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Chronic mucus hypersecretion in COPD and asthma

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CHAPTER 8

Profiling of healthy and asthmatic airway smooth muscle cells following IL-1 β treatment: a novel role for CCL20 in chronic mucus hypersecretion

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ABSTRACT

Chronic mucus hyper-secretion (CMH) contributes to the morbidity and mortality of asthma and remains uncontrolled by current therapies in the subset of patients with severe, steroid resistant disease. Altered crosstalk between airway epithelium and airway smooth muscle cells (ASMCs), driven by pro-inflammatory cytokines such as IL-1 β , provides a potential mechanism that influences CMH.

This study investigated mechanisms underlying CMH by comparing IL-1 β -induced gene expression profiles between asthma and control-derived ASMCs and the subsequent paracrine influence on airway epithelial mucus production *in-vitro*. IL-1 β -treated ASMCs from asthmatic and healthy donors were profiled using microarray analysis and ELISA. Air-liquid interface (ALI)-cultured CALU-3 and primary airway epithelial cells were treated with identified candidates and mucus production assessed.

The IL-1 β -induced CCL20 expression and protein release was increased in ASMCs from moderate compared to mild asthmatics and healthy controls. IL-1 β induced lower MIR146A expression in asthma-derived ASMCs compared to controls. Decreased MIR146A expression was validated *in-vivo* in bronchial biopsies from 16 asthmatic versus 39 healthy donors. MiR-146a-5p overexpression abrogated CCL20 release in ASMCs. CCL20 treatment of ALI-cultured CALU-3 and primary airway epithelial cells induced mucus production, while CCL20 levels in sputum were associated with increased levels of CMH in asthmatic patients.

Elevated CCL20 production by ASMC, possibly resulting from dysregulated expression of the anti-inflammatory miR-146a-5p, may contribute to enhanced mucus production in asthma.

INTRODUCTION

Asthma is a chronic inflammatory disease affecting 300 million people worldwide¹. Chronic mucus hyper-secretion (CMH) contributes to the morbidity and mortality of asthma² and remains uncontrolled by current therapies. There is an urgent need to identify new therapeutic targets.

Although mucus production increases in the airway epithelial layer during inflammation³, the underlying mechanism of CMH remains to be elucidated. Differentiation of airway epithelial cells into either ciliated or goblet cells is directed by other structural cells in the submucosa. The cross talk between the epithelial layer and airway smooth muscle cells (ASMC) may regulate mucus production, since the airway smooth muscle mass is enlarged in asthma⁴.

ASMC have long been thought to have a passive role, but accumulating evidence suggests that these cells play an important role in the inflammatory process that underlies CMH, providing an active source of cytokines and chemokines via a number of pathways⁵.

One of these inflammatory pathways known to be altered in asthma is the inflammasome, a multi-protein complex that plays an important role in the activation of pro-inflammatory cytokines, for example conversion of IL-1 β from its pro-form into its active state⁶. The activity of the inflammasome is enhanced in neutrophilic asthma⁷, leading to increased levels of active IL-1 β in sputum.

IL-1 β is a strong pro-inflammatory signaling molecule, the downstream mediators of which are associated with mucus production⁸. However, little is known about the influence of IL-1 β on the pro-inflammatory response of airway structural cells, especially ASMC and the potential role in CMH in asthma.

In this study, we identified CCL20 and MIR146A when comparing gene expression profiles between asthmatic and healthy ASMC *in vitro* in response to IL-1 β . Importantly, CCL20 had been shown to induce MUC5AC expression in epithelial cultures by binding to its only known receptor CCR6⁹. Furthermore, in murine models, anti-CCL20-treatment significantly decreased virus-induced mucus production. While previously a SNP in miR-146a has been associated with the presence of asthma and other pro-inflammatory diseases^{11,12}. Interestingly, we have recently shown that lower expression of miR-146a-5p in bronchial biopsies is inversely correlated with CMH in COPD¹³, highlighting miR-146a-5p as a regulator of mucus regulation in respiratory diseases. Based on CCL20 and miR-146a-5p known role in mucus production, we then investigated how these factors produced by ASMC influence mucus production in airway epithelial cells.

METHODS

Human tissue

Primary human ASMCs were obtained as described previously^{14,15} with ethical approval from The University of Sydney and participating hospitals (Concord Repatriation General Hospital, Sydney South West Area Health Service and Royal Price Alfred Hospital). All patients, or next of kin, provided written informed consent. An outline of the patients' characteristics is shown in Table 1.

Microarray processing and analysis

ASMCs were isolated from asthmatic patients (n=3) and healthy controls (n=3) and cultured as previously described^{14,15} (Dataset A). Cells were treated with 10 ng/ml IL-1 β ¹⁶ (R&D Systems) for 8 hours. Total cellular mRNA was isolated, labeled and run on an Affymetrix (Santa Clara, California, USA) GeneChip Human Gene 1.0 ST Array according to the manufacturer's instructions (GSE63383)⁴. For independent validation, ASMCs derived from 2 asthmatic patients and 4 healthy donors (Dataset B) were grown and treated with IL-1 β as above. Samples were labeled and run on an Affymetrix (Santa Clara, California, USA) Human U133Plus 2.0 Array according to the manufacturer's instructions. Microarray analysis is outlined in the online supplement.

Pathway analysis

Functional enrichment analysis to identify the overlapping genes altered by IL-1 β treatment in Datasets A and B was performed using Gene Set Enrichment Analysis (GSEA) V.2.0.14. Protein Network analysis was conducted using String v10 on the overlapping genes. GSEA was also used to investigate pathways regulated in Dataset A using the Biocarta database. Transcript factor binding site analysis was conducted using gprofiler.

Miroarray candidate validation

The validation of the microarray results was undertaken at a transcriptional (quantitative real-time PCR) and translational (ELISA) level as described in the online supplement.

