

# IL-17A Induces CCL28, Supporting the Chemotaxis of IgE-Secreting B Cells

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## Key Words

Allergy · Asthma · B cell · CCL28 · IL-17A

## Abstract

**Background:** Atopic asthma is an allergic disease typically associated with T<sub>H</sub>2 cytokines. IL-17A is also associated with asthma, through the induction of chemokines. Mucosal CCL28 concentrations correlate with cellular recruitment to inflamed airways and support migration of IgA<sup>+</sup> B cells. Here, a link between IL-17A, CCL28 and IgE-secreting B cell chemotaxis is examined. **Methods:** Primary human airway cells and the airway epithelial line A549 were used to characterize IL-17A receptor expression and the effect of IL-17A on CCL28 transcription and translation. B cells, differentiated to IgE<sup>+</sup> cells ex vivo, were assessed for CCR10 surface expression and chemotaxis to CCL28 by flow cytometry, transwell migration and ELISpot. **Results:** Human airway epithelium expressed both IL-17RA and IL-17RC, and was responsive to IL-17A stimulation. Cultured human IgE<sup>+</sup> B cells expressed surface CCR10 and displayed CCR10-dependent chemotaxis towards recombinant CCL28. Enhanced levels of CCL28 were observed upon A549 cell incubation with IL-17A, and this up-regulation significantly increased the migration of IgE<sup>+</sup> antibody-

secreting B cells. The specificity of chemotaxis was confirmed by migration blockade in the presence of anti-CCL28 or anti-CCR10. **Conclusions:** This work identifies a novel chemokine for the migration of IgE<sup>+</sup> B cells, in addition to characterizing induction of CCL28 by IL-17A. Taken together the results presented here propose a new role for IL-17A in the allergic airways, linking this cytokine with the recruitment of IgE<sup>+</sup> antibody-secreting B cells, via the induction of CCL28. These observations justify further in vivo studies of larger cohorts.

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## Introduction

CCL28 is a mucosal chemokine with chemoattractant and antimicrobial properties [1, 2]. It recruits eosinophils, IgA<sup>+</sup> antibody-secreting cells (ASC) and T cells via CCR3 and CCR10 [3–5]. This chemokine is elevated in human asthmatic biopsy [6] and is associated with increased allergic pathology in murine models of airway disease, with increased CCL28 expression linked to the recruitment of both CCR10- and CCR3-expressing cells [3, 7]. In addition, when CCL28 antisera were adminis-

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tered to mice with established airway inflammation, mucus cell metaplasia, airway hyper-responsiveness and peribronchial eosinophilia were reduced [3]. We and others have shown that enhanced expression of this chemokine is due in part to IL-1 $\beta$ , TNF- $\alpha$  or LPS [5, 6].

Allergic asthma is a complex syndrome associated with T<sub>H</sub>2-type immunity, linked to airway remodelling, accompanied by local infiltration of T cells [8], eosinophils [9] and neutrophils [10], and mast cell activation [11]. The role of IgE has long been established in this disease [12]. However, the conventional understanding of asthma has been revised with the discovery of T<sub>H</sub>17 cells [13]. T<sub>H</sub>17 cells produce IL-17A [14] and IL-17F [15]. In addition to diseases such as inflammatory bowel syndrome [16] and psoriasis [17], T<sub>H</sub>17 cells and the associated cytokines are increased in patients with allergic asthma [18]. T<sub>H</sub>17 cells enhance T<sub>H</sub>2 responses in asthma and increase eosinophil recruitment [19]. Neutralization of IL-17A reduces allergen-induced eosinophilia, T<sub>H</sub>2 cytokine levels and airway hyper-responsiveness [20]. IL-17A acts via an interaction with either a homodimeric IL-17 receptor A (IL-17 RA) or heterodimeric IL-17RC/IL-17RA complex [21]. These receptors are widely expressed, and increased expression of IL-17RA is observed following oncostatin M, IL-15 or IL-21 stimulation [22, 23]. IL-17A induces pro-inflammatory cytokines, antimicrobial peptides and neutrophil-recruiting chemokines, and has a role in antimicrobial defence [24–26]. However, the exact mechanism by which IL-17A contributes to allergic asthma remains unclear.

In this study, the links between human IL-17A and CCL28 were explored *in vitro* through the use of cell lines, and *ex vivo* by sampling sputum preparations from an asthmatic cohort and utilizing human primary B cells. We hypothesized that IL-17A would induce CCL28. The enhanced expression of CCL28 in response to IL-17A would be in line with the inflammatory and antimicrobial roles proposed for this cytokine, as CCL28 is hypothesized to act both indirectly via the recruitment of inflammatory cells and directly by killing a broad spectrum of microbes [1, 2]. Prior to this study, no chemoattractant responsible for the selective recruitment of IgE<sup>+</sup> B cells had been identified. Here we examined the capacity of CCL28 to activate the chemotaxis of IgE-secreting cells, and showed that stimulation of A549 cells with IL-17A enhanced the migration of IgE<sup>+</sup> ASC, in a CCL28-dependent manner. Taken together, these results indicate a role for IL-17A in the human lung by enhancing the expression of CCL28 and hence driving the recruitment of IgE-secreting B cells.

## Materials and Methods

### *Patients and Sampling*

Human material was obtained with informed consent according to protocols approved by the research ethics committee of NUI Maynooth and the Federated Dublin Hospitals. Lung biopsy sample was collected at the Adelaide and Meath National Children's Hospital, Dublin, Ireland, from a normal healthy control individual by bronchoscopy. Tissue from the bronchial region of the lung, containing a mixed population of cells, including epithelial and goblet cells, was collected and stored in RNALater (Ambion, Austin, Tex., USA) until subsequent RNA extraction was performed. In a pilot study on protein and gene expression among asthmatic subjects, 5 patients were randomly selected and assessed by sputum induction. The study population was steroid naive with bronchial asthma, as defined by the Global Initiative for Asthma guidelines. All were  $\geq 18$  years old, non-smokers, exhibiting  $\geq 3\%$  eosinophilia and free from upper or lower respiratory infection for a minimum of 1 month prior to inclusion. Sputum was induced using the method of Pizzichini et al. [27]. Differential cell counts were performed following Giemsa staining (Fluka, Dorset, UK) of prepared cytospin slides and remaining cells were examined for gene expression by qRT-PCR. CCL28 was measured from sputum supernatant by ELISA [6].

