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CCR3 induced-p42/44 MAPK activation protects against staurosporine induced-DNA fragmentation but not apoptosis in airway smooth muscle cells

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Summary

Background Chemokine receptors (CCRs) are expressed on airway smooth muscle (ASM) cells. As their ligands are present in the airways in asthma, we hypothesized that ASM CCR activation could promote the increase in ASM mass seen in patients with chronic asthma.

Objective To determine which CCRs are expressed by ASM cells and their potential functional relevance to the chronic airway changes seen in asthma.

Methods CCR expression in primary ASM cell cultures and airway biopsies from patients with and without asthma was examined by RT-PCR, fluorescence-activated cell sorting and immunohistochemistry. ASM p42/44 MAPK activity, proliferation, migration and apoptosis were examined by western blotting, thymidine incorporation, transwell assay and TUNEL assay respectively.

Results CCR3 was the most frequently expressed CCR protein and was present on $79 \pm 14\%$ of cells. CX3CR1 and CXCR6 were present on 6% and 11% of cells respectively. CCR3 ligands CCL11 and CCL24 caused rapid activation of p42/44 MAPK but not Akt. CCR3 activation did not affect ASM proliferation, migration or VEGF secretion. DNA fragmentation detected by TUNEL staining could be induced by staurosporine and Fas activation although only Fas activation resulted in caspase 3 cleavage. CCL11 and CCL24 protected ASM cells against DNA fragmentation dependent upon p42/44 MAPK activity only via caspase 3 independent pathways. CCR3 was expressed in the smooth muscle and epithelium in the airways of patients with and without asthma. Smooth muscle cell DNA fragmentation in the airways of patients with stable asthma and controls was very uncommon.

Conclusions and Clinical Relevance CCR3 is strongly expressed by ASM cells *in vitro* and *in vivo*. Protection against cell death by CCR3 activation is dependent on p42/44 MAPK but does not affect caspase 3 mediated apoptosis.

Keywords airway remodelling, asthma, chemokine

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Introduction

Patients with asthma develop airway obstruction at a faster rate than those without airways disease. This decline in lung function is thought to be due to a combination of structural changes collectively termed airway remodelling which occur in response to different aspects of the disease including chronic inflammation and possibly airway contraction [1, 2]. In asthma, air-

way remodelling is categorized by airway smooth muscle (ASM) hyperplasia, extra-cellular matrix (ECM) deposition, mast cell infiltration of ASM and epithelial metaplasia [3–5]. Airway remodelling contributes to airway hyperresponsiveness and airflow obstruction which result in worsening symptoms, increased use of medication and health care resources [1, 5]. ASM is a key player in airway remodelling [6, 7] being increased in mass [8] and capable of producing growth factors

[9–11], ECM components [12], pro and anti-inflammatory mediators [13]. The increase in ASM mass observed may be the result of one or more processes including, enhanced proliferation, reduced cell death/protection from apoptosis and possibly the recruitment of circulating fibrocytes [14]. Our group's overarching hypothesis is that inflammation in the airways of patients with asthma generates an environment which supports remodelling. Consistent with this, we have previously shown that proteins over-expressed in the asthmatic airway can affect ASM synthetic function and migration [15] and that the ECM proteins fibronectin, laminin and collagens I and IV can protect ASM cells from apoptosis [16]. In addition to structural proteins we reasoned that mediators causing or released in response to inflammation, would also contribute to airway remodelling. Recently various groups have shown that ASM cells express chemokine receptors including CCR3 and that CCR3 expression [17] and its ligands CCL11 (eotaxin-1), CCL24 (eotaxin-2), MCP-3, MCP-4 and RANTES are increased in asthma and associated with bronchial hyperresponsiveness [18, 19]. Gene deletion of CCR3 in models of allergic inflammation results in impaired eosinophil recruitment and reduced airway inflammation [20–22]. Although the contribution of ASM hyperplasia to remodelling is well recognized the main research focus has been towards ASM proliferation rather than protection against cell death [23]. Death of ASM cells via apoptosis has been reported in response to Fas cross-linking, serum withdrawal and simvastatin treatment [24–26]. Death of ASM by necrosis due to oxidative stress has also been reported *in vitro* [27]. The significance of these processes in human asthma and whether or not protection against ASM cell death could contribute to the enhanced ASM mass in asthma is not certain. Herein we use a combination of *in vitro* and *ex vivo* methods to address if ASM chemokine receptor signalling has the potential to impact upon these processes.

Methods

Airway smooth muscle cell culture

Primary human ASM cells were derived from bronchial smooth muscle obtained from patients undergoing surgical resection for carcinoma. ASM was dissected free from epithelium, digested with collagenase and sub-cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Calf Serum (FCS) and 2 mM Glutamine as previously described [28]. The resulting cells had the characteristic 'hill and valley' appearance by light microscopy and were >90% positive for α -smooth muscle actin and desmin by immunofluorescence. All cells were used at fourth passage or less.

Bronchial biopsies

Endobronchial biopsies were obtained from patients with asthma or control patients undergoing bronchoscopy for other reasons. Patients had physician diagnosed asthma at BTS stage II or III, without history of an exacerbation or change in therapy for at least 6 weeks, underwent spirometry and fibre-optic bronchoscopy according to standard procedures [29]. Up to six endobronchial biopsies were taken from a subcarina, fixed in formalin and paraffin embedded for immunohistochemistry. Control patients had no history of asthma, were not current smokers and had histologically normal airways. The use of both ASM cells and bronchial biopsies was approved by the Nottingham local research ethics committee and all patients gave informed consent.

Chemokine receptor RT-PCR

Primers were designed to target all known chemokine receptors and have been described previously [30]. RT-PCR was performed in primary ASM cells from three separate donors. All reactions were repeated from three independent cultures and determined as positive or negative when visualized using ethidium bromide after agarose gel electrophoresis. PCRs were run on a Tetrad PTC200 (MJ Research, Ramsey, MN, USA). The PCR programme was as follows: heat lid to 94°C, denature for 30 s at 94°C, anneal for 30 s at 60°C and elongate for 30 s at 72°C, for 30 cycles. Genomic DNA was used as a positive control.