Bronchial biopsies processing for quantification of MIR146A expression

Bronchial biopsies were collected from respiratory healthy subjects^[17] and current asthma patients^{18,19} with a previous doctor's diagnosis of asthma, documented reversibility and AHR to histamine (PC20 histamine (using 30-s tidal breathing) < 32 mg/ml). The analysis was conducted on 39 healthy subjects and 16 asthmatics,

who were all non-smokers and currently not taking inhaled corticosteroids (ICS). An outline of the patients' characteristics is shown in Table S1. All study protocols were approved by the UMCG medical ethics committee and all subjects provided written informed consent. RNA was isolated and sequenced as described in the online supplement.

MiR-146a-5p predicted targets

To identify downstream targets of miR-146a-5p, we used a publically available microarray dataset (GSE79340) of human hepatic Huh7.5.1 cells transfected with miR-146a-5p mimic (5 nM) compared to a negative control (n=3).

Functional analysis

Immortalised ASMC (IASMC) were grown to 80-90% confluence and serum deprived before transfection with either a miR-146a-5p (100 nM) mimic or mimic control using RNAimax (Invitrogen). Twenty four hours later, cells were treated with IL-1 β (10 ng/ml) or 0.1% BSA (control). Cell-free supernatants were collected at 24 hours and IL-8 levels assessed by ELISA.

The human lung epithelial cell line CALU-3 and primary airway epithelial cells were grown at air-liquid interface (ALI), treated with CCL20 (10 ng/ml) for 48 hours and mucus assessed by Alcian Blue staining as described in the online supplement.

CCL20 levels in sputum

Sputum was induced in a population of asthmatic patients (n=89), as previously described^{20,21}, with and without CMH. CCL20 was measured by ELISA.

Definition of CMH

To define CMH asthmatic patients were asked to respond to a clinical questionnaire: "How often did you cough up sputum during the last week?"²² This question had seven possible answers: i) never, ii) sometimes, iii) once in a while, iv) often, v) most of the time, vi) regularly, and vii) always. Patients who responded i) were classified as no CMH; ii) and iii) moderate CHM; and iv) to viii) severe CMH. Of the asthmatics with available sputum 80 gave answers to the questionnaire and were analysed in this study.

Statistics

Statistical tests and graph plotting were conducted using GraphPad Prism 6 (GraphPad Software, La Jolla, California USA). A probability (p) value of <0.05 was considered statistically significant.

Table 1. Demographics of individual patients from whom samples were obtained.

No.	Diagnosis	Age	Gender	Samples	FEV1% predicted	Experiments for which sample was used
1	Asthma	33	M	Bronchoscopy	N	1
2	Asthma	22	M	Bronchoscopy	N	1
3	Asthma	33	F	Transplant	N	1
4	Non diseased donor	31	M	Bronchoscopy	N	1
5	Non diseased donor	22	M	Bronchoscopy	N	1
6	Non diseased donor	27	F	Bronchoscopy	N	1
7	Asthma	20	M	Bronchoscopy	65	2
8	Unknown		F	Transplant	N	2
9	Asthma	19	F	Bronchoscopy	97	2
10	Non diseased donor	20	M	Bronchoscopy	N	2
11	Non diseased donor	30	M	Bronchoscopy	N	2
12	Non diseased donor	21	F	Bronchoscopy	N	2
13	Non diseased donor	31	F	Immortalised ASM cells	105	3,4
14	Non diseased donor	40	M	Immortalised ASM cells	131	3
15	Non diseased donor	23	M	Immortalised ASM cells	82	3,4
16	Non diseased donor	22	F	Immortalised ASM cells	87	3,4
17	Asthma	39	M	Immortalised ASM cells	84	3,4
18	Asthma	29	M	Immortalised ASM cells	89	3,4
19	Asthma	21	M	Immortalised ASM cells	108	3,4
20	Asthma	31	M	Immortalised ASM cells	85	3,4
21	Asthma	27	F	Immortalised ASM cells	78	3,4
22	Asthma	33	M	Immortalised ASM cells	78	3,4
23	Non diseased donor	69	M	Immortalised ASM cells	N	4
24	Non diseased donor	22	F	Immortalised ASM cells	N	4
25	Non diseased donor			Immortalised ASM cells	N	6
26	Cystic fibrosis	22	F	Paraffin embedded bronchus	N	5
27	Non diseased donor	16	M	Paraffin embedded bronchus	N	5
28	COPD	56	F	Paraffin embedded bronchus	N	5
29	Pulmonary Fibrosis	53	F	Paraffin embedded bronchus	N	5

Abbreviations used M = male, F = female, N= not available, COPD= Chronic obstructive pulmonary disease
 1= HuGene-1_0-st-v1 microarray (dataset A), 2= HG-U133_Plus_2 microarray (dataset B), 3= qReal Time-PCR validation, 4= CCL20 ELISA, 5= CCR6 IHC,6=miR-146A-5p functional work.

RESULTS

Response of ASMC to IL-1 β

To evaluate if IL-1 β is involved in the abnormal crosstalk between ASMC and airway epithelium, contributing to CMH in the asthmatic airway, we first examined the regulation of genes following IL-1 β treatment. Asthmatics (n=3) and controls (n=3) were pooled to obtain sufficient power to determine the effect of IL-1 β on gene expression (dataset A). Gene expression analysis identified 408 genes that were up-regulated and 143 genes downregulated upon IL-1 β treatment compared to baseline (Fold change \pm 2, FDR<0.05, Table S2). Figure 1A&B illustrate the genes significantly altered by IL-1 β and a volcano plot, respectively.

To validate these findings, we investigated a second independent dataset from 2 asthmatic and 4 healthy-derived ASMC cultures treated with IL-1 β 10 ng/ml for 8 hours (dataset B). Gene expression analysis identified 377 genes that were up-regulated and 98 genes down-regulated during IL-1 β treatment compared to baseline (Fold change \pm 2, FDR<0.05). Gene Set Enrichment Analysis (GSEA) of the two datasets revealed that upon IL-1 β treatment, 255 of the significantly up- and 111 of the significantly down-regulated genes were core enriched in the same direction in the two datasets (Figure 1C).

