

IL-4-expressing bronchoalveolar T cells from asthmatic and healthy subjects preferentially express CCR3 and CCR4

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Background: The concept of the polarization of chemokine receptor expression by T_H1 and T_H2 cells provides an attractive mechanism for their differential recruitment to tissue, which could be subject to disease-specific therapeutic intervention. The paradigm that T_H1 cells preferentially express CXCR3 and CCR5 and T_H2 cells preferentially express CCR3, CCR4, and CCR8 has been well established in the setting of *in vitro* polarized cell lines; however, the situation *in vivo* appears less clear-cut.

Objective: We sought to investigate whether this pattern of polarization can be demonstrated in human lung tissue.

Methods: We used single-cell analysis to investigate the relationship between chemokine receptor expression and cytokine production on peripheral blood and bronchoalveolar lavage fluid T cells in patients with asthma, a putative T_H2 disease, as well as in healthy control subjects.

Results: We have found in both asthmatic and control subjects that IL-4-expressing blood and bronchoalveolar lavage fluid T cells are significantly more likely to express the T_H2 type 2 chemokine receptors CCR3 and CCR4, with 10-fold and 2-fold differences in expression, respectively, compared with IFN- γ -expressing cells.

Conclusion: We have provided evidence that polarization of T_H2-type chemokine receptors on IL-4-expressing cells can be demonstrated in an *in vivo* setting and therefore that these cells might indeed be susceptible to differential patterns of recruitment as a result of expression of the relevant chemokines at inflammatory sites. (J Allergy Clin Immunol 2005;116:594-600.)

Key words: Lung, human, T_H1/T_H2 cells, T cells, chemokines

Memory lymphocytes migrate around the body in a programmed fashion, preferentially returning to the organ where they became sensitized. This process, known as

Abbreviations used

BAL: Bronchoalveolar lavage

BALF: Bronchoalveolar lavage fluid

lymphocyte homing, increases the chance of a T cell encountering its cognate antigen.¹ Patterns of migration are controlled by organ-specific expression of adhesion molecules and chemokines, which attract subsets of lymphocytes bearing the relevant ligand. The most well-described and specific pathways are those to the skin (involving CLA, CCR4, and CCR10),^{2,3} the small intestine ($\alpha 4\beta 7$ and CCR9),⁴ and the lymph nodes (L-selectin and CCR7).⁵ To date, no specific chemokine receptors have been identified that control homing of cells to the lung; however, it is apparent that the phenotype of lung-homing cells is distinct from that of both gut- and skin-homing T cells.⁶

Another pattern of lymphocyte migration involves differential trafficking of T_H1 and T_H2 cells. T_H1 cells are characterized by their ability to secrete IFN- γ and IL-2, whereas T_H2 cells secrete the cytokines IL-4, IL-5, and IL-13. T_H1 and T_H2 cells in both mice and human subjects were initially characterized in terms of the differential cytokine release of T-cell clones.^{7,8}

Subsequently, by using *in situ* hybridization techniques to characterize cytokine production, T_H1 and T_H2 cells were identified in inflammatory lesions.

Most notably, Robinson et al⁹ reported that up to 50% of bronchoalveolar lavage (BAL) fluid T cells in asthma expressed mRNA for IL-4, although we¹⁰ and others,¹¹ using single-cell cytokine staining, have found evidence for much lower numbers of IL-4-expressing T cells. More recently, investigators have generated *in vitro* T_H1- and T_H2-polarized cell lines from naive T cells by exploiting the cross-inhibitory effects of IFN- γ and IL-4 on T_H2 and T_H1 development, respectively. These cells show differential expression of chemokine receptors, with T_H1 cells showing a strong bias toward expression of CXCR3 and CCR5 and T_H2 cells showing a strong bias toward CCR3, CCR4, and CCR8. In contrast, analysis of the relationship between cytokine production and chemokine receptor expression in freshly isolated peripheral blood T cells showed only a limited bias toward the differential patterns described above.¹²

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We have previously observed that the expression of CCR5, CXCR3, CCR3, and CCR4 on BAL fluid (BALF) T cells did not differ between asthmatic subjects and healthy volunteers.⁶ However, because we found that only 5% to 10% of T cells were IL-4 producing, it remained possible that CCR3- and CCR4-expressing cells were preferentially contained within this subset. We have therefore undertaken a study to measure expression of the T_H1- and T_H2-associated chemokine receptors in BALF T cells expressing IL-4 and IFN- γ from patients with mild asthma and healthy control subjects.

