Glucocorticoids induce the production of the chemoattractant CCL20 in airway epithelium

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ABSTRACT Th17-mediated neutrophilic airway inflammation has been implicated in decreased response to glucocorticoids in asthma. We aimed to investigate the effect of glucocorticoids on the airway epithelial release of the neutrophilic and Th17-cell chemoattractant CCL20.

We studied CCL20 and CXCL8 sputum levels in asthmatic subjects using inhaled glucocorticoids or not, and the effect of budesonide on CCL20 and CXCL8 production in primary bronchial epithelial cells. The mechanism behind the effect of budesonide-induced CCL20 production was studied in 16HBE14o- cells using inhibitors for the glucocorticoid receptor, intracellular pathways and metalloproteases.

We observed higher levels of CCL20, but not CXCL8, in the sputum of asthmatics who used inhaled glucocorticoids. CCL20 levels correlated with inhaled glucocorticoid dose and sputum neutrophils. Budesonide increased tumour necrosis factor (TNF)-α-induced CCL20 by primary bronchial epithelium, while CXCL8 was suppressed. In 16HBE14o- cells, similar effects were observed at the CCL20 protein and mRNA levels, indicating transcriptional regulation. Although TNF-α-induced CCL20 release was dependent on the ERK, p38 and STAT3 pathways, the increase by budesonide was not. Inhibition of glucocorticoid receptor or ADAM17 abrogated the budesonide-induced increase in CCL20 levels.

We show that glucocorticoids enhance CCL20 production by bronchial epithelium, which may constitute a novel mechanism in Th17-mediated glucocorticoid-insensitive inflammation in asthma.

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Introduction

Asthma is a chronic obstructive airway disease affecting millions of people worldwide, characterised by airway hyperresponsiveness, remodelling and inflammation, the latter predominantly characterised by eosinophils and Th2 cells. Inhaled glucocorticoids are currently the cornerstone of asthma treatment due to their broad anti-inflammatory effects, including a suppressive effect on chemokine production by structural airway cells. Despite this, a subset of asthmatic subjects is relatively insensitive to glucocorticoid treatment. This insensitivity has been associated with a neutrophilic type of airway inflammation [1], which is thought to play a prominent role in acute exacerbations and chronic severe asthma [2].

Recently, neutrophilic airway infiltration has been associated with T-lymphocytes of the Th17 subset [3]. Th17 cells specifically secrete cytokines of the interleukin (IL)-17 family, although they are not the only source of these cytokines, which act on the airway epithelium to induce the secretion of pro-inflammatory cytokines (*e.g.* CXCL8, granulocyte–macrophage colony-stimulating factor and CCL20) that recruit neutrophils to the site of inflammation [4–6]. Interestingly, it has recently been demonstrated in mice that passive transfer of Th17 cells and subsequent airway challenge induces glucocorticoid-insensitive neutrophilic airway inflammation and hyperresponsiveness [7]. Despite these novel insights, it is still unknown how Th17-mediated inflammation develops and why glucocorticoids are unable to efficiently suppress Th17-mediated neutrophilic airway inflammation. Neutrophils are relatively insensitive to glucocorticoids; however, the production of their chemoattractants, including CXCL8, by airway epithelium is glucocorticoid sensitive [8].

In addition to CXCL8, chemoattraction of neutrophils as well as Th17 cells can be induced by CCL20 [9]. CCL20 acts on CCR6, which is expressed on memory T-lymphocytes, predominantly of the Th17 subtype, on a subset of neutrophils and on dendritic cells [10]. Airway epithelium is a major producer of CCL20 [11]. Interestingly, increased CCL20 levels have been observed in asthma patients, with a further increase upon allergen challenge [12]. In addition, severe asthma patients displayed higher CCL20 levels in sputum than nonsevere asthma patients, which was associated with higher neutrophil counts [13]. Moreover, increased levels of CCL20 mRNA have been observed in the bronchoalveolar lavage fluid of glucocorticoid-insensitive asthmatic subjects [14]. However, it is still unknown if and how airway epithelial CCL20 production is regulated by glucocorticoids.

In this study, we were interested in assessing whether the epithelial release of CCL20 is sensitive to glucocorticoids. We investigated CCL20 levels in sputum from asthmatics using inhaled glucocorticoids or not, as well as the release of CCL20 by primary bronchial epithelial cells from asthma patients upon treatment with glucocorticoids *in vitro*. Interestingly, we found that CCL20 levels were higher in the sputum of inhaled glucocorticoid-using subjects, and that glucocorticoids increased the release of CCL20 by primary bronchial epithelial cells rather than inhibiting it. Therefore, we further unravelled the mechanism of CCL20 upregulation by glucocorticoids in the bronchial epithelial cell line 16HBE.

Material and methods

Subjects

Samples from 89 asthmatic individuals were included in a cross-sectional, observational study and classified by the use of inhaled glucocorticoids, rendering a group of 50 subjects using inhaled glucocorticoids and a group of 39 subjects who did not use glucocorticoids. Table 1 presents the clinical characteristics of these subjects.

All subjects had stable asthma and did not use exacerbation treatment nor was maintenance therapy altered during the 6 months preceding their inclusion in the study.

