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

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ABSTRACT Chronic mucus hypersecretion (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 cross-talk between airway epithelium and airway smooth muscle cells (ASMCs), driven by pro-inflammatory cytokines such as interleukin (IL)-1β, provides a potential mechanism that influences CMH. This study investigated mechanisms underlying CMH by comparing IL-1β-induced gene expression profiles between asthma and control-derived ASMCs and the subsequent paracrine influence on airway epithelial mucus production *in vitro*.

IL-1β-treated ASMCs from asthmatic patients 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-1β-induced *CCL20* expression and protein release was increased in ASMCs from moderate compared with mild asthmatic patients and healthy controls. IL-1β induced lower *MIR146A* expression in asthma-derived ASMCs compared with controls. Decreased *MIR146A* expression was validated *in vivo* in bronchial biopsies from 16 asthmatic patients *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 ASMCs, possibly resulting from dysregulated expression of the anti-inflammatory miR-146a-5p, may contribute to enhanced mucus production in asthma.

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Introduction

Asthma is a chronic inflammatory disease affecting 300 million people worldwide [1]. Chronic mucus hypersecretion (CMH) contributes to the morbidity and mortality of asthma [2], 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 inflammation [3], 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 (ASMCs) may regulate mucus production, since the airway smooth muscle mass is enlarged in asthma [4].

ASMCs 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 pathways [5].

One of these inflammatory pathways know to be altered in asthma is the inflammasome, a multiprotein complex that plays an important role in the activation of pro-inflammatory cytokines, *e.g.* conversion of interleukin (IL)-1β from its pro-form into its active state [6]. The activity of the inflammasome is enhanced in neutrophilic asthma [7], leading to increased levels of active IL-1β in sputum.

IL-1β is a strong pro-inflammatory signalling molecule, the downstream mediators of which are associated with mucus production [8]. However, little is known about the influence of IL-1β on the pro-inflammatory response of airway structural cells, especially ASMCs 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 ASMCs *in vitro* in response to IL-1β. Importantly, CCL20 had been shown to induce MUC5AC expression in epithelial cultures by binding to its only known receptor CCR6 [9]. Furthermore, in murine models, anti-CCL20 treatment significantly decreased virus-induced mucus production [10]. Previously, a single nucleotide polymorphism (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 chronic obstructive pulmonary disease (COPD) [13], highlighting miR-146a-5p as a regulator of mucus regulation in respiratory diseases. Based on known roles of CCL20 and miR-146a-5p in mucus production, we then investigated how these factors produced by ASMCs 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) in Sydney, Australia. 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 (Dataset A) [14, 15]. Cells were treated with 10 ng·mL^{-1} IL-1β (R&D Systems, Minneapolis,

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

Patient	Diagnosis	Age years	Sex	Samples	FEV_1 % pred	Experiment(s) for which sample was used[#]
1	Asthma	33	Male	Bronchoscopy	NA	1
2	Asthma	22	Male	Bronchoscopy	NA	1
3	Asthma	33	Female	Transplant	NA	1
4	Nondiseased donor	31	Male	Bronchoscopy	NA	1
5	Nondiseased donor	22	Male	Bronchoscopy	NA	1
6	Nondiseased donor	27	Female	Bronchoscopy	NA	1
7	Asthma	20	Male	Bronchoscopy	65	2
8	Unknown	NA	Female	Transplant	NA	2
9	Asthma	19	Female	Bronchoscopy	97	2
10	Nondiseased donor	20	Male	Bronchoscopy	NA	2
11	Nondiseased donor	30	Male	Bronchoscopy	NA	2
12	Nondiseased donor	21	Female	Bronchoscopy	NA	2
13	Nondiseased donor	31	Female	Immortalised ASMCs	105	3, 4
14	Nondiseased donor	40	Male	Immortalised ASMCs	131	3
15	Nondiseased donor	23	Male	Immortalised ASMCs	82	3, 4
16	Nondiseased donor	22	Female	Immortalised ASMCs	87	3, 4
17	Asthma	39	Male	Immortalised ASMCs	84	3, 4
18	Asthma	29	Male	Immortalised ASMCs	89	3, 4
19	Asthma	21	Male	Immortalised ASMCs	108	3, 4
20	Asthma	31	Male	Immortalised ASMCs	85	3, 4
21	Asthma	27	Female	Immortalised ASMCs	78	3, 4
22	Asthma	33	Male	Immortalised ASMCs	78	3, 4
23	Nondiseased donor	69	Male	Immortalised ASMCs	NA	4
24	Nondiseased donor	22	Female	Immortalised ASMCs	NA	4
25	Nondiseased donor	NA	NA	Immortalised ASMCs	NA	6
26	Cystic fibrosis	22	Female	Paraffin-embedded bronchus	NA	5
27	Nondiseased donor	16	Male	Paraffin-embedded bronchus	NA	5
28	COPD	56	Female	Paraffin-embedded bronchus	NA	5
29	Pulmonary fibrosis	53	Female	Paraffin-embedded bronchus	NA	5

