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IL-1β and TNF-α induce increased expression of CCL28 by airway epithelial cells via an NFκB-dependent pathway

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Abstract

CCL28 is a mucosal chemokine that attracts eosinophils and T cells via the receptors CCR3 and CCR10. Consequently, it is a candidate mediator of the pathology associated with asthma. This study examined constitutive and induced expression of CCL28 by A549 human airway epithelial-like cells. Real-time RT-PCR and ELISA of cultured cells and supernatants revealed constitutive levels of CCL28 expression to be low, whereas IL-1β and TNF-α, induced significantly increased expression. Observations from induced sputum and human airway biopsies supported this. Signal transduction studies revealed that IL-1β and TNF-α stimulation induced NFκB phosphorylation in A549 cells, but antagonist inhibition of NFκB p50–p65 phosphorylation correlated with marked reduction of IL-1β or TNF-α induced CCL28 expression. Together these studies imply a role for CCL28 in the orchestration of airway inflammation, and suggest that CCL28 is one link between microbial insult and the exacerbation of pathologies such as asthma, through an NFκB-dependent mechanism.

Keywords: CCL28; IL-1β; TNF-α; Respiratory tract; NFκB; Asthma

1. Introduction

Chemokines are a family of 8–16 kDa proteins with critical roles in immune cell trafficking, recruitment and recirculation [1,2]. Their interaction with specific G-protein-coupled receptors (GPCRs) leads to paracrine, pleiotropic actions, resulting in the induction of inflammatory infiltrates to tissues [3]. CC chemokines such as CCL2, CCL3, CCL5 and CCL11/eotaxin attract lymphocytes, eosinophils and monocytes [4–7]. These cells play a central role in inflammation in the airways that leads to bronchial hyperresponsiveness (BHR) and mucus hypersecretion, seen during obstructive airway disorders that are reversible (asthma) or irreversible (COPD) [8–11].

A novel CC chemokine, CCL28, also known as mucosae-associated epithelial chemokine (MEC), is expressed in both murine and human mucosal tissues [1,12]. It displays chemotactic activity for eosinophils and both $CD4^+$ and $CD8^+$ T cells through CCR3 and CCR10 receptors [1,12,13]. Human CCL28 has a coding sequence of 373 nucleotide base pairs encoded by four exons separated by introns of varying size [1,12]. There is 76% nucleic acid identity between mouse and human sequences which results in 83% similarity at the amino acid level [1,12]. Sequence analysis has revealed CCL28 to be most similar to CCL27/CTACK (cutaneous T-cell attracting chemokine). CCL27 is constitutively expressed by keratinocytes but has both a homeostatic and inflammatory role in the skin [1,14–16]. It is not known if CCL28 plays similar roles at mucosal sites.

The association of CCL28 with the eotaxin receptor CCR3 [12], makes it a candidate chemokine for a role in eosinophil infiltration at mucosal sites [17]. Recent evidence has reappraised the eosinophil, which is now considered to play a critical role in the early events of airway inflammation [18,19]; one of the dominant characteristics of airway inflammation during respiratory distress [20].

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Although the asthmatic response is maintained by Th2-like cytokines (IL-4, 5 and 13) [10], it is apparent that asthma is more complex than an imbalance in the products of Th1 and Th2 cells [21]. Studies in knockout mice have revealed a role for Th1 cytokines in exacerbating airway pathology [22], and Th1-driving infections do not necessarily protect but can exacerbate allergic asthma in murine models [23]. The mechanism that links pro-inflammatory infections (often Th1-driven) and asthma (considered a Th2-driven pathology) is poorly understood. However, it is likely that monokines and cytokines such as IL-1β and TNF-α, produced early in the host pathogen encounter, determine the chemokine profile that ultimately recruits Th2 cells and eosinophils that mediate the pathology of allergic asthma [24,25].

Recently, it has been demonstrated that IL-1α, but not TNF-α, induced CCL28 transcription in a human colon epithelial cell line [26]. The difference is unusual given that both ligands share some signalling pathways and that IL-1α-mediated effects were shown to be dependent on the transcription factor NFκB. Secreted IL-1β and TNF-α are more central to the events of inflammation and can initiate transcription of chemokine genes via NFκB activation, albeit employing different cellular receptors and adaptor molecules [27,28], however there are unique signalling features to each cytokine [27,29]. Binding of IL-1β to the IL-1 receptor 1 initiates the arrangement of a signalling complex. The receptor accessory protein, IL-1RacP associates with the adaptors MYD88 and TOLLIP [30]. Engagement of this signalling complex allows recruitment of cellular IL-1 receptor-associated kinase (IRAK) [31]. Phosphorylation of IRAK ultimately activates the IκB kinase (IKK) complex. In contrast, TNF-α activates NFκB via association of the TNF receptor associated death domain (TRADD) adaptor with TRAF-2 and receptor interacting protein (RIP) and this complex interacts with IKK [29]. Both pathways converge on activated IKK which phosphorylates the NFκB inhibitor, IκB [28]. Subsequent polyubiquitination and degradation of phosphorylated IκB, releases NFκB to translocate to the nucleus in dimer form, and initiate gene transcription [27]. Five different protein sub-units, each containing conserved Rel homology regions, constitute the NFκB family (p50, p52, p65/RelA, RelB and c-Rel) [27]. Interaction between different sub-units results in dimers that are active upon phosphorylation. Phosphorylated p50–p65 nuclear dimers are transcriptionally active for genes related to inflammation, whereas other dimers (e.g., p50–p50 or p50–RelB) typically act in transcription of non-inflammatory genes or have a regulatory role [28].