Pathway analysis of Dataset A revealed that the majority of pathways increased by IL-1 β were pro-inflammatory, including the NF- κ B, Interleukin-1 receptor (IL1R) and T1D (Chaperones modulate interferon Signaling Pathway) pathways (Figure 1D). Protein network analysis (using STRING v10.0) showed that the increased IL-1 β expression signature in ASMC was enriched for protein-protein interactions, indicating that the identified genes may have similar functions (Figure 1E). Three distinct clusters were identified: interferon related genes, NF- κ B signaling related genes and pro-inflammatory cytokines. NF- κ B and IRF1 were identified as central hub proteins connecting a number of protein network clusters together, indicating a central role of these proteins during IL-1 β stimulation in ASMC (Figure 1E). Six of the top ten genes up-regulated by IL-1 β formed a clear individual cluster (cytokines), which included the CXCL family proteins CXCL8, a cytokine previously associated with neutrophilic airway inflammation in asthma²³, CXCL10, an interferon regulated cytokine associated with mast cell migration²⁴ and CCL20, a chemoattractant for CCR6⁺ immature dendritic cells, Th17 cells and neutrophils.

Transcription factor binding analysis conducted on the overlapping genes between Dataset A&B using gprofiler identified that the up-regulated genes were enriched for NF- κ B (FDR=7.84x10⁻¹⁰), RelA, a component of the NF- κ B complex (FDR=7.18x10⁻¹³) and IRF1 (FDR=1.72x10⁻¹³) transcription factor binding sites, while the down-regulated genes were enriched for ETF (FDR=5.57x10⁻⁷) and EGR1 (FDR=5.64x10⁻⁵) transcription factor binding sites. These results again identify NF- κ B and IRF1 as key regulators of IL-1 β signaling in ASMC.

Importantly, CCL20 has been shown to induce MUC5AC expression in epithelial cultures^{10,25}. Furthermore, in murine models, anti-CCL20-treatment significantly decreased virus-induced mucus production. Based on its known role in mucus production, we selected CCL20 for further functional studies.

