numbers of p40 and IL33 positive cells were counted for each of the selected airways, and then the fraction of p40+ cells per airway that were also positive for IL-33 was calculated. Only airways with more than eight basal cells were used for analysis, which led to the exclusion of 2 patients because no large enough airways were present. The results derived from the analyses were tested for significant differences between the four different groups using linear mixed model analysis in R software V.4.0.0 (Boston, Massachusetts, USA).

Western blotting

For western blot, snap frozen lung tissue from resected lung tissue was used from current (n=10) and ex smoker (n=9) COPD patients. Approximately 10 slices of 10µm were cut for the lung tissue samples, and collected in RIPA Lysis and Extraction buffer (89900, Thermo Scientific™, U.S.A.) and Halt™ Protease and Phosphatase Inhibitor Single-Use Cocktail (100X) (1861281, 1x, Thermo Scientific™, U.S.A.). To assure tissue degradation and lysis of all cells, the tissue samples were run in Bullet Blender® Gold (Next Advance, U.S.A.) for 1 minute on speed 8, using 0,2 mm stainless steel balls. After tissue samples, but not the beads, were put in new cups, samples were centrifuged (Eppendorf Centrifuge 5417 R) for 10 minutes at 4°C and 10.000 RPM. Protein assay was performed using Pierce™ BCA Protein Assay Kit (23227, Thermo Scientific™, U.S.A.), where the standard curve was determined using Pierce™ Bovine Serum Albumin Standard Ampules (23209, Thermo Scientific™, U.S.A.). Absorbance was measured with CLARIOstar Plus (BMG Labtech, Germany). Absorbance was converted to protein levels, which was then used to take 25µg per sample, and adjust the volume to 20µl using the RIPA buffer after which 5µl loading dye was added. These samples were heated for 5 minutes at 100 °C, before resting on ice and pipetting 20µl per sample in 12.5% SDS-polyacrylamide gels. Electrophoresis was run at 100V, before blotting proteins on 0.45μm nitrocellulose membranes for 75 minutes at 4 °C and 100V. After blotting, images of the membranes were taken with Gel Doc™ EZ Imager (Bio-Rad Laboratories, U.S.A.) to visualize protein loading, before blocking with 5% ELK in TBST for 1 hour. Membranes were incubated at 4 °C overnight with the primary IL-33 antibody (AF 3625, 1:1000, R&D Systems, U.S.A.), followed by incubation with a HRP labeled rabbit anti-goat antibody (p0449, 1:1000, Dako, Denmark). The antibodies were visualized with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (34578, Thermo Scientific™, U.S.A.), before blots were imaged/visualized with Invitrogen iBright 1500 (Thermo Scientific™, U.S.A.). After stripping for 10 min with stripping buffer, membranes were blocked again with 5% ELK in TBST before incubation with an anti-beta actin antibody (ab 8227, 1:6000, abcam, U.K.) for one hour at room temperature. After incubation with an HRP labeled goat anti-rabbit antibody (P0448, 1:1000, Dako, Denmark), Pierce™ ECL Western Blotting Substrate (32106, Thermo Scientific™, U.S.A.) was used to visualize antibody binding. Images of the bands were taken with Invitrogen iBright 1500 (Thermo Scientific™, U.S.A.).

Western blot analysis

Normalization of samples was performed using β-actin expression. In iBright Analysis Software, protein bands associated with IL-33 and the splice variants (31kDA, 20kDa, 16kDa) and b-actin were analyzed using local background corrected density, after which the ratios of IL-33/b-actin were calculated for each sample and each of the IL-33 bands. Statistical analysis between groups were performed using Mann-Whitney U tests in Prism 8.0.1 (Graphpad Software inc, U.S.A.).