In the present study, the control of CCL28 in airway inflammation was explored, and the link between the NFκB signal transduction pathway and CCL28 expression was examined. Both IL-1β and TNF-α induced NFκB-dependent CCL28 expression at a high level, suggesting a potential role for this chemokine in determining the quality of airway inflammation following microbial insult. CCL28 may thus provide a mechanistic link between pro-inflammatory, microbe-induced stimuli in the airways such as IL-1β or TNF-α, and the subsequent exacerbation of pre-existing pathologies such as asthma or COPD.

2. Materials and methods

2.1. Cell culture

A549 cells were cultured at 37 °C without CO_2 in D-MEM:F12 (1:1) media including 15 mM HEPES, and supplemented with 1% L-glutamine (Invitrogen, Paisley, UK) and 5% heat inactivated FCS (Sigma–Aldrich, Dorset, UK).

2.2. Human-induced sputum and airway biopsy

Sputum samples were collected from 11 healthy, 1 asthmatic and 1 subject with a respiratory infection following stimulation with inhaled sodium chloride according to previous protocols [32]. Samples were processed by centrifugation and the supernatants stored at −20 °C until required for use. Biopsies were collected from two healthy donors, six asthmatics and one subject with aspergillosis. Samples were removed to RNA later (Ambion, Austin, USA) and prepared for mRNA extraction. All subjects provided informed consent, and the local research ethics committees of both the collaborating institutions approved study design.

2.3. In vitro stimulation of cells

A549 cells were cultured with a variety of recombinant cytokines (R&D Systems, Oxford, UK and Immunotools, Friesoythe, Germany) at concentrations previously determined as optimal for induction of inflammatory responses; IL-4 and IL-5 (1 ng/ml), IL-13, SDF-1α, TGF-β1, IL-12p40 and LPS (10 ng/ml) and GMCSF (50 ng/ml) [4,33–39]. The pro-inflammatory cytokines, IL-1β or TNF-α (R&D Systems, Oxford, UK), were introduced into cultures either alone, in combination or following prior exposure of the cell cultures to the NFκB antagonist pyrrolidine-dithiocarbamate (PDTC) (Sigma–Aldrich, Dorset, UK). PDTC was used at concentrations described in figure legends. For analysis of CCL28 mRNA expression, 5×10^5 cells were harvested after 6 h stimulation, whereas protein determinations were from supernatants taken from cultures at 24 h. These time points were selected after optimisation studies (data not shown). No increase in apoptosis was observed at either time point under any stimulation condition. For analysis of NFκB activation, nuclear extracts were recovered from 8×10^6 cells, stimulated as described above and cultured for 6 h.

2.4. Real-time RT-PCR quantification of CCL28 mRNA

Reverse transcription of total isolated RNA was carried out as previously described [40] using Trizol™ (Molecular

Research Centre Inc., Cincinnati, USA). Quantitative real-time PCR was carried out using a quantitative thermocycler (MJ Research DNA Opticon™, Massachusetts, USA) in conjunction with the QuantiTect™ SYBR Green PCR Kit (Qiagen, Crawley, UK). Each reaction mixture contained 12.5 μl SYBR Green Master Mix and 0.4 pmol appropriate primer. PCR primers for CCL28 were based on the sequence of Pan et al. [12]; forward 5′-TACTTCC CATTGCCTCCAG-3′ and reverse 5′-ATGGTG TTTCTTCCTGTGG-3′. GAPDH primers were: forward 5′-GGTGAAGGTCGGAGTCAACG-3′ and reverse 5′-C AAAGTTGTCATGGATGACC-3′. Amplification was performed by denaturation (95 °C for 15 min at the first cycle and subsequently for 45 s), annealing (60 °C for 45 s, CCL28; or 55 °C, GAPDH) and extension (72 °C for 1 min or 10 min in final cycle). Amplification of equal concentrations of cDNA was performed over 36 cycles in a reaction volume of 25 μl.

CCL28 mRNA concentration was expressed as fg/100 ng cDNA or as a fold difference in CCL28 compared to GAPDH mRNA. Relative quantification was determined by the formula $2^{\Delta C_t}$ where $\Delta C_t = (C_{t\ \mathrm{CCL28\ control}} - C_{t\ \mathrm{CCl28\ stimulated}}) - (C_{t\ \mathrm{GAPDH\ control}} - C_{t\ \mathrm{GAPDH\ stimulated}})$. For this study, specific mRNA below 20 fg per 100 ng cDNA is defined as low, whereas expression above this level is termed high.

