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The CC chemokine Ligand (CCL)-1 is Released into the Airways of Atopic Asthmatics

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Abstract

CCL1/I-309 is a potent attractant for Th2 lymphocytes. We have investigated whether this cytokine is released in the bronchoalveolar (BAL) fluid of asthmatic patients. Measurements of CCL1 by ELISA showed that levels of this cytokine were significantly elevated in BAL fluid from asthmatics compared with normals (medians 193, range 120-449 pg/ml vs 30 pg/ml range 21-55 pg/ml). Differential cell counts in BAL fluid showed that either lymphocyte- or eosinophil numbers were elevated in asthmatic- compared with normal subjects (median 10.8 vs $1.0x10^3$/ml, $p<0.005$ and 1.7 vs $0.2x10^3$/ml, $p<0.001$, respectively). There was a trend towards a significant correlation between CCL1 levels and lymphocyte numbers in BAL fluid ($r=0.4$, $p=0.08$). Separation of BAL fluid by sequential CCL1 affinity column and reverse-phase HPLC chromatography allowed detecting biologically active CCL1. By immunohistochemistry, CCL1 immunoreactivity was localized predominantly to the airway epithelium. Interestingly, there was a significant correlation between CCL1 levels and epithelial cell numbers ($r=0.73$, $p=0.001$) in BAL fluid and between these cells and lymphocyte numbers ($r=0.6$, $p=0.02$). Moreover, IL-4, IL-13 or IFN-gamma stimulated primary bronchial airway epithelial cells to release CCL1. These findings suggest that CCL1 may play a role in lymphocyte recruitment in bronchial asthma.

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Keywords: CCL/I-309, asthma, lymphocytes, BAL fluid.

Introduction

It is now well recognized that asthma is a chronic inflammatory airway disease characterized by infiltration of eosinophils and T cells. CD4 T cells that produce Th2 type cytokines including IL-4, IL-5 and IL-13 play an important role in orchestrating immune and inflammatory processes that lead to immunoglobulin (Ig) E production and allergic inflammation (1). Activated CD4 T cells have been a consisting finding in the bronchoalveolar lavage (BAL) fluid and bronchial biopsies in stable asthma (2, 3). Furthermore, CD4 lymphocytes have been shown to increase further following allergen challenge (4, 5).

Lymphocyte recruitment from peripheral blood into the airways is regulated by chemokines. The chemokines are a large family of 8–14 kD heparin-binding peptides, which have been subdivided into four subfamilies on the basis of the position of either one or two cysteine residues located near the amino terminus of the protein (CXCL, CCL, CL, and CX3CL) (6, 7). The cytokine CCL1/I-309 is member of the CCL chemokine subfamily which, was initially discovered as a gene of unknown function by subtractive hybridization from the human IL-2-dependent γδ T cell line IDP2 (8), but subsequently shown to be monocyte- and Th2 cell attractant (9 – 11). CCL1 is located to human chromosome 17 (12). Upon activation CCL1 is produced by a number of cellular sources, including peripheral blood mononuclear cells (PBMC), monocytes, activated T lymphocytes and endothelial cells (8, 13, 14). *In vitro* studies have shown that CCL1 as well as both CCL22/MDC and CCL17/TARC induce migration of lymphocytes, especially of the Th2 phenotype (13, 14). CCL22 and CCL17 activate these cells through the CC chemokine receptor (CCR)-4 while CCL1 uses CCR8 (13, 16). It is now well established that CCL22 and CCL17 are released into the airways of

asthmatic patients (17 - 19). However, the role of CCL1 in asthma remains to be shown. Evidence for the participation of CCL1 for an *in vivo* role in allergic inflammation derives from an animal study showing that neutralization of CCL1 with an anti-CCL1 antibody significantly inhibits eosinophil recruitment into the lung of sensitized mice (20). Another approach has been the use of CCR8 knockouts mice. For example Chensue et al (21) demonstrated that $CCR8^{-/-}$ mice had impaired pulmonary eosinophilia. However, Chung et al were unable to show any impairment in both pulmonary Th2 cytokine responses and eosinophilia in CCR8 deficient mice (22). Although mice models of asthma have shed light into the pathogenesis of asthma, these models do not necessary mirror human asthma. In the present study we have investigated CCL1 in the BAL fluid of stable asthmatics.

Methods

Subjects

A group of 22 symptom free asthmatics treated with inhaled salbutamol alone and 13 healthy subjects volunteered to participate in the study. Clinical characteristics of both asthmatic and normal subjects are shown in table 1. Their atopic status was confirmed by skin-prick testing with different allergens (i.e. *Dermatophagoides pteronnisinus* and *farinae*, mixed grass, tree pollen, cat and dog dander and cockroach). The hospital's ethic committee approved the study.

Fiberoptic Bronchoscopy

Bronchoscopy was undertaken according to National Institutes of Health guidelines (23). Bronchoalveolar lavage (BAL) was performed with saline solution. The recovered BAL fluid was pooled and centrifuged, cells were separated and supernatant frozen. For differential cell counts, 100 μl of cells ($4x10^5$ cells) were subjected to cytocentrifugation, air dried and stained.

