IL-22 and its receptors are increased in human and experimental COPD and contribute to pathogenesis

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Supplementary methods

Ethics statement. This study was performed in accordance with the recommendations issued by the National Health and Medical Research Council of Australia. All experimental protocols were approved by the animal ethics committee of The University of Newcastle, Australia.

Gene Expression in Human COPD Microarray Datasets. Analysis of IL-22, IL-22RA1 IL-10RB and IL-22RA2 in published human array datasets (Affymetrix Human Genome U133 Plus 2.0 Array, Accession numbers: GSE5058 and GSE27597) [1-3] was performed using the Array Studio software (Omicsoft Corporation, Research Triangle Park, NC, USA) by applying a general linear model adjusting for age and gender and the Benjamini–Hochberg method for p-value adjustment. Data are expressed as $\log_2$ intensity robust multi-array average signals. The Benjamini–Hochberg method for adjusted P value/false discovery rate (FDR) was used to analyse differences between two groups. Statistical significance was set at FDR < 0.05.

In the GSE5058 dataset, gene arrays from small airway epithelial cells obtained from normal non-smokers (n = 12), healthy chronic smokers (n = 12),

smokers with early COPD (n=9), and smokers with established COPD (n = 6) were evaluated. The FEV1/FVC ratio of the subjects in these groups were 99 ± 7, 97 ± 7, 78 ± 4 and 66 ± 14, respectively.

In the GSE27597 dataset, gene arrays from 8 sample pairs from different lung slices from 6 subjects requiring lung transplant for COPD and 2 organ donors were analysed. The 6 subjects with COPD had a FEV1 <25% predicted (severe disease).

In addition, we examined gene expression from lung tissue specimens derived from 56 subjects (GSE8581 [4]). These subjects had undergone lobectomy for removal of a suspected tumour. Tissue was derived from histologically normal tissue distant from the tumour margin. COPD (cases, n = 15) were defined as subjects with FEV1<70% and FEV1/FVC<0.7 and controls (n = 18) as subjects with FEV1>80% and FEV1/FVC>0.7.

Mice. Female, 7-8-week-old, wild-type (WT) C57BL/6, *Il17a*$^{eGFP/+}$*;Il22*$^{td\text{-}tomato/+}$ reporter and *Il22*$^{-/-}$ mice were obtained from the Australian Bioresource Facility, Moss Vale, NSW, Australia. *Il17a*$^{eGFP/+}$*Il22*$^{td\text{-}tomato/+}$ dual reporter and *Il22*$^{-/-}$ and mice were generated as previously described [5]. Mice were housed under a 12-hour light/dark cycle and had free access to food (standard chow) and water. After a period of acclimatization (5 days), mice were randomly placed into experimental groups and exposed to either normal air or nose-only inhalation of CS for eight weeks as described previously [6-13].

Isolation of RNA and qPCR. Total RNA was extracted from whole lung tissue and blunt-dissected airways and parenchyma and reversed transcribed [8]. mRNA

transcripts were determined by real-time quantitative PCR (qPCR, ABIPrism7000, Applied Biosystems, Scoresby, Victoria, Australia) using custom designed primers (Integrated DNA Technologies, Baulkham Hills, New South Wales, Australia), normalized to the reference gene hypoxanthine-guanine phosphoribosyltransferase (*hprt*) (**supplementary table S1**).

Flow Cytometry Analysis. The numbers of IL-17A$^+$ and IL-22$^+$ CD4$^+$ T-cells, $\gamma\delta$ T-cells, NKT-cells and group 3 innate lymphoid cells in lung homogenates were determined based on surface marker expression using flow cytometry (**supplementary table S2**) [14-16]. Flow cytometric analysis was performed using a FACSAriaIII with FACSDiva software (BD Biosciences, North Ryde, Australia). Flow cytometry antibodies were purchased from Biolegend (Karrinyup, Western Australia, Australia) or BD Biosciences (**supplementary table S3**). BD compensation beads (BD Biosciences) were used to compensate for spectral overlap.