2.5. Detection of CCL28 protein

Measurement of CCL28 was carried out by sandwich-type ELISA (R&D Systems, Oxford, UK), as previously described [41]. Mouse anti-human CCL28 mAb (clone 62714) was used as capture antibody and biotinylated polyclonal goat anti-human CCL28 (R&D Systems, Oxford, UK) was used to detect CCL28 in cell supernatants. Human recombinant CCL28 protein (R&D Systems, Oxford, UK) was titrated from 8000 pg/ml in doubling dilutions and used to quantify CCL28 protein. For studies of tissue culture supernatant, a CCL28 protein concentration below 100 pg/ml was defined as low, however, concentrations below 4.0 ng/ml were considered low for studies of human sputum.

2.6. Nuclear extraction and measurement of NFκB phosphorylation

Nuclear extracts were obtained from 8×10^6 control or treated A549 cells after 6 h culture, using a commercial kit according to the manufacturer's instructions (Active Motif, Rixensart, Belgium). Extracts were normalised for protein content using the Biorad™ Protein Assay (Bio-Rad Laboratories, California, USA), and subsequently maintained at −80 °C until required for use. Subsequently, samples were assayed for the presence of phosphorylated forms of NFκB p50, p52, p65, RelB and c-Rel sub-units using the TransAm™ NFκB family transcription factor assay kit (Active Motif). This ELISA-like system detects only phosphorylated nuclear forms of NFκB bound to a fixed oligonucleotide specific for the NFκB consensus site. The protocol, including suitable standards and controls, was performed as recommended by the manufacturer.

2.7. Statistical analysis

Results were analysed by Student *t*-test or one-way ANOVA with Bonferroni's multiple comparison post-test for significance as appropriate.

3. Results

3.1. CCL28 is expressed at low levels by human airway epithelium-like cells and in induced human sputum

CCL28 may play a role in inflammatory airway disease. Therefore, the expression of CCL28 by airway epithelium was examined. Human airway epithelium-like A549 cells were cultured for 6 h in the absence of any stimulus and the isolated mRNA analysed. Using real-time quantitative methods, these cells were found to constitutively express CCL28 mRNA at low levels (Fig. 1). It is known that levels of cytokine and chemokine mRNA do not always correspond with protein expression. Therefore, CCL28 protein secretion by A549 cells was determined by ELISA. Low, but reproducibly positive, levels of CCL28 protein were detected (Fig. 1C) suggesting that the constitutive presence of mRNA is reflected in CCL28 protein expression by cultured airway epithelium. As A549 cells are a transformed cell line, it could be argued that expression by these cells does not reflect the scenario in vivo. Although this study was focused on the mechanisms of CCL28 control, airway biopsy tissue was sampled from normal healthy volunteers and from indicator patients with inflammatory conditions such as asthma or respiratory infection for confirmatory purposes. CCL28 mRNA extracted from human tissue was quantified by real-time RT-PCR and was significantly elevated in an asthmatic patient and an individual presenting with aspergillosis compared to normal healthy human tissue (Fig. 2A). Again, mRNA levels do not always correlate with protein expression, so CCL28 protein expression was examined ex vivo. Induced sputum was collected from 11 healthy volunteers and analysed by ELISA for the presence of CCL28 protein. Fig. 2B shows that sputum contained low levels of CCL28 protein a (1.7 ng/ml ± 0.23). Interestingly, the CCL28 protein content of sputum induced from an asthmatic patient prior to anti-inflammatory treatment was much higher (4.0 ng/ml ± 0.26, $p < 0.05$) as was sputum induced from an individual with an unidentified bacterial respiratory infection (8.0 ng/ml ± 0.73, $p < 0.001$). Although the human study can only be considered indicative, taken together these data are consistent with CCL28 being constitutively expressed at low levels by healthy human airway epithelium but suggest that this is increased during respiratory inflammation, supporting both a homeostatic and inflammatory role for CCL28 at this mucosal site.