Primary bronchial epithelial cells were obtained from bronchial brushings from four asthmatic patients and four healthy subjects. All subjects were nonsmokers (<10 pack-years, no smoking in the last year), and between 18 and 65 years. Asthma patients were free of other lung diseases and included on basis of the presence of allergy (either by skin test or phadiatop), forced expiratory volume in 1 s (FEV_1) >80% predicted, and documented bronchial hyperresponsiveness defined as either an adenosine monophosphate provocative concentration causing a 20% fall in FEV_1 (PC_{20}) <320 mg·mL^{-1}, a methacholine PC_{20} <8 mg·mL^{-1} or a histamine PC_{20} <8 mg·mL^{-1}.

The Medical Ethics Committee of the University Medical Center Groningen (Groningen, the Netherlands) approved the study and signed informed consent was given by participants.

Sputum induction and processing

Sputum was induced by inhalation of nebulised hypertonic saline (5%) for three consecutive periods of 5 min. Whole sputum samples were processed as described previously [15].

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TABLE 1 Subject characteristics

	Not using inhaled GC	Using inhaled GC
Subjects n	39	50
Females	19 (48)	25 (50)
Age years	50 (24–70)	51 (22–71)
Atopy	26 (68)	36 (75)
Current smokers	17 (43)	9 (18)*
Smoking exposure pack-years	8.4 (0–47.3)	0.2 (0–63.8)
Pre-BD FEV_1 L	2.9 (1.6–5.9)	2.7 (1.4–4.5)
Pre-BD FEV_1 % pred	90.3 (53.9–113.7)	86.3 (51.6–128.7)
Pre-BD FEV_1/VC %	69.6 (45.8–89.5)	66.5 (40.3–94.4)
Pre-BD PEF $L \cdot s^{-1}$	7.2 (4.7–14.9)	7.9 (4.0–14.2)
Pre-BD MEF_{50} $L \cdot s^{-1}$	2.4 (1.0–5.6)	2.3 (0.6–5.3)
Reversibility % pred	10.0 (-2.2–33.2)	9.5 (1.5–38.4)
AMP PC_{20} $mg \cdot mL^{-1}$	78.7 (0.02–>640)	51.5 (0.01–>640)
Total IgE $IU \cdot L^{-1}$	45 (0–604)	2 (0–1668)
Blood eosinophils $\times 10^9$ per L	0.20 (0.01–0.51)	0.20 (0.06–1.16)
Sputum eosinophils %	1.2 (0–7.3)	0.8 (0–65.8)
Sputum neutrophils %	53.0 (11.8–87.7)	54.0 (15.3–90.0)
Alveolar NO ppb	5.57 (2.13–18.34)	5.63 (1.49–51.72)
Bronchial NO $nL \cdot s^{-1}$	0.64 (0.06–3.17)	0.89 (0.20–10.38)
Control according to GOAL criteria	24 (72)	24 (51)

Data are presented as n (%) or median (range), unless otherwise stated. GC: glucocorticoid; BD: bronchodilator; FEV_1: forced expiratory volume in 1 s; VC: vital capacity; PEF: peak expiratory flow; MEF_{50}: half-maximal expiratory flow; AMP: adenosine monophosphate; PC_{20}: provocative concentration causing a 20% fall in FEV_1; GOAL: Gaining Optimal Asthma Control. *: $p \leqslant 0.05$ *versus* not using inhaled GC.

Cell culture and stimulation

Primary bronchial epithelial cells and human bronchial epithelial 16HBE14o- cells (16HBE) (kindly provided by D.C. Gruenert, University of California, San Francisco, CA, USA) were cultured in hormonally supplemented bronchial epithelium growth medium (BEGM, Lonza, Walkersville, MD, USA) containing bovine pituitary extract, epidermal growth factor (EGF), adrenaline, hydrocortisone, retinoic acid and triiodothyronine, or in Eagle's minimal essential median/10% fetal calf serum, in collagen/fibronectin- or collagen-coated flasks respectively, as previously described [16]. Primary cells were used for experimentation in passage 3. Cells were seeded in duplicates at a concentration of 10^5 cells$\cdot mL^{-1}$ in 24-well plates, grown to ~90% confluence and hormonally or serum-deprived (16HBE) medium overnight, pre-treated for 2 h with budesonide (AstraZeneca, Lund, Sweden) in concentrations ranging from 10^{-10} to 10^{-7} M, and subsequently stimulated with/without 10 ng$\cdot mL^{-1}$ tumour necrosis factor (TNF)-α (Sigma, St Louis, MO, USA) upon 60 min of pre-incubation with/without specific inhibitors for the ERK (extracellular signal-regulated kinase) (U0126, 10 μM), p38 (SB203580, 1 μM), STAT3 (signal transducer and activator of transcription) (S3I-201, 100 μM) and phosphoinositol 3-kinase (PI3K) (LY294002, 10 μM) pathways, glucocorticoid receptor inhibitor (RU486, 1 μM), general metalloprotease inhibitor (TAPI-2; Calbiochem, Omnilabo International BV, Breda, The Netherlands; 20 μM), ADAM10/17 (a disintegrin and metalloprotase) inhibitor (GW280264X, 10 μM) or ADAM10-inhibitor (GI254023X, 1 μM) prior to budesonide treatment. GW280264X and GI254023X were kindly provided by GlaxoSmithKline (London, UK). Unless stated otherwise, inhibitors were purchased from Tocris Bioscience (Bristol, UK).

