Supplementary materials

Microarray analysis

Microarray analysis was conducted in two stages.

First, asthmatics and controls were pooled to obtain sufficient power to determine the effect of IL-1β on gene expression in airway smooth muscle cells (ASMC) (n=6, dataset A).

To validate these findings, we investigated a second independent dataset from 2 asthmatic and 4 healthy-derived ASMC cultures treated with IL-1β 10 ng/ml for 8 hours (dataset B). Where again asthmatics and controls were pooled to determine the effect of IL-1β on gene expression in ASMC.

Second, subjects in dataset A were separated based on health status and analysis was conducted on the subset of genes altered by IL-1β, comparing the change in expression to IL-1β in asthmatics to the change in expression of IL-1β in healthy controls (Fold change ± 2, FDR<0.05). All microarray analysis was performed using the computing environment R (R Development Core Team, 2013, version 3.02). Additional software packages (limma) were taken from the Bioconductor project, and normalised using Robust Multi-array Average**.**

Cell culture

ASMC

ASMC n=6 were isolated from asthmatic and healthy controls and cultured as previously described[1, 2]. At passage 3-4, ASMC were seeded at $1x10^4$ cell/cm^2 and grown in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA), 5% fetal bovine serum FBS and 1% antibiotics (Invitrogen) for 3-4 days to confluence. Cells were quiesced for 72 hours in DMEM with 0.1% bovine serum albumin (BSA, (Sigma Aldrich, St Louis,

MO, USA) and then treated with vehicle (0.1% BSA) or, 10 ng/ml IL-1β (R&D Systems). Total cellular mRNA was isolated using the Qiagen total RNA isolation kit (Qiagen, Doncaster, VIC, Australia) following 0 or 8 hours stimulation and finally stored at -80^{o}C (dataset A). Total mRNA was collected for microarray analysis.

Immortalised ASMC

For *in vitro* validation and functional studies, primary ASMC were immortalised using the hTERT over expression system, as previously described [3]. Moderate asthmatic, mild asthmatic and healthy ASMC, defined by the Global Initiative for Asthma (GINA) guidelines were seeded at 1x10^4 cells per cm^2 in 6 well plates and grown to confluence in DMEM(Invitrogen) supplemented with 10% FBS (JRH Biosciences) and 1% antibiotics (Invitrogen). Cells were quiesced in 0.1% BSA in DMEM for 3 days and subsequently treated with IL-1β (10 ng/ml) and control. mRNA was collected at 8 hours for realtime PCR while supernatants for CCL20 ELISAs were collected at 24 hours.

CALU-3 cells

CALU- 3 cells were grown to confluence in 75cm^2 flasks in complete Dulbecco's Modified Eagle's medium (F-12 containing 10% (v/v) foetal bovine serum, 1% (v/v) nonessential amino acid solution and 1% (v/v) L-glutamine solution).

Microarray candidate validation

Real-time PCR was conducted on total mRNA using Taqman primer CCL20 (Life Technologies, Mulgrave, VIC, AUS). Samples with no expression were given the value Ct=35. Samples were normalised to their untreated controls. CCL20 protein levels were

accessed using CCL20/MIP-3α ELISA (R&D Systems). Samples below the detection limit were given the value 16 pg/ml the detection limit of the ELISA.

RNA extraction, Sample preparation and High-throughput sequencing

Bronchial biopsies were taken from segmental divisions of the main bronchi. Biopsies frozen in Tissue-Tek (VWR, Radnor, PA) at -80°C were thawed at room temperature and cut from the blocks when they were semi-solid. Total RNA was extracted using AllPrep DNA/RNA Mini kit (Qiagen, Venlo, the Netherlands). Samples were lysed in 600 µl RLT-plus buffer using an IKA Ultra Turrax T10 Homogenizer, and RNA was purified according to the manufacturer's instructions. RNA samples were dissolved in 30 µl RNAse free water. Concentrations and quality of RNA were checked using a Nanodrop-1000 and run on a Labchip GX (PerkinElmer, Waltham, MA).

RNA samples were further processed using the TruSeq Stranded Total RNA Sample Preparation Kit (Illumina, San Diego, CA), using an automated procedure in a Caliper Sciclone NGS Workstation (PerkinElmer, Waltham, MA). In this procedure, all cytoplasmic and mitochondria rRNA was removed (RiboZero Gold kit). The obtained cDNA fragment libraries were loaded in pools of multiple samples unto an Illumina HiSeq2500 sequencer using default parameters for paired-end sequencing (2 × 100 bp).