High performance liquid chromatography

HPLC was performed at room temperature with a HP1100 liquid chromatography system equipped with a quaternary pump and a variable wave length detector.

Purification of lymphocyte chemotactic activity (LCA) from BAL fluid.

BAL fluid from five subjects was combined and concentrated using Amicon YM3 filters before application to a CCL1 affinity column which had been previously prepared loading an anti-CCL1 antibody (AF272, R&D systems, Minneapolis, MN USA) on a NHS-activated sepharose column (HiTrap, 5 ml; Pharmacia, Uppsala, Sweden) (24). Bound material was eluted from the affinity column and separated by reverse-phase chromatography (RP-8) as we have previously described (24, 25). Fractions obtained from the column were lyophilized before assay for lymphocyte chemotaxis.

Isolation of $CD4^+$ lymphocytes

Human $CD4^+$ T cells were purified from healthy donor peripheral blood by Ficoll-Hypaque (Pharmacia Biotech AB, Uppsala, Sweden) density gradient centrifugation followed by isolation with immunomagnetic beads ($CD4^+$ T cell isolation kit purchased from Milteny Biotech, CA, USA). Purity was routinely >95%. For chemotaxis CD4+ cells were stimulated with IL-2 for 5 days. Consistent with a previous report (26) at day 5th of incubation with IL-2, CD4+ were found to express the CCL1 receptor CCR8 (figure 4A).

Chemotaxis assays

Chemotaxis on BAL HPLC fractions was performed using a multiwell microchemotaxis chamber as previously described (27). Recombinant CCL1 was used as a positive control. Chemotaxis was expressed as a chemotactic index. The Anti-CCL1 antibody MAB 272 was used for neutralizing experiments.

SDS-PAGE and western blot

SDS-PAGE was performed using Phast-Gel high density gel (Pharmacia) according to the manufacturer's protocol. Briefly, electrophoresis was performed at pH 6.4 with Phastgel SDS buffer strips that contained 0.112 M Tris/ 0.112M acetate buffer, and were visualized by the use of the silver staining kit (Sigma, St Louis MO). Western blot análisis was performed to detect CCL1 using a specific polyclonal antibody (Peprotech 500-P110Bt).

Immunohistochemistry

Bronchial biopsies were fixed in paraformaldehyde and paraffin embedded. Immunohistochemistry for CCL1 was performed by indirect immunoperoxidase staining as previously described (28). The sections were counterstained using Meyer's haematoxylin.

RNA Preparation and Analysis

Total RNA was isolated from BAL cells using TRIzol method. Total cellular RNA was quantified and reverse transcribed for CCL1, CCR8, GAPDH and beta 2 microglobuline PCR reaction as previously described (29). Specific primers had the following sequence: CCL1 (Forward 5'-GTTGCTTCTCATTTGCGG-3'; Backward 5'-GTAGGGCTGGTAGTTTCGG-3)'; CCR8 (Forward 5´-CATCACCCTCATGAGTGTGG-3´; Backward 5'- CACGTTGAATGGGACCCA GA-3´); beta 2 microglobuline (Forward 5'-GCTTACATGTCTCGATCCGACTTAA-3'; Backward 5'-CTCGCGCTACTCTCTCTTTCTGG-3').

Culture and Stimulation of Human Airway Epithelial Cells

Human bronchial airway epithelial cells (BAEC) were isolated from bronchial epithelia mucosa tissue and grown as previously described (24). Subsequently, cells were incubated with IL-4, IL-13 and IFN-gamma at concentrations of 10 ng/ml for 24 hours. The purity

and identity of the cells were checked by immunohistochemistry using the anti-citokeratin mAb AE1/AE3 (Zymed, USA).

Measurements of CCL1

Measurement of immunoreactive CCL1 in both 2 times concentrated BAL fluid and airway epithelial culture supernatant was performed using two-Ab sandwich ELISA kit following the manufacturer´s protocol. The primary anti-CCL1 capture antibody was the MAB272 while the secondary antibody was a Biotinylated anti-CCL1 antibody CCL1 (R&D Systems, USA). A list of the antibodies used in the different experiments is shown in table II. A standard curve consisting of serial dilutions from 1 to 0.007 ng/ml of recombinant human CCL1 (R&D Systems, USA) was included in the assay. Concentration of CCL1 in samples was calculated from the standard curve and in the case of BAL fluid concentrations of this cytokine were corrected for the initial 2-fold concentration. The lower limit of detection was 7 pg/ml, and the interassay coefficient variation was 5%.

Statistical analysis

Analysis of CCL1 in BAL fluid and BAL cell counts was performed with the Mann-Whitney U-test for unpaired data. Correlations between CCL1 and infiltrating cells in BAL fluid were evaluated by nonparametric Sperman's rank correlation coefficient test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Clinical findings

The median age for asthmatics and controls was 30 vs. 33 years, respectively. Lung function data showed that asthmatics suffered from mild to moderate asthma. The FEV1% (median) predicted in asthmatics and normals was 89.5% (range 70 - 120%) and 104.4%, (range 90 -138%), respectively. All asthmatic patients were atopic while

control subjects were non-atopic. Clinical data is shown in table III. No complications were seen in any of the patients following bronchoscopy. Minor complains such as sore throat and mild cough was seen in three patients.