Air–liquid interface culture

Normal human bronchial epithelial (NHBE) cells (Lonza) were grown on semipermeable collagen/fibronectin-coated membranes in a 1:1 mixture of Dulbecco's modified Eagle's medium (Lonza) and BEGM supplemented with retinoic acid (15 ng$\cdot mL^{-1}$; Sigma) and exposed to an air–liquid interface (ALI) for 4 weeks as described previously [17]. On day 14 of air exposure, cells were placed submerged in growth factor-deprived medium overnight and subsequently treated with 10^{-8} M budesonide for 24 h. Cell-free supernatants were harvested from the apical side.

Methods for cytokine measurements and real-time RT-PCR are described in the online supplementary material.

Statistics

We used the t-test for paired observations for differences between conditions within the cell experiments, the Mann–Whitney U-test for differences in continuous data between subject groups and the Chi-squared test for differences in ordinal data between groups. Spearman's rho (r_s) test was used for analysis of correlations in patient groups. When analysing the correlation with glucocorticoid dose, only subjects using glucocorticoids were tested.

Results

Higher sputum levels of CCL20 in asthma patients using inhaled glucocorticoids than in patients who do not

First, we tested CCL20 levels in sputum from asthmatic individuals using inhaled glucocorticoids and those who did not use inhaled glucocorticoids. Both groups had similar disease severity as ascertained by clinical parameters (table 1). Importantly, we observed significantly higher levels of CCL20 in the sputum of asthma patients using inhaled glucocorticoids than the subjects who did not (fig. 1a), while CXCL8 levels were not different (fig. 1b). Within the group of subjects using inhaled glucocorticoids, we observed a significant correlation between the dose of inhaled glucocorticoids and the level of CCL20 in the sputum samples (r_s=0.28, p=0.04; online supplementary fig. S1A). Moreover, CCL20 levels in sputum correlated with the number of neutrophils in sputum (r_s=0.34, p=0.01; online supplementary fig. S1B), although the numbers of sputum neutrophils did not differ between asthma patients using inhaled glucocorticoids and those who did not (table 1). As expected, sputum CXCL8 levels correlated significantly with sputum neutrophils (r_s=0.24, p=0.03; online supplementary fig. S1D).

Glucocorticoids increase CCL20 release in primary bronchial epithelial cells

Next, we examined whether the glucocorticoid budesonide regulates CCL20 secretion by primary bronchial epithelial cells from asthma patients. We used TNF-α as a relevant cytokine to induce a pro-inflammatory response. TNF-α significantly increased CCL20 and CXCL8 secretion (fig. 2a and b). Budesonide significantly inhibited TNF-α-induced CXCL8 secretion (fig. 2b). In striking contrast, the TNF-α-induced secretion of CCL20 was significantly increased upon treatment with BUD (fig. 2a). In addition, we observed that budesonide induced a significant increase of baseline CCL20 levels and significantly enhanced the house dust mite allergen-induced CCL20 secretion (fig. 2c). Budesonide did not significantly decrease levels of CXCL8, probably due to a lack of power (fig. 2d). Similar effects were observed in bronchial epithelial cells from healthy controls (fig. 2e and f), with no significant differences between asthma patients and healthy controls.

To increase the relevance of our findings, we also studied the effect of budesonide on CCL20 secretion in primary human bronchial epithelial cells cultured at ALIs to induce mucociliary differentiation, reflecting the *in vivo* situation better. Again, treatment with budesonide (10^{-8} M, 24 h) significantly increased CCL20 levels (fig. 2g), while CXCL8 levels were not affected (fig. 2h).

Mechanisms of glucocorticoid-induced CCL20 secretion in 16HBE cells

To further elucidate the underlying mechanisms of glucocorticoid-induced CCL20 upregulation in airway epithelium, we used the human bronchial epithelial cell line 16HBE due to the limited numbers of primary

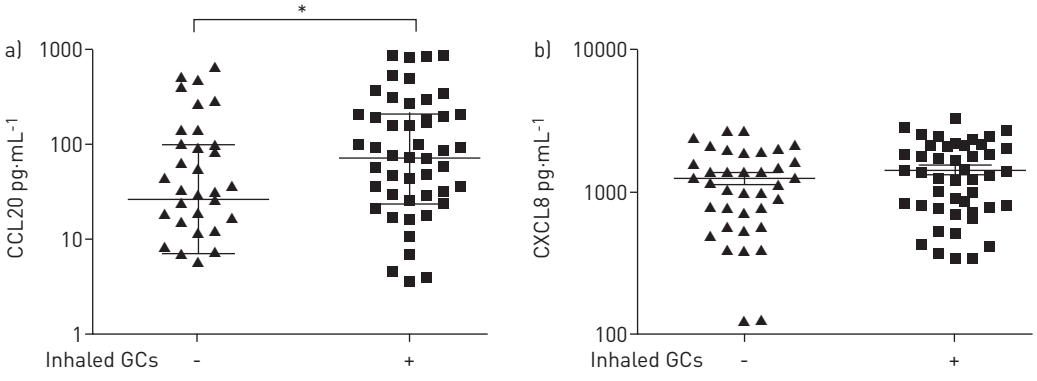

FIGURE 1 a) CCL20 levels are significantly higher in sputum of asthmatics using inhaled glucocorticoids (GCs) (n=39) than in those who did not (n=50), while b) CXCL8 levels are similar between groups. Levels of CCL20 and CXCL8 were measured in induced sputum by ELISA. Horizontal lines represent the median and error bars represent the interquartile range. *: p<0.05.