FEV_1: forced expiratory volume in 1 s; ASMC: airway smooth muscle cell; NA: not available; COPD: chronic obstructive pulmonary disease. [#]: 1, Human Gene 1.0 ST microarray (Dataset A); 2, Human U133Plus 2.0 microarray (Dataset B); 3, quantitative real-time PCR validation; 4, CCL20 ELISA; 5, CCR6 immunohistochemistry; 6, miR-146a-5p functional work.

MN, USA) for 8 h [16]. Total cellular mRNA was isolated, labelled and run on a GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions (Gene Expression Omnibus identifier GSE63383) [4]. For independent validation, ASMCs derived from asthmatic patients (n=2) and healthy donors (n=4) were grown and treated with IL-1β in the same manner (Dataset B). Samples were labelled and run on a Human U133Plus 2.0 Array (Affymetrix) according to the manufacturer's instructions. Microarray analysis is outlined in the supplementary material.

Pathway analysis

Functional enrichment analysis to identify the overlapping genes altered by IL-1β treatment in Datasets A and B was performed using gene set enrichment analysis (GSEA) software version 2.0.14 (http://software.broadinstitute.org/gsea). Protein network analysis was conducted using STRING version 10 (https://string-db.org) on the overlapping genes. GSEA was also used to investigate pathways regulated in Dataset A using the BioCarta database (www.biocarta.com). Transcript factor binding site analysis was conducted using g:Profiler (https://biit.cs.ut.ee/gprofiler).

Microarray candidate validation

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

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 airway hyperresponsiveness to histamine (provocative dose causing a 20% fall in forced expiratory volume in 1 s for histamine (using 30-s

tidal breathing) <32 mg·mL^{-1}). The analysis was conducted on 39 healthy subjects and 16 asthmatic patients, who were all nonsmokers and currently not taking inhaled corticosteroids. An outline of the patients' characteristics is shown in supplementary table S1. All study protocols were approved by the medical ethics committee of the University Medical Center Groningen (Groningen, The Netherlands) and all subjects provided written informed consent. RNA was isolated and sequenced as described in the supplementary material.

miR-146a-5p predicted targets

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

Functional analysis

Immortalised ASMCs 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, Carlsbad, CA, USA). 24 h later, cells were treated with 10 ng·mL^{-1} IL-1β or 0.1% bovine serum albumin (control). Cell-free supernatants were collected at 24 h and IL-8 levels assessed by ELISA.

The human lung epithelial cell line CALU-3 and primary airway epithelial cells were grown at the air–liquid interface (ALI), treated with 10 ng·mL^{-1} CCL20 for 48 h and mucus assessed by Alcian blue staining as described in the supplementary material.

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: 1) never, 2) sometimes, 3) once in a while, 4) often, 5) most of the time, 6) regularly and 7) always. Patients who responded 1) were classified as no CMH, patients who responded 2) and 3) were classified as moderate CMH, and patients who responded 4)–7) were classified as severe CMH. Of the asthmatic patients with available sputum, n=80 gave answers to the questionnaire and were analysed in this study.

Statistics

Statistical tests and graph plotting were conducted using Prism version 6 (GraphPad, La Jolla, CA, USA). A p-value <0.05 was considered statistically significant.

Results

Response of ASMCs to IL-1β

To evaluate if IL-1β is involved in the abnormal cross-talk between ASMCs and airway epithelium, contributing to CMH in the asthmatic airway, we first examined the regulation of genes following IL-1β treatment. Asthmatic patients (n=3) and controls (n=3) were pooled to obtain sufficient power to determine the effect of IL-1β on gene expression (Dataset A). Gene expression analysis identified 408 genes that were upregulated and 143 genes that were downregulated upon IL-1β treatment compared with baseline (fold change > ±2, false discovery rate (FDR) <0.05) (supplementary table S2). Figure 1a and b illustrate the genes significantly altered by IL-1β and a volcano plot, respectively.