Gene expression quantification

The trimmed fastQ files where aligned to build b37 of the human reference genome using HISAT (version 0.1.5) allowing for 2 mismatches (Kim et al. 2015). Before gene quantification SAMtools (version 1.2) was used to sort the aligned reads (Li et al. 2009). The

gene level quantification was performed by HTSeq (version 0.6.1p1) using Ensembl version 75 as gene annotation database.

Quality Control

Quality control (QC) metrics were calculated for the raw sequencing data, using the FastQC tool (version 0.11.3) (Andrews 2010). Alignments of 220 subjects were obtained. QC metrics were calculated for the aligned reads using Picard-tools (version 1.130) (http://picard.sourceforge.net) CollectRnaSeqMetrics, MarkDuplicates, CollectInsertSize-Metrics and SAMtools flagstat. We discarded 36 samples due to poor alignment metrics. In addition, we checked for concordance between sexlinked (*XIST* and Y-chromosomal genes) gene expression and reported sex. All samples were concordant. This resulted in high quality RNAseq data from 184 subjects.

Differential expression

Raw counts of expressed features were analysed using the R-package DESeq2 (Love et al. 2014). Feature counts were set as the dependent variable, with asthma status as the predictor variable. Sex, current smoking, and age were entered as co-variables.

Immunohistochemistry

Tissue staining for CCR6 with the rabbit anti-human CCR6 (R&D Systems) [0.2 μg/ml] was conducted as previously described [3].

Air liquid interface

CALU-3

To establish the air-liquid interface model CALU-3 cells were seeded onto Transwell polyester inserts (Sigma Aldrich) at a density of $5x10^5$ cells/cm^2 in 100 µL apical and 500 µL basolateral medium. The apical medium was removed 24 hours after seeding and cells were allowed to grow for 5-7 days, with basolateral medium changed at day 4. On day 5 of treatment the apical layer of the transwells was washed with HEPES for 1 hour to remove any mucus. Cells were treated with either CCL20 (10ng/ml), rabbit anti-human CCL20 antibody (Abcam), isotype control (DakoCytomation), rabbit anti human CCL20 antibody + CCL20 10ng/ml, Isotype control + CCL20 (10ng/ml) and complete DMEM (control) in the basolateral side for 48 hours.

Primary airway epithelium

Air liquid interface (ALI) cultured primary epithelial cells was conducted according to a previous publication [4]. Briefly, primary epithelial cells obtained from the enzymatic digestion of bronchial tissue were seeded at 75,000 in 200µl Bronchial Epithelial Cell Growth Medium (BEGM) in the apical part of the insert and 500µl BEGM at the basolateral part. When the cell-layer was confluent (3-5 days) the cells were exposed to air at the apical side and Dulbecco's Modified Eagle Medium (DMEM) / BEBM (1:1) with retinoic acid (15ng/ml) added to the basolateral side (500µl). Media was refreshed every 3 days with DMEM / BEBM (1:1) with retinoic acid (15ng/ml). Cells were quiesed on day 28 with BEBM medium for 24H and then treated with CCL20 10ng/ml or PBS for 48H. ALI apical washes (BEBM medium 5 minutes) were conducted following treatment and MUC5AC was measured by ELISA (SEA756Hu, Cloud-Clone Corp., China).

MUC5AC ELISA

MUC5AC levels in apical washes (2x dilution) were measured using the ELISA kit (SEA756Hu, Cloud-Clone Corp., China) according to the manufacturer's protocol.

Alcian Blue Staining

Following treatment transwells were washed with PBS. Cells were fixed with 4% (v/v) paraformaldehyde for 20 minutes. Transwells were then washed with PBS and stained using alcian blue (1% (w/v) alcian blue in 3% (v/v) acetic acid/water at pH 2.5)(Sigma-Aldrich) for 15 minutes. Following the staining the transwells were rinsed multiple times with PBS and allowed to air-dry overnight. The transwell's filter was then cut out with a sharp point scalpel and mounted on a glass slide using Entellan new mounting medium (Merck Millipore).

Sections were imaged on an Olympus BX60 microscope (Olympus, Hamburg, Germany) with manual light exposure and 'one push' white balance on a background region. Images were then taken using an attached DP71 camera (Olympus) at 20X magnification and recorded using Kodak software. Each image was analysed using Image J (v1.42q, NIH) with Colour deconvolution plugin. Images were separated based on alcian blue stain colours and densitometry mean was determined for 5 representative images of each insert and averaged.