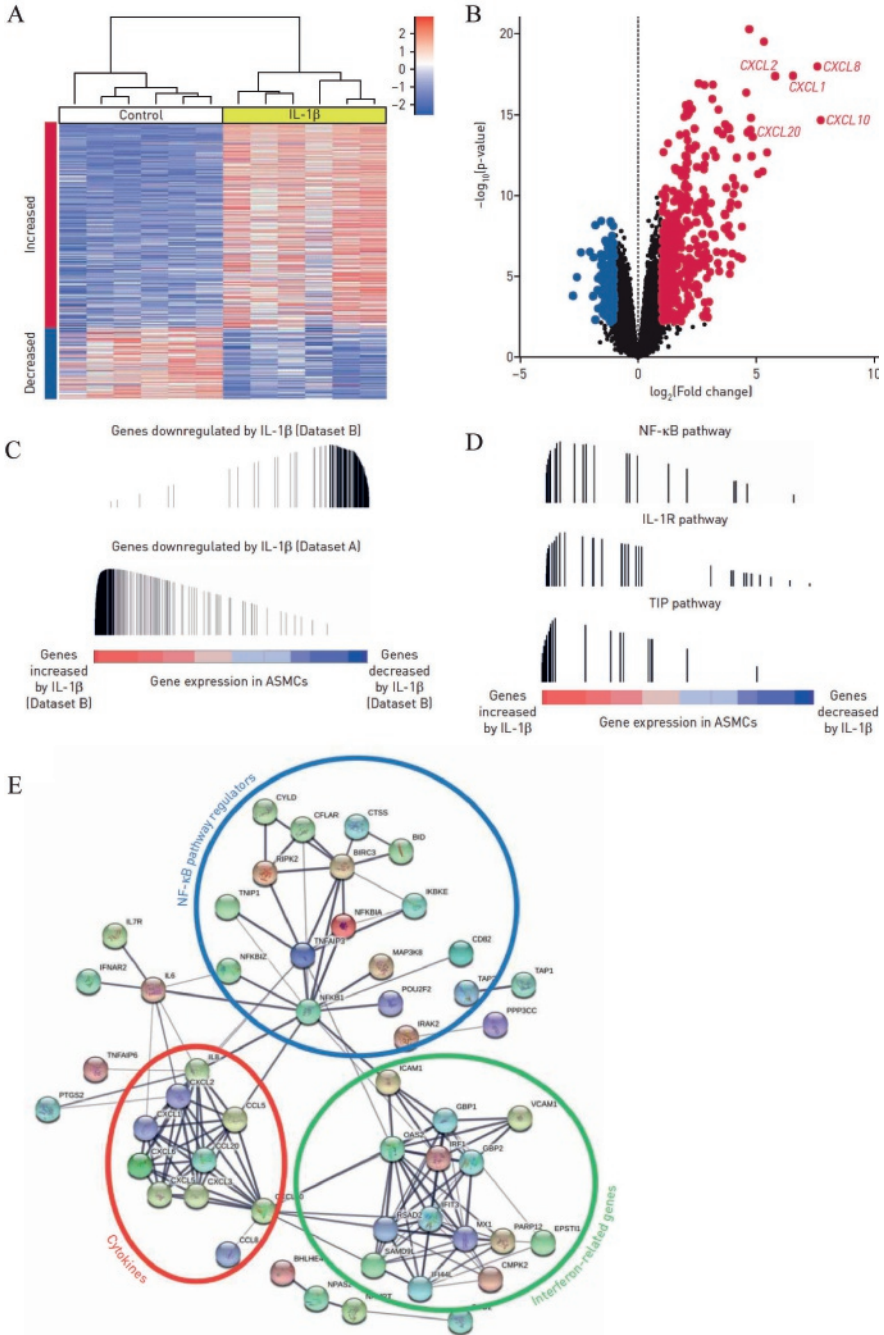


Figure 1. Treatment of ASMC with IL-1 β . [A] Heatmap and [B] Volcano plot of genes altered by IL-1 β treatment for 8 hours in airway smooth muscle cells (ASMC), (FDR<0.05, FC \geq 2). [C] Gene set enrichment analysis (GSEA): enrichment of genes up- and down-regulated by IL-1 β treatment in ASMC comparing two independent datasets (GSEA FDR<0.05). [D] GSEA: Enrichment of genes involved with the in the NF- κ B, IL1R and T1D pathways with genes up-regulated by IL-1 β treatment in ASMC (GSEA FDR<0.05). Coloured bar represents genes ranked based on their differential expression to treatment in ASMC. Vertical bars represent the running GSEA enrichment score and location (in the ranked gene list) of genes which are involved in the pathway being tested. [E] Protein interaction analysis

CCL20 protein release induced by IL-1 β in ASMC

To validate the microarray findings, CCL20 mRNA expression was measured following the stimulation of the asthmatic (mild and moderate) and healthy-derived ASMCs from Dataset A with IL-1 β (10 ng/ml) for 8 hours. IL-1 β significantly increased CCL20 expression, supporting the microarray results (Figure 2A). No differences were found in mRNA expression between asthmatic and healthy-derived ASMCs, nor between AMSCs from mild and moderate asthmatics. Next we confirmed our findings at the protein level and observed that IL-1 β significantly increased CCL20 release from ASMC after 24 hours (Figure 2B). The levels of CCL20 were more strongly elevated in moderate asthmatic ASMCs compared to those from both healthy controls and mild asthmatics. Levels of CCL20 released from ASMCs at baseline were equivalent to levels previously reported to be released by epithelial cells²⁰

MIR146A is decreased in asthma and regulates CCL20

Having seen that CCL20 protein was differentially regulated by IL-1 β in asthmatic compared to healthy-derived ASMC, we investigated other IL-1 β -induced genes.

This analysis was conducted on a subset of genes regulated by IL-1 β treatment in Dataset A (Fold change \pm 2, FDR<0.05). Only a single transcript, MIR146A had a significantly smaller increase in gene expression upon IL-1 β treatment in asthmatic-derived ASMC compared to ASMC from healthy controls (Fold change \pm 2, FDR<0.05, Figure 3A). MIR146A is the precursor transcript for miR-146a-3p and miR-146a-5p, the latter being a well-known anti-inflammatory miRNA, identified to be dysregulated in a number of inflammatory diseases[26]. To determine whether MIR146A expression was altered in asthmatics in-vivo, we investigated its expression in bronchial biopsies from 16 asthmatics and 39 healthy controls. MIR146A expression was decreased in asthmatic bronchial biopsies compared to healthy controls, reflecting the in-vitro results (Figure 3B).

To identify the function of MIR146A, we focused on the known anti-

inflammatory mature transcript miR-146a-5p and studied direct and indirect targets of this transcript using a publically available dataset of gene expression in Huh7.5.1 cells overexpressing miR-146a-5p. Gene expression analysis identified 5 genes (UBD, CXCL10, CXCL8, CCL20 and UCA1) that were down-regulated, but no genes were upregulated upon miR-146a-5p overexpression (Fold change $>\pm 2$, FDR < 0.05). A volcano plot is illustrated in Figure 3C, a table of significant genes in Table S3. One of the down-regulated genes, CCL20, is known to be modulated by miR-146a-5p[27]. Therefore we investigated whether miR-146a-5p negatively regulates IL-1 β -induced CCL20 protein release in ASMCs. Overexpression of the miR-146a-5p mimic in immortalised ASMCs led to a significant down-regulation of IL-1 β -induced CCL20 release (Figure 2C).

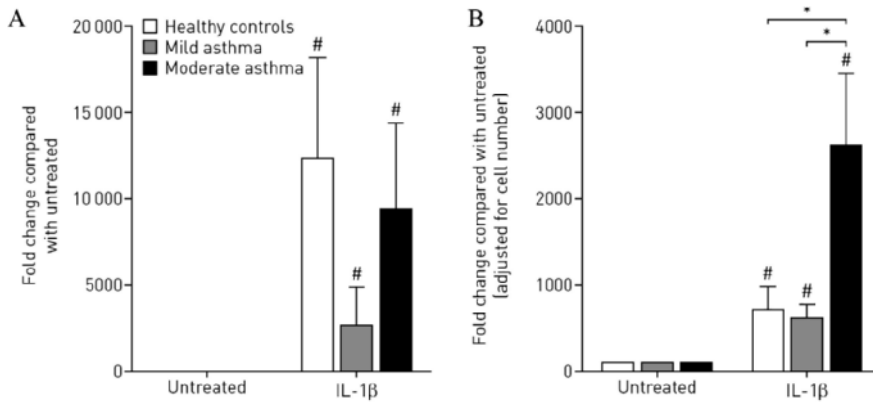


Figure 2. IL1 β induced production of CCL20 mRNA by ASMC. ASMC were grown to confluence in growth media and quiesced for 72 hours and then treated with 0.1% BSA (control) or IL-1 β (10ng/ml) for 8 or (mRNA) 24 hours (protein). [A] mRNA levels of CCL20 (healthy control n=4, mild asthma n=3 and moderate asthma n=3). [B] CCL20 protein levels were measured in cell-free supernatant (healthy control n=4, mild asthma n=3 and moderate asthma n=3). Data are expressed as a mean \pm standard error of the mean. Statistical analysis used was paired Student's t test and Student's t test for paired and unpaired samples, respectively (*=p<0.05 compared to healthy control, # = p<0.05 compare treatment to IL1 β). Abbreviations ASMC=airway smooth muscle cells, IL-1 β = Interleukin-1beta.