To validate these findings, we investigated a second independent dataset from n=2 asthmatic and n=4 healthy-derived ASMC cultures treated with 10 ng·mL^{-1} IL-1β for 8 h (Dataset B). Gene expression analysis identified 377 genes that were upregulated and 98 genes that were downregulated during IL-1β treatment compared with baseline (fold change > ±2, FDR <0.05). GSEA of the two datasets revealed that upon IL-1β treatment, 255 of the significantly upregulated genes and 111 of the significantly downregulated 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-1β were pro-inflammatory, including the NF-κB, IL-1 receptor (IL-1R) and TID (chaperones that modulate interferon (IFN) signalling pathway) pathways (figure 1d). Protein network analysis (using STRING version 10.0) showed that the increased IL-1β expression signature in ASMCs was enriched for protein–protein interactions, indicating that the identified genes may have similar functions (figure 1e). Three distinct clusters were identified: IFN-related genes, NF-κB signalling-related genes and pro-inflammatory cytokines. NF-κB and IFN regulatory factor 1 (IRF1) were identified as central hub proteins connecting a

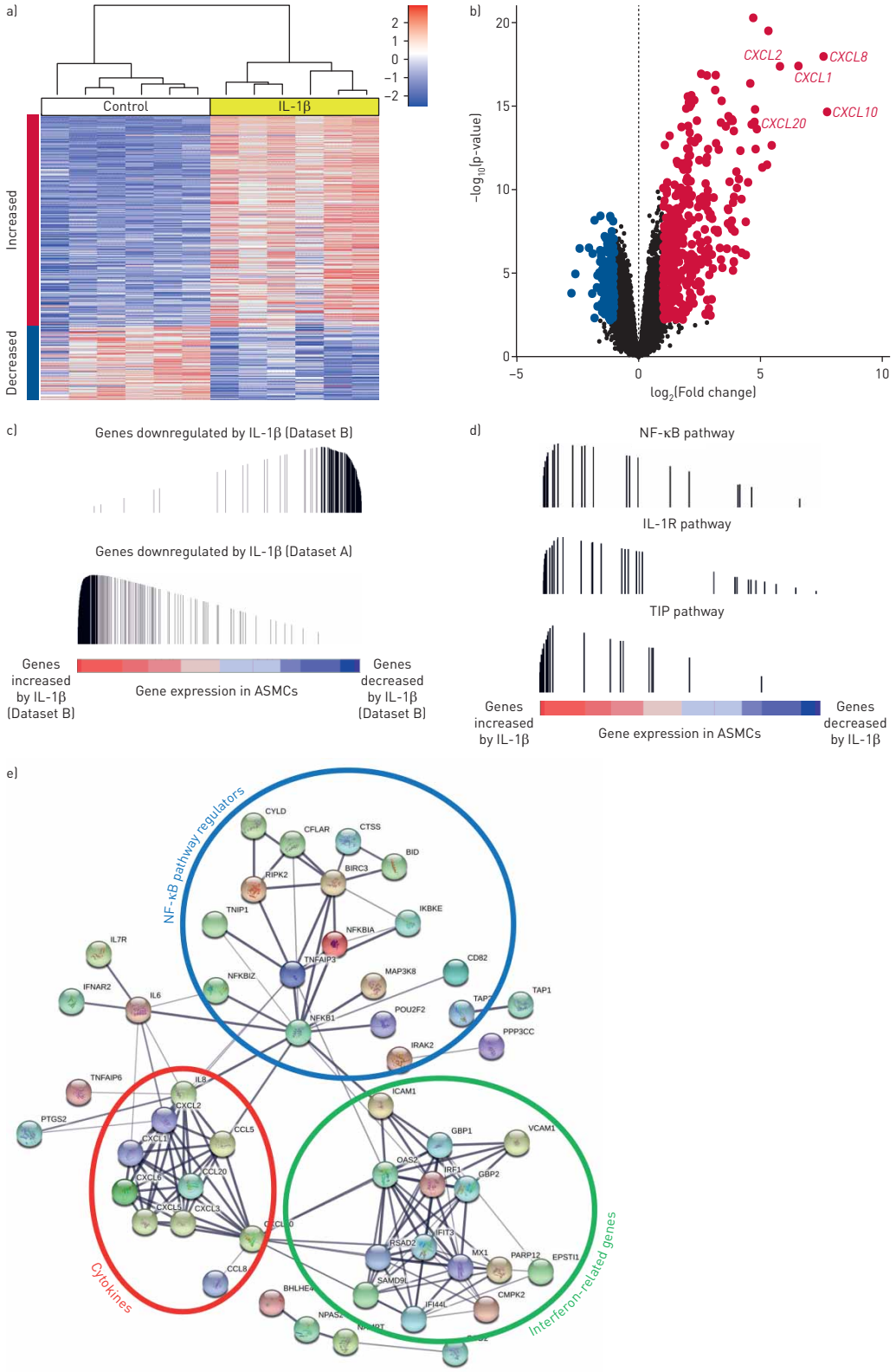

FIGURE 1 Treatment of airway smooth muscle cells (ASMCs) with interleukin (IL)-1β. IL-1R: IL-1 receptor; GSEA: gene set enrichment analysis; FDR: false discovery rate. a) Heatmap and b) volcano plot of genes altered by IL-1β treatment for 8 h in ASMCs (fold change >±2, FDR <0.05). c) GSEA: enrichment of genes upregulated and downregulated by IL-1β treatment in ASMCs comparing two independent datasets (GSEA FDR <0.05). d) GSEA: enrichment of genes involved in the NF-κB, IL-1R and TID pathways with genes upregulated by IL-1β treatment in ASMCs (GSEA FDR <0.05). Coloured bars in (c) and (d) represent genes ranked based on their differential expression to treatment in ASMCs; vertical bars represent the running GSEA enrichment score and location (in the ranked gene list) of genes that are involved in the pathway being tested. e) Protein interaction analysis.