Fluorescence activated cell sorting (FACS)

ASM cells from three separate donors were grown in T75 cm² tissue culture flasks, removed using enzyme-free cell dissociation media (Sigma), pelleted, re-suspended in ice cold 3% BSA/PBS and fixed in 2% paraformaldehyde. Cells were incubated for 30 min with antibodies against CCR1, CCR7, CXCR4 (MAbs 171, 172, and 173), CXCR6 and rat monoclonal anti-human CCR3 (all from R&D Systems, Oxford, UK) and rabbit polyclonal anti-CX3CR1 (Abcam, Cambridge, UK). Rat anti-human CMKLR1 antibody and rat IgG2a isotype control were obtained from Dr Brian Zabel (Stanford University, Stanford, CA, USA). Rabbit, rat and mouse IgG isotype were used as controls. After incubation with the primary antibody, cells were washed in BSA/PBS and incubated with the appropriate Alexa-Fluor 488 conjugated secondary antibody (Invitrogen, Nottingham, UK). Flow cytometry was performed using a Coulter Epics Altra HyPerSort System (Beckman Coulter, High Wycombe, UK) and data analysed using WinMDI software (Scripps Research Institute, La Jolla, CA, USA).

Western blotting

ASM cells were incubated with recombinant human PDGF-BB, CCL11, CCL24, CCL27, CCL28, CXCL16 and CX3CL1 (Peprotech, London, UK) for 5–120 min, lysed and subjected to SDS-PAGE/western blotting as described [31]. Filters were stripped for reprobing using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Pierce Protein Research, Cramlington, UK). Antibodies used were anti-total p42/44, phospho-p42/44, total Akt and phospho-Akt^(Ser473), anti-Caspase 3 (Cell Signaling Technologies, Danvers, MA, USA). Where indicated, results were quantitated by densitometry of at least three independent experiments using Image J version 1.40g (Wayne Rasband, NIH, Bethesda, MD, USA) and means compared using one-way ANOVA with a Dunnett's post test correction in Graphpad Prism 4 (GraphPad Software, San Diego, CA, USA).

Immunohistochemistry

Sections were de-waxed in HistoClear (R.A. Lamb, Fisher Scientific, Loughborough, UK) subjected to antigen retrieval using citrate buffer and endogenous peroxidases blocked using hydrogen peroxide as described [31]. Rat monoclonal anti-human CCR3 (R&D Systems) was used at 1 : 100 diluted in blocking reagent and incubated overnight at 4°C. Antibody binding was identified using a Vectastain ABC kit for rat IgG (Vector Laboratories, Peterborough, UK) according to the manufacturer's instructions and sections were counterstained with haematoxylin.

Proliferation

ASM proliferation was examined by tritiated thymidine incorporation as described [15]. Briefly cells were grown to confluence, serum depleted for 24 h and treated with 10–500 ng/mL of recombinant chemokine for 24 h. Fetal bovine serum (10%) was used as a positive control stimulus. Experiments were run in triplicate wells with at least three independent experiments performed. Data were compared using one-way ANOVA with a Dunnett's post test in Graphpad Prism 4.

Migration

Migration assays were performed as described previously [15]. Recombinant chemokines (200 ng/mL) were placed in serum-free media in the lower chamber of a Transwell insert (Invitrogen) with 8 µm pores. PDGF-BB (10 ng/mL) was used as a positive control.

VEGF ELISA

Production of VEGF after chemokine stimulation was measured by specific ELISA (R&D systems). ASM cells

were incubated with recombinant chemokines for 24 h in serum-free medium and VEGF production from three independent ASM donors measured as described [9].

Cell death assays

For the detection of apoptosis DNA fragmentation was assessed using a Fluorescein *In Situ* Cell Death Detection Kit (Roche Diagnostics, Burgess Hill, UK) according to the manufacturer's instructions. For cell culture experiments, cells were plated in eight well chamber slides and serum-starved. Fas-dependent cell death was induced using an activating anti-Fas antibody (clone CH11; Millipore Corporation, Watford, UK) at a concentration of 1 µg/mL for 24 h under serum free conditions. After 24 h in serum free medium cells were treated with staurosporine or anti-Fas and recombinant chemokines and in some experiments, the p42/44 MAPK inhibitor UO126 (10 µM) or vehicle for 18 h. Cells were then fixed with 4% formaldehyde in PBS for 20 min at room temperature, permeabilized with 0.1% Triton X-100 (Sigma-Aldridge Ltd, Gillingham, UK) for 10 min and treated with 50 µL TUNEL reaction mixture in a dark, humidified atmosphere for 60 min at 37°C. Positive controls were treated with DNase I for 10 min at room temperature prior to the addition of the TUNEL reaction mixture. Negative controls include samples treated with staurosporine but with the addition of label solution without terminal transferase, and samples treated with serum-free medium instead of staurosporine. After incubation, cells were stained for 2 min with 1 µg/mL DAPI. The solution was replaced with PBS and cells viewed under an inverted wide field fluorescence microscope at 200× magnification (Nikon Diaphot 300, Nikon Instruments Europe BV, Kingston, UK). Six random fields from triplicate experiments were counted using an Insight QE camera by an observer blind to the experimental details. Analysis was performed using one-way ANOVA with a Dunnett's post test correction using Graphpad Prism 4.

For measurement of smooth muscle DNA fragmentation *in vivo*, tissue sections from endobronchial biopsies from seven patients with asthma and seven control patients were processed for immunohistochemistry as described above. Smooth muscle bundles were identified by staining with anti α -smooth muscle actin (Sigma, Poole, Dorset, UK). In the same sections, apoptosis was examined using the Fluorescein *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's instructions for immunohistochemistry and nuclei were identified by staining with DAPI. Treatment of tissue sections with DNase I prior to addition of the TUNEL reagent was used as a positive control. Tissue sections were viewed under an inverted wide field fluo-

rescence microscope at 200 $\times$ magnification (Nikon Diaphot 300).

Results

Chemokine receptor expression in airway smooth muscle

We initially examined chemokine receptor expression in ASM cells by PCR. In ASM cultures from three different normal donors we observed that the transcripts for CCR1, CCR3, CCR7, CCR9, CCR10, CXCR6, CXCR7 and CX3CR1 were present in the majority of ASM cells examined (Fig. 1a). Chemokine receptors of relevance to ASM and asthma were selected for study at the protein level based on previous literature. CCR3 was the

most frequently expressed receptor protein and was present on the surface of 79% of cells by flow cytometry. CXCR6 and CX3CR1 proteins were present on 11% and 7% of cells respectively. Despite the presence of the transcript CCR1, CCR7 and CMKLR1 proteins were detected at low levels or not at all (Fig. 1b).