Bronchoalveolar lavage fluid and cell population

Bronchoalveolar lavage was obtained from all 35 subjects taking part in the study. There was no significant difference in the volume of fluid recovered between asthmatic and normal subjects (75 ±10 ml vs. 62 ± 12 ml, $p>0.05$).

Differential cell counts in BAL showed that lymphocytes, eosinophils and epithelial cells numbers were significantly elevated in asthmatics compared with normals (medians 10.8 vs. 1.0 $x10^3$ /ml, $p<0.005$; 1.7 vs. 0.24 $x10^3$ /ml, $p<0.01$ and 1.3 vs. 0.02 $x10^3$ /ml, $p <0.05$, respectively), table III. There were no differences in the number of macrophages and total cell counts between asthmatic- and normal subjects.

Measurements of CCL1 in BAL fluid

Measurements of CCL1 by ELISA showed that levels of this cytokine were significantly elevated in BAL fluid from asthmatics compared with the normals (medians 193 pg/ml, range 108 – 449 pg/ml vs. 30 pg/ml range 15 - 55 pg/ml, $p<0.001$) (figure 1A).

There was a trend towards a correlation between CCL1 levels and lymphocyte numbers in BAL fluid although this just failed to reach statistical significance ($r=0.4$, $p=0.08$), figure 2A. Interestingly, there was a significant correlation between CCL1 levels and epithelial cells ($r=0.73$, $p=0.001$) (figure 2B) and between these cells and lymphocytes ($r=0.6$, $p=0.02$), figure 2C. However, there was not a significant correlation between CCL1 and any other infiltrating leukocytes including macrophages and eosinophils. Neither, there was a significant correlation between concentrations of CCL1 and bronchial hyperreactivity (PC_{20} FEV1 methacholine).

By SDS-PAGE and western blot CCLI was detected in BAL fluid derived from asthmatics but not in control subjects (Fig. 3).

CCL1 mRNA in BAL cells

CCL1 mRNA was investigated in BAL cells derived from normal and asthmatic subjects. Constitutive CCL1 mRNA was observed in BAL cells from normal subjects. However, increased CCL1 mRNA expression was seen in BAL cells derived from asthmatic- compared with normal subjects (Figure 1B).

Biochemical and biologic characterization of LCA from BAL fluid.

To investigate lymphocyte chemotactic activity (LCA), bronchoalveolar lavages from five asthmatics were combined (250 ml of BAL fluid). Subsequent analysis of BAL fluid by sequential CCL1 affinity column and reverse-phase chromatography allowed partially purifying a peak of LCA (Figure 4), By ELISA it was demonstrated that this peak of activity contained CCL1. Similar LCA profiles were obtained in three separate experiments using different pooled BAL fluid samples. Incubation of BAL CCL1 with a neutralizing anti-CCL1 antibody (MAB272) blocked the BAL-LCA (Figure 5B). For chemotaxis assays CCR8 was induced in CD4 lymphocytes by incubating these cells with IL-2 for 5 days (Figure 5A).

CCL1 immunoreactivity in bronchial biopsies

To investigate potential cellular sources of CCL1 the technique of immunohistochemistry was applied to bronchial biopsies. CCL1 immunoreactivity was localized predominantly to the airway epithelium (Figure 6A and C). Similar, findings were observed in biopsies derived from 4 different asthmatics. In contrast, not CCL1 immunoreactivity was seen in bronchial biopsies derived from normal subjects (data not shown). To further investigate this cytokine nasal polyps were also analysed by

immunohistochemistry. Interestingly, CCL1 immunoreactivity was also localized to the airway epithelium of nasal polyps (Figure 6D).

Bronchial airway epithelial cells release CCL1 *in vitro.*

Having demonstrated CCL1 immunoreactivity localized to the bronchial airway epithelium we have next investigated whether primary BAEC release this chemokine upon stimulation with either Th1 (IFN-gamma) or Th2 type cytokines (IL-4 and IL-13). Measurements of CCL1 in the culture supernatant showed that these cells produced low levels of CCL1 constitutively. Among the cytokines, IL-4 was the strongest stimulus for the release of CCL1 followed by IL-13 and IFN-gamma (Figure 7).

Discussion

A novel finding in the present study has been the demonstration that CCL1 is released in high concentrations into the airway epithelial lining fluid of asthmatic patients. Interestingly, BAL CCL1 was found to activate CD4 cells as assessed in chemotaxis assays. By immunohistochemistry, CCL1 was localized predominantly to the bronchial airway epithelium. And human bronchial epithelial cells were found to release high concentrations of CCL1 upon cytokine stimulation *in vitro*.

CCL1 was originally characterized as a chemotatic factor for monocytes, and subsequently shown to be a potent Th2 lymphocyte attractant (9 - 11). Because Th2 lymphocytes play an important role in asthma this cytokine has attracted major attention in allergic inflammation. The murine homologue of CCL1/I-309 is CCL1/TAC-3 (30, 31). CCL1/TAC-3 has been implicated in several models of inflammatory diseases including inflammatory bowel disease, experimental allergic encephalomyelitis and allergic asthma (20, 32, and 33). To date however, the role of this cytokine in asthma remains to be shown.