### *Cell Culture*

B cells were purified from PBMC to  $>95\%$  using MagCollect human B cell isolation kit (R&D Systems, Abingdon, UK). Purified B cells were cultured in 10% v/v heat-inactivated FCS, RPMI with L-glutamine, and stimulated with 30 ng/ml IL-4 (Mabtech, Nacka Strand, Sweden) and 1  $\mu\text{g/ml}$  anti-CD40 (Mabtech) for 5 days to induce class switching to IgE. Purity was verified by flow cytometry. The human alveolar epithelial cell line, A549 (EACC No. 86012804) was maintained at low passage in DMEM/F-12 containing HEPES supplemented with 10% v/v heat-inactivated FCS and 2 mM L-glutamine.

### *RNA Extraction and RT-PCR*

A549 cells were untreated or cultured in the presence of IL-17A (Peprotech EC, London, UK), or 10 ng/ml IL-1 $\beta$ , 10 ng/ml TNF- $\alpha$ , 10 ng/ml LPS, 50 ng/ml GM-CSF, 1 ng/ml IL-4 or 100 ng/ml IFN- $\gamma$  (R&D Systems) using times and concentrations previously optimized [6]. Some studies included 600 ng/ml anti-human IL-17A (eBioscience, Hatfield, UK). Total mRNA extraction, reverse transcription and preparation for qRT-PCR was performed as previously described [8]. Primer pairs were: CCR10: 5'-GGG TTT CTC CTT CCA CTC CT-3' and 5'-TAT TCC CCA CAT CCT CCT TG-3'; IL-17RA: 5'-AGA CAC TCC AGA ACC AAT TCC-3' and 5'-TCT TAG AGT TGC TCT CCA CCA-3'; IL-17RC: 5'-ACC AGA ACC TCT GGC AAG C-3' and 5'-GAG CTG TTC ACC TGA ACA CA-3'; IL-17A: 5'-GCA ATG AGG ACC CTG AGA GA-3' and 5'-CCC ACG GAC ACC AGT ATC TT-3'; CCL28: 5'-CAT GCC TCA GAA GCC ATA CT-3' and 5'-CCC TGA TGT GCC CTG TTA CT-3'.

### *Western Blotting*

Protein concentrations were quantified by Bradford assay (Bio-Rad, Hercules, Calif., USA) and normalized to 50  $\mu\text{g}$  per sample. Culture supernatant was resolved on a 15% SDS-PAGE gel and transferred onto nitrocellulose. Blots were blocked in 0.05%

v/v Tween 20, PBS containing 10% w/v milk powder and probed with 100 ng/ml anti-human CCL28 (R&D Systems). Rabbit anti-mouse IgG conjugated to HRP (Sigma-Aldrich) was the secondary antibody. The reaction was detected with ECL reagent (Pierce, Rockford, Ill., USA). Densitometric analyses of bands were performed by trace quantity using QuantityOne quantitation software (BioRad).

#### *Chemotaxis Assay*

B cell CCL28-dependent migration was determined using a 24-well Transwell plate with a 5.0- $\mu$ m pore polycarbonate insert (Corning Costar, Cambridge, Mass., USA). Recombinant human CCL28 (R&D Systems) or CXCL12 (R&D Systems) were adjusted to 250 and 100 nM, respectively, in serum-free RPMI. Six hundred microliters of recombinant protein, A549-conditioned or serum-free (control) medium were added directly into the lower chambers and  $2 \times 10^5$  B cells added to the upper chamber. To verify the specificity of migration, B cells were incubated with 250 ng/ml anti-CCR10 [7] for 30 min prior to addition or CCL28 was neutralized by 30  $\mu$ g/ml anti-human CCL28 (lower chamber; R&D Systems). Cells were allowed to migrate for 2 h at 37°C in 5% CO<sub>2</sub> and counted from 5 random fields of view imaged by an inverted microscope with a digital camera. A chemotactic index was calculated by expressing the number of migrated cells in sample wells compared to controls.

#### *IgE ELISpot*

ELISpot was performed using ELISpot<sup>PLUS</sup> for human IgE (Mabtech) according to the manufacturer's instructions. Plates were coated with 15  $\mu$ g/ml coating antibody and blocked with 10% FCS RPMI. Migrated cells, from the lower chemotaxis chambers, were washed and resuspended in complete medium. These cells were incubated on the plate at 37°C with 5% CO<sub>2</sub> for 24 h. IgE+ ASC were detected with 1  $\mu$ g/ml biotinylated antibody and streptavidin-HRP. A 40-min incubation with TMB substrate was carried out and a total spot count was performed.

#### *Flow-Cytometric Analysis*

Cell surface expression of proteins was characterized by flow cytometry (FACSCalibur, San Jose, Calif., USA) and analysed using CellQuest software (BD Biosciences, Oxford, UK). Cells were labelled with 5  $\mu$ g/ml anti-CCR10 PE (R&D Systems) and 10  $\mu$ g/ml mouse anti-human IgE (BD Biosciences) followed by a secondary goat anti-mouse FITC antibody (Immunotools, Friesoythe, Germany) or matched isotype controls (Immunotools).

#### *Statistics*

Data were represented as mean values  $\pm$  SEM and interpreted using the Student t test as well as regression analysis. The small sample sizes for the human pilot study support the limited use of these data as indicative, justifying larger confirmatory study. Pearson's correlation coefficient was used to measure the dependence between variables. Statistical analyses and graphical representations were performed using GraphPad Prism<sup>TM</sup> software (GraphPad, San Diego, Calif., USA).

