Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/j.1365-2249.2008.03657.x

Elevated CCR6+ CD4+ T lymphocytes in tissue compared with blood and induction of CCL20 during the asthmatic late response

J. N. Francis,* I. Sabroe,† C. M. Lloyd,‡ S. R. Durham* and S. J. Till*

*Sections of Allergy and Clinical Immunology and ‡Leukocyte Biology, Imperial College London, London, and †Academic Unit of Respiratory Medicine, School of Medicine and Biological Sciences, University of Sheffield, Sheffield, UK

Accepted for publication 27 February 2008

Correspondence: S. Till, Section of Allergy and Clinical Immunology, Sir Alexander Fleming Building, Imperial College London, Exhibition Road, London SW7 2AZ, UK.

E-mail: stephen.till@imperial.ac.uk

Summary

CCR6 is expressed by multiple leucocyte subsets, including peripheral blood memory T cells, and mouse models implicate a role for this receptor in diverse inflammatory responses that include allergic airway disorders, inflammatory bowel disease and autoimmune encephalitis. In order to study the role of CCR6 in humans, we have investigated the patterns of CCR6 expression and function on T cells from the peripheral blood, skin, nose and lung, in health and in allergic disease. Results show that CCR6 was expressed consistently on a higher proportion of tissue *versus* peripheral blood-derived CD4+ T cells ($P < 0{\cdot}01$). CCR6 was expressed predominantly on CD4+ compared with CD8+ cells in both blood- and tissue-derived T cells ($P < 0{\cdot}001$). The number of cells showing CCR6 expression was not proportionally greater in peripheral blood or nasal mucosal T cells of subjects with symptomatic allergic rhinitis. CCR6+ cells demonstrated enhanced functional responses to CCL20 and CCL20 was increased in bronchoalveolar lavage fluid of asthmatics following endobronchial allergen provocation ($P < 0{\cdot}05$). Thus, CCR6 may be important in the regulation of T cell recruitment to tissue and up-regulation of CCL20 expression may contribute to the recruitment and/or retention of effector T cells in allergic asthma.

Keywords: allergy, CCL20, CCR6, chemokine receptor, T cells

Introduction

Chemokine receptors have the potential to direct selective leucocyte recruitment through restricted patterns of cell surface expression. For example, T helper 2 (Th2) cells express CCR3, CCR4 and CCR8 preferentially *in vitro* [1–3] and Th1 cells express CCR5 and CXCR3 [4], although the specificity of these receptors in directing human Th2 and Th1 responses *in vivo* remains unclear. Some receptors, including CCR3 [5], CXCR1 [6] and CX3CR [7], appear to be up-regulated on T cells in allergic responses, although their roles in other T cell-mediated inflammatory conditions is unknown. Other receptors appear to exert homeostatic functions, such as CCR7, which mediates T cell recruitment to lymph nodes via high endothelial venules [8].

The contribution of CCR6 to regulating recruitment of leucocyte subpopulations is only partially understood. CCR6 is expressed on human peripheral blood memory CD45RO+ T cells [9,10], B cells [11] and certain dendritic cell subsets [12]. Ligation of CCR6 by the chemokine CCL20 results in firm arrest of memory T cells on activated endothelial cells [13]. Nevertheless, CCR6 function may well extend beyond simple recruitment of memory T cells, as CCR6$^{-/-}$ mice are characterized by impaired dendritic cell recruitment into Peyer's patches and increased intestinal epithelial T cell infiltration with dysregulation of mucosal, although not systemic, immune responses to antigen [14]. In a murine model of asthma, CCR6$^{-/-}$ animals showed reduced airway inflammatory responses with lower interleukin (IL)-5 production [15]. Similarly, CCR6 knock-out mice have suggested roles for this receptor in other inflammatory responses including graft-*versus*-host disease [16] and inflammatory bowel disease [17]. In humans, CCR6 is expressed on a higher proportion of CD3+ T cells from bronchoalveolar lavage than peripheral blood [18]. CCR6 is also expressed on a higher proportion of skin-homing cutaneous lymphocyte-associated antigen (CLA+) memory T cells in peripheral blood, and both CCR6 and CCL20 mRNA expression are increased in psoriatic skin lesions [19].

In the present study, our aim was to test the hypothesis that certain chemokine receptors are associated with T cell localization in the nasal mucosa and also the presence of active allergic disease. Having identified higher levels of CCR6 expression on T cells expanded from nasal mucosal

© 2008 The Author(s)
Journal compilation © 2008 British Society for Immunology,

Table 1. Subject characterization. Data are shown as median (lower quartile, upper quartile).