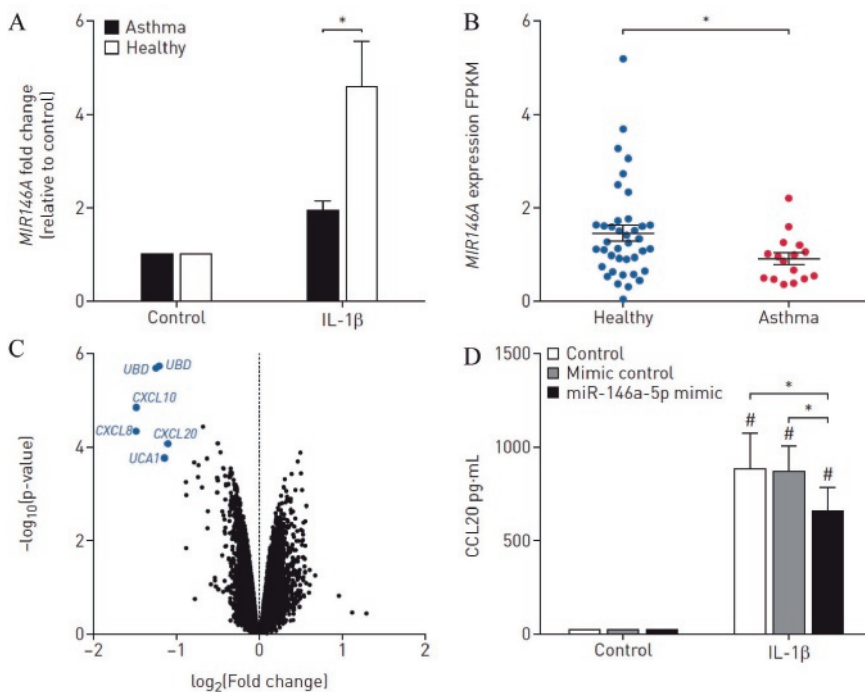


Figure 3. Function of miR-146a-5p in ASMC. [A] Microarray results of ASMC treated with IL-1 β for 8 hours from asthmatics (n=3) and healthy controls (n=3). [B] MIR146A expression in bronchial biopsies from asthmatic and healthy control. [C] Volcano plot of Huh7.5.1 cells transfected with a miR-146a-5p mimic (5nM) compared to a negative control (n=3). [D] CCL20 levels from ASMC treated with IL1 β in the presence and absence of miR-146a-5p (mimic) over expression compared to mimic control (n=5). Data are expressed as a mean \pm standard error of the mean. Statistical analysis used was paired Student's t test and Student t test for paired and unpaired samples, respectively (*=p<0.05, #=p<0.05 treatment compared to control).

CCL20 receptor CCR6 is present on structural cells of the airways and CCL20 induces mucus production by CALU-3 cells grown at air-liquid interface

Having identified CCL20 as a mediator that is differentially expressed in asthmatic compared to healthy-derived ASMC, we next wanted to understand the functional consequences of increased CCL20 in the asthmatic airways. First, to determine whether structural cells in the airways are able to respond to CCL20, the expression of CCR6, its unique receptor, was investigated in airway cells. Initially, we performed realtime PCR in human primary ASMCs, IASMCs and CALU-3 cells, which showed

detectable levels of CCR6 mRNA (Figure S1). Immunohistochemistry staining for CCR6 in human bronchial sections confirmed expression on both ASMCs and airway epithelium (Figure S2).

Previously, in murine models, anti-CCL20-treatment significantly decreased mucus production in response to RSV infection, and CCL20 has been shown to induce MUC5AC expression in submerged culture[9, 10]. To determine whether CCL20 can directly promote mucus production in a model of differentiated epithelial cells, CALU-3 cells were grown at ALI and allowed to differentiate into mucus producing cells before being treated basolaterally with physiologically relevant levels of CCL20. CCL20 treatment of CALU-3 cells for 48 hours increased the production of mucus measured by alcian blue staining (Figure 4A). CCL20-induced mucus production was significantly reduced with the specific anti-CCL20 antibody in CALU-3 cells, while the isotype control had no significant effect (Figure 4B). A trend in the same direction was found by Alcian blue staining upon CCL20 treatment of ALI-differentiated primary human airway epithelial cultures ($p=0.0625$, Figure 4C-E). MUC5AC protein levels in apical were also found to be increased by CCL20 treatment (Figure 4F).

Sputum levels of CCL20 are associated with mucus hypersecretion in asthma

Previous studies have shown that mesenchymal factors can cross the basal lamina propria and be found in lung fluids[28]. This phenomenon is thought to be enhanced in asthmatic patients due to the documented leaky nature of the epithelial layer[29], allowing trafficking to the mucosal layer, where it may induce mucus production. To determine whether CCL20 levels are associated with mucus production in asthmatics, we investigated sputum levels of CCL20 in asthmatic patients with and without CMH. Of the 80 patients included, 24 were classified with having no CMH, 32 with mild CMH, and 19 with moderate to severe CMH. CCL20 protein levels in sputum were found to be significantly increased comparing moderate-to-severe CMH with no CMH ($p<0.05$), and a trend towards an increase was observed between mild and no CMH ($p=0.062$, Figure 4G), further supporting the role of CCL20 in mucus production. As smoking may be a confounding factor, this analysis was repeated in non-smoking patients only, where CCL20 sputum levels were also significantly increased in moderate to severe CMH ($n=11$) compared with no CMH ($n=21$).