METHODS

Subject selection

Sixteen asthmatic subjects and 9 healthy control subjects participated in the study (see Table I for clinical characteristics). Each subject underwent spirometry, skin prick testing to 4 common aeroallergens to determine atopic status, methacholine challenge testing to determine PC₂₀ FEV₁ methacholine values, and chest radiography.

Asthmatic subjects were defined as having a compatible history, such as cough, episodic wheeze, or breathlessness relieved by β_2 -agonists. All demonstrated a PC₂₀ FEV₁ methacholine value of less than 8 mg/mL (performed at least 2 weeks before bronchoscopy to minimize the potential for airway inflammation caused by the procedure) or greater than 15% reversibility to an inhaled β_2 -agonist. All asthmatic subjects were receiving intermittent treatment with only a β_2 -agonist and were defined as having stage 1 disease (mild disease) on the basis of British Thoracic Society criteria.¹³ None had received inhaled or oral corticosteroids within the 6 weeks before the study.

Control subjects had no history of respiratory disease, a PC₂₀ FEV₁ methacholine value of greater than 16 mg/mL, and normal spirometry.

All subjects involved in the study had normal chest radiographic results.

Clinical investigations

The PC₂₀ FEV₁ (concentration of methacholine required to cause a 20% decrease in FEV₁) was measured with a tidal breathing method as per European Respiratory Society guidelines on airway responsiveness testing.¹⁴

Skin prick testing for atopy was performed to *Dermatophagoides pteronyssinus*, cat fur, grass pollen, and dog fur, with histamine and saline as positive and negative controls, respectively. A wheal of more than 2 mm in diameter greater than that elicited by the negative control was considered a positive test result.

A 15-mL heparinized blood sample was obtained from all volunteers immediately before the bronchoscopy.

All patients underwent fiberoptic bronchoscopy (Olympus company, Tokyo, Japan) and lavage in accordance with standard procedure¹⁵ as a day case. Asthmatic subjects were premedicated with 2.5 mg of nebulized salbutamol to reduce BAL-associated bronchospasm. All subjects received topical lignocaine applied to the inside of the nose and the back of the throat. If requested, subjects were lightly sedated with 2.5 mg of intravenous midazolam. Supplemental oxygen through nasal prongs was administered at a rate of 2 L/min. Pulse oximetry and blood pressure were monitored throughout the procedure. Four milliliters of 2% lignocaine was applied to the vocal cords, followed by aliquots of 2 mL to the trachea and the right and left main bronchi. The scope was wedged in the middle lobe bronchus, and a 20-mL bronchial wash was performed. For the

TABLE I. Clinical characteristics of asthmatic and control subjects

	Asthmatic subjects	Control subjects
N	16	9
Age, y	47 (22-69)	43 (22-61)
Sex, F/M	9/7	5/4
Atopic, %	64	0
FEV ₁ , % predicted	93 (69-131)	107 (92-125)
PC ₂₀ , mg/mL	1.9	All >16

Values are presented as means (ranges).

*Determined by means of skin prick testing; at least one positive test result.

BAL, 180 mL of warmed 0.9% saline was instilled into the middle lobe bronchus in aliquots of 60 mL. Gentle suction was applied to retrieve as much fluid as possible.

All subjects provided written informed consent. All clinical studies were reviewed and approved by the Leicestershire and Rutland Research and Ethics Committee.

Laboratory methods

Mononuclear cells were isolated from blood after density gradient centrifugation with Histopaque 1077 (Sigma, Dorset, United Kingdom) and washed with PBS (Invitrogen, Paisley, United Kingdom) and 0.5% BSA (Sigma) before further labeling of cells.

The BALF sample was filtered through a 48- μ m mesh to remove debris. If necessary, a red blood cell depletion step was then carried out with a standard osmotic shock technique. Briefly, this involved the resuspension of the cell pellet in 5 mL of distilled water for exactly 30 seconds, followed by quenching with a large volume of PBS buffer before further centrifugation. A differential cell count for each BAL sample was obtained by using the Romanowsky stain and counted by an individual blinded to the subjects' clinical status. Differential counts were expressed as the percentage of nonsquamous cells.