number of protein network clusters together, indicating a central role of these proteins during IL-1β stimulation in ASMCs (figure 1e). Six of the top 10 genes upregulated by IL-1β formed a clear individual cluster (cytokines), which included the CXCL family proteins CXCL8 (a cytokine previously associated with neutrophilic airway inflammation in asthma [23]), CXCL10 (an IFN-regulated cytokine associated with mast cell migration [24]) and CCL20 (a chemoattractant for $CCR6^+$ immature dendritic cells, T-helper 17 cells and neutrophils).

Transcription factor binding analysis conducted on the overlapping genes between Datasets A and B using g:Profiler identified that the upregulated genes were enriched for NF-κB (FDR 7.84×10^{-10}), RelA (a component of the NF-κB complex; FDR 7.18×10^{-13}) and IRF1 (FDR 1.72×10^{-13}) transcription factor binding sites, while the downregulated genes were enriched for ETF (FDR 5.57×10^{-7}) and EGR1 (early growth response 1; FDR 5.64×10^{-5}) transcription factor binding sites. These results again identify NF-κB and IRF1 as key regulators of IL-1β signalling in ASMCs.

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.

CCL20 protein release induced by IL-1β in ASMCs

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 10 $ng{\cdot}mL^{-1}$ IL-1β for 8 h. IL-1β significantly increased CCL20 expression, supporting the microarray results (figure 2a). No differences were found in mRNA expression between asthmatic and healthy-derived ASMCs or between ASMCs from mild and moderate asthmatic patients. Next, we confirmed our findings at the protein level and observed that IL-1β significantly increased CCL20 release from ASMCs after 24 h (figure 2b). The levels of CCL20 were more strongly elevated in moderate asthmatic ASMCs compared with those from both healthy controls and mild asthmatic patients. Levels of CCL20 released from ASMCs at baseline were equivalent to levels previously reported to be released by epithelial cells [20].

MIR146A *is decreased in asthma and regulates CCL20*

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

This analysis was conducted on a subset of genes regulated by IL-1β treatment in Dataset A (fold change > ±2, FDR <0.05). Only a single transcript, *MIR146A*, had a significantly smaller increase in gene expression upon IL-1β treatment in asthmatic-derived ASMCs compared with ASMCs from healthy controls (fold change > ±2, FDR <0.05) (figure 3a). *MIR146A* is the precursor transcript for miR-146a-3p