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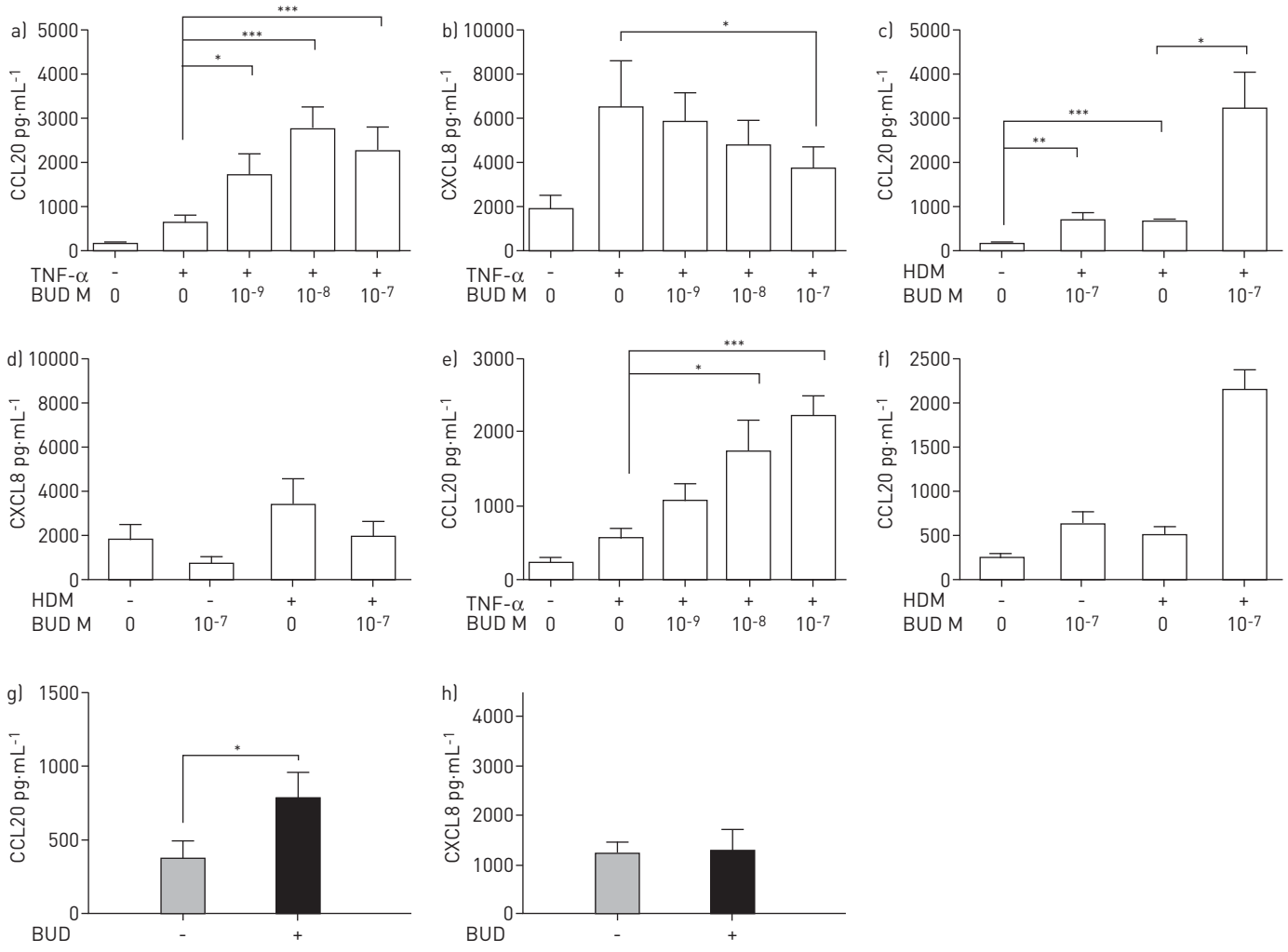