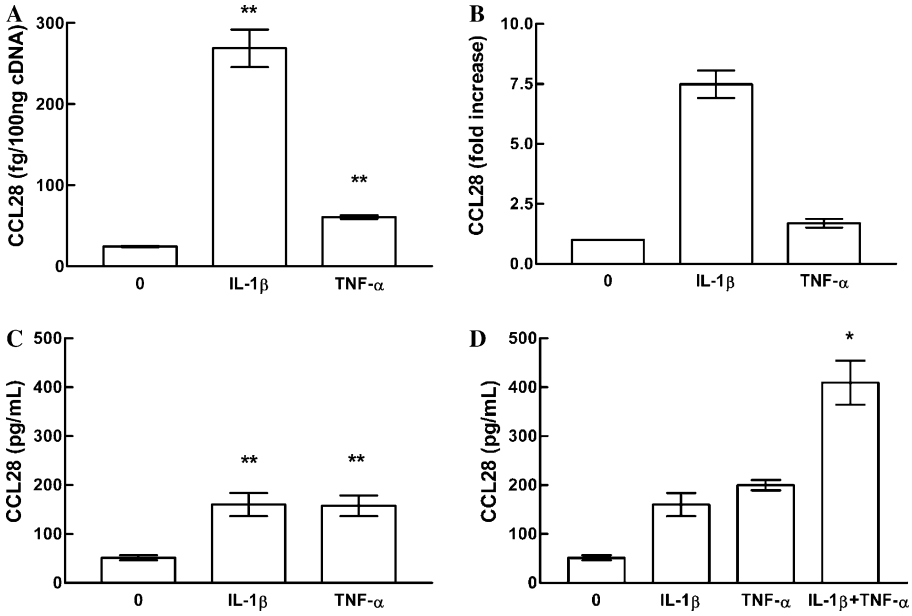

Fig. 1. IL-1β and TNF-α induce upregulation of constitutive CCL28 expression in vitro. A549 airway epithelial-like cells were cultured in the absence or presence of IL-1β (10 ng/ml) or TNF-α (100 ng/ml) for 6 h. CCL28 and GAPDH mRNA were amplified and quantified by real-time RT-PCR. Data are expressed in absolute quantities per 100 ng of cDNA (A) or normalised to GAPDH and expressed as a fold increase relative to control (B); CCL28 protein present in culture supernatant after 24 h was measured by ELISA (C and D). mRNA quantities are expressed as mean (±SEM) from duplicate determinations of a representative experiment (performed three times). Protein levels represent mean concentration (±SEM) of triplicate determinations, from a representative experiment (performed at least three times), $^{*}p < 0.05$, $^{**}p < 0.01$.

3.2. IL-1β and TNF-α increase CCL28 mRNA and protein expression in vitro

IL-1β and TNF-α can both be present in the airways during infections that are known to exacerbate asthma [23,42]. The presence of CCL28 in the respiratory tract, its association with the receptors CCR3 and CCR10 [1,12,17], and the indicative data from inflamed tissue, point to a role for the chemokine during airway inflammation. To further elucidate the effects of an inflammatory environment on CCL28 expression, A549 cells were exposed to a variety of inflammatory stimulants and analysed for the presence of mRNA and secreted protein as described above. Contrary to observations from colon cells, exposure to IL-1β or TNF-α caused a significant increase in CCL28 mRNA and protein relative to non-treated cells (Figs. 1A–C) although the increase in mRNA after TNF-α stimulation was less dramatic than with IL-1β. Increased CCL28 mRNA and protein induction was dependent upon the degree of stimulation and concentrations of IL-1β or TNF-α above 0.1 or 1.0 ng/ml, respectively, were required to achieve significance (results not shown). No significant apoptosis was observed within these cultures. Combined stimulation of A549 cells with IL-1β and TNF-α, enhanced CCL28 protein expression above that seen with either cytokine alone (Fig. 1) but only at TNF-α concentrations below 1 ng/ml (Fig. 1). Interestingly, other factors associated with bronchial inflammation, asthma or eotaxin induction did not significantly increase either CCL28 mRNA or protein expression above controls (Table 1).

3.3. IL-1β and TNF-α induce phosphorylation of NFκB in vitro

The NFκB signal transduction pathway is involved in both IL-1β- and TNF-α-mediated induction of chemokines [43]. Recently it has been demonstrated that antagonists of NFκB successfully inhibit IL-1α induced CCL28 transcription in a human colon epithelial cell line [26]. Since IL-1β and TNF-α are also known to signal via the NFκB pathway, cytokine induction of transcription factor phosphorylation was assessed in A549 cells. An ELISA-like assay was used to analyse the phosphorylation of NFκB sub-units, p50, p52, p65, RelB, and c-Rel, isolated from cells cultured in the presence or absence of either IL-1β or TNF-α. Following 6 h stimulation, A549 cells were removed from culture and nuclear extracts isolated. Fig. 3 shows that IL-1β significantly increased phosphorylation of NFκB p50 and p65 relative to control cells ($p < 0.01$) as detected in nuclear extracts (Figs. 3A and B). Significant but smaller increases were observed for the phosphorylated p52 and RelB sub-units, while no change was detected in c-Rel phosphorylation in nuclear extracts (results not shown). Exposure to TNF-α (10 ng/ml) increased phosphorylation of p50 ($p < 0.01$) and p65 ($p < 0.01$) (Figs. 3C and D) with no significant effect on the other components examined. Thus although there may be differences in scale and concentration, both cytokines are effective in inducing transcriptionally active NFκB p50–p65 in A549 cells.