Cell samples from both peripheral blood and BALF were incubated with 10% FCS to reduce nonspecific binding before incubation with one of 5 primary unlabelled monoclonal chemokine receptor antibodies. In each case, the entire BAL sample was used. The number of cells analyzed varied for each patient and chemokine receptor depending on the number of cells available; in some cases where there was insufficient cell recovery from the BALF, fewer than 5 chemokine receptors were studied. Chemokine receptor antibodies used were CCR3 and CCR4 (a kind gift of Millennium Pharmaceuticals), CCR5 (BD Pharmingen, Oxford, United Kingdom), and CXCR3 and CXCR6 (R&D, Oxford, United Kingdom); all were mouse mAbs and were used at a final concentration of between 5 and 10 μ g/mL. Staining was visualized with an FITC-labeled rabbit anti-mouse antibody (DAKO, Glostrup, Denmark) at a dilution of 1:20.

Half of each chemokine receptor-labeled cell aliquot was then subjected to stimulation for 4 hours at 37°C in medium (RPMI plus 10% FCS) containing 5 ng/mL phorbol 12-myristate 13-acetate (Sigma), 250 ng/mL calcium ionophore (Sigma), and 10 μ g/mL brefeldin (Sigma). The other half of the sample was not subjected to this stimulation procedure to obtain a resting sample.

Subsequently, after blocking with 10% normal mouse serum, staining for CD3 was performed with directly labeled antibody (CD3PerCP, BD Pharmingen). Cells were then fixed and permeabilized with 4% paraformaldehyde (Sigma)/0.1% saponin (Sigma) for 15 minutes at 4°C before direct labeled anticytokine antibody (either IFN- γ phycoerythrin or IL-4 phycoerythrin, both BD Pharmingen) was added to half of the cells of each resting or stimulated chemokine receptor-labeled sample. Isotype controls were used at each stage of the experiment.

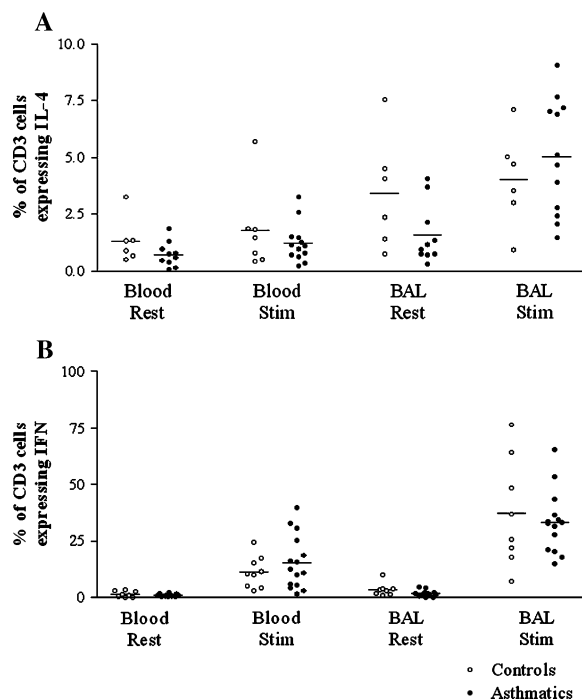


FIG 1. Scattergraph showing the average expression of IL-4 (**A**) and IFN- γ (**B**) by T cells derived from blood and BALF with and without stimulation with phorbol 12-myristate 13-acetate/ionophore. Individual points are derived from the mean values obtained within experiments from each separate chemokine-cytokine pairing analyzed. Values for asthmatic subjects are shown as filled circles, and values for control subjects are shown as open circles.

Because of technical difficulties, we were unable to successfully stain for IL-4 cytokine in the first 5 subjects (3 control and 2 asthmatic subjects) and therefore have not presented these data, although staining for IFN- γ was successful in these cases.

All samples were acquired with a BD FACScan flow cytometer, and results were analyzed with CellQuest Pro software, version 4.0.2 (BD Biosciences, Cowley, Oxford, United Kingdom). Lymphocytes were initially gated on the basis of their forward-scatter/side-scatter properties. Within this gated area, cells were assessed for CD3 expression. Positive cells were gated and used for further analysis. Values obtained for isotypic controls were subtracted from each quadrant to give a final value for expression (see Fig E1 in the Online Repository in the online version of this article at www.mosby.com/jaci for an example).

Where data were log normally distributed, geometric means were used for analysis, and results are presented as geometric means $\pm$ logSEM. Where data were normally distributed, values are expressed as arithmetic means $\pm$ SEM. The Student *t* test was used to compare differences between groups, and *P* values of less than .05 were considered significant. All statistical analysis was done with Graphpad Prism (Graphpad Software Inc, San Diego, Calif).