	Atopic	Normal
Gender	2 male/5 female	2 male/5 female
Age	29 (25, 33)	32 (29, 40)
Skin prick test to *Phleum pratense* (mm)	7 (5·5, 8)	0 (0, 0)
RAST to *P. pratense* (IU/ml)	63 (30, 75)	< 0·34 (< 0·34, < 0·34)
Total IgE (IU/ml)	214 (108, 343)	10 (8·5, 78)

RAST, radioallergosorbent test.

biopsies than matched cells expanded from peripheral blood, we went on to analyse CCR6 expression on CD4$^+$ and CD8$^+$ T lymphocytes expanded from cutaneous and lung biopsies, as well as obtained from fresh bronchoalveolar lavage fluid. We report that CCR6 is a correlate of non-specific tissue localization and that local production of CCL20 during asthmatic allergen challenge suggests that the CCL20–CCR6 axis has the potential to contribute to effector T cell recruitment in allergic inflammation.

Materials and methods

Reagents and antibodies

We used the following anti-chemokine receptor monoclonal antibodies (mAbs), all of which were from Millennium Pharmaceuticals (with grateful thanks especially to Drs Greg LaRosa and Lijun Wu) except where indicated: anti-CCR1 2D4, anti-CCR2 1D9, anti-CCR3 7B11, anti-CCR4 1G1, anti-CCR5 2D7, anti-CCR6 11A9, anti-CCR7 3G4, anti-CXCR1 5A12, anti-CXCR2 6C6, anti-CXCR3 1C6 and CCR6-phycoerythrin (PE) (Pharmingen, Cowley, Oxon, UK). Cell culture and general laboratory reagents were from Sigma (Poole, Dorset, UK). Anti-human integrin $\alpha 4\beta 1$ (P4G9), CD11a (MHM24), CD44 (DF1485), CD29 (K20), CD103 (Ber-ACT8), CD49f (M3511), CD45RO, CD4-fluorescein isothiocyanate (FITC), CD4-R-PE (RPE)-Cy5, CD8-FITC and secondary antibodies anti-mouse immunoglobulin G (IgG)-FITC, anti-mouse IgG-PE and anti-mouse IgG-RPE-Cy5 were all obtained from DakoCytomation (Ely, East Anglia, UK). Control antibodies (mouse IgG1 clone MOPC-21 and IgG2a clone UPC-10) were purchased from Sigma. Recombinant human IL-2 and CCL20 were from PeproTech EC (London, UK).

Study participants

Atopic and normal subjects are characterized in Table 1. For experiments aimed to assess differences between normal and atopic donors, samples were collected during the UK grass pollen season and allergic subjects were symptomatic at the time of participation. Other experiments, using samples collected from only normal donors, were collected throughout the year. The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals National Health Service Trust and subjects provided written informed consent.

Isolation of cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density gradient centrifugation over Histopaque (Sigma), washed twice with RPMI-1640 (Invitrogen, Paisley, UK) and resuspended at 10^6 cells/ml in RPMI-1640 supplemented with 5% human antibody serum, 100 U/ml penicillin/streptomycin and 2 mM L-glutamine (all Invitrogen). In some experiments PBMC were incubated with 0, 2 or 20 µg/ml of *Phleum pratense* allergen extract (Aquagen, ALK-Abelló, Horsholm, Denmark) and after 6 days cells were assessed for CCR6 expression, as below. For flow cytometric analysis on freshly isolated bronchoalveolar lavage (BAL) T cells, seven non-allergic non-asthmatic subjects underwent fibreoptic bronchoscopy, bronchoalveolar lavage and bronchial biopsy, as described previously [20]. BAL fluid was filtered through two layers of sterile gauze to remove mucus and cells were washed twice in RPMI-1640 before being stained with mAbs for flow cytometry as described below.

Culture of T cells from nasal biopsies and peripheral blood

Nasal mucosal, bronchial mucosal or skin biopsy specimens were collected as described previously [21] for generation of short-term T cell lines. Biopsies were placed in a 24-well culture plate containing 2 ml of complete medium and supplemented with 10 ng/ml of IL-2. In all cases, a sample of peripheral blood was collected for analysis at the same visit as the biopsy was performed. PBMC from these specimens were resuspended at 10^6 cells/ml and cultured (in parallel with biopsy material) in the presence of IL-2 as above. Following incubation for 5 days, biopsy tissue was removed from culture wells, and the remaining lymphocytes restimulated with irradiated PBMC (3000 rads) at 10^6 cells/ml in the presence of 10 ng/ml of IL-2 and 1 µg/ml phytohaemagglutinin. Peripheral blood T cell lines were derived in parallel cultures under identical conditions. T cells were expanded for an additional 8 days, with fresh complete media and cytokine added every 2–3 days.