## **Results**

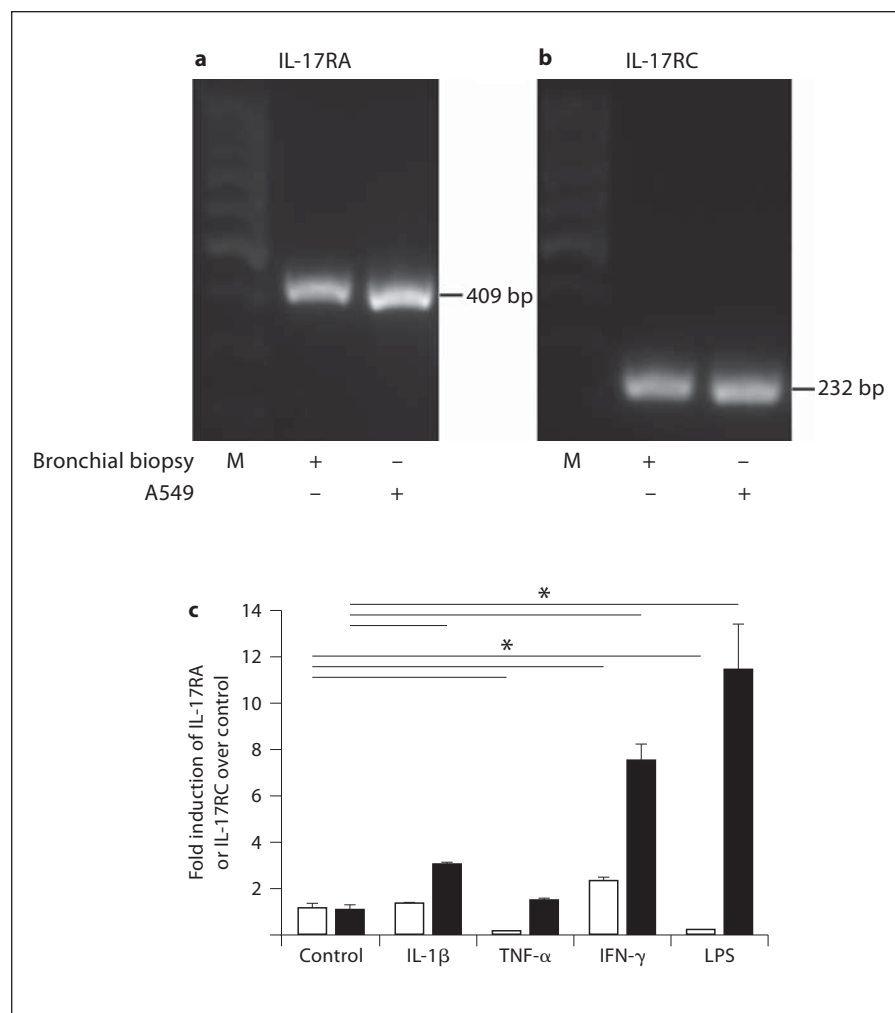
### *IL-17A Induces Elevated CCL28 Expression by Human Airway Epithelium*

CCL28 and IL-17A are elevated in the inflamed airway [3, 6, 7, 15, 28] and IL-17A is a potent inducer of chemokines [26], therefore the role of IL-17A in the regulation of CCL28 was examined. Firstly, the expressions of IL-17RA and IL-17RC, the two receptors that mediate cellular responses to IL-17A, were verified in airway cells. The expressions of both receptors by human primary airway cells were confirmed at mRNA level from a normal lung biopsy (fig. 1a, b). The alveolar epithelial-like cell line A549 also expressed both of the IL-17A receptors (fig. 1a, b). Murine lung tissue was used as a negative control sample for each of the primer sets, due to the high distribution of IL-17RA and IL-17RC expression among different cell and tissue types (negative results not shown). Whilst A549 cells may not fully represent the behaviour of complex airway epithelial populations, they have been widely used in models of airway biochemistry and as a model system for the study of lung CCL28 expression [6]; further in vitro experiments were performed using this cell line.

IL-17RA expression by fibroblasts, CD8+ T cells and intestinal epithelial cells is increased following pro-inflammatory cytokine stimulation [22, 23, 29]. Therefore, it was possible that the effector function of IL-17A might be modulated by enhanced or reduced expression of its corresponding receptors within an inflammatory setting. As airway inflammation is defined by high levels of pro-inflammatory stimuli, a quantitative analysis of IL-17RA and IL-17RC mRNA from A549 cells unstimulated or stimulated with inflammatory cytokines or LPS was performed. The expressions of IL-17RA and IL-17RC were not altered by the presence of IL-4, IL-17A or GM-CSF (data not shown). However, the expression of each receptor was altered differentially by the addition of other inflammatory cytokines or bacterial components, and a common induction was observed for both IL-17RA and IL-17RC when stimulated with IFN- $\gamma$  (fig. 1c). IFN- $\gamma$  increased epithelial expression of IL-17RA, whereas TNF- $\alpha$  or LPS significantly reduced this (fig. 1c, open bars;  $p < 0.05$ ). IL-17RC was up-regulated by IFN- $\gamma$  or IL-1 $\beta$  (fig. 1c, closed bars;  $p < 0.05$ ). However, in contrast to IL-17RA, LPS exposure greatly enhanced IL-17RC expression by these epithelial like cells (fig. 1c;  $p < 0.05$ ).

The expression of CCL28 following IL-17A stimulation was examined. A549 cells were stimulated with increasing concentrations of IL-17A and assessed for the

**Fig. 1.** Bronchial tissues and A549 cells express the receptors for IL-17A. Expression of IL-17RA and IL-17RC mRNA from primary lung cells from bronchial biopsy or airway A549 epithelial-like cells was examined by RT-PCR. Biopsy tissues and the cell line both expressed IL-17RA (**a**) and IL-17RC (**b**) at the transcriptional level. M indicates the position of a DNA marker, while the product size of each band is indicated by a line and base pair value. To assess the effect of inflammatory cytokine or LPS stimulation on IL-17 receptor expression, A549 cells were stimulated for 24 h and examined by quantitative RT-PCR (**c**). The expressions of IL-17RA (open bars) or IL-17RC (closed bars) mRNA were modulated following stimulation by IL-1 $\beta$  (10 ng/ml), TNF- $\alpha$  (10 ng/ml), IFN- $\gamma$  (100 ng/ml) or LPS (10 ng/ml). Graph illustrates triplicate analysis of duplicate experiments (n = 6), each treatment was compared with the respective control unstimulated sample. \* p < 0.05 compared to control values.



levels of CCL28 mRNA after 6 h and protein after 24 h. In a dose-dependent manner, IL-17A significantly increased the expression of CCL28 at both mRNA and protein levels (fig. 2a, b; p < 0.05 and p < 0.01, respectively). Up-regulation was attributable to IL-17A stimulation, as addition of an anti-IL-17A antibody resulted in a significant reduction in CCL28 expression (fig. 2c; p < 0.05).