FIGURE 2 Budesonide (BUD) enhances the tumour necrosis factor (TNF)-α- and house dust mite (HDM) allergen-induced CCL20 release, but suppresses the TNF-α-induced CXCL8 release in primary bronchial epithelial cells from a–d) asthma patients and e, f) healthy donors. Cells were obtained from four donors per group. Cells were pre-treated for 2 h with or without BUD (10^{-7}–10^{-9} M) and left unstimulated or stimulated for 24 h with a, b, e) 10 ng·mL^{-1} TNF-α or c, d, f) 50 μg·mL^{-1} HDM. g, h) cells were grown in air–liquid interface culture for 2 weeks. CCL20 and CXCL8 levels were measured in cell-free supernatants from the apical side after treatment with or without BUD (10^{-8} M) for 24 h. Data are presented as mean±SEM (n=4). *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

cells. In these cells, TNF-α also induced a significant increase in CCL20 secretion, which was again further upregulated by budesonide (fig. 3a), while CXCL8 secretion was strongly reduced (fig. 3b). Furthermore, budesonide induced a significant increase in baseline levels of CCL20 (data not shown). To determine whether the increased CCL20 secretion induced by budesonide was mediated by glucocorticoid receptor activation, we used the competitive glucocorticoid receptor antagonist mifepristone (RU486) and found that the presence of RU486 completely prevented the budesonide-induced increase in CCL20 secretion (fig. 3c). Next, we studied whether CCL20 was regulated at the transcriptional level and we observed that budesonide was able to increase CCL20 mRNA levels (fig. 3d).

TNF-α-induced CCL20 production is dependent on ERK, p38 and STAT3

As CCL20 was regulated by budesonide at the transcriptional level, we aimed to further unravel the signal transduction pathways involved in these effects. Since the STAT3, ERK and p38 pathways have been implicated in CCL20 transcription as well as in glucocorticoid-insensitive airway inflammation [18–20], we tested the effect of their specific inhibitors on the TNF-α- and budesonide-induced CCL20 production in 16HBE cells. Pre-incubation with the inhibitors of the ERK (U0126), p38 (SB203580) and STAT3 (S3I-201) pathways significantly reduced the TNF-α-induced CCL20 production, indicating a role for these signalling molecules in CCL20 production (fig. 4a). Inhibition of the PI3K pathway did not affect CCL20 production, although it significantly inhibited IL-8 secretion under the same conditions (online supplementary fig. S2A). Next, we determined whether the budesonide-induced increase was dependent on the aforementioned