Airway smooth muscle chemokine receptors signal via p42/44 MAPK

To determine if chemokine receptor expression had functional consequences we examined the activity of p42/44 MAPK, a key proliferative molecule for ASM cells [32] which is downstream of CCR3 in other cell types [33] and Akt a central regulator of apoptosis

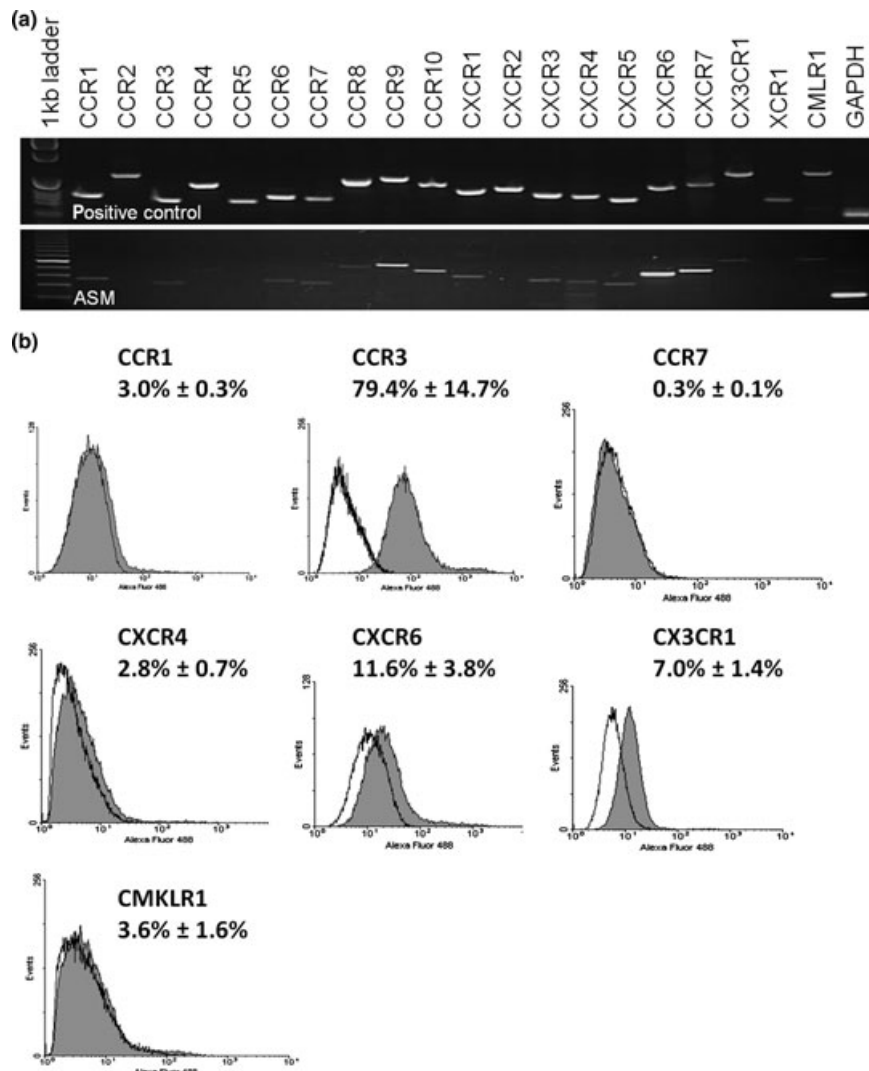


Fig. 1. Expression of chemokine receptors in airway smooth muscle (ASM). (a) Expression of all chemokine receptor transcripts by RT-PCR in control genomic DNA (top panel) and mRNA from primary ASM cells (lower panel). Figure shown is representative of one of three donors each repeated three times. (b) FACS analysis of selected chemokine receptors in a representative ASM culture. Figures show mean $\pm$ SE of three independent donors.

and survival. Addition of the CCR3 ligands CCL11 (eotaxin 1) and CCL24 (eotaxin 2) resulted in significant and robust phosphorylation of p42/44 MAPK with a peak activation at 5 min. The magnitude of the response was similar to that achieved by PDGF which is known to activate p42/44 MAPK in ASM. Neither agonist resulted in Akt phosphorylation (Fig. 2). CCL28 (CCR3 and CCR10 agonist) also caused p42/44 MAPK activation by 5 min. CXCL16 (CXCR6 agonist) and CX3CL1 (CX3CR1 agonist) caused p42/44 MAPK activation with a peak at 60 min. CCL27

Table 1. Fold increase in baseline p44/42 MAPK activity after incubation with chemokine ligands (200 ng/mL)

Chemokine	CCL11	CCL24	CCL28	CCL27	CXCL16	CX3CL1
Incubation (min)	Phospho-p44/42 activity (fold unstimulated)					
0	1	1	1	1	1	1
1	0.74	0.85	0.91	0.32	0.31	0.56
5	5.45**	3.06**	2.37	0.90	1.08	2.21
20	5.22**	2.57**	2.71*	0.91	1.99	2.09
60	4.91**	3.02**	2.67*	1.10	2.82	2.20
120	2.27*	0.63	1.99	0.43	1.38	1.67

* $P < 0.05$, ** $P < 0.001$.