IL-1 $\beta$  synergizes with IL-17A to up-regulate CXCL8 expression [30]. This cytokine also induces airway CCL28 expression [6] and enhanced IL-17RC mRNA (fig. 1c); therefore, synergies between IL-1 $\beta$  and IL-17A for CCL28 induction were examined. A549 cells were cultured with IL-1 $\beta$  for 24 h to allow enhancement of IL-17RC surface expression [23] and then stimulated with 10 ng/ml IL-17A for a further 6 h. This lower concentration of IL-17A was utilized to assess the responsiveness of the cells to sub-maximal stimulation. Although stimulation with either

IL-1 $\beta$  or IL-17A up-regulated CCL28 mRNA expression, no enhanced or synergistic effect was observed (fig. 2d). In contrast, IFN- $\gamma$  (that increased expression of both IL-17RA and IL-17RC; fig. 1c) synergized with IL-17A to induce CCL28 expression (fig. 2d; p < 0.01). A549 cells do not express constitutive or IFN- $\gamma$ -inducible IL-17A (data not shown); hence, these results support a model whereby increased expression of either IL-17RA alone or IL-17RA and IL-17RC in response to IFN- $\gamma$  enhances the cellular response to IL-17A.

Interestingly, in a pilot study investigating protein and gene expression in the sputum of steroid-naïve asthmatic patients, CCL28 protein levels were determined to correlate with IL-17A mRNA concentrations from sputum-derived cells (fig. 3; r = 0.9611, p < 0.01), suggesting that these findings are not confined to the A549 system. This pilot study examined only 5 patients; although linear re-

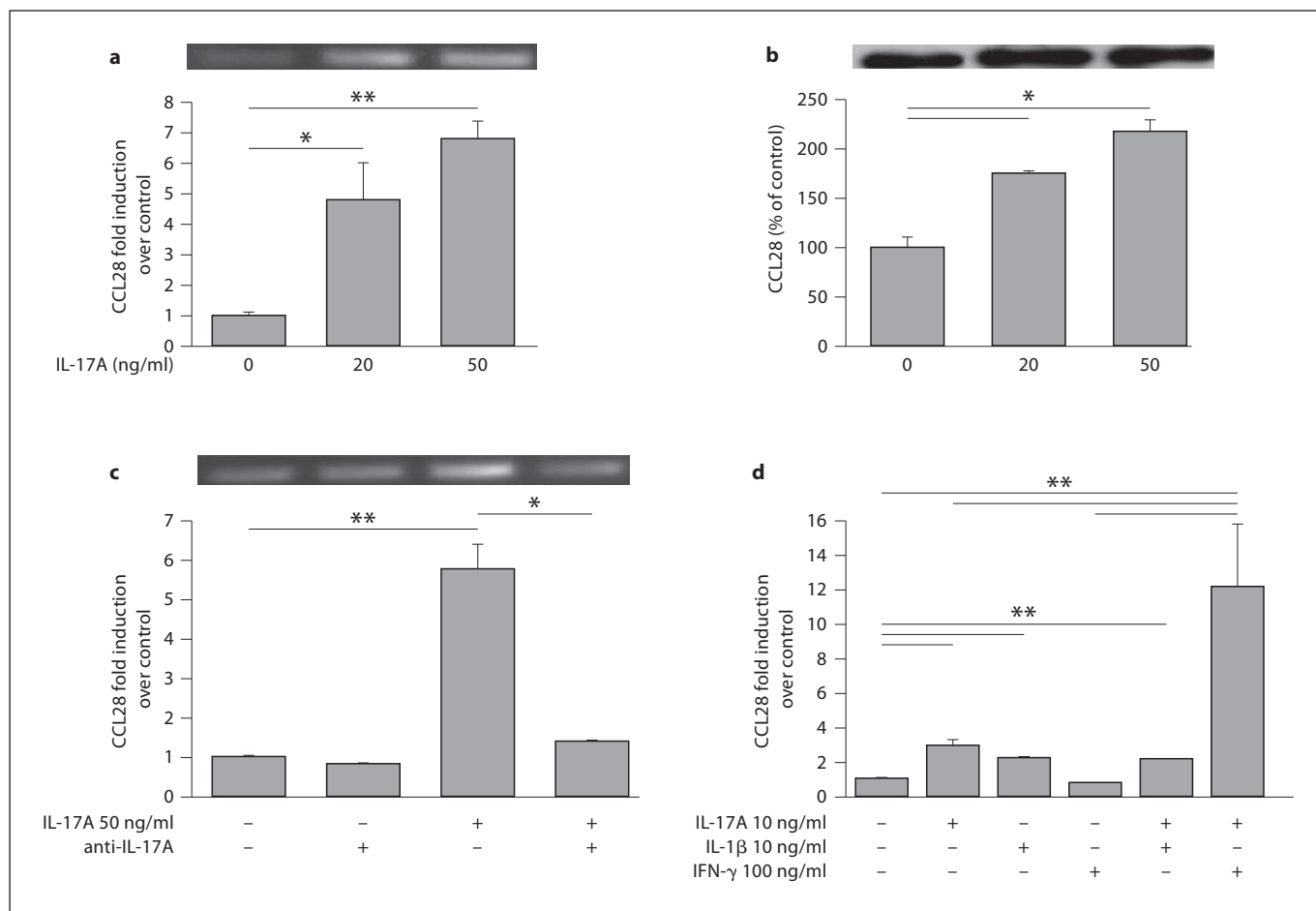


**Fig. 2.** IL-17A induces CCL28 in an epithelial cell line and synergizes with IFN- $\gamma$ . A549 cells were cultured with IL-17A at the concentrations indicated and CCL28 mRNA was measured after 6 h (a); CCL28 protein was measured from culture supernatant after 24 h (b). Data from semi-quantitative RT-PCR (a, upper panel) and quantitative RT-PCR (a, bar chart) are shown. Western blot is displayed in b (upper panel) and lower panel indicates the quantitative band intensities compared to controls. Addition of anti-IL-17A (600 ng/ml) to stimulated A549 cells (IL-17A at 50 ng/ml) reduced