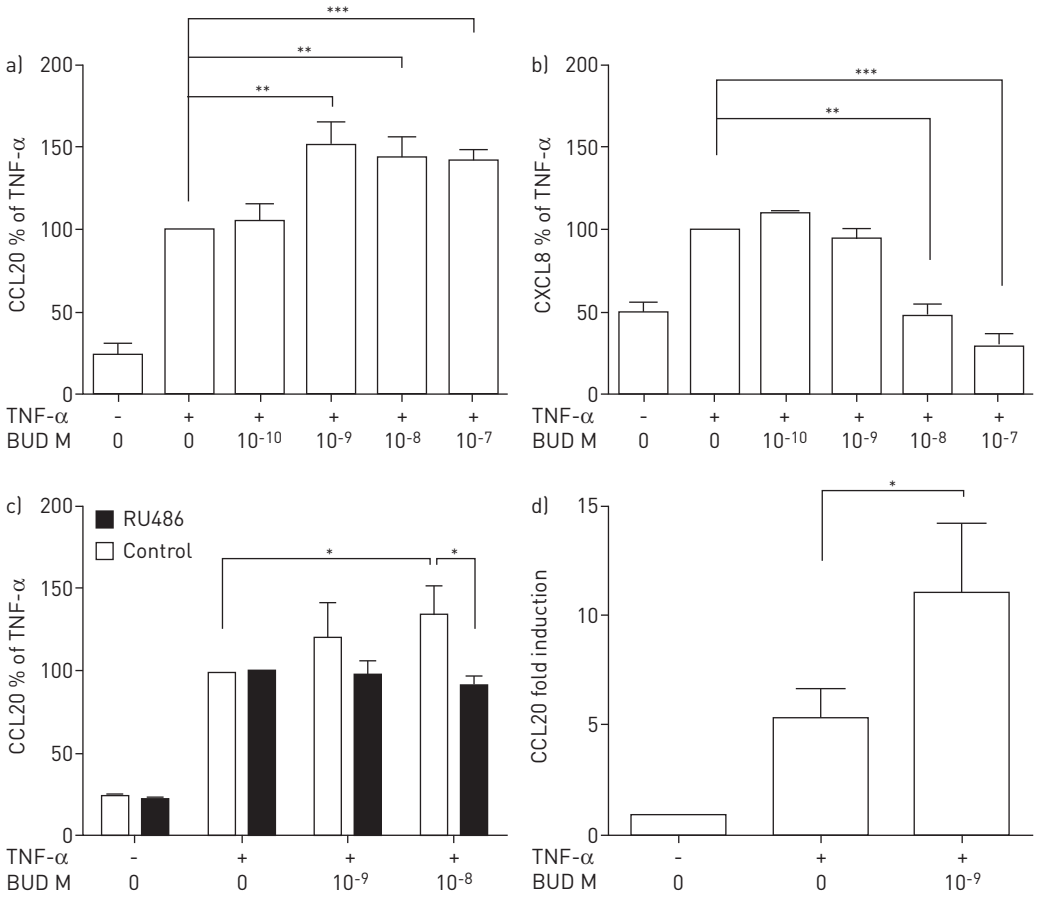

FIGURE 3 Budesonide (BUD) enhances tumour necrosis factor (TNF)-α-induced CCL20 release and mRNA expression, which is dependent on glucocorticoid receptor activity, but suppresses TNF-α-induced CXCL8 release in 16HBE cells. Cells were pre-treated with BUD (10^{-7}–10^{-10} M) for 2 h, stimulated with 10 ng·mL^{-1} TNF-α, and mRNA and cell-free supernatants were collected after 6 h and 24 h, respectively. a) CCL20 and b) CXCL8 were measured in cell-free supernatants and expressed as percentage of the TNF-α levels without BUD. c) Prior to BUD, cells were treated for 1 h with 10 μM RU486 and CCL20 levels are expressed as percentage of the TNF-α levels without BUD. d) CCL20 mRNA levels were related to a housekeeping gene and expressed as fold change compared with the unstimulated control ($2^{-\Delta\Delta Ct}$). Data are presented as mean±SEM of four independent experiments. Ct: threshold cycle. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

pathways observed. However, no decrease in the upregulatory effect of budesonide was found upon the use of LY294002, U0126, SB203580 or S3I-201 (fig. 4b), nor did budesonide increase the phosphorylation of p38, ERK or STAT3 (data not shown). Thus, our data indicate that while TNF-α-induced CCL20 production is dependent on the ERK, p38 and STAT3 pathways, the additional upregulatory effect of glucocorticoids is not mediated by these pathways in human bronchial epithelium, suggesting the involvement of additional pathways.

Glucocorticoid-induced CCL20 secretion is ADAM17 dependent

Previously, KIM *et al.* [21] have described that ADAM17-dependent EGF receptor (EGFR) stimulation can increase CCL20 production, while glucocorticoids have been reported to increase EGFR activity [22]. Indeed, ADAM17 has been described as a key sheddase of ligands of EGFR [23]. Therefore, we used the broad-spectrum metalloprotease inhibitor TAPI-2, the selective ADAM10/17 inhibitor GW280264X and the selective ADAM10 inhibitor GI254023X, as well as the EGFR inhibitor AG1478 to determine if an ADAM/EGFR-dependent mechanism could be involved in glucocorticoid-induced CCL20 secretion. TAPI-2 did not significantly inhibit TNF-α-induced CCL20 secretion but completely abrogated the upregulatory effect of budesonide. A similar effect was observed for the selective ADAM10/17 inhibitor GW280264X, while the more ADAM10-specific inhibitor GI254023X did not show a significant effect on the secretion of CCL20 (fig. 5a). Since both GW280264X and TAPI-2 have a higher affinity for ADAM17 than ADAM10, these data suggest that the budesonide-induced increase in CCL20 is dependent on ADAM17 activity. In contrast to the data of KIM *et al.* [21], we did not observe an effect of the EGFR inhibitor AG1478 on the

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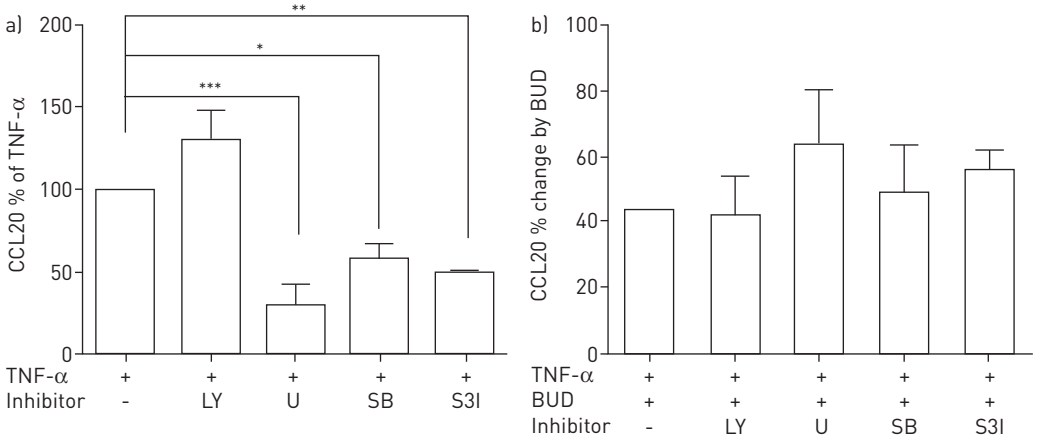

FIGURE 4 Inhibition of the ERK (extracellular signal-regulated kinase), p38 and STAT3 (signal transducer and activator of transcription) pathways reduces tumour necrosis factor (TNF)-α-induced CCL20 release, but did not block the upregulatory effect of budesonide (BUD) in 16HBE14o- cells. Cells were treated with LY294002 (LY; 10 μM), U0126 (U; 10 μM), SB203580 (SB; 1 μM) or S3I-201 (S3I; 100 μM) for 30 min prior to pre-treatment with BUD (10^{-8} M) for 2 h and subsequently stimulated with TNF-α (10 ng·mL^{-1}) for 24 h. a) Effect of the inhibitors on TNF-α-induced CCL20 release. CCL20 levels are expressed as percentage of the TNF-α levels without inhibitors. b) Effect of the inhibitors on BUD-induced CCL20 release. CCL20 levels are expressed as percentage increase over the levels with TNF-α alone. Data are presented as mean±SEM of four independent experiments. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

budesonide-induced increase in CCL20 release (fig. 5a), although it significantly inhibited CXCL8 secretion under the same conditions (online supplementary fig. S2B). This excludes the involvement of EGFR ligand shedding and subsequent EGFR activation in budesonide-induced CCL20 secretion.