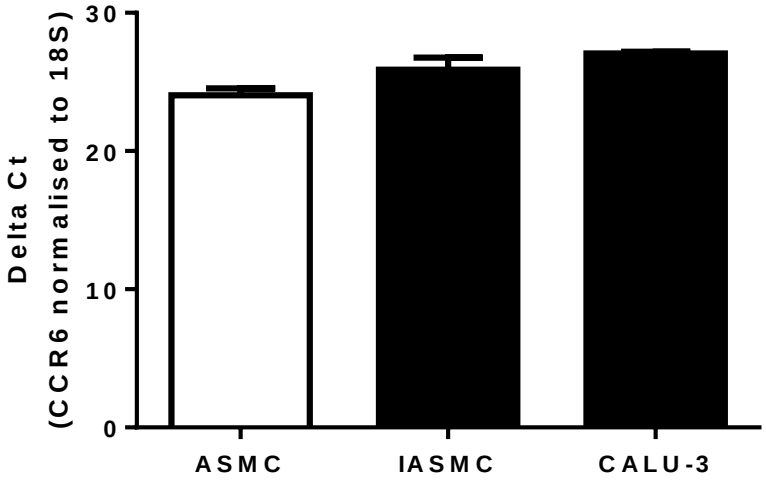

Figure S1 Gene expression of CCR6 in structural cells in the airways.
CCR6 mRNA levels were measured by Real time PCR in quiesced cells (ASMC (n=4), IASMC (n=3), and CALU-3 cells (n=3)). Data are expressed as mean ± standard error of the mean. Abbreviations ASMC=airway smooth muscle cells, IASMC=immortalised airway smooth muscle cells, DMEM= Dulbecco's Modified Eagle Medium, FBS= Foetal bovine serum and BSA= bovine serum albumin.