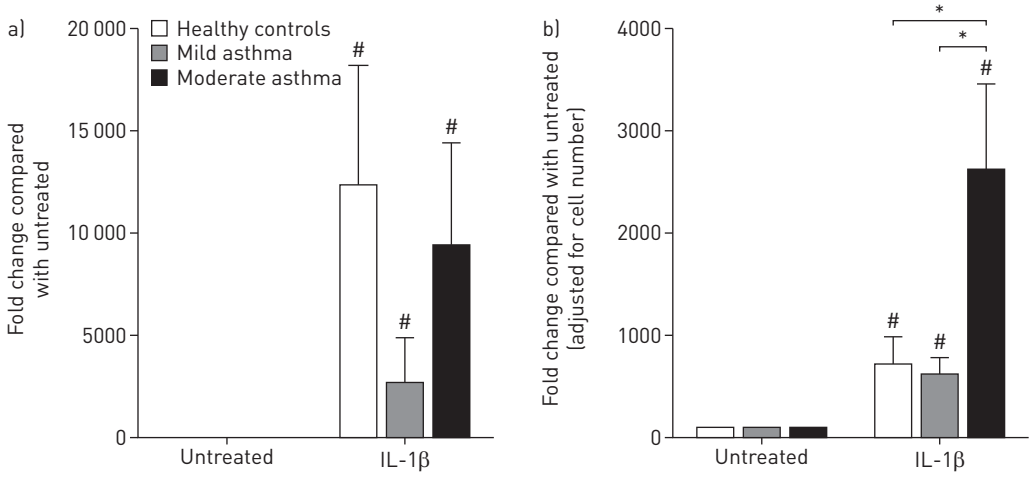

FIGURE 2 Interleukin (IL)-1β induced production of CCL20 mRNA by airway smooth muscle cells (ASMCs). ASMCs were grown to confluence in growth media, quiesced for 72 h and then treated with 0.1% bovine serum albumin (control) or 10 $ng{\cdot}mL^{-1}$ IL-1β for 8 h (mRNA) or 24 h (protein). a) mRNA levels of CCL20 (healthy controls n=4, mild asthma n=3 and moderate asthma n=3). b) CCL20 protein levels were measured in cell-free supernatant (healthy controls n=4, mild asthma n=3 and moderate asthma n=3). Data are presented as mean±SEM. Statistical analysis was performed using the paired t-test and t-test for paired and unpaired samples, respectively. *: p<0.05, compared with healthy controls; #: p<0.05, compared with IL-1β treatment.