This is the first study to demonstrate that CCL1 is released into the airways of asthmatic patients. Indeed, levels of this cytokine were 6-fold greater in asthmatics compared with normals. A previous group has failed to detect CCL1 in BAL fluid from asthmatics exposed to allergen challenge while detected other chemokines such as CCL17 (TARC), CCL22 (MDC) and CXCL10 (IP-10) (18). Their failure to detect CCL1 could be explained by the fact that CCL1 was investigated in neat BAL fluid. Alternatively, antibodies used in their ELISA were not sensitive enough to detect CCL1. In the present study we have concentrated BAL fluid 2 - 10 times and demonstrated that 2 times concentrated BAL fluid was good enough to detect CCL1. Moreover, the presence of CCL1 in BAL fluid was further confirmed by western blot. It is well established that BAL fluid is a dilution of airway epithelial lining fluid of 1:100 to 1:200 (34-37). A finding of 193 pg/ml thus, reflects a concentration of 3.8 to 38 ng/ml in airway lining fluid. Expressing cytokines levels per ml of BAL fluid after correction for the initial concentration is a well established method (25, 37 - 39), although few researchers correct inflammatory mediators such as eosinophil cationic protein, IgE and other immunoglobulins according to either albumin or urea concentrations (40, 41). CCL1 levels were also calculated according to albumin concentrations in BAL fluid. However, there was not statistical difference from that expressed per ml of BAL fluid (data not shown). Interestingly, the report of ERS task force concludes that expressing results of acellular components as amounts per milliliter approach should be used in order to facilitate comparison of data from different workers until a reliable external marker can be defined (42). In previous studies we and other groups have detected other CD4 activating chemokines including CCL17 and CCL22 in stable asthmatics (18, 37). Thus, we hypothesize that CCL1 in concert with other CD4

activating chemokines such as CCL22 and CCL17 may be involved in the recruitment of lymphocytes into the airways of asthmatic patients.

In order to further investigate CCL1 we have studied the gene encoding this cytokine and shown increased CCL1 mRNA expression in BAL cells from asthmatic patients compared with that observed in cells obtained from normal subjects. This finding further probes that CCL1 is highly regulated in asthma.

Having unequivocally, shown increased immunoreactive CCL1 and CCL1 gene upregulation we wondered whether biological active CCL1 could be identified in BAL fluid. We have previously shown that BAL fluid constitutes an excellent tool to study cell attractants (25, 27). Indeed, separation of BAL fluid by sequential CCL1 affinity chromatography and reverse phase-chromatography enabled the identification of a peak of LCA, which by ELISA was found to correspond to CCL1. Moreover, the biological activity associated with these fractions could be abolished completely using a neutralizing antibody to CCL1. Thus, the finding that BAL CCL1 activates CD4 cells expressing CCR8 *in vitro* (fig 3) together with the demonstration that CCL1 levels showed a trend towards a correlation with lymphocyte numbers in BAL fluid suggest that when released locally, this cytokine may attract CD4 cells into the airways of asthmatics.

To investigate the cellular source of the CCL1 that we have detected in BAL fluid, we have then performed immunohistochemical staining of bronchial biopsies. This has allowed us to demonstrate that CCL1 immunoreactivity was predominantly localized to the airway epithelium. Moreover, levels of CCL1 correlated with the number of airway epithelial cells in BAL fluid and these cells correlated with lymphocytes which, suggests that during the inflammatory process airway epithelial cells may release CCL1 and this cytokine in turn may recruit lymphocytes into the

airways of asthmatics. Interestingly, the number of airway epithelial cells in BAL fluid from asthmatics was greater compared with those observed in BAL fluid from control subjects and it is likely that they may have accounted for the increased CCL1 mRNA expression observed in BAL cells. Thus, it could be hypothesized that epithelial cells shed from the airways could be major cell source of CCL1 *in vivo*. To further investigate CCL1 production, primary BAEC were stimulated with either Th1 or Th2 cytokines Interestingly, IL-4 was found to be strong stimulus for CCL1 release from BAEC followed by IL-13 and IFN-gamma. Both IL-4 and IL-13 are known to play an important role in allergic inflammation. These cytokines activate B cells inducing: class switching to IgE, expression of both CD23 and class II MHC antigens (43, 44). Increased mRNA encoding IL-4 and IL-13 has been reported in both bronchial biopsies and BAL cells derived from asthmatics (45). Taken these observations all together suggest that the cytokines IL-4, IL-13 and IFN-gamma may induce CCL1 release into asthmatic airways .although further studies must investigate in more detail the production of this cytokine by BAEC.