Flow cytometry. For assessment of chemokine receptor expression, cells were washed with staining buffer [phosphate-buffered saline (PBS) containing 0·1% bovine serum albumin (BSA) and 0·09% sodium azide] and incubated with an anti-chemokine receptor antibody or control antibody for 30 min on ice. Cells were washed and incubated

© 2008 The Author(s)

with RPE-conjugated goat anti-mouse antibodies. A blocking step was performed with mouse IgG prior to labelling with CD8 and CD4. Some experiments utilized directly conjugated CCR6 antibodies. Cells were finally resuspended in PBS containing To-Pro 3 (Molecular Probes, Eugene, OR, USA) for exclusion of dead cells. The lymphocyte region was defined by gating on a forward-/side-scatter plot and lymphocytes were gated for expression of either CD4 or CD8. Cells that stained positively for To-Pro 3 were excluded from analysis. Chemokine receptor expression was assessed by gating on either $CD4^+$ or $CD8^+$ cells and cells stained with an isotype control antibody were used to define the negative population [21]. Samples were analysed using a FACSCalibur flow cytometer with appropriate compensation settings (Becton-Dickinson, Mountainview, CA, USA) and analysed with WinMDI (J. Trotter, The Scripp Research Institute, La Jolla, CA, USA).

Chemotaxis assay

Cells were resuspended at 5×10^6 viable cells/ml in RPMI-1640 containing 0·5% BSA. In duplicate assays, 600 μl of diluted recombinant human CCL20 (at a final concentration of 10, 100 or 1000 ng/ml) was added to the lower half of transwell inserts (Corning Costar, NY, USA) and 100 μl of cell suspension was added to the upper chamber. The assay was incubated for 3 h at 37°C and the cells were collected from the lower chamber after washing the filter underside. The total number of cells in the lower chamber was assessed by duplicate haemacytometer measurements. In order to correct for chemokinesis (enhanced random migration) of cells, the data are expressed as a migration index, calculated by dividing the percentage migration obtained in the presence of chemokine divided by the percentage migration obtained in the absence of chemokine.

Intracellular actin polymerization

Freshly isolated PBMC were labelled with anti-CCR6-PE and CD4–Cy5 as described and incubated at 5×10^6 cells/ml for 10 min at 37°C in RPMI-1640. Recombinant human CCL20 (at a final concentration of 1,10 or 100 ng/ml) was added to the cell suspension and every 15 s aliquots of $0{\cdot}5 \times 10^6$ cells were removed and mixed with 400 μl of 10^{-7} M FITC-phalloidin, 0·125 mg/ml L-α-lysophosphatidylcholine and 4·0% paraformaldehyde and analysed by flow cytometry.

Measurement of CCL20 in bronchoalveolar lavage fluid

Bronchoalveolar lavage samples were obtained from allergen-sensitive asthmatics before and after segmental allergen challenge with 100 BU of *Dermatophagoides pteronyssinus*, as described previously [22]. Concentrations of CCL20 in BAL samples were measured in duplicate by means of enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs (R&D Systems, Abingdon, UK) and human recombinant cytokine as a standard (PeproTech EC). The limit of detection for the assay was 0·05 pg/ml. It is noteworthy that using standard lavage procedures, the volume of lung epithelial lining fluid is calculated to be 1·0 ± 0·1 ml per 100 ml of recovered BAL [23].

Statistical analysis

Data were distributed normally as determined by the Kolmogorov–Smirnov normality test, and therefore all statistical analyses were performed by use of parametric tests. Comparisons were performed with the unpaired or paired Student's *t*-test as appropriate (GraphPad Software, San Diego, CA, USA) without correction for multiple analyses. *P*-values of less than 0·05 were considered to indicate statistical significance.