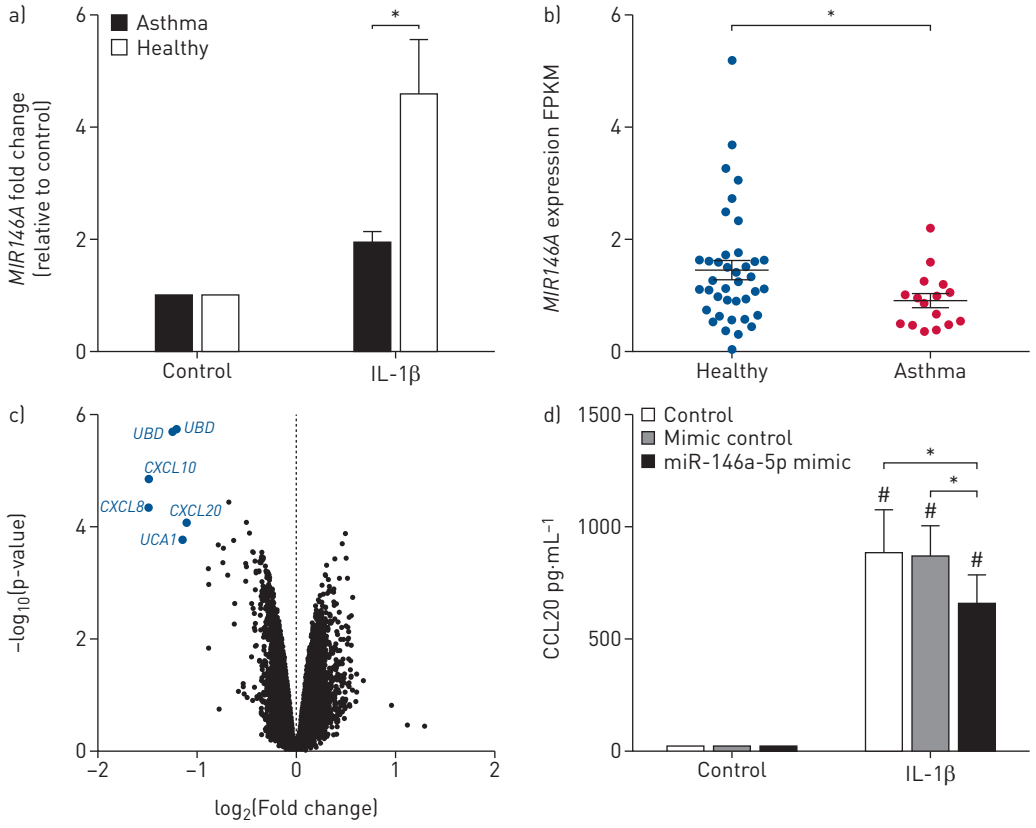

FIGURE 3 Function of miR-146a-5p in airway smooth muscle cells (ASMCs). IL: interleukin; FPKM: fragments per kilobase of transcript per million mapped reads. a) Microarray results of ASMCs treated with IL-1β for 8 h from asthmatic patients (n=3) and healthy controls (n=3). b) *MIR146A* expression in bronchial biopsies from asthmatic patients and healthy controls. c) Volcano plot of Huh7.5.1 cells transfected with a miR-146a-5p mimic (5 nM) compared with a negative control (n=3). d) CCL20 levels from ASMCs treated with IL-1β in the presence and absence of miR-146a-5p (mimic) overexpression compared with a mimic control (n=5). Data are presented as mean±SEM. Statistical analysis was performed using the paired t-test and t-test for paired and unpaired samples, respectively. *: $p<0.05$; #: $p<0.05$, treatment compared with control.

and miR-146a-5p, the latter being a well-known anti-inflammatory microRNA (miRNA), identified to be dysregulated in a number of inflammatory diseases [26]. To determine whether *MIR146A* expression was altered in asthmatic patients *in vivo*, we investigated its expression in bronchial biopsies from 16 asthmatic patients and 39 healthy controls. *MIR146A* expression was decreased in asthmatic bronchial biopsies compared with 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 five genes (*UBD* (ubiquitin D), *CXCL10*, *CXCL8*, *CCL20* and *UCA1* (urothelial cancer associated 1)) that were downregulated, but no genes that were upregulated upon miR-146a-5p overexpression (fold change >±2, FDR <0.05). A volcano plot is shown in figure 3c and a table of significant genes is given in supplementary table S3. One of the downregulated genes, *CCL20*, is known to be modulated by miR-146a-5p [27]. Therefore, we investigated whether miR-146a-5p negatively regulates IL-1β-induced CCL20 protein release in ASMCs. Overexpression of the miR-146a-5p mimic in immortalised ASMCs led to a significant downregulation of IL-1β-induced CCL20 release (figure 3d).

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

Having identified CCL20 as a mediator that is differentially expressed in asthmatic compared with healthy-derived ASMCs, 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 real-time PCR in human primary ASMCs, immortalised ASMCs and CALU-3 cells, which showed detectable levels of *CCR6* mRNA (supplementary figure S1). Immunohistochemistry staining for CCR6 in human bronchial sections confirmed expression on both ASMCs and airway epithelium (supplementary figure S2).

Previously, in murine models, anti-CCL20 treatment significantly decreased mucus production in response to respiratory syncytial virus 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 the 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 h 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 measured in ALI apical wash 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 asthmatic patients, we investigated sputum levels of CCL20 in asthmatic patients with and without CMH. Of the 80 patients included, 24 were classified as having no CMH, 32 were classified as having mild