Mouse lung IHC. Lungs were perfused, inflated, formalin fixed, paraffin embedded, and sectioned (4μm)[8, 9]. Longitudinal sections of the left lung were deparaffinised by placing on a heating block at 70°C for 15mins then sections were immersed in fresh xylene for 10mins then 5mins. Rehydration was performed using a series of ethanol gradients (100% twice, 90%, 80%, 70%) and 0.85% saline for 5mins each. Heat-induced antigen retrieval was performed in citrate buffer (10mM citric acid, 0.05% Tween 20, pH 6.0) at 100°C for 30mins. Sections were blocked with casein blocker (Thermo Fisher Scientific, Scoresby, Victoria, Australia) for 1h. Sections were washed with PBS-T and incubated overnight at 4°C with either rat anti-Il22ra1

(MAB42941; R&D Systems, Minneapolis, Minnesota, United States) or rabbit anti-Il22ra2 (ab203211; Abcam, Melbourne, Victoria, Australia) antibodies. Following washing with PBS-T, sections were incubated for 30mins at 37°C with either goat anti-rat (HAF005; R&D Systems) or goat-anti-rabbit (ab7090; Abcam) secondary antibodies conjugated to horseradish peroxidase. Each primary and secondary antibody was diluted 1:100 in PBS-T. Following washing with PBS-T, sections were incubated for 20 mins with 3,3'-diaminobenzidine chromogen-substrate buffer (Aligent Technologies, Mulgrave, Victoria, Australia) according to the manufacturer's instructions. Sections were washed with ddH_2O then counterstained with standard haematoxylin for 5mins. Sections were washed with tap H_2O and were dehydrated by immersion in a series of saline, ethanol then xylene, inverse to that described above. Coverslips were mounted with standard non-aqueous medium and slides imaged using a Zeiss Axio microscope with ZEN-blue edition software V2.5 (Carl Zeiss Microscopy, Thornwood, New York, United States). Unless otherwise stated, each incubation was at room temperature protected from light in a humidified chamber. All wash steps were performed 5 times for 3mins each.

Airway remodelling. Airway epithelial (μm^2) and collagen deposition area (μm^2) were assessed in a minimum of four small airways (basement membrane [BM] perimeter <1,000μm) per section [7-9, 12, 13]. Lung sections were stained using Masson's trichrome stain, and photographs of small intact airways were taken at 40x magnification. These photographs were then analysed in ImageJ software (Version 1.50, NIH).

Airway epithelial thickness analysis was performed by carefully tracing the BM and inner epithelial surface perimeters. Airway epithelial area was calculated by

subtracting the inner airway area from the outer airway area. This was then expressed as area per µm of BM.

For collagen analysis, a colour deconvolution method was used to isolate the collagen, stained blue. This method breaks the original photograph into three images, containing three separate colour ranges. In this manner, the blue-stained areas of the images (representing collagen) could be isolated and quantified separately. The BM was traced and measured as described above. Collagen deposition immediately surrounding the airway was traced and measured, but only in images that isolated the blue-stained pixels. We could then reach a quantitative 'collagen per airway' measurement by expressing the area of blue-stained pixels per µm of BM.

Pulmonary Inflammation. Airway inflammation was assessed by differential enumeration of inflammatory cells in bronchoalveolar lavage fluid (BALF) [7, 8, 17-19]. BALF supernatants were stored at -20°C for assessment of IL-22 protein levels. Lung sections were stained with periodic acid-Schiff (PAS) and tissue inflammation assessed by enumeration of inflammatory cells [7, 8, 17, 18]. Histopathological score was determined in lung sections stained with hematoxylin and eosin (H&E) based on established custom-designed criteria [19].

ELISA. Right lung lobes were homogenised on ice in 500uL of PBS supplemented with Complete mini protease inhibitor cocktail (Roche Diagnostic, Sydney, NSW, Australia) and PhosphoSTOP tablets (Roche Diagnostic). Lung homogenates were incubated on ice for 5 mins and subsequently centrifuged (8,000x*g*, 15 mins).

Supernatants were collected, stored at -20°C overnight and total protein levels were determined using Pierce BCA assay kit (Thermo Fisher Scientific) prior to ELISA. IL-17A, IL-22, MPO and neutrophil elastase protein levels were quantified with commercially available ELISA kits (R&D Systems or Biolegend) [5]. IL-22 protein levels were normalised to total protein in lung homogenates.