CCL28 expression (c), while IFN- $\gamma$ , but not IL-1 $\beta$ , synergized with IL-17A to increase CCL28 transcription (d). A549 cells were cultured in the presence of either 10 ng/ml IL-1 $\beta$  or 100 ng/ml IFN- $\gamma$  for 24 h, then stimulated with 10 ng/ml IL-17A for 6 h (to assess cell responses to low-level stimulation) and assessed for mRNA expression. Quantitative PCR graphs represent triplicate analysis of experiments performed 3 times, Western blot densitometry demonstrates the analysis of 2 blots run in parallel and is representative of an experiment performed at least 3 times. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

gression was performed, these findings should only be considered as indicative data justifying future, more extensive, studies in larger clinical populations.

#### *CCL28 Supports Chemotaxis of CCR10+ IgE+ B Cells*

CCL28 levels in murine hyper-responsive airways are increased compared to controls and correlate with the localized expression of the receptors CCR3 and CCR10; this has been attributed to the infiltration of eosinophils and T cells bearing these receptors [3, 7]. An examination of sputum, from a small cohort of steroid-naïve human asth-

matic subjects (fig. 3), suggested that CCL28 protein concentrations correlated with both CCR10 and CCR3 mRNA expressions (fig. 3b, c;  $r = 0.898$ ,  $p < 0.05$  and  $r = 0.9676$ ,  $p < 0.01$ , respectively). It is important, however, to note that these data are not informative of the specific role of these proteins in asthma, which will require larger studies with control data from healthy volunteers and parallel studies in knockout animal models. Whilst care needs to be exercised in extrapolating from small patient cohorts, these results are consistent with previous mouse studies [3, 7] and suggest a comparable response in humans.

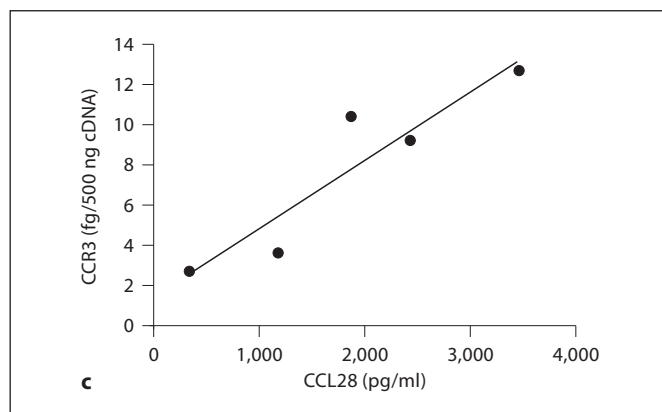
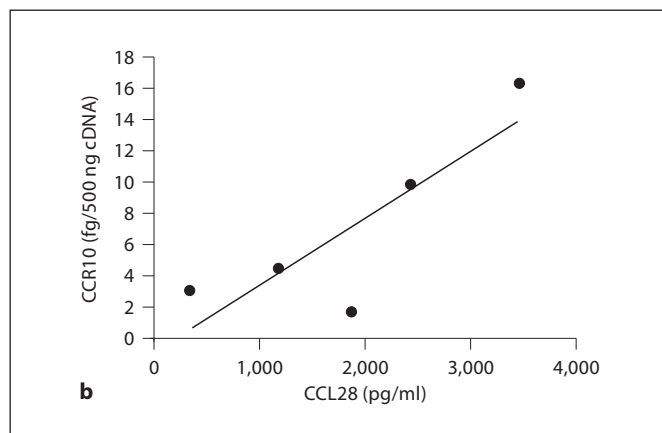
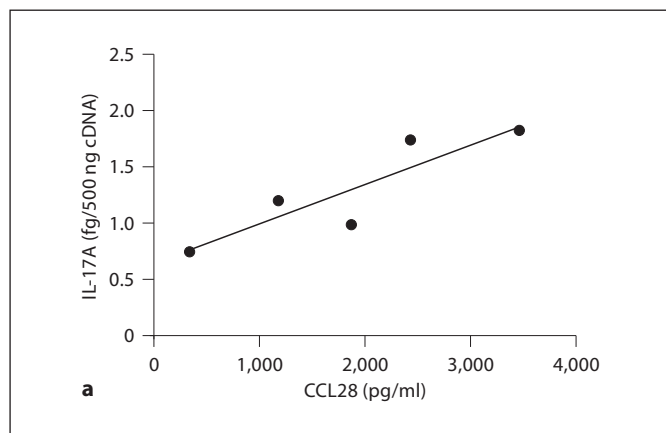


**Fig. 3.** Assessment of the IL-17A-CCL28-CCR3/CCR10 association in asthmatic patients. Induced sputa from a small pilot cohort of steroid-naïve asthmatic patients ( $n = 5$ ) were assessed for the levels of CCL28 (determined by ELISA), and IL-17A mRNA (**a**), CCR10 mRNA (**b**) and CCR3 mRNA (**c**) concentrations were determined by quantitative RT-PCR. Each patient sample ( $n = 5$ ) was examined in triplicate by ELISA and qRT-PCR. Regression in such sample sizes is limited and therefore data are considered as indicative.