Subsequently, we aimed to determine whether the glucocorticoid-induced CCL20 secretion is due ADAM17-mediated shedding of CCL20 itself or is a consequence of downstream signalling induced by the shedding of an ADAM17 substrate other than EGFR ligands. We assessed this by studying the effect of ADAM17 inhibition at the CCL20 mRNA level. Notably, budesonide was no longer able to upregulate CCL20 mRNA when cells were pre-treated with GW280264X (fig. 5b). Thus, our results indicate that the upregulatory effect of glucocorticoids on CCL20 is dependent on ADAM17 activity and downstream signalling of an as yet unknown substrate of ADAM17.

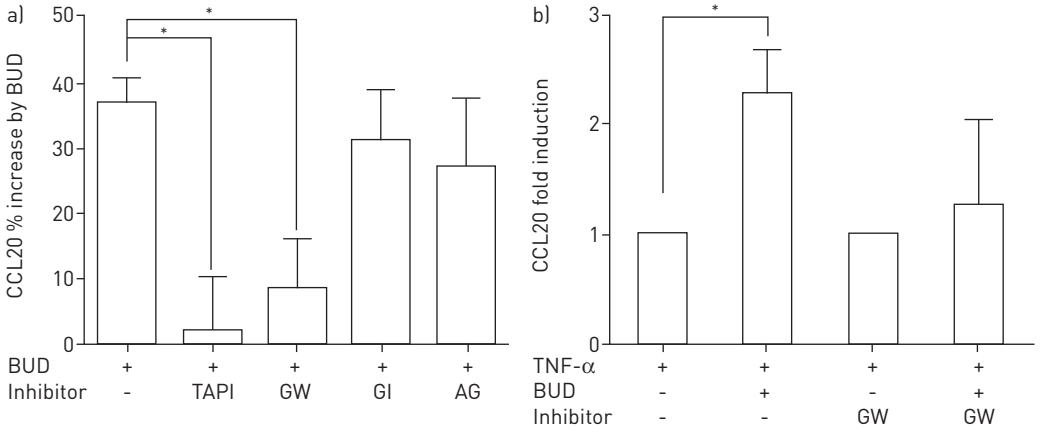

FIGURE 5 The effect of budesonide (BUD) is dependent on activity of ADAM17 (a disintegrin and metalloprotease). Cells were pre-treated for 1 h with the broad-spectrum metalloprotease inhibitor TAPI-2 (TAPI; 20 μM), ADAM17 and ADAM10 inhibitor GW280264X (GW; 10 μM), ADAM10 inhibitor GI254023X (GI; 1 μM), or EGFR inhibitor AG1478 (AG; 1 μM), and subsequently incubated with BUD (10^{-9} M) for 24 h (cell-free supernatants) or stimulated with tumour necrosis factor (TNF)-α for 6 h and harvested for mRNA isolation. a) CCL20 was measured in cell-free supernatants and levels are expressed as the percentage increase over the levels with BUD alone. Data are presented as mean±SEM of four independent experiments. b) CCL20 mRNA levels were related to a housekeeping gene and expressed as fold change compared with the levels in the absence of BUD ($2^{-\Delta\Delta Ct}$). Data are presented as mean±SEM of three independent experiments. Ct: threshold cycle. *: $p<0.05$.

Discussion

The mechanism of glucocorticoid-insensitive Th17-mediated neutrophilic airway inflammation in asthma has remained unclear, and the effect of glucocorticoids on the airway epithelial secretion of the Th17 cell and neutrophil chemoattractant CCL20 has not been studied before. We show for the first time that asthma patients using inhaled glucocorticoids display higher sputum levels of CCL20 than asthmatics who do not use inhaled glucocorticoids, while CXCL8 levels did not differ between the groups. Furthermore, we demonstrate that glucocorticoids upregulate CCL20 secretion in cultured bronchial epithelial cells from asthma patients, whereas CXCL8 is inhibited by glucocorticoids. Our experiments in 16HBE cells further reveal that this effect of glucocorticoids is regulated at the transcriptional level by an ADAM17- and glucocorticoid receptor-dependent mechanism.