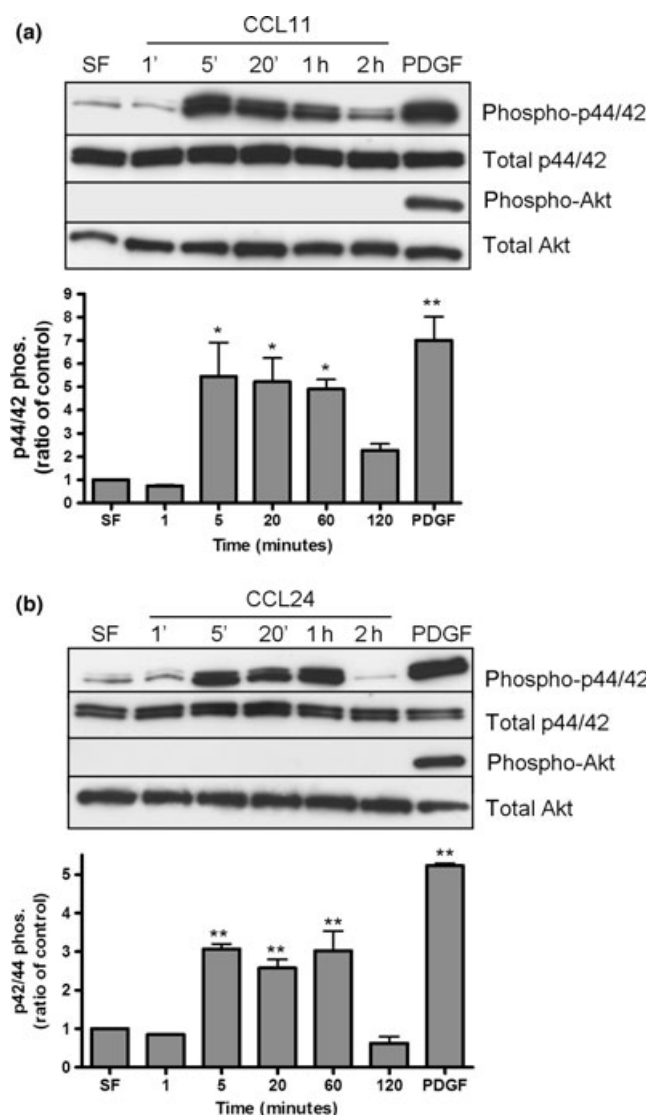


Fig. 2. Activation of p42/44 MAPK but not Akt by CCR3 ligands. Primary airway smooth muscle (ASM) cells were treated with recombinant CCL11 or CCL24 and p42/44 MAPK and Akt phosphorylation measured by western blotting. Graphs show mean $\pm$ SE for three independent primary cultures from separate donors. PDGF (10 ng/mL for 20 min) is used as positive control. * $P < 0.05$, ** $P < 0.001$.

(CCR10 agonist) had no effect on either p42/44 MAPK or Akt. CXCL16, CX3CL1 and CCL27 also did not cause Akt phosphorylation (Table 1; Fig. S1).

Chemokines do not significantly affect airway smooth muscle proliferation, migration or VEGF production

To determine if activation of these receptors affected ASM biology we studied the effect of chemokine receptor activation on processes of relevance to airway remodelling: namely proliferation, migration and synthetic function. The CCR3 agonist CCL11 (eotaxin 1) had no significant effect on ASM proliferation, migration or VEGF secretion (Fig. 3). In addition CCL5, CX3CR1 and CXCL16 also did not stimulate proliferation or migration of ASM (Figs S2 and S3). As CCR3 has previously been reported to have a modest effect on these processes [17] we then tested the hypothesis that the lack of effect seen in our system may be due to protease-mediated degradation of chemokines. Matrix metalloproteinases (MMPs) are expressed by ASM cells [12] and can influence the activity of chemokines by cleavage of the terminal receptor-binding domain resulting in receptor antagonists [34]. The membrane-bound serine protease di-peptidyl peptidase IV (DPPIV) can also cleave chemokines including CCL11 [35, 36]. We therefore incubated ASM cells with recombinant chemokines in the presence of the protease inhibitors ilomastat (matrix metalloproteinase inhibitor, 10 μ mol), KR62436 (DPPIV inhibitor, 10 μ mol), AEBSF (serine protease inhibitor, 100 μ mol) and a broad spectrum protease inhibitor cocktail (Roche Complete protease inhibitor cocktail, CPI, manufacturer's recommended dilution). The response to serum was used as a positive control. None of the protease inhibitors increased the level of p42/44 MAPK phosphorylation in response to CCL11 (Fig. 3). Similar results were seen with CCL24, CCL28, CX3CL1 and CXCL16 (Fig. S4).

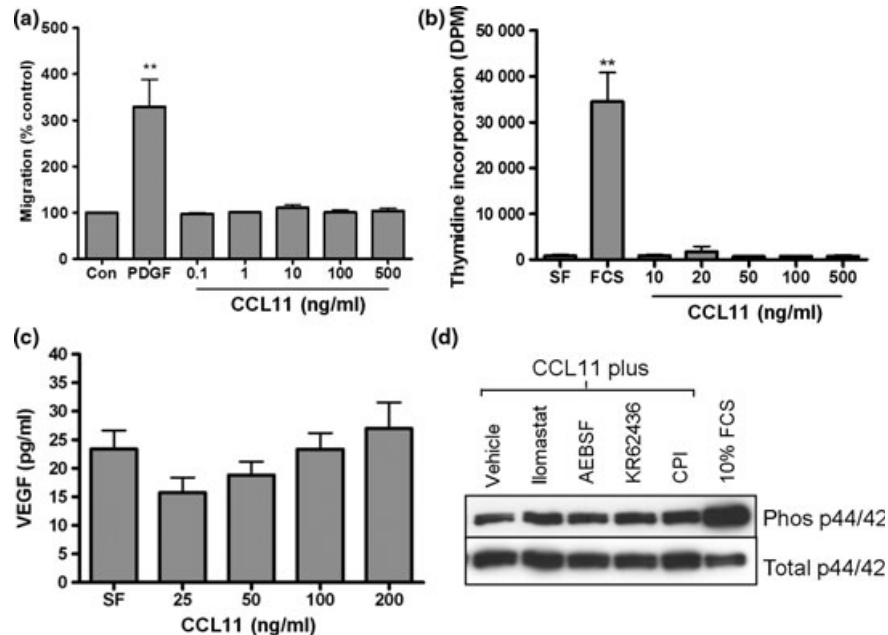


Fig. 3. Chemokines do not affect airway smooth muscle (ASM) proliferation, migration or synthetic function. Primary ASM cells were treated with various concentrations of recombinant CCL11. (a) Migration was measured in a transwell assay with PDGF (10 ng/mL) as positive control. (b) Proliferation was measured by thymidine incorporation (disintegrations per minute, DPM) with fetal calf serum (FCS) as positive control. (c) VEGF secretion was measured by ELISA. All graphs show mean $\pm$ SE of three independent primary cultures from separate donors. (d) ASM cells were pre-incubated for 24 h with protease inhibitors selective for MMPs (ilomastat, 10 μ mol), serine proteases (AEBSF, 100 μ mol), di-peptidyl peptidase IV (KR62436, 10 μ mol) or a broad spectrum protease inhibitor cocktail (Roche Complete protease inhibitor, CPI, manufacturer's recommended dilution). The effect of protease inhibition on CCL11 induced p42/44 activation was then measured by phospho-western blotting.

Effect of CCR3 ligands on DNA fragmentation in airway smooth muscle *in vitro*

As one of the cardinal features of airway remodelling in asthma is an increase in ASM mass which could arise by enhanced ASM survival, we examined if chemokine receptor activation could result in protection from cell death. The effect of pre-incubation of ASM cells with recombinant chemokines (200 ng/mL) for 24 h on ASM DNA fragmentation in response to staurosporine, an anti-Fas cross-linking antibody and serum deprivation was examined. As observed in other studies serum deprivation resulted in apoptosis in only 1–2% of cells and this method was not used in further experiments [26]. Addition of the CCR3 ligands CCL24 and CCL11 resulted in a 40% ($P < 0.05$) and 30% (not significant after Bonferroni correction) reduction in DNA fragmentation in response to staurosporine over vehicle control respectively (Fig. 4a). CCL28, CX3CL1 and CXCL16 had no significant effect.