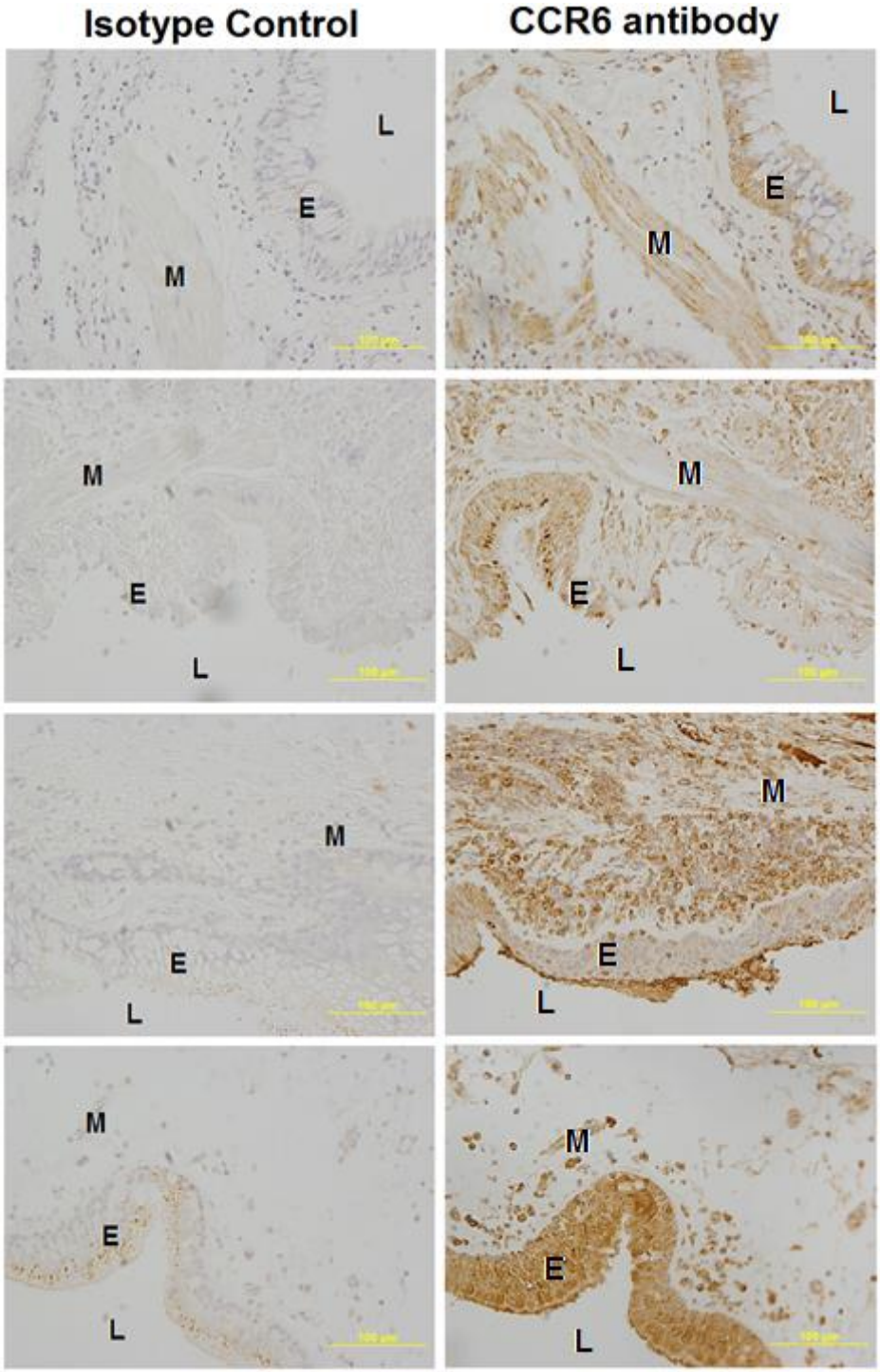

Figure S2 Immunohistochemistry on paraffin embedded bronchus (>2mm) for CCR6. CCR6 immunohistochemistry was conducted on non cancerous sections following lung resections (n= 4)(representative images). Specific staining was detected using a chemical chromophore DAB (brown) and cell nucleus was counterstained with haematoxylin (blue)(scale 100μm). Abbreviations M= Airway Smooth Muscle, L= Lumen, E = Epithelium

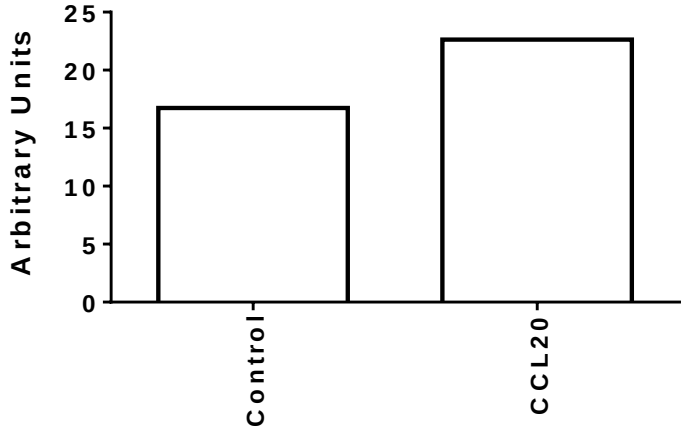

Figure S3 Densitometry analysis of Alcian blue staining in ALI's derived from primary airway epithelial cells treated with CCL20. Primary airway epithelial cells grown in air-liquid interface treated on day 28 with either C) PBS or CCL20 10ng/ml for 48 hours (n=1).

Table S1. Patient characteristics for RNA-Seq results

	Asthma	Healthy
N	16	39
Age (years)	44.72±12.93	38.95±18.9
Gender male n(%)	8 (50)	19(48.7)
FEV1 % predicted	84.45±9.63**	101.95±11.67
PC20	218.61±296.29**	630.52±59.23

Data are presented as mean± SD unless stated otherwise. Differences in variables before and after treatment were analysed using a two-sided, Student's t test.

**p<0.01 versus healthy.

FEV1, forced expiratory volume in one second; PC20, provocative dose of Adenosine 5′-Monophosphate (AMP) causing a 20% fall in FEV1

Table S2. Top 50 genes altered by IL1β compared to baseline (FDR adjusted p value<0.05)