Lung Function. Mice were anaesthetised with ketamine (100mg/kg) and xylazine (10mg/kg, Troy Laboratories, Smithfield, Australia) prior to tracheostomy. Tracheas were then cannulated and attached to Buxco® Forced Manoeuvres systems apparatus (DSI, St. Paul, Minnesota, USA) to assess total lung capacity [7, 8]. Mice were then attached to a FlexiVent apparatus (FX1 System; SCIREQ, Montreal, Canada) to assess lung volume, airway resistance, inspiratory capacity, forced vital capacity and compliance (tidal volume of 8mL/kg at a respiratory rate of 450 breaths/mins) [7, 20, 21]. All assessments were performed at least three times and the average was calculated for each mouse.

Human lung tissue study population. Peripheral lung samples were obtained from subjects undergoing lung resection for peripheral lung carcinoma from the Respiratory Unit of the University Hospital of Ferrara, Italy (supplementary table S4). Smokers with mild-to-moderate stable COPD (n=12) were compared with age- and smoke history-matched smokers with normal lung function (NLF) (n=12). Diagnosis of COPD was defined according to international guidelines as the presence of post-bronchodilator FEV1/FVC ratio <70% or the presence of cough and sputum production for at least 3 months in each of two consecutive years [22]. All patients were in stable condition at the time of the surgery and had not suffered acute

exacerbations or upper respiratory tract infections in the preceding two months. None had received glucocorticoids or antibiotics within the month preceding surgery, or inhaled bronchodilators within the previous 48 h. Patients had no history of asthma or other allergic diseases. All former smokers had stopped smoking for more than one year. Each patient was subjected to medical history, physical examination, chest radiography, electrocardiogram, routine blood tests, and pulmonary function tests during the week prior to surgery. Pulmonary function tests (Biomedin Spirometer, Padova, Italy) were performed as previously described [23] according to published guidelines.

Lung sample preparation and IHC. Collection, processing, immunohistochemical analysis of lung tissue samples as well as data analysis were performed as previously published [24, 25]. The primary antibodies (anti-human) used are summarised in supplementary table S5. Negative antibody controls used were nonspecific isotype matched Ig at their respective primary antibody concentrations**.** Image analysis was performed [24] using an integrated microscope (Olympus, Albertslund, Denmark), video camera (JVC Digital color, Tatstrup, Denmark), automated microscope stage (Olympus) and PC running Image pro-Plus Software (Media Cybernetics) to quantify the RBP staining areas. Immunostaining counting and interpretation were performed blinded without prior knowledge of clinical-pathologic parameters.

Scoring system for IHC in peripheral lung. Staining analysis was performed as previously published [24, 25]. A bronchiole was taken to be an airway with no

cartilage and glands in its wall. According to a validated method [24] the number of positively stained endoalveolar macrophages was expressed as a percentage of the total cells with the morphological appearance of alveolar macrophages counted inside of the alveoli. The number of bronchiolar epithelial cells with positive staining was expressed as a percentage of the total number of epithelial cells counted in each bronchiolar section and group data were expressed as mean and standard error of the mean (SEM). Airway epithelial-specific IL-22RA1 protein intensity was quantified using the Aperio imaging system and normalized to the length of the basement membrane.

Statistical analyses. Unless otherwise stated, data are presented as means ± standard error of mean (SEM) and are representative of two independent experiments with 6 mice per group. The two-tailed Mann-Whitney test was used to compare two groups. The one-way analysis of variance with Bonferroni post-test was used to compare 3 or more groups. Statistical significance was set at $P < 0.05$ and determined using GraphPad Prism Software version 6 (San Diego, CA, USA).