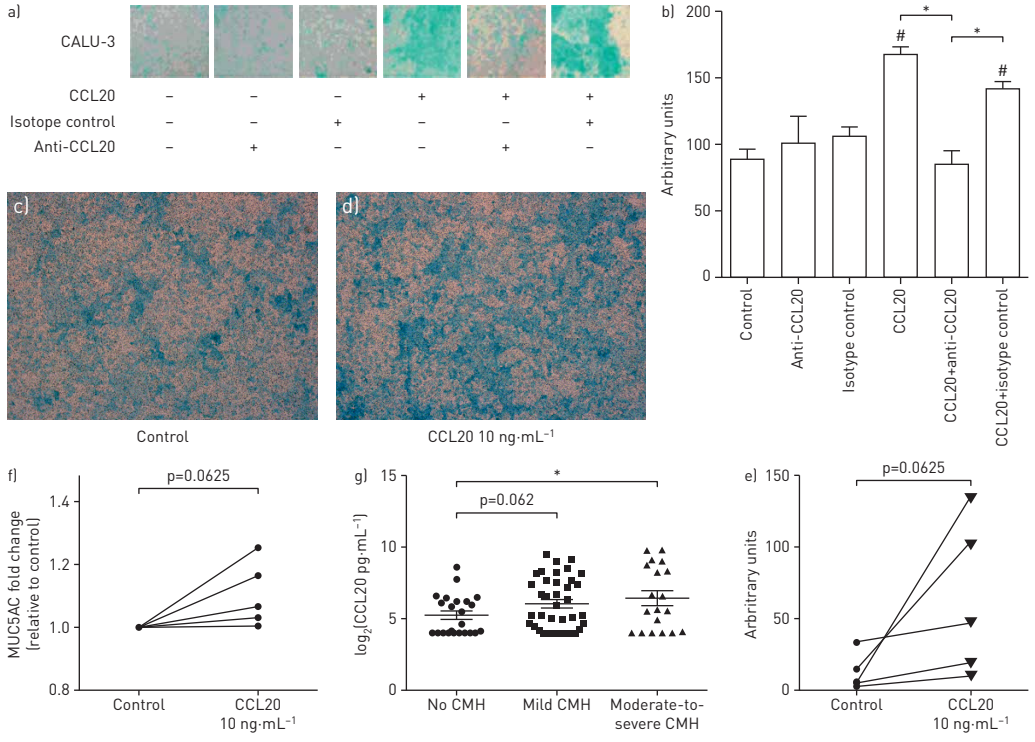

FIGURE 4 CCL20 effect on mucus production in CALU-3 and primary airway epithelial cells grown at the air–liquid interface (ALI). CMH: chronic mucus hypersecretion. a) Representative images of Alcian blue staining of CALU-3 cells grown at the ALI treated on day 5 with either complete DMEM (control), rabbit anti-human CCL20 antibody, isotype control, 10 ng·mL^{-1} CCL20, rabbit anti-human CCL20 antibody+10 ng·mL^{-1} CCL20 and isotype control+10 ng·mL^{-1} CCL20 for 48 h (n=3) for each. b) Densitometry analysis of Alcian blue staining in CALU-3 cells. c–e) Representative images of Alcian blue staining of primary airway epithelial cells grown at the ALI treated on day 28 with either c) PBS or d) 10 ng·mL^{-1} CCL20 for 48 h and e) densitometry analysis (n=5). f) MUC5AC protein measurement from ALI washes (n=5). g) CCL20 levels in sputum from patients with no CMH, mild CMH or moderate-to-severe CMH. Data are presented as mean±SEM. Statistical analysis was performed using the paired t-test and t-test for paired and unpaired samples, respectively. *: p<0.05; #: p<0.05, compared with control. A Wilcoxon analysis was conducted on matched samples of primary airway epithelial cells grown at the ALI.

CMH and 19 were classified as having moderate-to-severe CMH. CCL20 protein levels in sputum were found to be significantly increased when 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 nonsmoking patients only, where CCL20 sputum levels were also significantly increased in moderate-to-severe CMH (n=11) compared with no CMH (n=21).

Our current findings indicate that IL-1β produced by the airway epithelium following insult induces CCL20 production by the airway smooth muscle mass that is increased in asthmatic ASMCs. 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-κB activation. However, this miR-146a-5p induction is lower in asthmatic ASMCs (figure 5)

Discussion

In this study we compared gene expression profiles between asthmatic and healthy ASMCs *in vitro* in response to the NLRP3 inflammasome downstream mediator IL-1β. Through this analysis, we provide genome-wide evidence that ASMCs respond to the active form of IL-1β, levels of which are increased in the sputum of asthmatic patients [7], providing a source of inflammatory chemokines and cytokines in the airways. Furthermore, we observed enhanced expression of *CCL20* and *MIR146A* in response to IL-1β in ASMCs, with a lower increase in *MIR146A* in asthmatic ASMCs. Furthermore, IL-1β induced a stronger increase in CCL20 protein secretion by ASMCs from moderate compared with mild asthmatic patients 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 asthmatic patients. 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 airway smooth muscle 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 stimulation, mirroring the results in this study [27]. However, this repression of inflammatory cytokines is not limited to CCL20, as the overexpression of miR-146a-5p led to the decrease of well-known NF-κB-regulated pro-inflammatory cytokines UBD, CXCL10 and CXCL8. Previous studies have identified miR-146a-5p as an anti-inflammatory miRNA that inhibits NF-κB signalling by targeting the IL-1R downstream signalling molecules IL-1 receptor-associated kinase 1 and tumour necrosis factor receptor-associated factor 6 for degradation, key genes in the activation of the NF-κB pathway [32].