RESULTS

Cytokine production in resting and stimulated cells from asthmatic subjects and healthy control subjects

The mean total cell number in recovered BALF was 8.52 million (range, 4 million to 13 million) for control subjects and 9.26 million (range, 1.5 million to 21 million)

for asthmatic subjects. The percentage of lymphocytes in these samples ranged from 0.8% to 16.8%. There was no significant difference in these results between the asthmatic and control samples. Mean numbers of T cells analyzed from asthmatic BALF for each chemokine receptor-cytokine combination was 3397 ± 638 for IFN- γ -chemokine receptor combinations, and 3351 ± 631 for IL-4-chemokine receptor combinations. These numbers were not significantly different for control subjects (2153 ± 608 and 2232 ± 617 , respectively).

In all subjects studied, the majority of the T cells from both blood and BALF samples did not express IL-4 or IFN- γ , even after stimulation. In stimulated BALF samples from asthmatic and control subjects, the mean percentages of IFN- γ -expressing T cells were $33.1\% \pm 3.7\%$ ($n = 14$) and $37.1\% \pm 8.5\%$ ($n = 8$), respectively, and for IL-4 they were $5.0\% \pm 0.7\%$ ($n = 12$) and $4.1\% \pm 0.9\%$ ($n = 6$), respectively (Fig 1).

Within both groups studied, there were significantly more cytokine-expressing T cells in the stimulated BALF samples compared with stimulated blood samples, with the exception of the IL-4 expression in control subjects, which showed a trend toward an increase in the BALF samples compared with the blood samples but which did not reach significance ($P = .06$, Fig 1).

Comparison of asthmatic subjects with control subjects did not reveal any difference in the percentages of cells able to express either cytokine, with or without stimulation, in either peripheral blood or BALF.

Chemokine receptor expression of T cells from blood and BALF in asthmatic and control subjects

The pattern of chemokine receptor expression on freshly isolated unstimulated blood and BALF T cells is shown in Fig 2. As has been previously reported for BAL,⁶ there were no significant differences in the pattern of chemokine receptor expression between healthy or asthmatic subjects in the BALF. This similarity of chemokine receptor expression between subject groups was also seen in the unstimulated peripheral blood samples.

Expression of CCR3 and CCR4 on BALF T cells was low in both groups ($7.1\% \pm 4.1\%$ and $29.6\% \pm 5.7\%$ for control subjects and $2.9\% \pm 0.7\%$ and $20.7\% \pm 2.7\%$ for asthmatic subjects, respectively) and was of a similar magnitude on the blood T cells ($0.6\% \pm 0.3\%$ and $22.3\% \pm 3.0\%$ for control subjects and $1.1\% \pm 0.3\%$ and $23.1\% \pm 3.3\%$ for asthmatic subjects, respectively). As previously reported,⁶ expression of CCR5 and CXCR3 was higher on T cells from BALF compared with T cells from blood in both groups.

Association between IFN- γ and IL-4 expression and expression of the T_H2-associated chemokine receptors CCR3 and CCR4 on BALF T cells

T cells from each sample were identified during the analysis process as either IFN- γ expressing (putative T_H1)

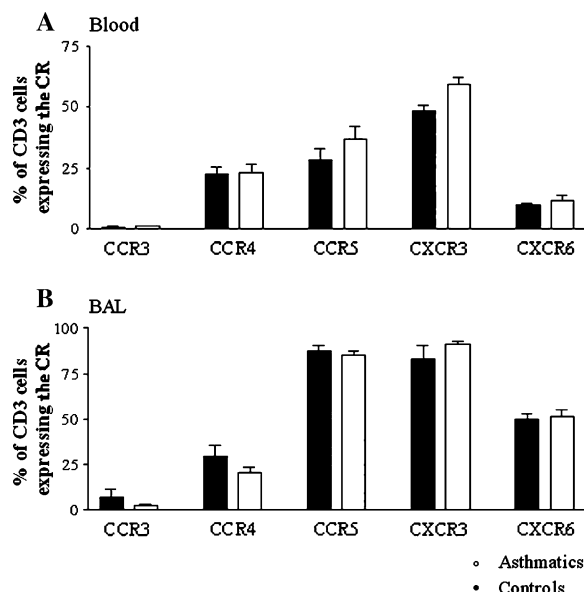


FIG 2. Histograms showing the expression of individual chemokine receptors in CD3⁺ T cells from peripheral blood (**A**) and BALF (**B**) samples for asthmatic (open bars) and control (filled bars) patients. Error bars show the SEM. Significant differences between groups by unpaired *t* test are marked. Numbers vary from 6 to 14 for each individual receptor within a subject group. CR, Chemokine receptor.