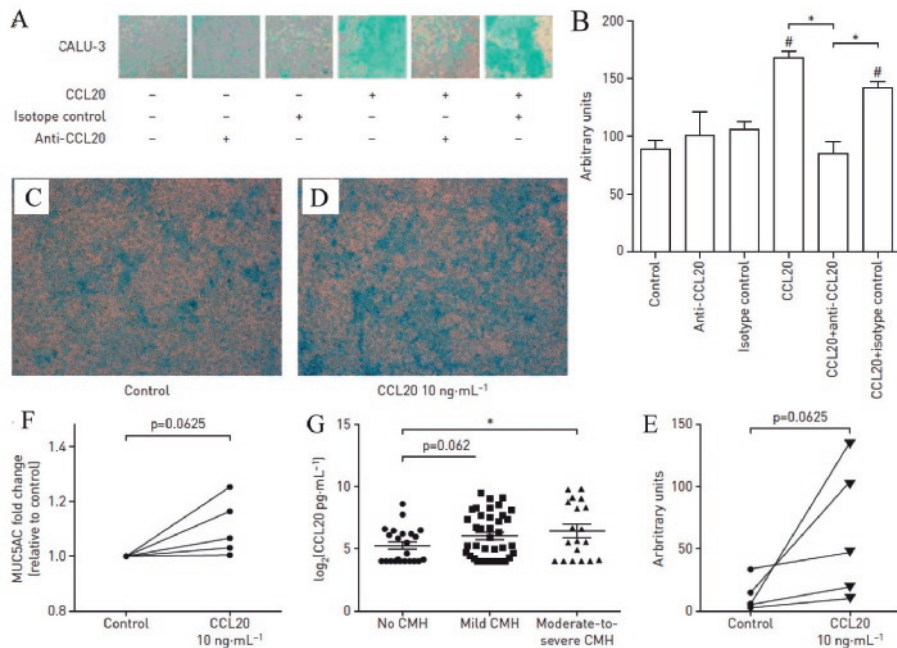


Figure 4. CCL20 effect on mucus production in CALU-3 and primary airway epithelial cells grown at air-liquid interface. [A] Representative images of alcian blue staining of CALU-3 cells grown in air-liquid interface treated on day 5 with either complete DMEM (control), rabbit anti-human CCL20 antibody, Isotype control, CCL20 10ng/ml, rabbit anti-human CCL20 antibody + CCL20 10ng/ml or Isotype control + CCL20 10ng/ml for 48 hours (n=3) for each. [B] Densitometry analysis of Alcian blue staining in CALU-3 cells. Representative images alcian blue staining of primary airway epithelial cells grown in air-liquid interface (ALI) treated on day 28 with either [C] PBS or [D] CCL20 10ng/ml for 48 hours and [E] Densitometry analysis (n=5). [F] MUC5AC protein measurement from ALI washes (n=5). [G] CCL20 levels in sputum from patients with i) No CMH, ii) mild CMH and ii) moderate-to-severe CMH. Statistical analysis used was paired Student's t test and Student's t test for pair and unpaired samples, respectively (*=p<0.05, #=p<0.05 compared to control). A Wilcoxon analysis was conducted on matches samples of primary airway epithelial cells grown at ALI. Data are expressed as a mean \pm standard error of the mean. Abbreviations: DMEM= Dulbecco's Modified Eagle Medium.

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Our current findings indicate that IL1 β produced by the airway epithelium following insult induces CCL20 production by the ASM mass which is increased in asthmatic ASM. This CCL20 can then act on the airway epithelium by binding to CCR6, resulting in increased mucus production. CCL20 production is inhibited by miR-146a-5p expression, which is induced by NF κ B activation. However this miR-146a-5p induction is lower in asthmatic ASM (Figure 5)

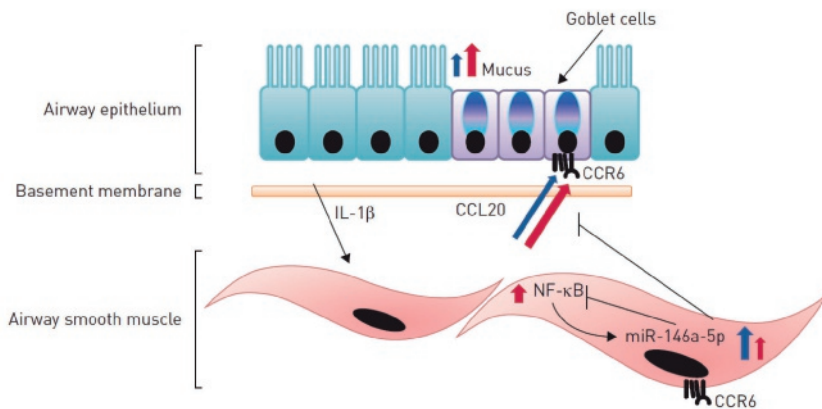


Figure 5. Summary of the crosstalk between the airway epithelium and airway smooth muscle cells (ASM) in the asthmatic airway. Basolaterally secreted IL-1 β produced by the damaged airway epithelium induces CCL20 production by ASM cells, which is increased in asthmatic ASM. CCL20 can subsequently act on the airway epithelium by binding to its only known receptor CCR6, resulting in increased mucus production. CCL20 production is inhibited by miR-146a-5p expression, which is induced upon NF- κ B activation. The miR-146a-5p induction is lower in asthmatic ASM, leading to reduced suppression of CCL20. Red arrows = asthma, Blue arrows = healthy.

DISCUSSION

In this study, we compared gene expression profiles between asthmatic and healthy ASMC *in vitro* in response to the NLRP3 inflammasome downstream mediator IL-1 β . Through this analysis, we provide genome-wide evidence that ASMCs respond to the active form of IL-1 β , levels of which are increased in the sputum of asthmatics⁷, providing a source of inflammatory chemokines and cytokines in the airways. Furthermore, we observed enhanced expression of CCL20 and MIR146A in response to IL-1 β in ASMCs, with a lower increase in MIR146A in asthmatic ASMC. Furthermore, IL-1 β induced a stronger increase in CCL20 protein secretion by ASMCs from moderate compared to mild asthmatics and healthy controls. Interestingly, CCL20 release was reduced following overexpression of miR-146a-5p, providing evidence that this miRNA may be a dysregulated inhibitor of CCL20 production in ASMCs from asthmatics. Recombinant CCL20 directly induced mucus production from differentiated airway epithelial cells, indicating that CCL20 may contribute to CMH in asthma. The importance of this finding was corroborated by our observation that CCL20 levels in sputum were associated with increased levels of CMH in asthmatic patients. The current study reinforces the hypothesis that ASM is not a passive bystander in inflammation and CMH, but a key driver^{30,31}.