As CCR3 activates p42/44 MAPK, we then used the p42/44 inhibitor U0126 to test if the protection against DNA fragmentation by CCL24 was dependent upon this pathway. U0126 alone had no effect on DNA fragmentation but completely blocked the protective effect of CCL24 (Fig. 4b).

As DNA fragmentation may occur as a result of apoptosis and necrosis we also used an anti-Fas cross-linking antibody to induce DNA fragmentation via a classical caspase-mediated apoptosis pathway. The anti-Fas antibody induced similar levels of DNA fragmentation as assessed by TUNEL staining to staurosporine (Fig. 5a) although where anti-Fas induced caspase 3 cleavage, staurosporine did not (Fig. 5b). Interestingly neither CCL11 nor CCL24 prevented anti-Fas induced apoptosis, nor reduced caspase 3 cleavage (Fig. 5c).

DNA fragmentation in airway smooth muscle is very low *in vivo*

To determine if CCR3 is expressed in ASM *in vivo* and could affect ASM DNA fragmentation in human asthma we then examined the expression of CCR3 in bronchial biopsies from seven patients with asthma and seven control patients. CCR3 was expressed in the bronchial smooth muscle and epithelium of all patients (Fig. 6). We then performed *in situ* staining for DNA fragmentation in the smooth muscle bundles of these bronchial biopsies. DNA fragmentation, as defined by staining of α -smooth muscle actin positive regions with TUNEL label was highly infrequent and not quantifiable in patients both with and without asthma (Fig. 7).

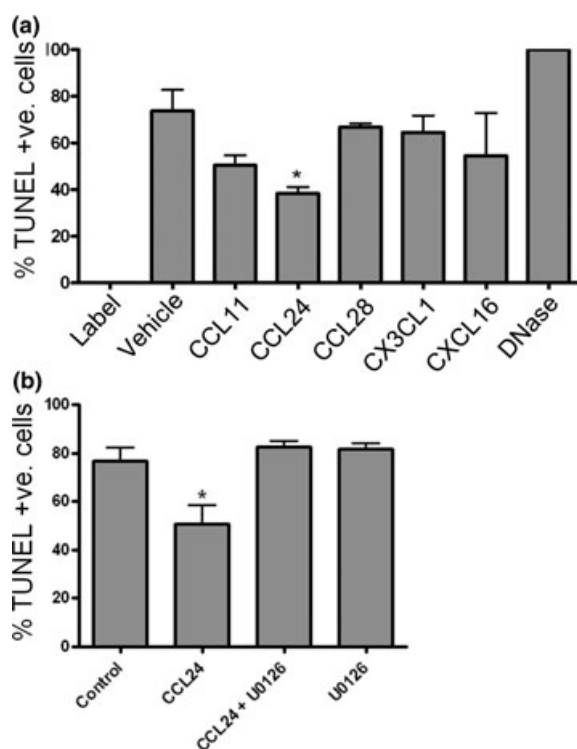


Fig. 4. CCR3 ligands protect airway smooth muscle (ASM) cells from staurosporine induced DNA fragmentation. (a) Primary ASM cells were treated with staurosporine (1 μ M) and recombinant chemokines or vehicle control. 'Label' is the negative control and 'DNase' the positive control, 'vehicle' is the an equal volume of diluents for the chemokines. (b) CCL24 prevents ASM DNA fragmentation induced by staurosporine. Protection against DNA fragmentation is abolished by the p42/44 MAPK inhibitor U0126. In both experiments graphs show mean $\pm$ SE of three independent experiments from different donors. * P < 0.05 vs. vehicle.

Discussion

We have shown that CCR3 is expressed by ASM cells, both in culture and within the airway smooth muscle bundles of patients. Activation of CCR3 by CCL11, CCL24 and CCL28 (eotaxins 1, 2 and 3) results in strong activation of p42/44 MAPK and protection of ASM cells from caspase 3 independent DNA fragmentation induced by staurosporine but not apoptosis induced by Fas cross-linking. Protection against apoptosis by eotaxin has been described in eosinophils, mast cells and recently cancer cells [33]. One report, published very recently, has described protection against apoptosis *in vitro* where eotaxin at concentrations ranging from 0.1 to 100 ng was associated with a 0.5% absolute reduction in apoptosis in ASM *in vitro* [26]. Our data show that Fas activation induces apoptosis to a greater degree than serum withdrawal in ASM and likely through different pathways, only some of which may be eotaxin sensitive. Furthermore, our findings suggest that DNA fragmentation in

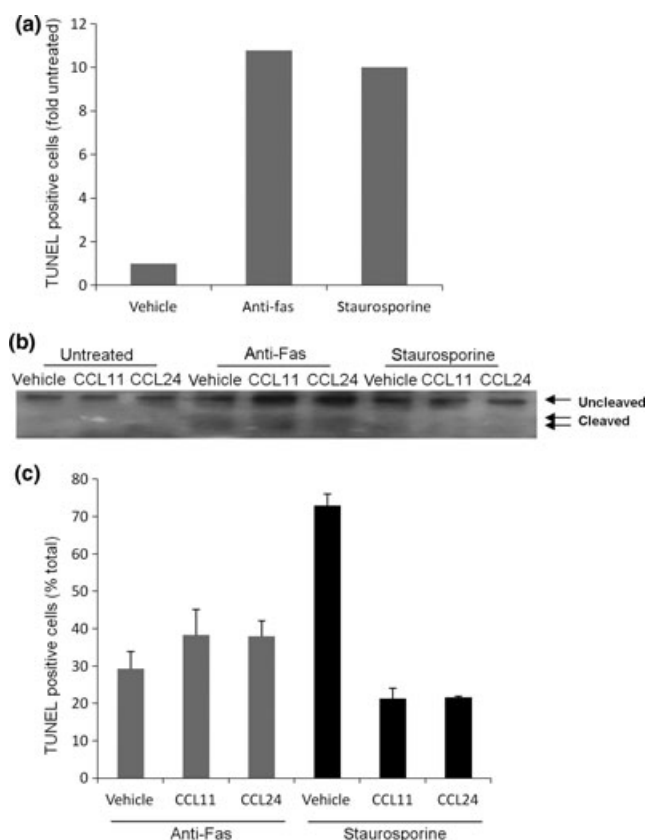


Fig. 5. CCL11 and 24 protect against staurosporine but not anti-Fas-mediated DNA fragmentation. (a) Both Fas activation and staurosporine treatment result in DNA fragmentation as measured by TUNEL. (b) Fas activation but not staurosporine caused caspase 3 cleavage which was not attenuated by treatment with CCL11 or CCL24. (c) CCL11 and CCL24 reduced DNA fragmentation caused by 10 nM staurosporine, but not by anti-Fas treatment.