or IL-4 expressing (putative T_H2). These subgroups were then further analyzed for their chemokine receptor expression. For an example of the gating strategy used to correlate chemokine receptor expression with cytokine production, see Fig E1 in the Online Repository in the online version of this article at www.mosby.com/jaci.

There was a striking difference in the percentage of IL-4-expressing BALF T cells expressing CCR3 and CCR4 compared with IFN- γ -expressing BALF T cells in both asthmatic and healthy subjects. In BALF from control subjects, the percentage of IL-4- and IFN- γ -expressing T cells also expressing CCR3 was $31.4\% \pm 8.1\%$ and $2.4\% \pm 1.1\%$, respectively (mean difference, 29.0; 95% CI, 44.5-13.6; $P = .0015$; Fig 3, A). The percentages of IL-4- and IFN- γ -expressing T cells also expressing CCR4 were $46.7\% \pm 1.1\%$ and $18.3\% \pm 1.4\%$, respectively (mean fold difference, 2.55; 95% CI, 0.16-0.98; $P = .046$; Fig 3, B). In asthmatic subjects the same pattern of chemokine receptor polarization was seen in that $32.5\% \pm 10.5\%$ of IL-4-expressing cells expressed CCR3 compared with $2.3\% \pm 0.8\%$ of IFN- γ -expressing cells (mean difference, 30.2; 95% CI, 50.2-10.2; $P = .005$; Fig 3, A) and $43.7\% \pm 1.1\%$ of IL-4-expressing cells expressed CCR4 compared with $5.4\% \pm 1.5\%$ of IFN- γ -expressing cells (mean fold difference, 8.1; 95% CI, 0.05-0.32; $P = .0002$; Fig 3, B).

There was no significant difference in the degree of polarization of CCR3 and CCR4 expression on IFN- γ - and IL-4-expressing BALF T cells in control subjects compared with that seen in asthmatic patients.

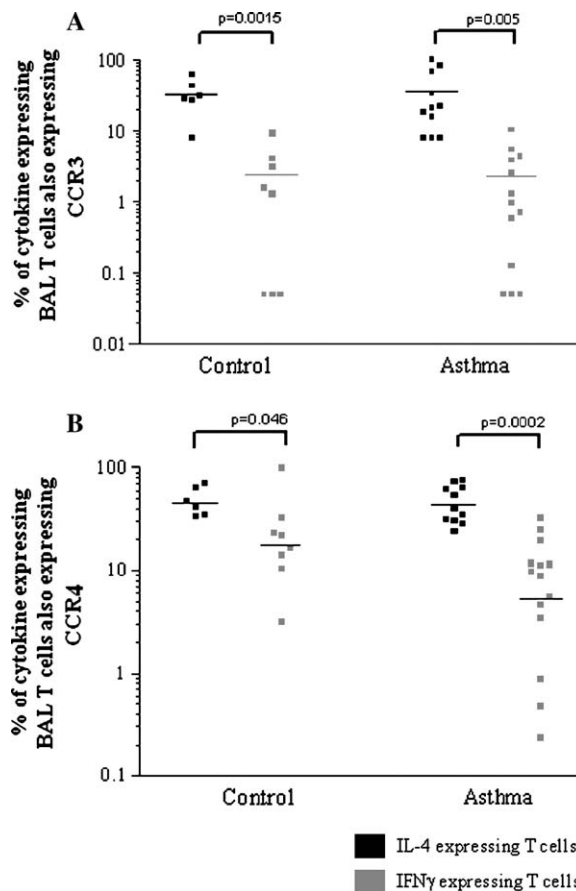


FIG 3. Scattergraphs showing percentages of asthmatic and control BAL T cells that express either IFN (gray) or IL-4 (black) and that also express the chemokine receptor CCR3 (**A**) or CCR4 (**B**). Significant differences in CCR3 and CCR4 expression for IL-4-secreting cells compared with IFN-expressing cells are shown. Control subjects, *n* = 6 to 8 for each chemokine receptor-cytokine pairing; for asthmatic subjects, *n* = 11 to 14.