Gene Symbol	Gene Name	log FC	p value
CXCL10	chemokine (C-X-C motif) ligand 10	7.75	2.87E-12
IL8	interleukin 8	7.60	1.02E-14
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	6.58	2.43E-14
CXCL2	chemokine (C-X-C motif) ligand 2	5.82	2.43E-14
CXCL6	chemokine (C-X-C motif) ligand 6	5.48	1.50E-10
TNFAIP2	tumor necrosis factor, alpha-induced protein 2	5.33	4.56E-16
GBP4	guanylate binding protein 4	4.87	1.91E-11
IL6	interleukin 6 (interferon, beta 2)	4.80	2.28E-10
GCH1	GTP cyclohydrolase 1	4.79	2.17E-12
CCL20	chemokine (C-C motif) ligand 20	4.77	9.56E-12
CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1	4.72	1.55E-16
BIRC3	baculoviral IAP repeat containing 3	4.64	1.17E-11
TNFAIP3	tumor necrosis factor, alpha-induced protein 3	4.61	1.44E-13
ELOVL7	ELOVL fatty acid elongase 7	4.19	2.69E-10
MFSD2A	major facilitator superfamily domain containing 2A	3.92	2.53E-11
CFB	complement factor B	3.86	7.73E-12
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	3.69	5.16E-12
MARCH3	membrane-associated ring finger (C3HC4) 3, E3 ubiquitin protein ligase	3.60	1.46E-11
SLC39A14	solute carrier family 39 (zinc transporter), member 14	3.39	9.64E-12
C15orf48	chromosome 15 open reading frame 48	3.28	2.41E-10
IRAK2	interleukin-1 receptor-associated kinase 2	3.17	1.22E-10
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3.17	5.27E-14
RIPK2	receptor-interacting serine-threonine kinase 2	3.15	3.17E-13
GBP3	guanylate binding protein 3	2.88	2.40E-10
ZC3H12A	zinc finger CCCH-type containing 12A	2.83	5.27E-14
IRF1	interferon regulatory factor 1	2.81	7.16E-10
CXCL3	chemokine (C-X-C motif) ligand 3	2.81	1.24E-09
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	2.56	5.27E-14
PARP14	poly (ADP-ribose) polymerase family, member 14	2.49	9.02E-10
GBP2	guanylate binding protein 2, interferon-inducible	2.45	6.18E-11
SLC43A2	solute carrier family 43 (amino acid system L transporter), member 2	2.41	6.18E-11
RELB	v-rel avian reticuloendotheliosis viral oncogene homolog B	2.40	8.08E-12
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	2.30	9.04E-13
TAP2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	2.19	6.11E-13

WTAP	Wilms tumor 1 associated protein	2.16	1.34E-12
IFNAR2	interferon (alpha, beta and omega) receptor 2	2.14	7.85E-11
HIVEP3	human immunodeficiency virus type I enhancer binding protein 3	2.13	4.88E-10
TNIP1	TNFAIP3 interacting protein 1	2.10	1.58E-12
MT2A	metallothionein 2A	2.10	3.05E-11
GPR37L1	G protein-coupled receptor 37 like 1	2.07	2.36E-10
WTAP	Wilms tumor 1 associated protein	2.05	9.91E-13
MT2A	metallothionein 2A	2.04	1.46E-11
IL32	interleukin 32	2.03	5.85E-10
MT1JP	metallothionein 1J, pseudogene	2.02	6.53E-13
MT2A	metallothionein 2A	1.99	2.23E-10
NINJ1	ninjurin 1	1.96	2.04E-12
BID	BH3 interacting domain death agonist	1.77	1.54E-11
STX11	syntaxin 11	1.57	1.06E-09
TRAF3	TNF receptor-associated factor 3	1.28	4.79E-11
UXS1	UDP-glucuronate decarboxylase 1	1.08	1.46E-10

Table S3.

Gene	logFC	p value	FDR
UBD	-1.20299	2.19E-08	0.000419
UBD	-1.24331	2.52E-08	0.000419
CXCL10	-1.4818	3.38E-07	0.003749
CXCL8	-1.48886	1.64E-06	0.010912
CCL20	-1.10357	3.76E-06	0.017906
UCA1	-1.14167	9.29E-06	0.028705

References

1. Faiz A, Tjin G, Harkness L, Weckmann M, Bao S, Black JL, et al. The expression and activity of cathepsins D, H and K in asthmatic airways. PloS one 2013; 8:e57245.
2. Faiz A, Donovan C, Nieuwenhuis MA, van den Berge M, Postma D, Yao S, et al. Latrophilin receptors: novel bronchodilator targets in asthma. Thorax 2017; 72:74-82.
3. Bossé Y, Paré PD, Seow CY. Airway wall remodeling in asthma: from the epithelial layer to the adventitia. Current allergy and asthma reports 2008; 8:357-66.
4. Hackett T-L, de Bruin HG, Shaheen F, van den Berge M, van Oosterhout AJ, Postma DS, et al. Caveolin-1 controls airway epithelial barrier function. Implications for asthma. American journal of respiratory cell and molecular biology 2013; 49:662-71.