Table E1: Baseline characteristics of additional studies

Study	Sample Types	Smoking Status	n (%)	Mean Age (SD)	Gender male n(%)	Mean Pack years (SD)	Mean FEV1 % (SD) Prediction	Mean FEV1/FVC (SD)	Platform	Profiling Assay
TIP study(1)	Bronchial brushings	Current smokers	63 (100)	40.8 (19.2)	49 (78)	17.6 (16.8)	95.6 (20.5)	74 (14)	Microarray	Affymetrix HG 1.0 ST microarrays
Bulk RNA-seq in-vitro study (2)	Primary bronchial epithelial cells differentiated in Air-liquid interface	1/3/4 (non/ex/ current smokers)	8	66.6 (6.2)	7(87.5)	N/A	N/A	68.9 (11.7)	Bulk RNA-Seq	Illumina NovaSeq6000 sequencer
GSE8545 Small airways Microarray (3)	Bronchial brushings of small airway epithelial cells	Current Smokers (non-COPD)	18 (33.3)	46 (5)	12 (66.7)	31 (16)	109 (15)	82 (5)	Microarray	Affymetrix Human Genome U133 Plus 2.0 Array
		Current Smokers (COPD)	18 (33.3)	50 (6)	15 (83.3)	34 (17)	82 (18)	64 (6)		
		Never Smokers	18 (33.3)	41 (7)	14 (77.8)	0	106 (9)	82 (5)		

Figure Legends

Figure E1. Normalized IL1RAP expression levels in bronchial datasets. A IL1RAP expression levels from the STOP study of bronchial biopsies (n=16) pre and post 12 months smoking cessation. B IL1RAP expression levels of bronchial biopsies from the GLUCOLD study of ex-smokers (n=33) and current smoker (n=46) COPD patients. C IL1RAP expression levels of bronchial brushes from the COPD microarray study of ex-smokers (n=57) and current smokers (n=30) COPD patients (GSE37147). D IL1RAP expression of bronchial biopsies from the NORM study of never (n=40) and current (n=37) healthy smokers. E IL1RAP expression of bronchial biopsies from the CRUKPAP study of never (n=8), ex- (n=151) and current (n=77) smokers. All analyses were corrected for age and gender. The numbers shown are p-values taken from results of differential expression analyses. Error bars are representing the standard deviation.

Figure E2. IL33 and related genes expression in small airways samples. Expression of A IL-33 B IL1RL1 and C IL1RAP in microarray data of small airway epithelium (GSE8545, 18 never smokers, 18 non-COPD current smokers and 18 COPD current smokers). Non-COPD [black] and COPD current smokers [green] data were merged. P-values shown on the figure were calculated from differential gene expression analysis using the limma R package which was corrected for age and gender.

Figure E3. Expression of IL-33 after acute cigarette smoke exposure. A Expression of IL-33 in a longitudinal dataset of bronchial brushings collected following 24 hours smoking cessation versus 24 hours after smoking 3 cigarettes. B Expression of IL-33 in primary bronchial epithelial cells (n=8 donors) differentiated air-liquid interface were exposed to cigarette smoke or air for 24h. The error bar indicates the standard deviation. Gene expression was not significant between air and cigarette smoke exposure in any of the time points. C IL-33 gene expression derived from bronchial biopsies from ex- (n=151) and current (n=77) smokers separated by their rs992969 genotype. P-values were acquired from Mann-Whitney for unpaired analyses.

Figure E4. Association between smoking and IL1RL1 variants. A Ratio of membrane and common (soluble and membrane) variant of IL1RL1 in the COPD ex (n=18) and current smokers (n=38) (GLUCOLD study) and the normalized gene expression (CPM normalization) of IL1RL1 variants: B membrane and C common (soluble and membrane) variant. D Ratio of membrane and common (soluble and membrane) variant of IL1RL1 in healthy never (n=40) and current smokers (n=37) and the gene expression of both E) membrane and F) common (soluble and membrane) variant (NORM study).

Figure E5. Single cell analysis of Human Lung Cell Atlas. Violin plots of A IL-33 and B IL1RL1 in cell derived from nasal, bronchial and lung samples of the Human Lung Cell Atlas 1.0.

Figure E6. Summary of why anti-IL-33 treatment is more effective in ex- smokers than compared to current smokers. Higher number of basal cells at the pre-differentiation state where IL-33 is primarily expressed in ex- smokers (right) than current smokers (left). Images were acquired from Servier Medical Art by(https://smart.servier.com/)

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