To date, there are not *in vivo* studies showing the direct effect of CCL1 on normal airways. Thus, it is not known whether CCL1 may induce bronchial hyperreactivity. In the present study we did not find a correlation between CCL1 levels and bronchial hyperreactivity (PC_{20} FEV1 methacholine), which is not surprising. We hypothesize that CCL1 induces recruitment of Th2 cells, which in turn may release cytokines such as IL-4, IL-5 and IL-13. These cytokines are potent inductors of bronchial hyperreactivity. Thus, it is tempting to hypothesize that neutralizing the effects of CCL1/CCR8 may have substantial impact in allergic disease. Indeed, a previous study has shown that the use of an anti-CCL1 antibody inhibits the eosinophil recruitment into the lung of sensitized mice (20). Similarly, Chensue et al (21)

demonstrated that CCR8$^{-/-}$ mice had impaired pulmonary eosinophilia although other authors have failed to reproduce these findings (22). Interestingly, increased CCR8 immunoreactivity, localized to Th2 cells, has been previously found in bronchial biopsies derived from asthmatics and its expression correlated with bronchial hyperreactivity (18). More recently, CCR8 has been reported to be expressed in both Th1 and Th2 skin lymphocytes (46). The development of small CCL1 antagonists with clinical efficacy will define the role of this cytokine in asthma. On the other hand, future studies most investigate the effect of corticosteroids, mainstay treatment of asthma, on CCL1 release.

In summary, our study has demonstrated that asthmatics release increased concentrations of CCL1 in BAL fluid. And the levels of this cytokine were found to be associated with an increased number of lymphocytes in BAL fluid. BAL CCL1 induced migration of CD4 cells expressing CCR8 in a chemotaxis assay. By immunohistochemistry, CCL1 immunoreactivity was localized predominantly to the bronchial airway epithelium. Interestingly, both CCL1 levels and lymphocyte numbers correlated with airway epithelial cell numbers in BAL fluid. Moreover, BAECs were found to release CCL1 *in vitro*. Taken together these findings suggest that CCL1 may play a role in the recruitment of lymphocytes that characterizes bronchial asthma.

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Legends to figures

Figure 1. **A**) Levels of immunoreactive CCL1 in the BAL fluid of asthmatic patients after correcting for for the initial 2-fold concentration.. Horizontal line represents the median values. **B**) CCL1 mRNA expression in BAL cells from normal and asthmatic subjects. Black bar represents CCL1 gene expression in PMA-stimulated U937 cells as a positive control (+). Densitometric analysis of CCL1 mRNA expression normalized to GAPDH is shown.

Figure 2 shows correlations between: **A**) CCL1 levels and lymphocyte numbers, **B**) CCL1 and epithelial cells and **C**) lymphocytes and epithelial cells.

Figure 3. Western Blot for CCL-1. Lane 1 MW markers, lane 2 and 3 BAL fluid derived from asthmatics, lane 3and 4 BAL fluid derived from control subjects.

Figure 4. Lymphocyte chemotactic activity (LCA) from BAL fluid after sequential CCL1/I-309 affinity chromatography and reverse-phase chromatography resolved into a peak of LCA (■). CD4 chemotaxis in response to individual fractions is given as a chemotactic index. Negative control (buffer) per definition, gave a chemotactic index of 1(□). Recombinant CCL1 was used as a positive control (■). CCL1 ELISA measurement (right line).

Figure 5. **A**) IL-2 stimulated CD4+ cells express CCR8 (lane 1). Non-stimulated lymphocytes do not express this receptor (lane 2). β2-m= beta 2 macroglobulin, bp = base pairs. **B**) Neutralization of lymphocyte chemotactic activity (LCA) of BAL CCL1 (mean of three separate experiments). BAL CCL1 (5 ng/ml) was incubated with 1 μg of

the MAB272 antibody (neutralizing Ab to CCL1) for 15 min before the chemotaxis assay. Chemotactic activity is expressed as a chemotactic index. Recombinant (r) CCL1 (10^{-8} M) was used as a positive control. Negative control (buffer) per definition, gave a chemotactic index of 1.

Figure 6. Localization of immunoreactive CCL1 to the airway epithelium of bronchial biopsies derived from two asthmatics (**A** and **C**). **D** shows immunoreactive CCL1 to the airway epithelium of a nasal polyp. **B** shows a negative control of a bronchial biopsy (instead of the anti- CCL1/I-309 antibody the isotype control antibody goat IgG 15256 was used). Details of immunohistochemistry are given in methodology.

Figure 7. Levels of immunoreactive CCL1 in the culture supernatants (n = 4) derived from both the cytokine-stimulated primary bronchial airway epithelial cells (BAEC) as well as the non-stimulated BAEC supernatants (control). Bars represent median values.

Table I

Clinical characteristics of subjects

	Sex	Age (yrs)	FEV-1 %	PC_{20}
Asthmatics	**19 F**	**33**	**89.5**	**1.5**
	3 M	**(24 - 49)**	**(70-120)**	**(0.8-4.5)**
Controls	**6 F**	**30**	**104.4**	**32**
	7 M	**(18 - 55)**	**(90-138)**	

Table II

Antibodies used in experiments

Antibody	Catalogue number	Source	Ig Class	Experiment
MAB272 R&D	Anti-CCL1 human monoclonal	Mouse	IgG1	ELISA Neutralization Quimiotaxis
BAF27 R&D 2	Anti-CCL1 human biotinylated	Goat	IgG	ELISA
AF272 R&D	Anti CCL1 human polyclonal	Goat	IgG	Inmunohistochemistry
500-P110 Peprotech	Anti-human I-309 biotinylated	Rabbit	IgG	Westernblot immunohistochemistry