Supplementary table S1. Custom-designed primers used in qPCR analysis

Primer	**Primer sequence (5' → 3')**
Il22ra1 forward	GTTTTACTACGCCAAGGTCACG
Il22ra1 reverse	CACTTTGGGGATACAGGTCACA
Il10rb forward	ATTCGGAGTGGGTCAATGT
Il10rb reverse	CTGAGAAACGCAGGTGTAAAG
Il22ra2 forward	CTCTTCTGTGACCTGACCAATGA
Il22ra2 reverse	TTATAGTCACGACCGGAGGATCT
Cxcl1 forward	GCTGGGATTCACCTCAAGAA
Cxcl1 reverse	CTTGGGGACACCTTTTAGCA
Cxcl2 forward	TGCTGCTGGCCACCAACCAC
Cxcl2 reverse	AGTGTGACGCCCCCAGGACC
Il17a forward	GTGTCTCTGATGCTGTTGCT
Il17a reverse	GTTGACCTTCACATTCTGGA
Hprt forward	AGGCCAGACTTTGTTGGATTTGAA
Hprt reverse	CAACTTGCGCTCATCTTAGGATTT

Supplementary table S2. Surface antigens used to characterise mouse IL-17A$^+$ IL-22$^+$ lung cell subsets by flow cytometry

Cell subset	Cell surface antigens
CD4$^+$ T cells	CD45$^+$CD3$^+$CD4$^+$CD8$^-$
$\gamma\delta$ T cells	CD45$^+$CD3$^+$$\gamma\delta$TCR$^+$
NKT cells	CD45$^+$CD3$^+$$\alpha$GalCer tetramer$^+$
ILC3	CD45$^+$CD3$^-$Ly6C/G$^-$CD11b$^-$B220$^-$TER119$^-$IL-7Rα^+CD90.2$^+$
IL-17A and IL-22	Reported by eGFP and td-tomato, respectively

Supplementary table S3. Antibodies used in flow cytometry analysis

Cell surface antigens	Clone	Fluorophore	Company
CD45	30-F11	PerCP-Cy5.5	Biolegend
CD3	17A2	AF700	Biolegend
CD4	RM4-5	APC-Cy7	Biolegend
CD8	53-6.7	BV510	Biolegend
γδTCR	GL3	BV421	Biolegend
αGalCer Tetramer	N/A	BV605	N/A
Lineage cocktail (CD3, Ly6C/G, CD11b, B220, TER119)	17A2, RB6-8C5, M1/70, RA3-6B2, Ter-119	AF700	Biolegend

Supplementary Table S4. Characteristics of subjects for the immunohistochemical study of interleukins on peripheral lung

Subjects	N.	Age	Sex	Smoking history	Pack-years	Chronic bronchitis	FEV_1 % pred	FEV_1/ FVC %
Control smokers	12	70.8 ±2.3	10M/ 2F	8 Ex smokers 4 Current smokers	41.9 ±11.4	0	104.3±4.0	76.7±1.3
COPD	12	72.4 ±1.5	12M	7 Ex smokers 5 Current smokers	40.6 ±3.3	4 with chronic bronchitis	76.9±6.2	61.6±2.7

Supplementary table S5. Primary antibodies and immunohistochemical conditions used for identification of interleukins in the peripheral lung

Antigen	Company	Catalogue	Host	Concentration	Secondary antibody
IL10Rb	MyBio Source	MBS2003603	Rabbit	1.8 µg/ml	Goat anti-rabbit IgG, Vector (BA 1000); 1:200
IL-22	R&D	AF782	Goat	4 µg/ml	Rabbit anti-goat IgG, Vector (BA 5000); 1:200
IL22RA1 / IL22R	EMD Millipore/LSBio	06-1077-I/LS-B1365	Rabbit	2.2 µg/ml	Goat anti-rabbit IgG, Vector (BA 1000); 1:200
IL22RA2	Atlas	HPA030582	Rabbit	1 µg/ml	Goat anti-rabbit IgG, Vector (BA 1000); 1:200

Supplementary table S6. Immunohistochemical percentage of peripheral lung IL-22-positive cells

Localization and antigen	Control smokers	COPD	Mann-Whitney test p value
Bronchiolar epithelium			
Nuclear	8.3±2.8	9.0±2.5	0.6427
	5.0 (9.6)	5.0 (8.7)	
	1.0-13.8	2.0-18.0	
Cytosolic	48.5±7.0	60.8±6.6	0.2037
	54.5 (24.1)	67.5 (22.8)	
	25.3-70.3	44.5-76.3	
Alveolar macrophages			
Nuclear	16.7±4.1	46.5±7.5	0.0130
	11.5 (14.1)	51.0 (26.1)	
Cytosolic	9.0-23.0	28.3-63.5	0.0602
	62.0 (20.3)	58.0 (13.8)	

	40.8-76.5	27.0-48.5

Data expressed as mean ± SEM (first line), median (SD) (second line) and interquartile range (third line). Data expressed as mean ± SEM (first line), median (SD) (second line) and interquartile range (third line).