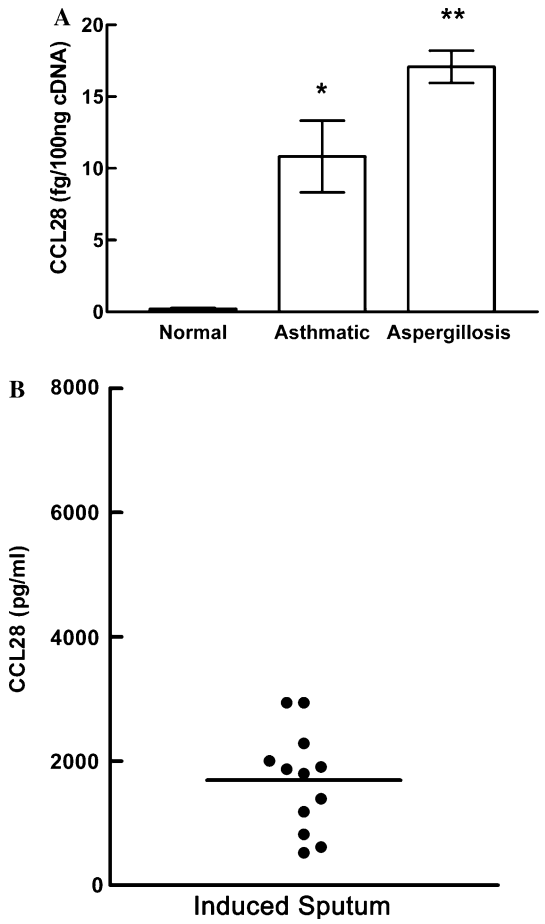

Fig. 2. CCL28 is expressed in human airway tissue and induced sputum. CCL28 mRNA extracted from normal or inflamed human airway, was quantified by real-time RT-PCR and data expressed in absolute quantities per 100 ng cDNA (A). Values are expressed as means (±SEM) from triplicate ($n = 3$) determinations. mRNA from inflamed airway tissue shows significantly higher CCL28 expression than normal healthy tissue, $^{*}p < 0.05$, $^{**}p < 0.01$. Induced sputum from 11 healthy non-smoking control volunteers were processed and analysed by ELISA for CCL28 protein content (B). Each point represents the mean CCL28 concentration determined in triplicate for each patient. The solid line represents the mean CCL28 concentration detected overall.

3.4. Inhibition of NFκB reduces cytokine-induced CCL28 in vitro

It was possible that IL-1β or TNF-α induced CCL28 by a pathway independent of NFκB activation. To confirm the involvement of the NFκB signal transduction cascade in cytokine-mediated CCL28 induction, A549 cells were treated with the NFκB inhibitor, PDTC, 30 min prior to IL-1β or TNF-α exposure. Nuclear extracts of cells pre-treated with PDTC, displayed significantly lower levels of phosphorylated NFκB p50 and p65 relative to cells treated with IL-1β ($p < 0.001$, Figs. 4A and B) or TNF-α alone ($p < 0.001$, Figs. 4C and D). PDTC treated A549 cells also displayed significantly reduced CCL28 mRNA, despite stimulation with IL-1β or TNF-α ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 5). Levels of secreted CCL28 protein were also significantly reduced following pre-treatment with PDTC, when compared to cytokine treatment alone (Figs. 5C and F). These studies confirm that IL-1β induces high levels of CCL28 expression in A549 cells via an NFκB-dependent pathway and demonstrates that TNF-α behaves in a similar manner to IL-1β in these cells.

Table 1
Inflammatory mediators associated with infection or asthma do not upregulate CCL28 expression from airway epithelial-like cells

Stimulus[a]	Significant induction above background expression[b]	
	mRNA	Protein
Medium	–	–
CCL28	–	–
IL-4	–	–
IL-5	–	–
IL-13	–	–
IL-4 + IL-5	–	–
IL-4 + IL-13	–	–
IL-5 + IL-13	–	–
IL-4 + IL-5 + IL-13	–	–
SDF-1	–	–
TGF-β1	–	–
GMCSF	–	–
IL-12p40	–	–
LPS	–	–
TNF-α	+	+
IL-1β	+	+
TNF-α + IL-1β	+	+

[a] Human airway epithelial-like A549 cells were cultured in the presence or absence of inflammatory mediators at concentrations and times given in methods; IL-1β (10 ng/ml) is included as a positive control.

[b] Expression of CCL28 was compared to non-stimulated cells by quantitative real-time PCR (mRNA) following 6 h culture, or by CCL28 specific ELISA (protein) on culture supernatant after 24 h.

4. Discussion

We report the constitutive expression of CCL28 mRNA and protein by the A549 human airway epithelial-like cell line. Ex vivo, we have shown low levels of CCL28 mRNA to be expressed in healthy, non-asthmatic human control airway biopsy tissue, while induced sputum from healthy human volunteers also contained CCL28 protein at low levels. Biopsy tissue and induced sputum from sample patients with asthma or respiratory infection presented higher levels of CCL28. Unstimulated airway epithelium-like A549 cells expressed constitutive but low levels of CCL28 mRNA and protein. However, exposure to the pro-inflammatory cytokines IL-1β or TNF-α significantly increased CCL28 expression, whereas IL-4, IL-5 and IL-13 did not. TNF-α enhanced IL-1β induction of CCL28 protein but this effect was concentration dependent. Both cytokines induced phosphorylation of NFκB p50 and p65 in A549 cells and this correlated with increased CCL28 expression. Inhibition of NFκB p50–p65 activation by an antagonist correlated with reduced cytokine-mediated CCL28 expression confirming that CCL28 induction is NFκB-dependent.