Table III

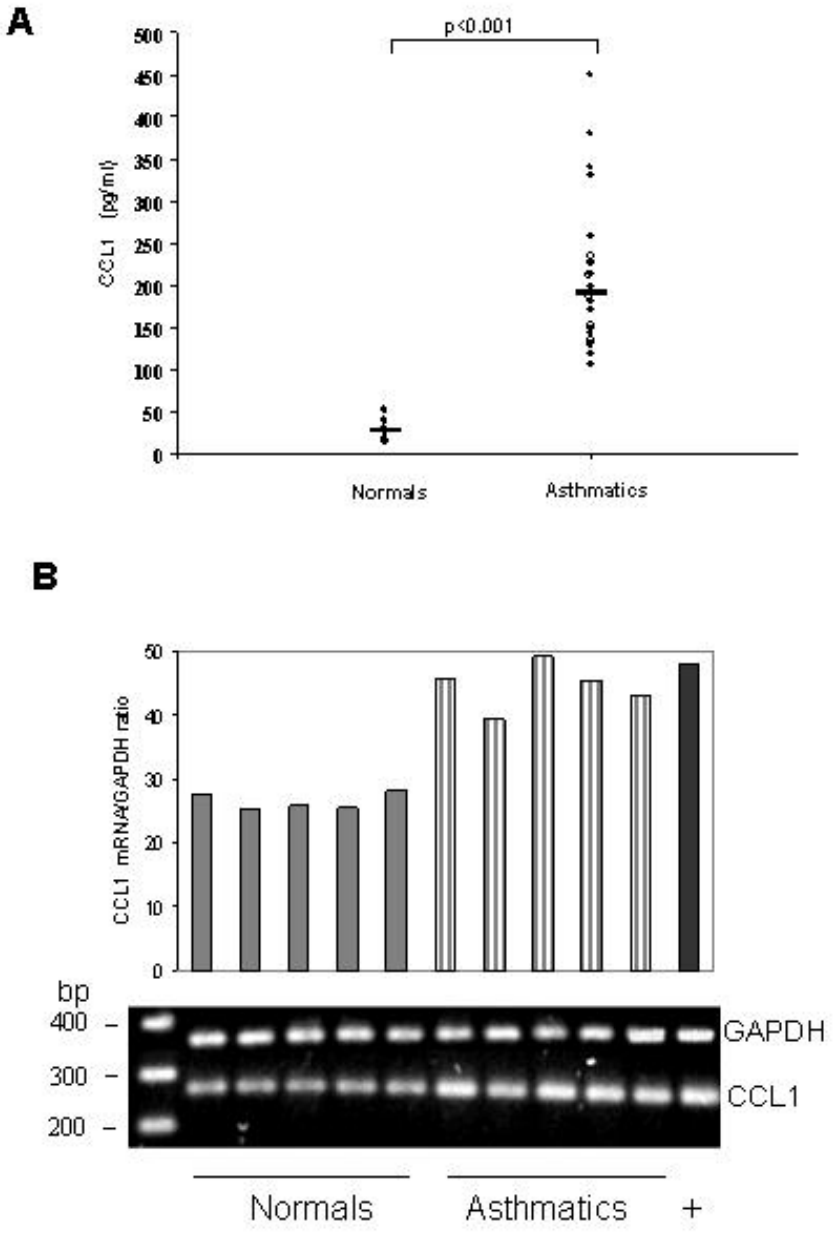
Differential and total cell counts in BAL fluid ($X10^3$/ml)

	Asthmatics	Normals
Macrophages	21.8 (17-60)	32.6 (1.8-129)
Lymphocytes	*10.8 (0-84)	1 (0-3.4)
Neutrophils	5.8 (0-15)	2.1 (0-10.8)
Eosinophils	†1.7 (0-8)	0.24 (0-0.8)
Epithelial cells	‡1.3 (0-9.6.2)	0.02 (0-0.35)
Total cell count	38 (3-100)	42.1 (1.9-140)

BAL cells are expressed as median (range)

*p<0.005, †p<0.001, ‡p<0.05.

Figure 1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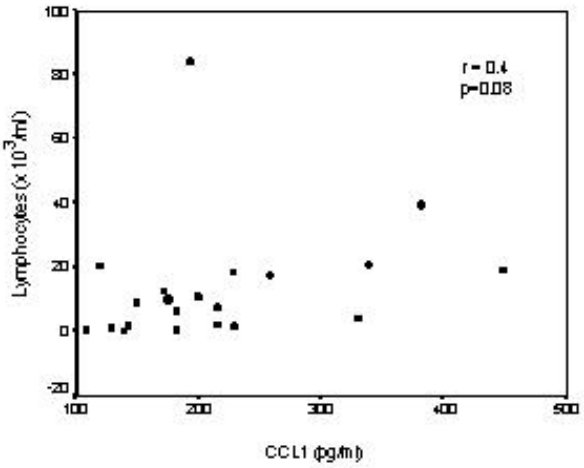
p<0.001
CCL1 (pg/ml)
500
450
400
350
300
250
200
150
100
50
0
Normals
Asthmatics
B
CCL1 mRNA/GAPDH ratio
50
40
30
20
10
0
bp
400
300
200
GAPDH
CCL1
Normals
Asthmatics
+