In the allergic hyper-responsive lung, high levels of allergen-specific IgE contribute to airway damage [12]. However, the receptors that are involved in the recruitment of IgE-secreting B cells to the lungs have, thus far, remained poorly described. CCL28 is known to induce the chemotaxis of CCR10+ IgA+ ASC, and this interaction is critical in the accumulation of these cells in lactating mammary tissues and to the gastric intestinal tract during infection [31, 32]. Hence, it was hypothesized that CCL28 might also play a role in the recruitment of IgE+ B cells in atopy/allergy and that localization of these cells to the lungs might contribute to the population of CCL28 receptor-expressing cells observed. IgE+ B cells were obtained ex vivo at high frequency (fig. 4a), and surface expression of the CCL28 receptor CCR10 was examined by flow cytometry (fig. 4b). A subpopulation of IgE+ B cells (24%) co-expressed the CCR10 receptor for CCL28 (fig. 4d). However, surface receptor expression does not always equate to function, so the ability of these cells to migrate towards CCL28 was examined. In chemotaxis assays, both CXCL12 (positive control [33]) and CCL28

induced migration of B cells as expected, with chemotactic indices of 6 and 10, respectively, the latter remained at background levels on addition of CCL28-specific antibody (data not shown). To assess the proportion of IgE-secreting B cells responding to CCL28, migrated cells from chemotaxis assays were assessed for IgE production by ELISpot. Only CCL28 led to the statistically significant chemotaxis of IgE+ ASC above control background (fig. 4e;  $p < 0.001$ ), suggesting that CCL28 selectively recruits this population. Enrichment of IgE+ cells was specific, as a reduction in migrating IgE+ B cells resulted following CCR10 receptor blockade (fig. 4e;  $p < 0.01$ ), using an antibody raised against a peptide fragment of CCR10 [7]. Taken together, these results support a functional role for CCL28 in the recruitment of IgE+ B cells.

#### *IL-17A-Conditioned Medium Recruits IgE+ B Cells in a CCL28-Dependent Manner*

IL-17A within the inflamed lung recruits cells via chemokine induction [34]; it induces CCL28 (fig. 2) and CCL28 supports chemotaxis of IgE+ plasma cells (fig. 4).

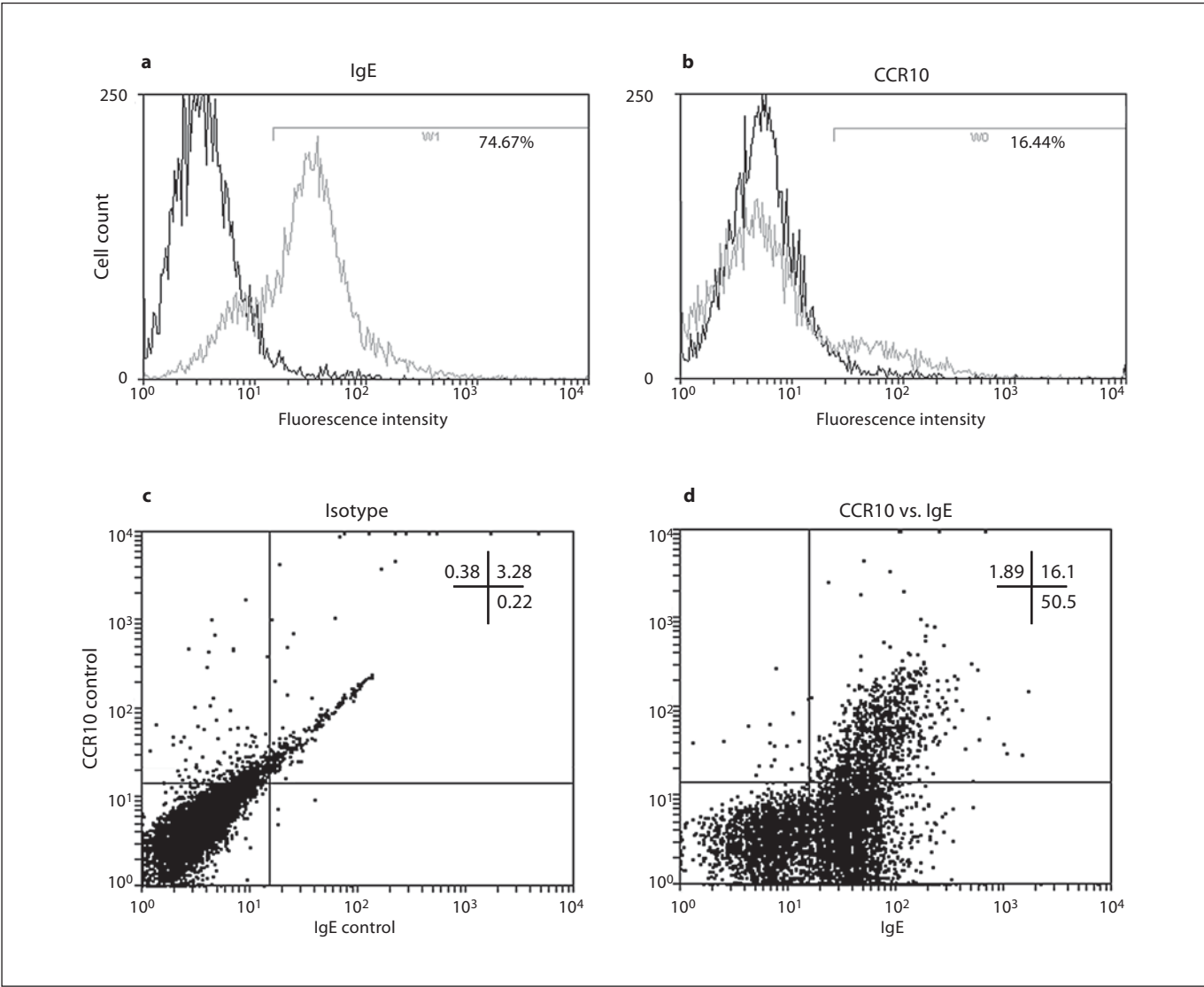
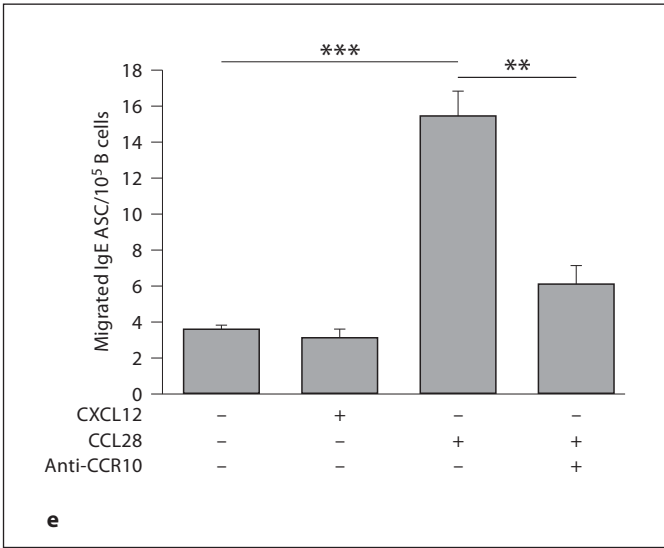