Association between IFN- γ and IL-4 expression and expression of the T_H2-associated chemokine receptors CCR3 and CCR4 in blood

Similar patterns of expression were also seen in peripheral blood from control and asthmatic subjects, with IL-4-expressing T cells having higher levels of expression of CCR3 and CCR4 than IFN- γ -expressing T cells (Fig 4). In control subjects $16.3\% \pm 1.4\%$ of IL-4-expressing T cells expressed CCR3 compared with $0.84\% \pm 1.7\%$ of IFN- γ -expressing T cells (mean fold difference, 19.2; 95% CI, 0.01-0.2; $P = .0004$; Fig 4, A). Corresponding values for asthmatic subjects were $8.4\% \pm 1.4\%$ and $0.5\% \pm 1.4\%$, respectively (mean fold difference, 17.1; 95% CI, 0.02-0.17; $P \leq .0001$; Fig 4, A). In control subjects CCR4 was expressed on $33.7\% \pm 1.1\%$ of IL-4-expressing cells compared with $13.9\% \pm 1.2\%$ of IFN- γ -expressing cells (mean fold difference, 2.43; 95% CI, 0.26-0.65; $P = .0008$; Fig 4, B), and in asthmatic

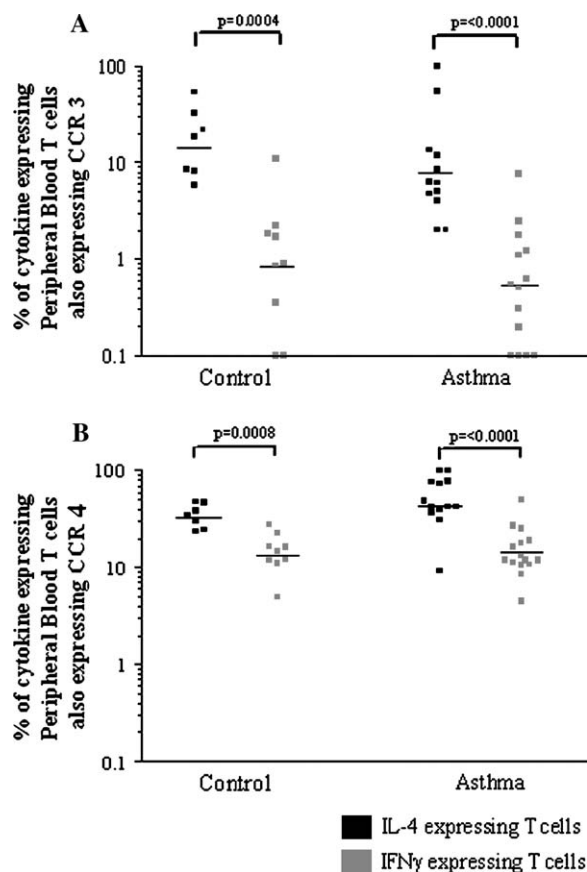


FIG 4. Scattergraphs showing percentages of asthmatic and control peripheral blood T cells that express either IFN (gray) or IL-4 (black) and that also express the chemokine receptors CCR3 (A) or CCR4 (B). Significant differences in CCR3 and CCR4 expression for IL-4-secreting cells compared with IFN-expressing cells are shown. For control subjects, $n = 7$ to 9 for each chemokine receptor-cytokine pairing; for asthmatic subjects, $n = 12$ to 15 .

subjects equivalent values were $47.6\% \pm 1.2\%$ and $14.2\% \pm 1.2\%$, respectively (mean fold difference, 3.35; 95% CI, 0.19-0.47; $P \leq .0001$; Fig 4, B).

Association between IFN- γ and IL-4 expression and expression of the T_H1 -associated chemokine receptors CCR5, CXCR3, and CXCR6 in BALF

There were no significant differences in the expression of CXCR3, CCR5, or CXCR6 on IFN- γ -secreting versus IL-4-secreting BALF T cells from either asthmatic or control subjects (Fig 5, A).