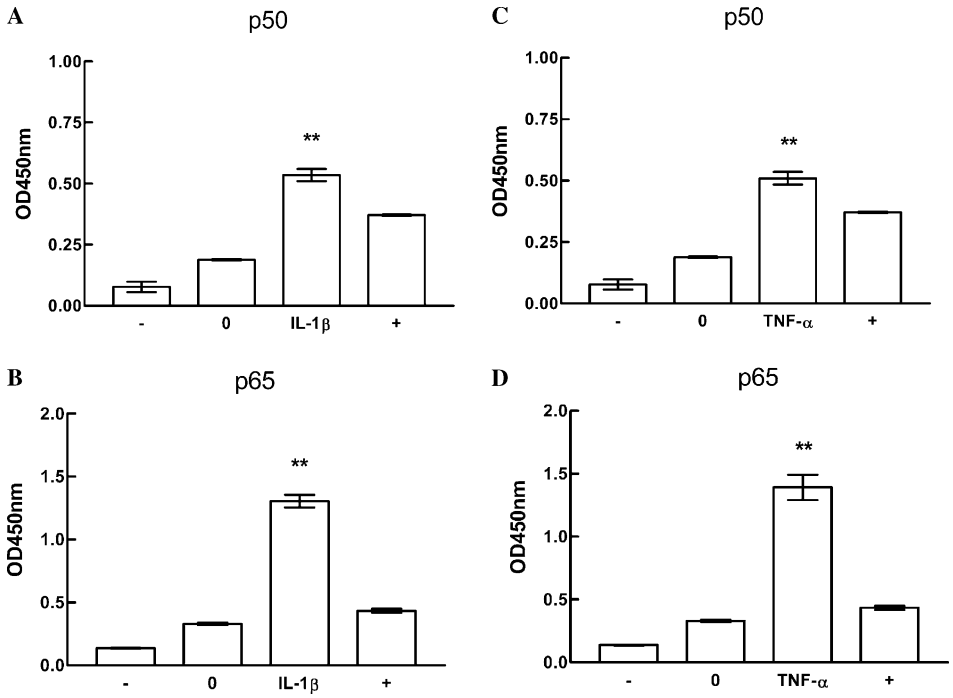

Fig. 3. IL-1β (A and B) and TNF-α (C and D) induce phosphorylation of NFκB sub-units in A549 cells. Exposure to IL-1β or TNF-α (10 ng/ml) induced significant activation of p50 (A and C) and p65 (B and D). Phosphorylated NFκB components were measured in nuclear extracts by phospho-ELISA following exposure of A549 cells to cytokine or from extracts of non-stimulated control cells (0). Nuclear extract from RAJI cells (+) was used as a positive control for comparison against background optical density (−). Absorbance values are expressed as mean optical density at 450 nm (±SEM) for duplicate samples from representative experiments performed three times. Statistical significance was measured relative to non-stimulated controls (0), $^{**}p < 0.01$.