ASM can occur through both caspase dependent and independent pathways which are likely to represent not only apoptosis but also necrosis.

Under appropriate circumstances, protection of ASM death either by apoptosis or necrosis could provide a mechanism which contributes to the increased ASM mass seen in airway remodelling. In the airways of patients both with and without mild stable asthma, we observed that levels of smooth muscle cell DNA fragmentation were almost undetectable. Others have shown that human ASM apoptosis *in vitro* does not vary between patients with and without asthma [37]. In a murine airway challenge model, smooth muscle apoptosis was detectable *in vivo* in control animals: in those subjected to airway antigen challenge there was a four-fold reduction in DNA fragmentation associated with an increase in smooth muscle mass [38]. In addition to species differences, various factors may account for these contrasting findings. In the mouse model, a strong and reproducible airway challenge was mounted which

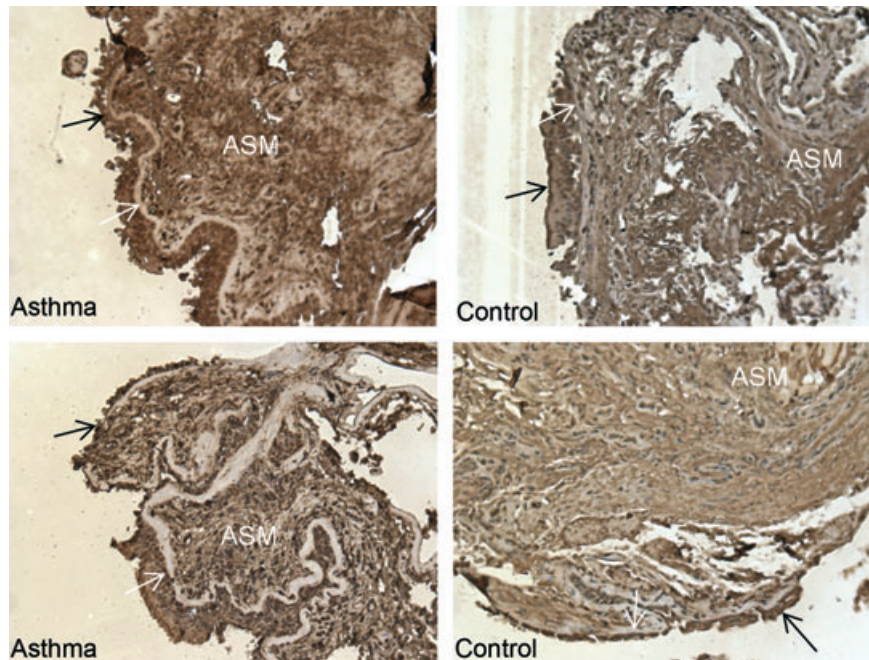


Fig. 6. Expression of CCR3 in the airways of patients with and without asthma. Immunohistochemical staining of CCR3 in representative airway biopsies of two patients with asthma and two control patients. CCR3 is represented by brown staining. Black arrow denotes the epithelium, white arrow subepithelial basement membrane, ASM denotes airway smooth muscle bundles.

resulted in increased smooth muscle mass. *In vitro* studies have implicated neutrophil elastase and TNF- α as inducers of ASM apoptosis [24, 39], produced by neu-

trophils and lymphocytes, respectively, which are released during exacerbations of asthma. It is possible that ASM apoptosis occurs predominantly during inflammatory stress such as bronchial infections or other pro-inflammatory stimuli and is mainly limited to asthma exacerbations. Our patients had relatively mild asthma and were free from exacerbations at the time of study. Studies examining patients with severe asthma or during exacerbations are difficult to perform due to patient safety concerns but may be required to determine the significance of our observations in human asthma.

Activation of p42/44 MAPK by chemokines was a mechanism common to other chemokine receptor/ligand pairs including CX3CL1/CX3CR1 and CXCL16/CXCR6. Although these chemokines had a significantly longer time to peak p42/44 activity than the CCR3 agonists; possibly suggesting an indirect mechanism of activation. The CCR10 agonist CCL27 had no significant effect upon p42/44 activation. As CCL28 activates CCR10 in addition to CCR3 this observation suggests that the main action of CCL28 in ASM is via CCR3. Activation of p42/44 was required for protection of ASM from DNA fragmentation induced by staurosporine, however, not all chemokines which activated p42/44 had this activity suggesting that although p42/44 MAPK activity is required, it is not sufficient, for protection against cell death in ASM. In many cases protection against cell death is mediated via Akt, how-

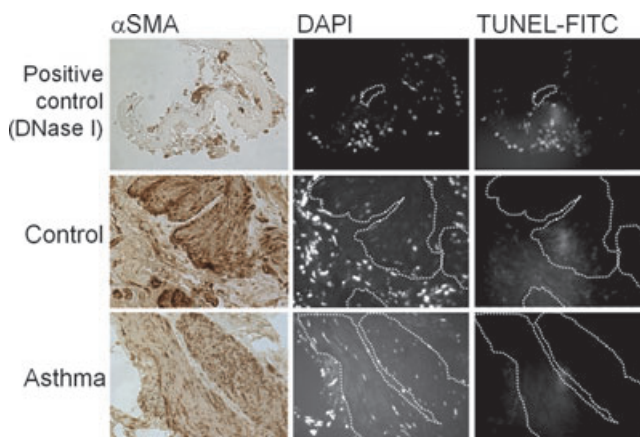


Fig. 7. Low levels of airway smooth muscle (ASM) apoptosis in the airways of patients. Airway biopsies were stained for α -smooth muscle actin (α -SMA) to identify ASM cells and nuclei counterstained with DAPI. In the same sections, a TUNEL label linked to FITC (TUNEL-FITC) was used to identify apoptotic cells. Slides were photographed three times with appropriate imaging systems for each label. Representative patients from the seven with asthma and seven controls are shown. The positive control has been incubated with DNase I. For clarity, α -SMA positive ASM, not visualized on the DAPI and FITC sections are enclosed within broken white lines. In the sections shown, no TUNEL positive nuclei are located in the α -SMA positive regions.

ever, none of the chemokines examined activated Akt. In our system, p42/44 MAPK activation inhibits staurosporine-induced cell death. The mechanism of this finding is not clear: although p42/44 MAPK inhibition protects against hydrogen peroxide induced cell death in fibroblasts and necrosis in neurotrophin-treated cortical neurons [40, 41].