Association between IFN- γ and IL-4 expression and expression of the T_H1 -associated chemokine receptors CCR5, CXCR3, and CXCR6 in blood

In asthmatic subjects there was no difference in the expression of CCR5 on IFN- γ -expressing compared with IL-4-expressing cells. There was, however, a significant

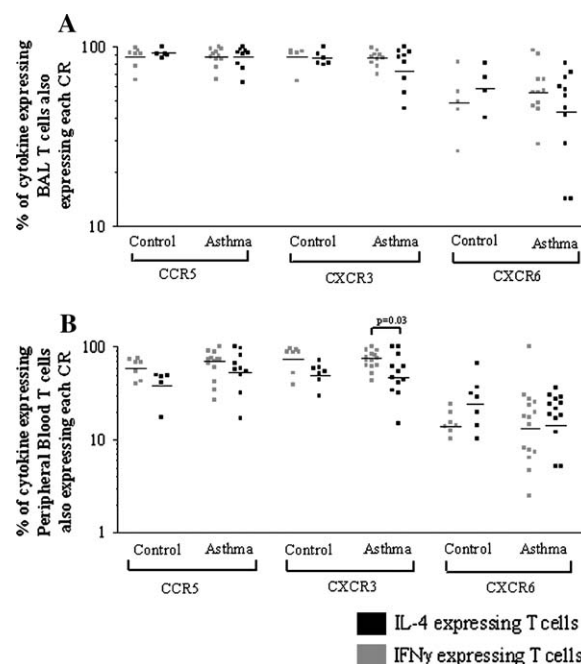


FIG 5. Scattergraphs showing the percentages of BALF T cells (A) and peripheral blood T cells (B) that express cytokine, either IFN (gray symbols) or IL-4 (black symbols), and that also express the chemokine receptors CCR5, CXCR3, or CXCR6. Values for both asthmatic and control samples are shown. Significant differences in expression of chemokine receptors on cytokine-expressing cells are shown. CR, Chemokine receptor.

difference in the expression of CXCR3, with $72.3\% \pm 1.1\%$ of IFN- γ -expressing T cells stained for CXCR3 compared with $50.4\% \pm 1.2\%$ of IL-4-secreting cells (mean fold difference, 1.43; 95% CI, 0.5-0.97; $P = .03$; Fig 5, B).

In the control group there was a trend toward greater expression of CCR5 on IFN- γ -expressing cells, with $59.6\% \pm 1.1\%$ of IFN- γ -expressing cells stained for CCR5 compared with $39.1\% \pm 1.2\%$ of IL-4-expressing cells (mean fold difference, 1.52; 95% CI, 0.42-1.04; $P = .052$). The control group also showed a trend toward greater expression of CXCR3 on IFN- γ -expressing cells ($74.8\% \pm 1.1\%$ and $51.9\% \pm 1.4\%$, respectively; mean fold difference, 1.4; 95% CI, 0.48-1.003; $P = .07$); however, neither of these differences reached statistical significance ($P = .052$ and $P = .07$, respectively; Fig 5, B).

There was no significant difference in CXCR6 expression on IFN- γ -expressing versus IL-4-expressing T cells in either subject group.

DISCUSSION

The possibility that T_H1 and T_H2 lymphocytes undergo differential patterns of migration as a result of variable chemokine receptor expression is of considerable interest, not least because selective antagonism of T_H1 - and T_H2 -related chemokine receptors could reduce inappropriate

inflammation without compromising immunity. For example, in asthma, a disease thought to be due to exaggerated T_H2 -mediated immune responsiveness in the airways,¹⁶ selective inhibition of T_H2 cell migration could ameliorate the disease without increasing the risk of respiratory infection. In this article we have shown, for the first time, that CCR3 and CCR4 are preferentially expressed by IL-4-secreting (putative T_H2) BALF T cells in the lung in both healthy subjects and patients with clinical mild asthma.

A number of groups have previously demonstrated that T cells polarized *in vitro* toward a T_H1 or T_H2 pattern of secretion preferentially express CXCR3 and CCR5 (T_H1) or CCR4 and CCR8 (T_H2),¹⁷⁻²¹ although the extent to which these *in vitro* polarized cells represent the position *in vivo* is unclear.

We did not see any difference in cell numbers or lymphocyte proportions in the BALF samples recovered from asthmatic and control subjects, and this finding is consistent with those of all other studies in clinical asthma, which do not generally show increased numbers of T cells either in BALF or bronchial biopsy specimens. As we have previously found,⁶ there was also little difference in the chemokine receptor expression on resting blood and BALF T cells in asthmatic compared with control subjects.

The majority of the T cells isolated from both blood and BALF in our study produced neither IL-4 nor IFN- γ , a finding that has been previously documented in other studies.¹² In the absence of stimulation, there was virtually no cytokine expression by blood or lung T cells (<5%).