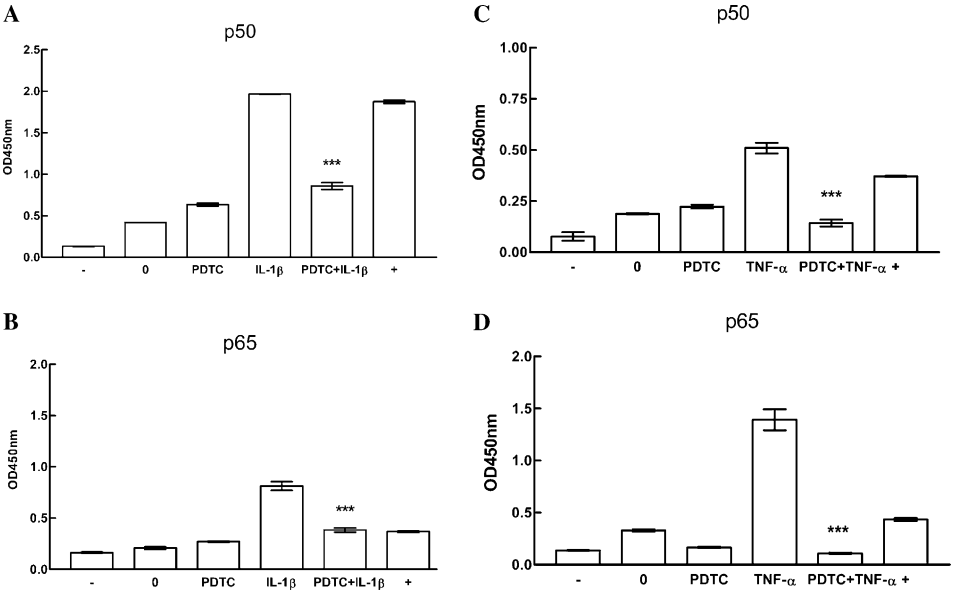

Fig. 4. IL-1β (A and B) and TNF-α (C and D) phosphorylation of p50 and p65 is inhibited by an NFκB antagonist. Cytokine-induced phosphorylation of p50 (A and C) and p65 (B and D) was measured by ELISA following exposure of A549 cells to nuclear extracts from non-stimulated control cells (0), or from cells cultured with combinations of the NFκB antagonist PDTC (100 μM) and /or IL-1β (10 ng/ml) or TNF-α (10 ng/ml). A nuclear extract from RAJI cells (+) was used as a positive control for comparison against background optical density (−). Absorbance values are expressed as mean optical density at 450 nm (±SEM) for duplicate samples from representative experiments performed at least three times. *** represents significant reduction from stimulated cells ($p < 0.001$ in each case).

Whilst there has been interest in the roles of CCL28 as a homeostatic chemokine with inflammatory roles in the gut, little is known of its regulation or involvement in mucosal immunity in the airways [44,45]. Similar to CCL25, CCL28 is capable of enhancing adhesion of IgA+ antibody secreting cells in the intestine, allowing increased extravasation

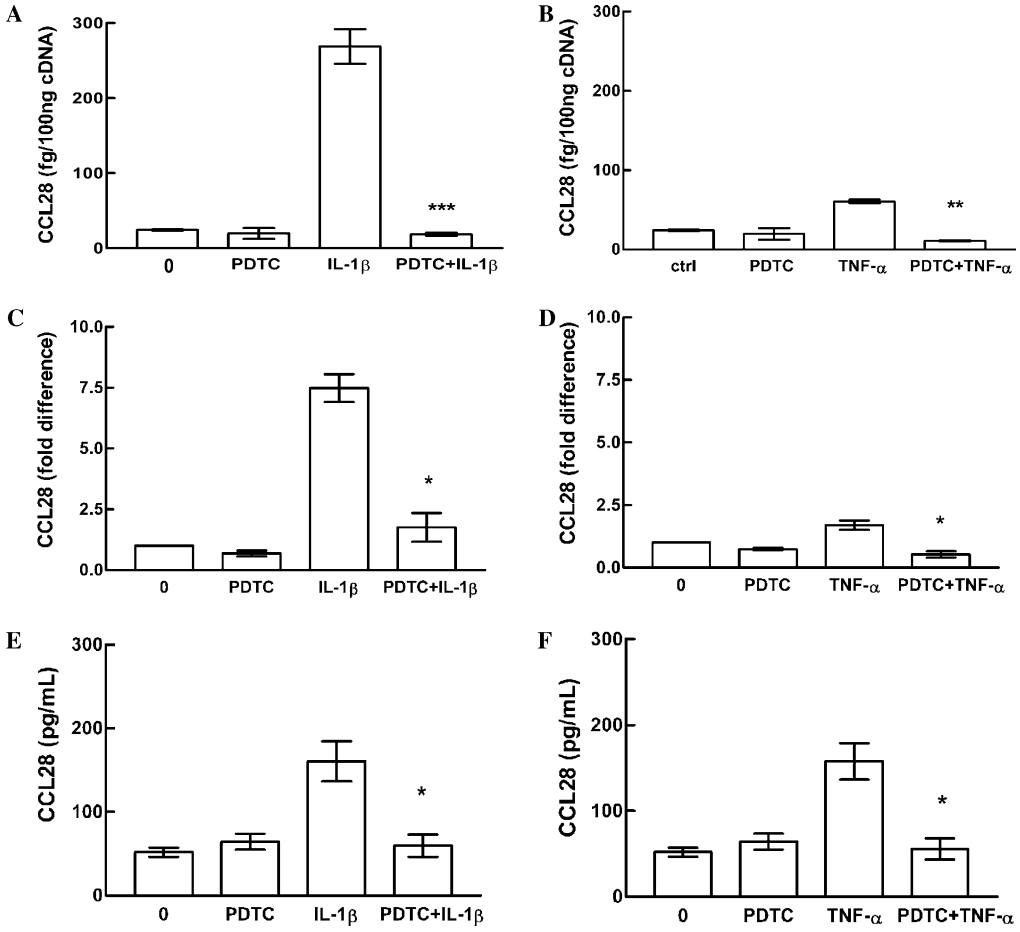

Fig. 5. Reduction of CCL28 expression following NFκB inhibition in vitro. A549 cells were exposed to the NFκB antagonist, PDTC (100 μM) 30 min prior to treatment for 6 h with 10 ng/ml IL-1β or 100 ng/ml TNF-α. Results were compared to cells treated with cytokine alone. CCL28 and GAPDH mRNA expression were measured by real-time quantitative PCR. Data were expressed in absolute quantities per 100 ng of cDNA (A and B) or normalised to GAPDH and expressed as a fold increase relative to control (C and D). CCL28 protein present in supernatant culture fluid was measured by ELISA (E and F). Results are from representative experiments, performed three times. Data are expressed as means (±SEM) for duplicate (mRNA) or triplicate (protein) samples.