**Fig. 4.** Cultured IgE+ B cells express CCR10 and chemotax to CCL28. B cells were isolated from peripheral blood, cultured for 5 days in the presence of 30 ng/ml IL-4 and 1  $\mu$ g/ml anti-CD40, and measured for surface expression of CCR10 and IgE by flow cytometry. Subsets of the B cell population expressed IgE (a) and CCR10 (b). Black histogram displays appropriate isotype control. Isotype controls for CCR10 and IgE co-expression are displayed (c), 16% of the B cell population were IgE+ CCR10+ B cells (d). Following chemotaxis towards 250 nM CCL28 or 100 nM CXCL12 migrated cells were analysed by IgE ELISpot (e). Cells were incubated on the ELISpot plate for 24 h and probed with 1  $\mu$ g/ml biotinylated anti-IgE antibody. B cell pre-treatment with 250 ng/ml anti-CCR10 confirmed the specificity of this migration. Flow cytometry plots are representative of 3 replicate analyses, ELISpot plots illustrate the results from triplicate wells of an experiment performed 3 times. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .



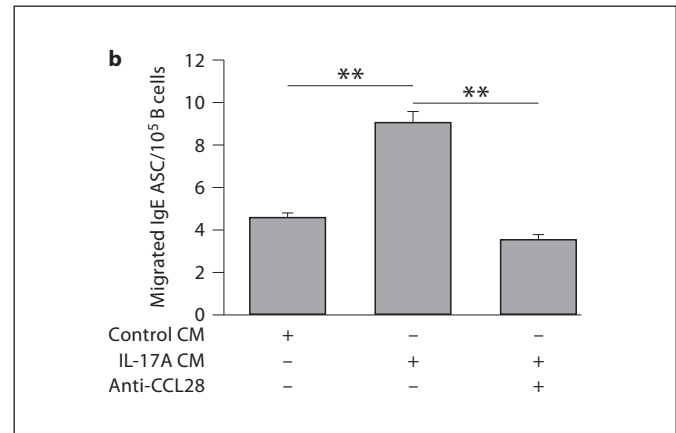
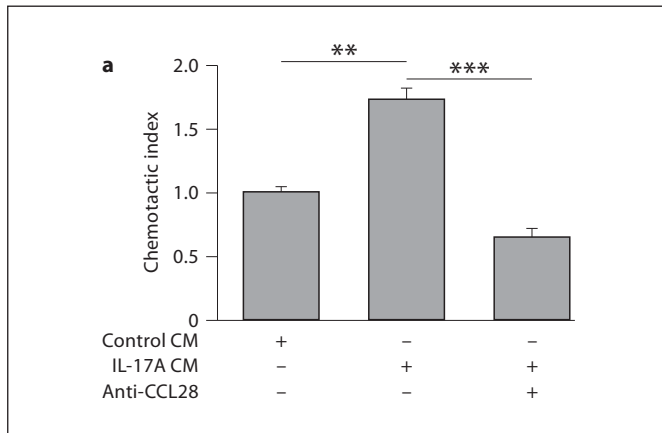


**Fig. 5.** IL-17A stimulation of lung epithelial cells enhanced the migration of IgE+ B cells. A549 cells were either unstimulated or stimulated with 50 ng/ml IL-17A for 24 h and the resulting conditioned media (600  $\mu$ l, undiluted) from unstimulated cells (CM) or from cells cultured with IL-17A (IL-17A CM) were assessed in a chemotaxis assay for their ability to recruit B cells over a 2-h pe-

riod (a), and by ELISpot to determine the number of IgE-specific ASC (b). Addition of 30  $\mu$ g/ml anti-CCL28 significantly reduced B cell and IgE-secreting B cell numbers below the levels observed for controls. Data represent triplicate analyses of experiments repeated 3 times. \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

Therefore, it was important to assess whether the levels of CCL28 resulting from IL-17A stimulation were relevant and sufficient to support enhanced B cell migration. Conditioned medium from control, unstimulated A549 cells (control CM) or medium from cells cultured for 24 h in the presence of 50 ng/ml IL-17A (IL-17A CM) were evaluated for the ability to support B cell chemotaxis. IL-17A CM induced a 2-fold increase in the number of migrating B cells, compared to unstimulated controls (fig. 5a;  $p < 0.01$ ). To eliminate the possibility that either direct IL-17A or other chemokines induced by IL-17A were responsible for this migration, an anti-CCL28 antibody was added to IL-17 CM. Addition of an anti-CCL28 antibody significantly reduced chemotaxis below control CM levels, verifying that the chemotaxis was CCL28 dependent (fig. 5a;  $p < 0.001$ ). Analysis of the numbers of IgE-secreting B cells showed similar results; the numbers of IgE+ ASC were 2-fold higher in IL-17 CM than basal levels and this was also CCL28 dependent (fig. 5b;  $p < 0.01$ ). While IL-17A stimulation of airway epithelium may result in the up-regulation of other known B cell chemoattractants, including CCL25 and CXCL12, in this study, the neutralization of IgE+ ASC migration in the presence of anti-CCL28 indicates direct contribution by this chemokine upon these cells. These results reflect the 2-fold increase in CCL28 levels above the basal level produced by epithelial-like cells in response to IL-17A (from 860 to 1,620 pg/ml; fig. 2b).

## Discussion

This study determined the effect of human IL-17A on CCL28 expression by an airway epithelial cell line and one functional outcome of this. Lung-derived primary and transformed epithelial cells expressed IL-17RA and IL-17RC, and these were enhanced in the presence of IFN- $\gamma$ . IL-17A induced the expression of CCL28 by these cells. IL-17A stimulation of lung epithelial cells supported the chemotaxis of IgE+ B cells in a CCL28-dependent manner. The levels of IL-17A and CCL28 in the sputum of a small cohort of allergic asthma patients correlated with the presence of CCR3 and CCR10 in the lungs. Hence, this study suggests a novel mechanistic link between enhanced IL-17A in the lung and increased infiltration of IgE+ B cells in asthma.