We have shown that ASM cells express CCR3, CXCR6 and CX3CR1 proteins but a larger number of mRNAs. This difference may be due to PCR detecting basal transcription of mRNAs which are rapidly degraded during unstimulated conditions. The expression of these chemokine receptor transcripts may be under tight regulation but can be rapidly induced under stress conditions such as hypoxia [42, 43] or stimulation with TNF- α [44]. Protection of mRNA from degradation may be a mechanism by which chemokine receptors can be rapidly expressed under certain conditions. In our primary ASM cells, we could not detect the expression of CCR1, CCR7, CXCR4, and CMKLR1 on >3% of cells. To date, ASM cells have been shown to express the chemokine receptors CXCR1, CXCR2 [45], CCR1 [46], CCR3 [17] and CCR7 [47, 48]. Variation between these studies and our own could be explained by heterogeneity between donors, differences in the isolation and expansion protocols, number of passages and the detection methods used.

Although we have observed changes in cell survival in ASM cells we did not observe significant changes in migration, proliferation or VEGF secretion, which sug-

gests that chemokines alone are insufficient to fully induce synthetic and functional responses in these cells. Where studies have shown significant changes in the behaviour of ASM in response to chemokines, these changes are often small [17, 45, 47]. We hypothesize that in order for chemokines to fully promote a functional response in normal and disease-related ASM cells additional environmental factors are required, such as hypoxia [42] and/or growth factors including EGF and VEGF [43, 49–52].

In summary, we have shown that the main chemokine receptor expressed by ASM is CCR3 and that *in vitro*, CCR3 can protect ASM against staurosporine-induced DNA fragmentation which is dependent on p42/44 MAPK. Although DNA fragmentation was not seen in ASM of patients with mild stable asthma, additional studies are required to determine if CCR3 mediated protection from cell death could contribute to airway remodelling during asthma exacerbations.

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Conflict of interest: None of the authors has any conflict of interest with the findings of this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Activation of p42/44 MAPK but not Akt by chemokine ligands.

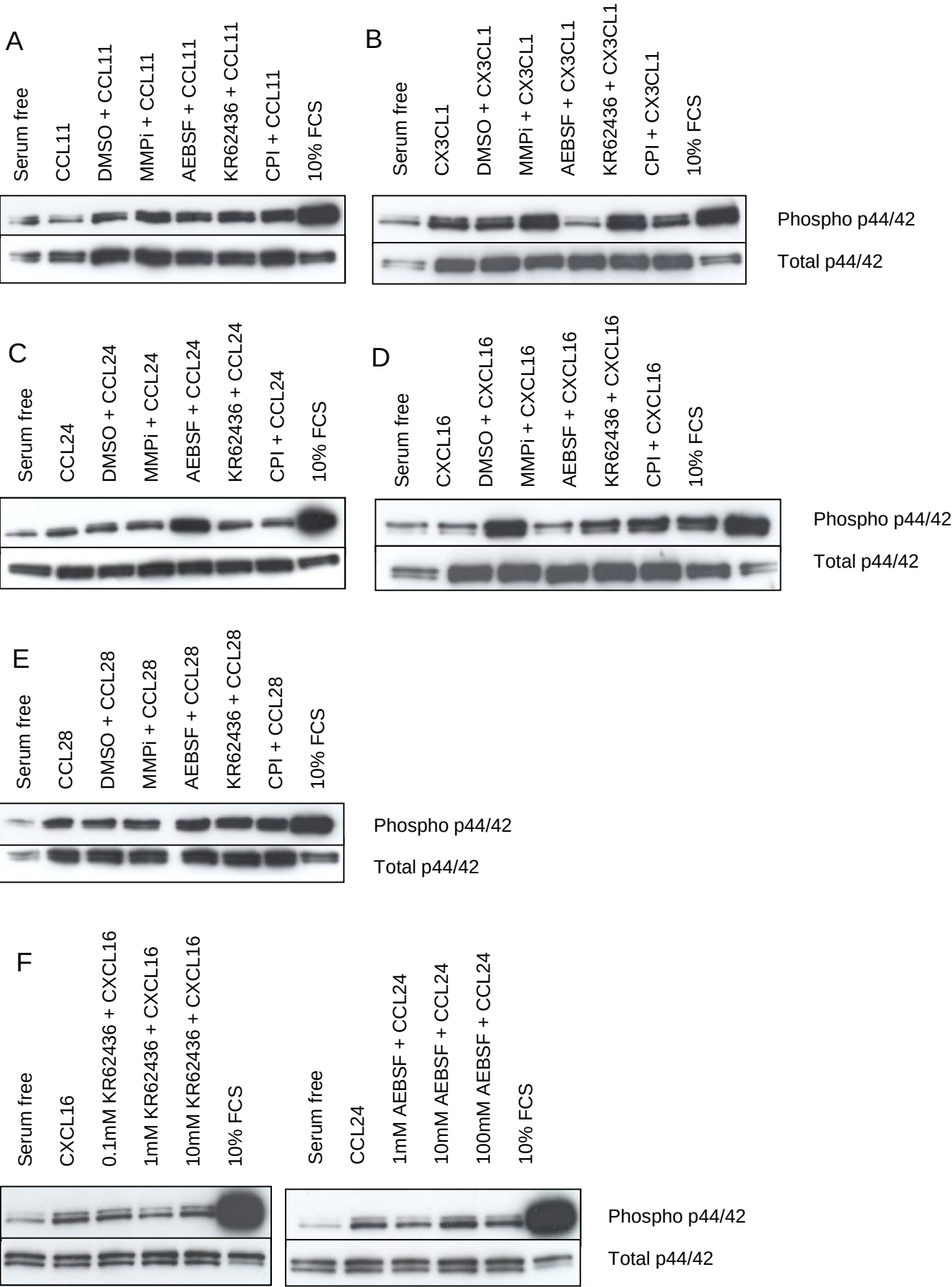
Fig. S2. Chemokines do not affect ASM migration.

Fig. S3. Chemokines do not affect ASM proliferation.