Supplementary table S7. Immunohistochemical percentage of peripheral lung IL22RA1-positive cells

Localization and antigen	**Control smokers**	**COPD**	**Mann-Whitney test p value**
Bronchiolar epithelium			
Nuclear	3.8±2.3	24.9±4.6	0.0009
	0.8 (7.9)	24.5 (15.9)	
	0.0-4.0	16.8-27.5	
Cytosolic	30.5±7.8	8.2±3.5	0.0123
	21.5 (27.1)	2.0 (12.1)	
	7.0-57.3	0.0-16.8	
Alveolar macrophages			
Nuclear	1.9±0.9	20.7±4.3	0.0005
	0.5 (3.2)	21.0 (15.0)	
	0.0-2.8	5.5-35.3	
Cytosolic	0.0-2.8	5.5-35.3	0.0022
	72.5±3.9	52.0±3.9	
	75.0 (13.4)	49.0 (13.5)	

Data expressed as mean ± SEM (first line), median (SD) (second line) and interquartile range (third line). Data expressed as mean ± SEM (first line), median (SD) (second line) and interquartile range (third line).

Supplementary table S8. Immunohistochemical percentage of peripheral lung IL22RA2-positive cells

Localization and antigen	Control smokers	COPD	Mann-Whitney test p value
Bronchiolar epithelium			
Nuclear	0	0	-----
	0	0	
	0	0	
Cytosolic	25.7±7.0	11.8±3.7	0.1645
	19.0 (24.1)	8.0 (12.8)	
	3.5-49.5	1.3-20.5	
Alveolar macrophages			
Nuclear	0	0	-----
	0	0	
	0	0	
Cytosolic	47.5±5.0	48.2±8.5	0.8173
	46.5 (17.5)	52.5 (29.3)	
	34.3-59.8	22.5-73.3	

Data expressed as mean ± SEM (first line), median (SD) (second line) and interquartile range (third line). Data expressed as mean ± SEM (first line), median (SD) (second line) and interquartile range (third line).

Supplementary table S9. Immunohistochemical percentage of peripheral lung IL10Rb-positive cells

Localization and antigen	Control smokers	COPD	Mann-Whitney test p value
Bronchiolar epithelium			
Nuclear	1.8±0.8	3.4±1.1	0.1259
	0.5 (2.6)	2.0 (3.8)	
	0.0-2.8	1.0-4.8	
Cytosolic	27.0±7.6	26.9±5.3	0.7505
	16.0 (26.5)	18.5 (18.4)	
	2.8-58.3	13.0-41.8	
Alveolar macrophages			
Nuclear	5.5±1.7	19.1±3.7	0.0044
	5.0 (5.9)	19.0 (12.8)	
	0.0-11.8	7.5-25.0	
Cytosolic	59.8±5.9	59.6±4.0	0.9769
	62.0 (20.3)	58.0 (13.8)	
	38.5-76.5	49.3-66.8	

Data expressed as mean ± SEM (first line), median (SD) (second line) and interquartile range (third line).

Supplementary Table S10. Characteristics of subjects for the IL-22RA1 intensity in airway epithelial cells

Subjects	Non-smokers	Healthy smokers	GOLD 2	GOLD 3, 4
Sex (M/F)	2/4	2/4	6/3	4/5
Smoking status (current/ex/NA)	0/0/0	4/2/0	4/3/2	1/8/0
Age (mean ± SD)	58.0±18.1	65.8±9.2	63.7±9.0	60.3±6.0
FEV1/FVC % (mean ± SD)	82.9±4.4	76.5±3.5	57.1±5.6	33.5±11.1

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