of IgA to the intestinal lamina propria [45–47]. Furthermore, CCL28 present in mammary tissue plays a role in accumulation of IgA plasma cells during lactation and promotes the transfer of IgA-mediated immunity to the neonate [48]. CCL28 is also a chemoattractant for CLA+ T cells [1,12]. Thus, CCL28 is likely to play an important role in governing homeostasis and inflammation at mucosal sites. The constitutive expression of CCL28 mRNA and protein by human airway-derived A549 cells observed here is consistent with experiments by Pan et al., showing CCL28 mRNA in human bronchial epithelial cell lines [12]. Recently published work by John and colleagues and also from our laboratory, support a role for CCL28 in murine airway inflammation with low constitutive levels of CCL28 mRNA [17,39]. Our detection of CCL28 in healthy human airway tissue (mRNA) and in sputum from healthy volunteers supports a role for CCL28 in the homeostatic and innate mechanisms operating in human respiratory tract. The full nature of this role is yet to be elucidated, but as CCL28 is known to exhibit anti-microbial activity [41], it is likely that the homeostatic function of CCL28 in sputum is in part to provide an innate or "frontline" immune response at the mucosal surfaces of the airways.

The similarity between CCL28 and the keratinocyte CCR10 ligand CCL27 [1], suggested that there may be an additional inflammatory role for this chemokine in the airways. In the present study, we show that a variety of inflammatory mediators including IL-4, IL-5 and IL-13 do not induce high levels of CCL28 expression, nor does CCL28 act in an autocrine fashion (Table 1). Taken together with data suggesting that CCL28 is chemotactic for eosinophils [17,46] this suggests that CCL28 is acting earlier in airway inflammation than these mediators. This is consistent with the observation that CCL28 is produced by airway epithelium, acting not simply as an inert barrier, but as an important signalling cell in the early events of airway inflammation [21]. We show that IL-1β and TNF-α are capable of upregulating CCL28 expression from human airway epithelial cells. These cytokines are produced by circulating macrophages and monocytes following exposure to microbial stimuli, and are associated with bacterial

infections that exacerbate asthma [23,42]. Recently, Ogawa and colleagues have demonstrated that IL-1α alone or in combination with a variety of microbial products, upregulated CCL28 in human colon epithelial cell lines [26]. While surface expressed IL-1α shares some functions with secreted IL-1β, the latter has a more defined role in airway pathology. It has been implicated in asthma through induction of the CXC chemokine RANTES [10,49]. IL-1β can enhance eosinophil trafficking to the lung by increasing the expression of adhesion molecules on respiratory endothelial cells and TNF-α acts in similar fashion [49]. Both cytokines also upregulate the expression of a variety of genes that contribute to the alteration of airway smooth muscle during the asthmatic response [50,51]. Thus, these data support a role for IL-1β or TNF-α produced as a result of microbial insult inducing high levels of CCL28 and exacerbating asthma. We have observed elevated CCL28 expression in inflamed human airway tissue when compared to normal healthy control biopsy tissue. Also induced sputum from representative asthmatic or infected patients contained higher quantities of CCL28 protein than control volunteers. These data are merely indicative and to support the present in vitro study but similar observations have been made in animal models of asthma, where constitutive CCL28 is upregulated following allergen challenge [17,39].

Our observation that TNF-α can upregulate CCL28 in a human airway epithelial cell line is consistent with previous work from our laboratory demonstrating that TNF-α increases CCL28 mRNA from the murine airway epithelial cell line, MLE12 [39]. However, it is in contrast to the observations of Ogawa using colon derived human cells [26]. One possibility was that CCL28 in airway epithelium cells was regulated in a different manner to that in intestinal epithelium, suggestive of a functionally distinct, tissue-specific role for the chemokine. However, our demonstration that both IL-1β and TNF-α induce expression through an NFκB-dependent pathway would argue against this. Nonetheless, we did observe that TNF-α-mediated induction of CCL28 tended to be more sensitive to concentration and timing effects and typically resulted in lower chemokine production when compared to IL-1β.

IL-1β and TNF-α can both initiate NFκB activation through various signalling pathways to allow phosphorylation of Rel protein species and the formation of active transcription factor dimers within the nucleus [43]. In this study, we find that IL-1β and TNF-α activate similar patterns of NFκB phosphorylation, predominantly the p50–p65 heterodimer. This data is the first to show TNF-α and IL-1β regulation of human CCL28 and to show that this pathway is dependent on p50–p65 activation. However, inhibition of NFκB phosphorylation did not result in complete ablation of cytokine-induced CCL28 mRNA or protein (Fig. 5). Whilst low-level constitutive production may account for some residual expression, it also suggests that other signalling pathways contribute to CCL28 regulation. LPS was not capable of inducing epithelial production of CCL28 in this system, however, we have previously shown that oncostatin-M, can induce murine CCL28 from airway epithelial cells and it is possible that IL-6-like cytokines or other inflammatory mediators induce CCL28 via NFκB-independent pathways in humans.

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