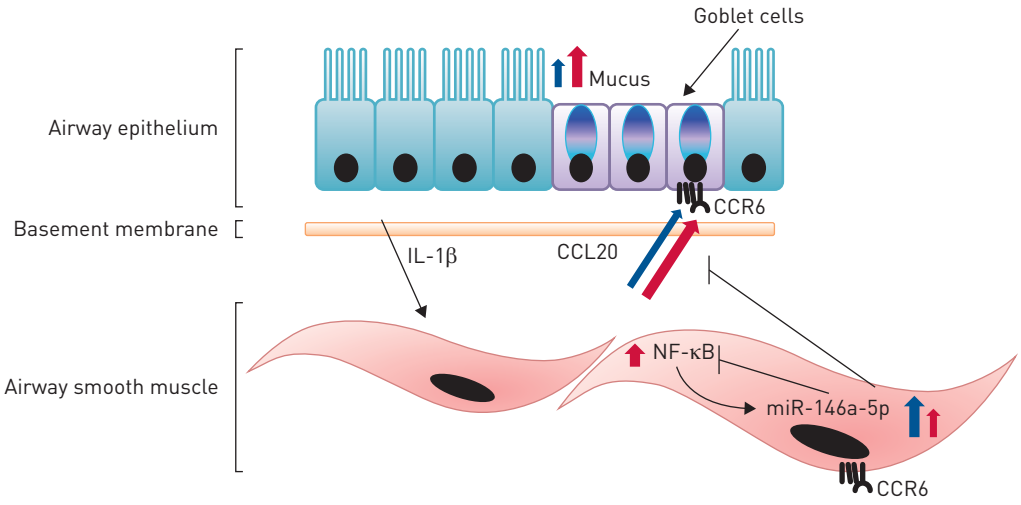

FIGURE 5 Summary of the cross-talk between the airway epithelium and airway smooth muscle cells (ASMCs) in the asthmatic airway. IL: interleukin. Basolaterally secreted IL-1β produced by the damaged airway epithelium induces CCL20 production by ASMCs, which is increased in asthmatic ASMCs. CCL20 can 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-κB activation. The miR-146a-5p induction is lower in asthmatic ASMCs, leading to reduced suppression of CCL20. Red arrows: asthma; blue arrows: healthy

In this study we found that induction of *MIR146A* in response to IL-1β was lower in asthmatic ASMCs compared with healthy controls, which may thus be responsible for the increased secretion of CCL20 from these cells. Notably, we validated the lower *MIR146A* expression in asthmatic patients using bronchial biopsies from asthmatic patients and healthy controls. Similar findings have been reported in human inflammatory cells, where circulating $CD4^+$ and $CD8^+$ T-cells of severe asthmatic patients expressed less miR-146a-5p than healthy controls [33]. A likely rationale for the decrease of *MIR146A* in asthmatic patients 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 COPD [13], 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 h miR-146a-5p overexpression. We postulate that only once the IL-1β-induced increase in miR-146a-5p starts to repress CCL20 expression does this lead 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 h used in this study may have been too early to detect differences in CCL20 gene expression levels between ASMCs from asthma patients and healthy controls due to the absence of miR-146a-5p suppression.

CCL20 was identified as a key chemokine for immature dendritic cells [35], and has recently been described as an antimicrobial protein [36] and regulator of mucus production [9, 25]. Overall, the function of CCL20 appears to be pro-inflammatory in nature and it is upregulated in the sputum in a number of inflammatory respiratory diseases, including asthma [20, 37], COPD [38] and cystic fibrosis [39]. Of interest, CMH is a feature of all of these diseases in at least a subset of patients [40–42]. 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 CCL20 [20], 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 screening 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 chemokine [43]. Despite this, the increased sensitivity and size of the muscle mass in asthmatic airways provides a potential reservoir of CCL20. Furthermore, due to remodelling of the airways, the airway smooth muscle mass is in closer proximity to the epithelial layer in asthma, which may increase the cross-talk 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 airway smooth muscle 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 cocultures 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 airway smooth muscle mass may contribute to the exaggerated mucus production by airway epithelium in asthmatic airways, due to reduced suppression by miR-146a-5p.

Author contributions: A. Faiz participated in project design, microarray analysis, *in vitro* cellular work, writing and proofreading of the manuscript. C.J. Vermeulen, C-J. Xu and M. Van den Berge participated in miRNA analysis, writing and proofreading of the manuscript. N.H.T. ten Hacken, G.G. King, C.S. Farah, A.J. Halayko, T.H. Lee and J.P.T. Ward provided either immortalised ASMC samples or sputum samples and participated in the proofreading of the manuscript. G. Tjin helped with immunohistochemical analysis. J.K. Burgess, M. Weckmann, J.L. Black and B.G. Oliver participated in project design, providing funding for the project, writing and proofreading of the manuscript.

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