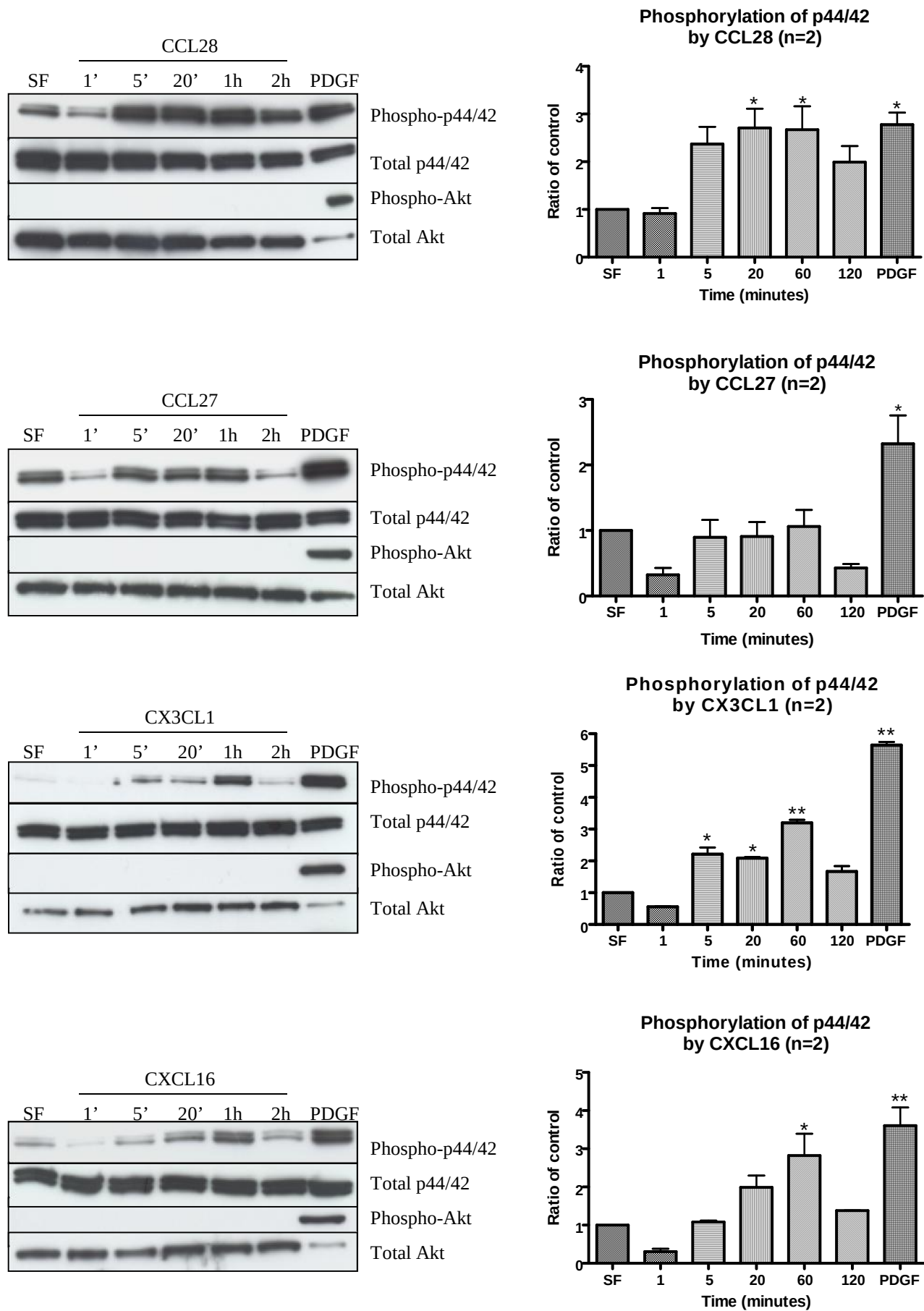
Fig. S4. The effect of protease inhibitors on the activation of p44/42 MAPK by CCL11, CX3CL1, CCL24, CXCL16, and CCL28.

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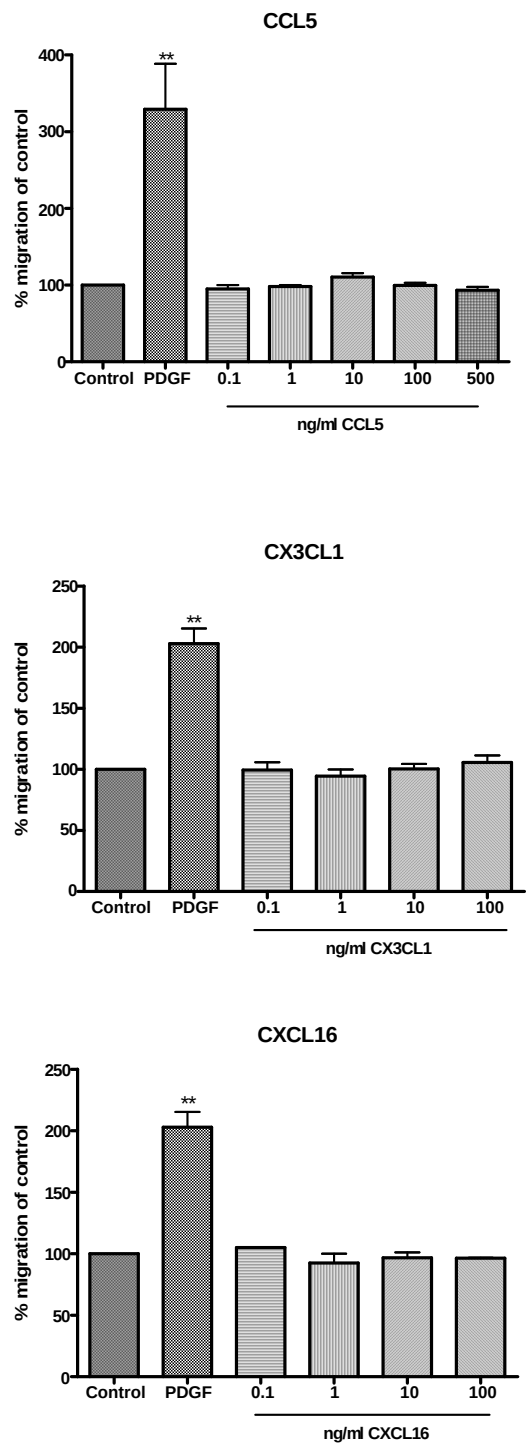
Supplementary figure 4



Supplementary figure 1



Supplementary figure 2



Supplementary figure 3