Figure 2

A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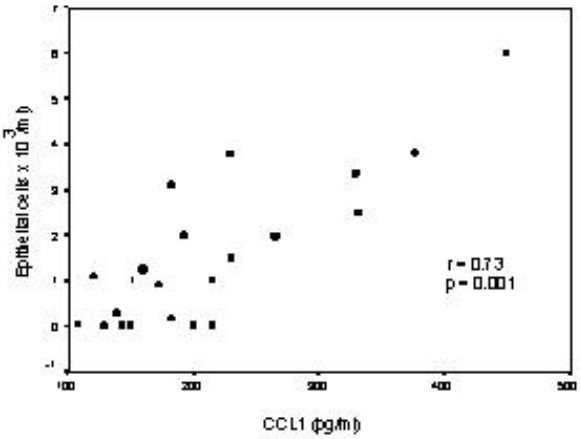
p=0.08
Lymphocytes (x 10^3/ml)
CCL1 (pg/ml)

B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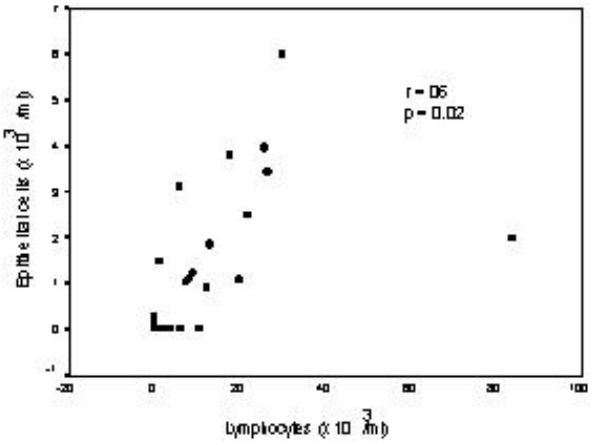
p = 0.001
CCL1 (pg/ml)

C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p = 0.02

Figure 3

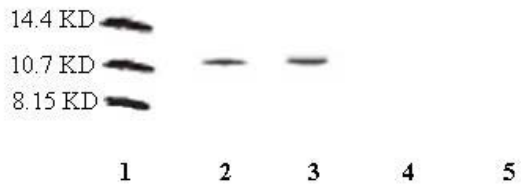


Figure 4

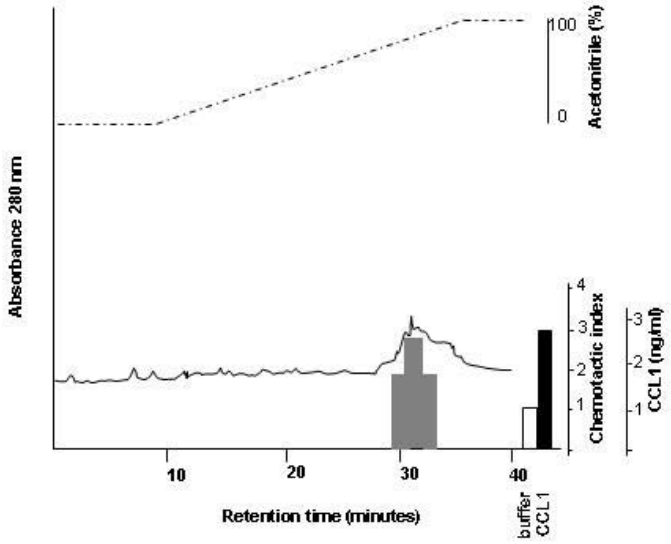
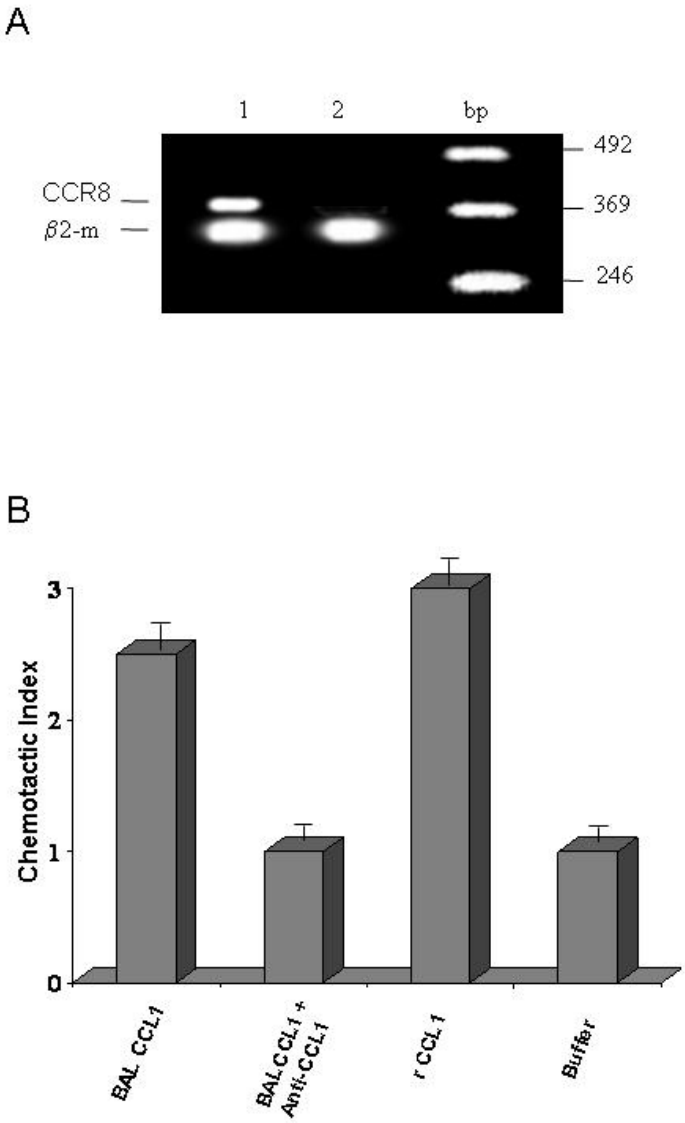