MiR-146a-5p has previously been found to regulate CCL20 production in skin keratinocytes after Toll-like receptor 2 (TLR2) stimulation, mirroring the results in this study²⁷. However, this repression of inflammatory cytokines is not limited to CCL20, as the over-expression of miR-146a-5p led to the decrease of well-known NF- κ B-regulated pro-inflammatory cytokines UBD, CXCL10 and CXCL8. Previous studies have identified miR146a-5p as an anti-inflammatory miRNA that inhibits NF- κ B signaling by targeting the IL1R downstream signaling molecules IRAK1 and TRAF6 for degradation, key genes in the activation of the NF- κ B pathway³².

In this study we found that induction of MIR146A in response to IL-1 β was less in asthmatic ASMCs compared to healthy controls, which may thus be responsible for the increased secretion of CCL20 from these cells. Excitingly, we validated the lower MIR146A expression in asthmatics using bronchial biopsies from asthmatics and healthy controls. Similar findings have been reported in human inflammatory cells, where circulating CD4⁺ and CD8⁺ T cells of severe asthmatics expressed less miR-146a-5p than healthy controls³³. A likely rationale for the decrease of MIR146A in asthmatics may be the presence of the SNP (rs2910164), which is known to influence the levels of both the pre and mature MIR146A transcripts[34]. This SNP has previously been associated with the presence of asthma and other pro-inflammatory diseases^{11,12}. Interestingly, we have recently shown that lower expression of miR-146a-5p in bronchial biopsies is inversely correlated with CMH in COPD¹³, highlighting miR-146a-5p as a consistent regulator of mucus regulation in respiratory diseases.

Although we observed increased CCL20 secretion from ASMCs derived from asthma patients, we did not find a significant difference in CCL20 gene expression between asthmatic and healthy controls. Furthermore, we observed a decrease in CCL20 gene expression following 72 hours miR-146a-5p overexpression. We postulate that only once the IL-1 β -induced increase in miR-146a-5p starts to repress CCL20 expression, this leads to differences between the asthma and control groups, with an insufficient level of miR-146a-5p in asthma-derived ASMCs. The selected time point of 8 hours used in this study may have been too early to detect differences in CCL20 gene expression levels between ASMCs from asthmatic and healthy controls due to the absence of miR-146a-5p suppression.

CCL20 was identified as a key chemokine for immature dendritic cells³⁵, and has recently been described as an anti-microbial protein³⁶ and regulator of mucus production^{9,25}. Overall, the function of CCL20 appears to be pro-inflammatory in nature and it is up-regulated in the sputum in a number of inflammatory respiratory diseases including asthma^{20,37}, COPD³⁸ and cystic fibrosis³⁹. Of interest, CMH is a feature of all of these diseases in at least a subset of patients⁴⁰⁻⁴². In the current study we found that CCL20 promoted mucus production from an ALI-differentiated airway epithelial cell line. Furthermore there was a direct link between sputum CCL20 levels and mucus production in asthma patients. Of note, data from our group have shown that treatment with inhaled corticosteroids increases sputum levels of CCL20²⁰, offering an explanation why current anti-inflammatory therapies are unable to revert mucus hypersecretion.

The main strength of this study is the multidisciplinary approach used to identify a novel gene target using mass screen approaches including microarray analysis followed by the functional interrogation of the candidate using *in vitro* models. There are some limitations to this study, as we were unable to determine the origin of the CCL20 levels in the sputum of the asthmatic patients. A number of cell types in addition to ASMCs, including airway epithelial cells, produce this chemokine⁴³. Despite this, the increased sensitivity and size of the muscle mass in asthmatic airways provides a potential reservoir of CCL20. Furthermore, due to remodeling of the airways, the ASM mass is in closer proximity to the epithelial layer in asthma, which may increase the crosstalk between epithelial cells and ASMCs. The measurement of MIR146A in the bronchial biopsies is reflective of its expression within a mixed population of cells in the asthmatic airways rather than a reflection of expression in the ASM alone. Finally, our finding that recombinant CCL20 increases mucin expression does not directly prove that airway smooth muscle-derived CCL20 drives epithelial mucus production *in vivo*. In future studies, we will use epithelium and smooth muscle co-cultures to further support our current findings.

In conclusion, in this study we identified a novel pathway leading to mucus production in asthma, where increased CCL20 released from the enhanced ASM

mass may contribute to the exaggerated mucus production by airway epithelium in asthmatic airways, due to reduced suppression by miR-146a-5p.

CONTRIBUTIONS

AF participated in project design, microarray analysis, *in vitro* cellular work, writing and proofreading of the manuscript. CJV, CX and MvdB participated in miRNA analysis writing and proofreading of the manuscript. NHTH, GGK, CF, AJH, THL and JPTW provided either IASMC samples or sputum samples, and participated in the proofreading of the manuscript. GT helped with IHC analysis. JKB, MW, JLB and BGO participated in project design, provided funding for project, writing and proofreading of the manuscript.

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SUPPLEMENTARY DATA

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-1 β 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-1 β 10 ng/ml for 8 hours (dataset B). Where again asthmatics and controls were pooled to determine the effect of IL-1 β 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-1 β , comparing the change in expression to IL-1 β in asthmatics to the change in expression of IL-1 β in healthy controls (Fold change \pm 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 1x10⁴ cell/cm² 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-1 β (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 -80oC (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 described³. Moderate asthmatic, mild asthmatic and healthy ASMC, defined by the Global Initiative for Asthma (GINA) guidelines were seeded at 1x10⁴ cells per cm² 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-1 β (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 75cm² 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-3 α 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 μ l 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 μ l 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 into an Illumina HiSeq2500 sequencer using default parameters for paired-end sequencing (2 \times 100 bp).