Increased levels of IL-17A are important in the clearance of many microbial pathogens through neutrophil recruitment and induction of antimicrobial proteins [24, 35]. However, high levels of this cytokine have also been implicated in the exacerbation of a number of inflammatory pathologies. As with pathogen clearance, IL-17A is thought to potentiate allergic inflammation via the recruitment of innate immune cells. Here, a novel role for IL-17A in the up-regulation of CCL28 by alveolar epithelial-like cells was demonstrated. CCL28 recruits T cells, IgA+ B cells and eosinophils via CCR10 and CCR3 [3–5]. This chemokine also contributes to the

development of allergic airway disease, with increased production identified in both asthmatic patients [6] and mouse models, correlating with the levels of CCR10 and eosinophilia in the lungs of mice [3, 7]. Hence, through the enhancement of CCL28 expression, increased IL-17A secretion may also contribute to the influx of these cells at mucosal surfaces. This may help explain the elevated numbers of IgA+ B cells and concentration of IgA in murine lung mononuclear cells and bronchoalveolar lavage fluid following adoptive transfer and activation of IL-17A-secreting T<sub>H</sub>17 cells [36]. That study did not report the concentration of IgE. CCL28 is also expressed by skin keratinocytes [37]. In a T<sub>H</sub>17-dominant skin inflammatory condition, stimulation of the skin with OVA enhances the levels of both IL-17A and OVA-specific IgE [38], thus the data herein provide a mechanistic basis for that observation. However, there are conflicting data on the effect of IL-17A on IgE production in the allergic airways, and the role of IL-17A in atopic and non-atopic asthma has been the subject of recent review [39]. In a study by Park et al. [20], addition of anti-IL-17 reduced the infiltration of inflammatory cells, airway hyperresponsiveness, T<sub>H</sub>2-associated cytokines and OVA-specific IgE. In contrast, others have demonstrated the requirement of IL-17R for initiation of allergic asthma but showed no reduction in the serum levels of this antibody in the presence of anti-IL-17 [34, 40]. The use of non-standardized mouse models and efficacy of IL-17 antibody administration may partly explain these discrepancies [41]. Resolution of these contradictory observations may require measurement of mucosal/local and systemic IgE in more standardized models. The study presented here suggests that the analysis of systemic IgE expression should be supported by quantification of the local IgE levels. Systemic IgE levels are not fully indicative or prognostic of the inflammatory response, as evidenced by a study of more than 90 subjects, where mucosally expressed IgE proved of greater relevance to determination of atopy and asthma severity [42]. Analysis of the IgE+ B cell populations, localized and circulating, would advance this area of research, although the low quantities of these cells may make this task difficult. Little difference has been observed in the number of CD20+ B cells between asthmatic patients compared to controls, while a significant increase in  $\epsilon$ -heavy chain mRNA has been observed [43].

Enhanced expressions of IL-17RA and IL-17RC are observed in peripheral whole blood and the synovium of patients with rheumatoid arthritis [26, 44], and, while the levels of IL-17A are increased in psoriatic lesional

skin, the expressions of IL-17RA and IL-17RC are either unchanged or reduced, respectively [45]. These reports advocate a role not only for the modulation of IL-17A expression in pathology but also for the specific receptors. IL-17RA expression by foetal intestinal epithelial cells shows a weak increase when stimulated by IFN- $\gamma$  [29]. Interestingly, the same study detected a synergistic relationship between IL-17A and IFN- $\gamma$  in the induced production of CXCL8 and CCL2 [29]. In the work presented here, both receptors were shown to be up-regulated upon stimulation with IFN- $\gamma$ , while LPS inhibited IL-17RA but enhanced IL-17RC expression. IFN- $\gamma$  and IL-17A synergized to enhance the expression of CCL28. It is most likely that this was due to increased IL-17A receptor expression caused by IFN- $\gamma$ . Neither control unstimulated A549 cells nor IFN- $\gamma$ -stimulated cells expressed IL-17A, therefore this effect cannot be the result of increased IL-17A levels in the culture. Hence, this study supports previous work where IL-17RA and IL-17RC enhancement increased sensitivity towards IL-17A [46]. This also suggests that T<sub>H</sub>1 immune responses would promote this effect. This result does not, however, explain what, if any, role this action plays in asthma or whether CCL28 is essential.

In  $\alpha_4\beta_7$  B cells, CXCR4 (the CXCL12 receptor) is expressed on approximately 90% of CD19+ cells, but is low on terminally differentiated IgA+ subsets. IgA+ B cells have comparatively higher levels of CCR3 and CCR10, and migrate to CCL28 [47]. Prior to this work, little was known about receptor expression on IgE+ B cells. Here, it was demonstrated that a subpopulation of IgE+ B cells co-expressed CCR10 and this enabled IgE-secreting B cells to migrate towards CCL28. In addition, assessment of enhanced migration of IgE+ ASC cells towards conditioned medium (from IL-17A-stimulated A549 cultures) indicated that the increased production of CCL28 by IL-17A was at a level to support a functional effect. These results suggest a mechanistic link between increased levels of IL-17A [28], the enhanced production of CCL28 [3, 6, 7] and consequently the recruitment of B cells to respiratory epithelium in asthma. IgE-switched B cells were investigated here, however, it is clear that IgA+ B cells are recruited by CCL28 [4] and that these cells may also be recruited, although they were not the subject of this study. IgG-secreting B cells do not respond to CCL28 (data not shown) [32, 48]. Previously, antibodies against CCR4 and CCR3 have been assessed for their capacity to inhibit T cell and eosinophil infiltration, respectively, into the allergic lung [49, 50]; this study adds the blockade of CCL28 or CCR10 as addi-

tional targets for the treatment of asthma. However, studies in large human cohorts will be required to test this.

Here we propose a novel link between IL-17A and IgE+ ASC recruitment via CCL28. A role for IL-17A in the induction of the dually functional, chemotactic and antimicrobial chemokine CCL28 in the asthmatic airway epithelium is proposed, associating IL-17A with the migration of IgE+ B cells. Taken together, this work suggests

CCL28 to be an important mediator of human allergic asthma, and supports the further evaluation of this role in humans and murine models.

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