Absorbance 280 nm
100
0
Acetonitrile (%)
4
3
2
1
Chemotactic index
3
2
1
CCL1 (ng/ml)
10
20
30
40
Retention time (minutes)
buffer
CCL1

Figure 5


A
1
2
bp
492
369
246
CCR8
β2-m
B
Chemotactic Index
3
2
1
0
BAL CCL1
BAL CCL1 + Anti-CCL1
r CCL1
Buffer

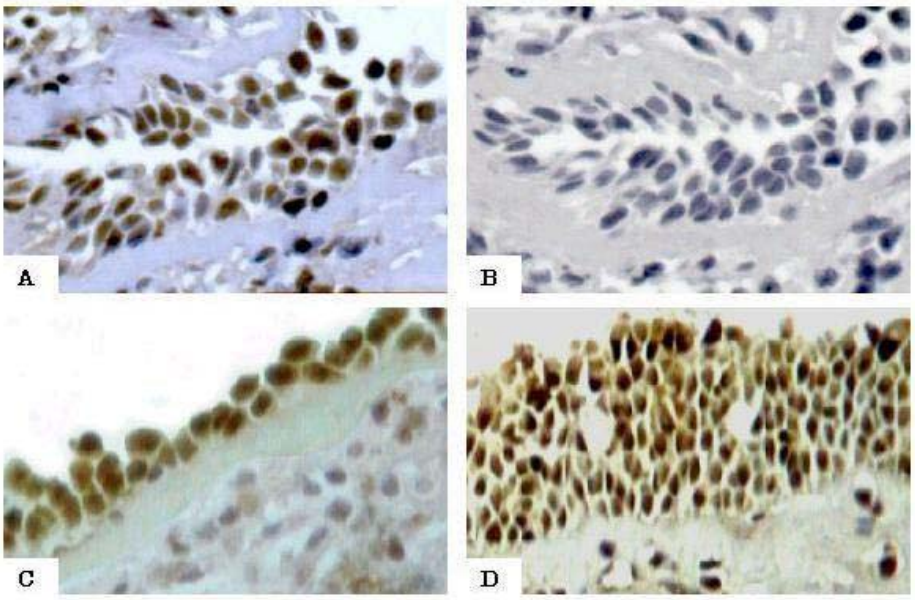
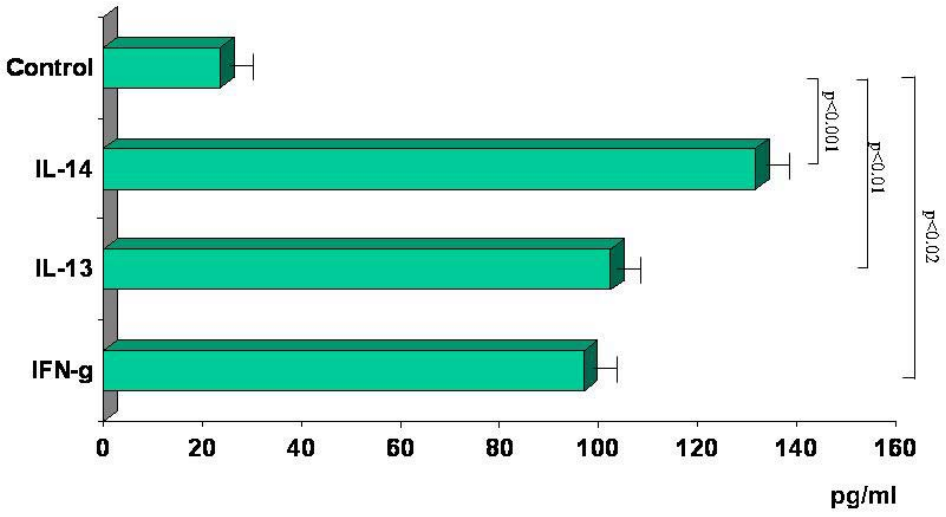

A
B
C
D

Figure 7


Control
IL-14
IL-13
IFN-g
0
20
40
60
80
100
120
140
160
pg/ml
p<0.001
p<0.01
p<0.02