Gene expression quantification

The trimmed fastQ files were 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 μ g/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 5×10^5 cells/cm² in 100 μ L apical and 500 μ L 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 200 μ l Bronchial Epithelial Cell Growth Medium (BEGM) in the apical part of the insert and 500 μ l 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 (500 μ l). Media was

refreshed every 3 days with DMEM / BEBM (1:1) with retinoic acid (15ng/ml). Cells were quiesced 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.

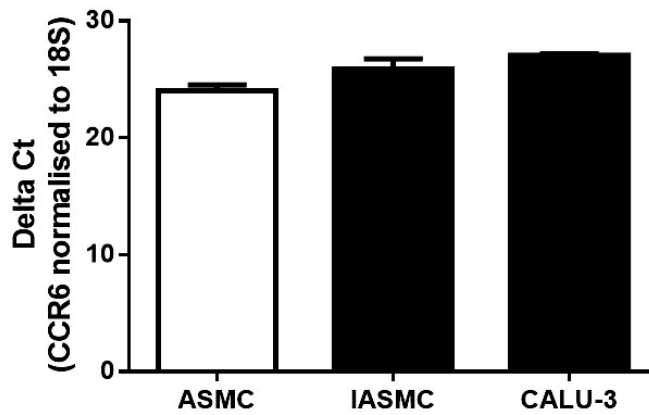


Figure S1. Gene expression of CCR6 in structural cells in the airways. CCR6 mRNA levels were measured by Real time PCR in quiesced cells (ASMC (n=4), IASMC (n=3), and CALU-3 cells (n=3)). Data are expressed as mean \pm standard error of the mean. Abbreviations ASMC=airway smooth muscle cells, IASMC=immortalised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

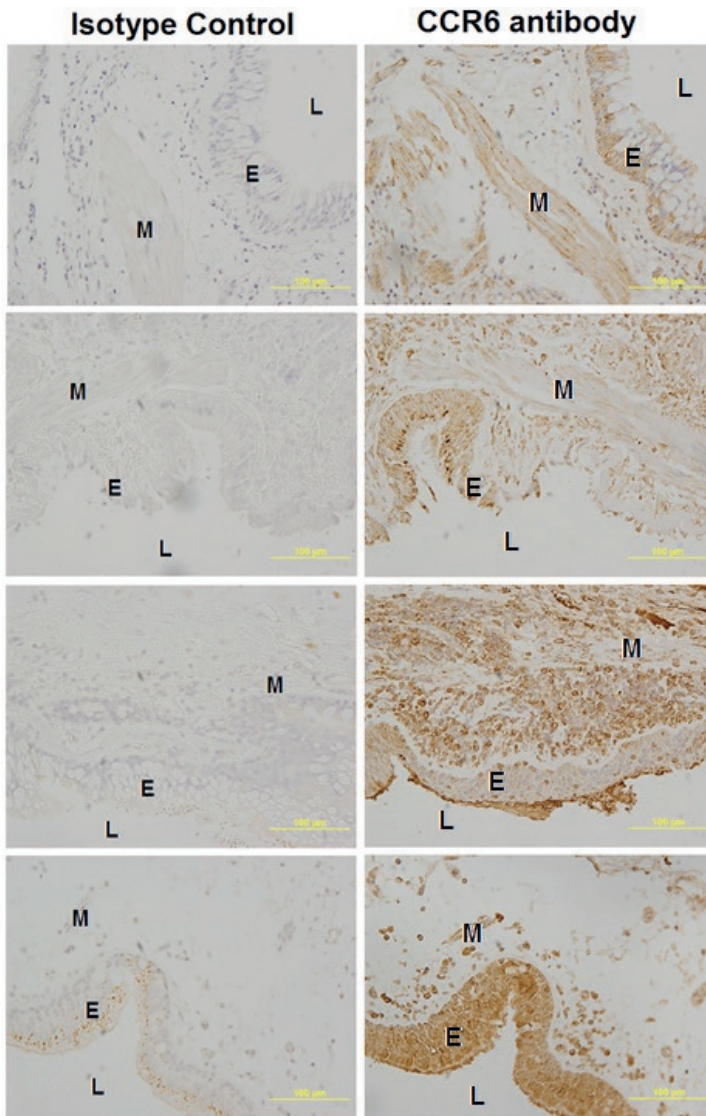


Figure S2. Immunohistochemistry on paraffin embedded bronchus (>2mm) for CCR6.

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 100 μ m). Abbreviations M= Airway Smooth Muscle, L= Lumen, E = Epithelium

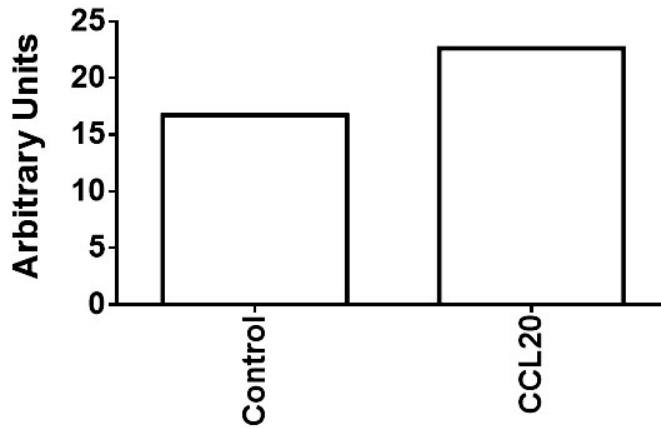


Figure S3. Densitometry analysis of Alcian blue staining in ALI's derived from primary airway epithelial cells treated with CCL20. Primary airway epithelial cells grown in air-liquid interface treated on day 28 with either C) PBS or CCL20 10ng/ml for 48 hours (n=1).

Table S1. Patient characteristics for RNA-Seq results

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

Data are presented as mean \pm 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 IL1 β 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

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