Even in the lung there were only small numbers of IL-4-expressing cells, with no difference between asthmatic and healthy subjects, possibly reflecting the fact that these asthmatic patients had only mild disease. However, they did have a significant airway eosinophilia as evidenced by cell counts of induced sputum from the asthmatic group in our study, which revealed an average eosinophil count of 5.3% (normal range in our laboratory of up to 3%; data not shown). These findings are consistent with other reports suggesting that T_H2 cells make up only a small minority of T cells in BALF.^{10,11}

Polarization of chemokine receptor expression on IL-4-producing cells has also been reported by Panina-Bordignon et al.²² They reported a bias in the expression of CCR4 and CCR8, but not CCR3, on IL-4-producing cells in the bronchial submucosa in patients with asthma, especially after allergen challenge. There are, however, a number of differences between their findings and ours. Using immunohistochemistry, they found very high levels of IL-4 expression by T cells, even in sham-challenged patients, whereas we¹⁰ and others,¹¹ using flow cytometry, have found very little constitutive expression of IL-4 or INF- γ . In addition, they found that 100% of CD3 T cells expressed CCR4 in asthma but found no expression in chronic obstructive pulmonary disease or sarcoidosis, whereas we have found moderately low levels of CCR4 expression and have not observed a difference between healthy subjects and subjects with asthma.⁶ They found no

expression of CCR3, whereas we have consistently found expression of CCR3 by a small percentage of BALF T cells. It is possible that these differences were due to the different compartments studied (endobronchial biopsy versus BAL), although the T-cell counts in their study were several-fold higher than in other comparable studies,²³ raising the possibility that they were investigating an unusual asthma phenotype. We have been unable to study CCR8 because in our hands none of the available anti-CCR8 antibodies worked in flow cytometry.

The detection of only small numbers of T cells expressing IL-4 in both blood and BALF samples after stimulation, combined with the low levels of CCR3 expression seen on these cells, means that the associated analysis of CCR3 expression in IL-4-expressing cells involves small numbers of cells. However, there was low background staining, and with careful attention to the use of isotypic mouse controls, we believe that the data and conclusions regarding the comparison of the relative numbers of CCR3 cells expressing IL-4 and IFN- γ are robust.

One problem with measuring coexpression of chemokine receptors and cytokines is that the level of chemokine receptor expression often changes during stimulation. By staining for chemokine receptors before stimulation, as described by Kim et al,¹² we were able to minimize this effect. Our work shows that the prestaining of chemokine receptors has no effect on the subsequent measurement of cytokine expression by the same cell.

Kim et al¹² have described CXCR3⁻CCR4⁺ cells that were heavily biased toward a T_H2 profile. We were unable to investigate this relationship in our study because we only had access to 3-color flow cytometry. In addition, we cannot exclude the possibility that some of the T cells were expressing both IL-4 and IFN- γ (putative T_H0 cells), although there are relatively few of these cells (around 1%) in peripheral blood,¹² and they are therefore unlikely to be present in high numbers in the airways. CXCR6 was also reported by this group as showing a T_H1 bias,¹⁸ but we found no evidence of this in our study either in BALF or blood. We were also unable to demonstrate the presence of chemokine receptor expression polarization of CCR5 and CXCR3 on BALF samples. This relationship has not been previously assessed in lung tissue, and these findings might suggest that the previously documented polarization patterns seen in peripheral blood T cells are not retained within the lung.

Recruitment of CCR3- and CCR4-expressing IL-4-secreting cells to the lung would require the generation of the relevant chemokine ligand. CCL17 (TARC) and CCL22 (MDC) are both found in BALF after allergen challenge but are not prominent in mild clinical disease. Similarly, most of the CCR3-binding chemokines have only been described in the lung in the context of more severe disease. It is possible that low levels of these chemokines are responsible for the recruitment of this minor population of lung T cells or that alternative novel CCR3- and CCR4-binding chemokines with a more homeostatic role are responsible.

In conclusion, we have provided evidence for the differential expression of the T_H2-type chemokine receptors CCR3 and CCR4 on IL-4-expressing blood and BALF T cells from both asthmatic subjects and healthy control subjects, thus providing a mechanism by which these cells might be differentially recruited to tissues and perhaps providing potential therapeutic targets for those diseases in which excessive trafficking becomes pathologic in nature.

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