Our findings may have important implications for our understanding of the initiation of glucocorticoid-insensitive Th17 and neutrophilic airway inflammation in asthma, as CCL20 has been known to attract both Th17 cells and neutrophils. In addition to allergen-induced airway inflammation, a crucial role for CCL20 has been demonstrated in cigarette smoke-induced airway infiltration of neutrophils, T-lymphocytes and dendritic cells in a mouse model of chronic obstructive pulmonary disease (COPD) [24]. Importantly, both Th17-mediated neutrophilic airway inflammation and cigarette smoking have been related to glucocorticoid insensitivity in asthma [25] and smoking has been shown to induce airway infiltration of both neutrophils and Th17-type cells [26]. Thus, we propose a novel paradigm for the development of glucocorticoid-insensitive airway inflammation in both asthma and COPD, where glucocorticoids enhance CCL20 release, inducing airway infiltration of $CCR6^+$ neutrophils and Th17 cells. In line with this hypothesis, the increased sputum levels of CCL20 in asthma patients using inhaled glucocorticoids were associated with neutrophil counts. Furthermore, COPD patients were found to display higher sputum levels of CCL20 than never-smokers and smokers without COPD. Here, the majority of COPD patients, but none of the control subjects, used inhaled glucocorticoids. It is tempting to speculate that glucocorticoid use contributes to the increased levels of COPD observed in this study, although the comparison of CCL20 levels in COPD patients using and not using inhaled corticosteroids would be required to support this.

We observed that the upregulatory effect of glucocorticoids on CCL20 was mediated at the transcriptional level and involved glucocorticoid receptor activation. Glucocorticoids have been shown to induce gene transcription through binding to a glucocorticoid response element (GRE) in the promoter region. A GRE has been described in an intron downstream of the transcription start site of the CCL20 gene [27]. The regulatory properties of this GRE have not been extensively studied to our knowledge, but our results suggest that ADAM17 activity is indispensable for the effect of glucocorticoids on CCL20. To our knowledge, GRE binding has not been described to be metalloprotease dependent. As ADAM17 inhibition also abrogated the upregulatory effect of budesonide at the transcriptional level, we anticipate that the upregulatory effect of budesonide is not mediated by ADAM17-dependent shedding of CCL20 itself, but rather involves downstream signalling of an as yet unknown ADAM17 substrate. ADAM17 plays a role in the shedding of many signalling molecules [23], such as Notch [28] and EGFR ligands. The latter has been implicated in many autocrine loops involving pro-inflammatory transcription, including CCL20 [21]. However, our results do not support a role for EGFR activation in glucocorticoid-induced CCL20 production. Indeed, the EGFR-induced CCL20 release described in H292 cells by KIM *et al.* [21] could not be confirmed in NHBE cells. Further studies will be of interest to elucidate which specific pathways downstream of ADAM17 substrates are affected by budesonide and are involved in the upregulatory effect on CCL20.

Our findings exclude a role for the STAT3, p38 ERK and PI3K pathways in glucocorticoid-induced CCL20 upregulation. We observed that TNF-α-induced CCL20 production was dependent on STAT3, p38 and ERK. In line with this, IL-17-induced CCL20 was shown to be dependent on ERK activity in primary human tracheal cells [18], on both ERK and p38 activity in human gingival fibroblasts [19], and on phosphorylation of STAT3 in naïve T-lymphocytes [29]. ERK and p38 phosphorylation have previously been shown to be inhibited by glucocorticoids [30], while glucocorticoids induce IL-10 in a STAT3-dependent way in B-lymphocytes [31]. In our setting, budesonide did not affect phosphorylation of STAT3 or the p38/ERK pathway, and the use of their inhibitors revealed that these pathways were not involved in the glucocorticoid-mediated enhancement of CCL20 release in human bronchial epithelium.

LANNAN *et al.* [32] have shown a possible mechanism of co-regulation between TNF-α and glucocorticoids. In their study, the upregulation of serpin A3 required both glucocorticoid activation and the soluble TNF receptor (TNFSR1). TNFSR1 can be shed by ADAM17 [33]. However, we render it unlikely that this mechanism plays a major role in the glucocorticoid-induced increase of CCL20, as the effect occurred regardless of the presence of TNF-α.

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Although epithelial cells play an emerging role in the regulation of airway inflammation in asthma [34], we must acknowledge the possibility of other cell types playing a role in the CCL20-induced chemotaxis of Th17 cells and neutrophils to the inflamed lungs in asthma as well. In particular, macrophages have been shown to produce CCL20 [35]. We cannot exclude that macrophages also contribute to the increased levels of CCL20 in sputum of asthma patients using inhaled glucocorticoids. The same mechanisms could also apply in macrophages as higher levels of CCL20 mRNA have been described in macrophages from glucocorticoid-insensitive subjects compared with glucocorticoid-sensitive subjects [14].

As the data on CCL20 levels in sputum of asthma patients were obtained in a cross-sectional, observational study, we cannot be sure whether glucocorticoid treatment will indeed increase CCL20 levels in the airways. Nevertheless, our sputum data in combination with our *in vitro* findings strongly suggest that glucocorticoid use in asthma patients leads to increased sputum levels of CCL20 as a consequence of direct effects of the glucocorticoids, inducing CCL20 release by airway epithelium. To confirm this, a future randomised clinical trial on the effect of glucocorticoids on CCL20 and Th17 cells will be required.

In conclusion, we show that levels of CCL20 are higher in asthmatic subjects using inhaled glucocorticoids and that glucocorticoids increase the production of CCL20 in human bronchial epithelium, which is mediated by the glucocorticoid receptor and dependent on ADAM17 activity. Our data may provide new opportunities for therapeutic intervention of glucocorticoid-insensitive asthma.

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