Results

CCR6 expression on $CD4^+$ cells derived from the nasal mucosa

Initial experiments were performed on T cells expanded from nasal mucosal biopsies and peripheral blood with the aim of identifying any restricted patterns of chemokine receptor expression associated with either tissue compartmentalization (nasal mucosa *versus* blood) or allergic disease (atopic allergic subjects *versus* healthy non-atopic controls). Nasal biopsies (2·5 mm) were taken from the inferior nasal turbinate of subjects with active seasonal allergic rhinitis and non-atopic control subjects ($n = 7$) under local anaesthesia and T cells were expanded *in vitro*, as described previously [21]. T cells were also expanded from peripheral blood of the same donors in parallel under identical conditions. Of the chemokine receptors tested, only CCR6 was expressed consistently on a higher proportion of tissue $CD4^+$ T cells derived from nasal mucosal tissue rather than peripheral blood of all subjects (nasal, 67·0 ± 30·7%, mean ± standard deviation; peripheral blood, 30·6 ± 9·1%: $P < 0{\cdot}001$; Fig. 1). CCR6 expression on $CD4^+$ cells was similar in atopic and normal subjects on T cells derived from nasal tissue (atopic, 62·9 ± 21·7%; normal, 70·6 ± 15·8%, $P = 0{\cdot}47$) and peripheral blood (atopic, 30·7 ± 6·4%; normal, 30·6 ± 11·6%, $P = 0{\cdot}98$). Of the other receptors analysed, only increased expression of CXCR1 was found on $CD4^+$ T cells from atopic subjects compared with normal controls, as reported previously [6]. CCR6 was found to be expressed predominantly on tissue $CD4^+$ cells, although $CD8^+$ cells derived from tissue also showed some expression of this receptor. While CCR6 expression was markedly lower on $CD8^+$ cells, it was consistently higher on nasal mucosal rather than peripheral blood cells (Fig. 2a, $P < 0{\cdot}001$). Of other chemokine receptors studied, no differences in expression between nasal and

© 2008 The Author(s)
Journal compilation © 2008 British Society for Immunology,

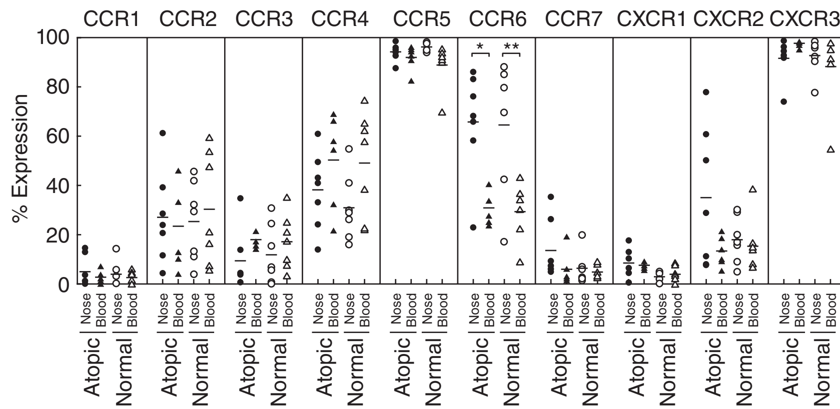

Fig. 1. CCR6 expression is increased on nasal-derived T cells. Nasal punch biopsies and peripheral blood were taken from normal (open symbols, $n = 6–7$) and atopic donors (closed symbols, $n = 7$). Short-term T cell lines were derived from nasal tissue (circles) and blood (triangles) and were assessed for expression of chemokine receptors (CCR1–7 and CXCR1–3) by flow cytometry. The figure shows the percentage of $CD4^+$ T cells expressing individual chemokine receptors where * and ** denote significant differences ($P < 0·05$ and $P < 0·01$ respectively) between chemokine receptor expression on nose and blood-derived T cells using a Student's paired *t*-test.

blood $CD8^+$ T cell lines were observed for either normal or atopic subjects (data not shown). Thus CCR6 was expressed by a higher percentage of nasal mucosal T cells than matched peripheral blood T cells isolated and cultured in parallel. Moreover, this phenomenon was observed for both $CD4^+$ and $CD8^+$ T cell lines, although CCR6 was expressed at two–threefold higher levels in the former population.

$CCR6^+$ and $CCR6^-$ nasal and blood $CD4^+$ T cells were also examined for expression of various adhesion molecules including CLA, CD11a, α4β1 integrin, CD29, CD44, CD49f (α6 integrin) and CD103. Only CD49f was expressed by a modestly higher proportion of $CCR6^+$ than $CCR6^-$ cells from the nasal mucosa (37·0 ± 11·7% of $CCR6^+$ cells *versus* 32·2 ± 10·8% of $CCR6^-$ cells; $n = 7$; $P = 0·02$) and peripheral blood (21·6 ± 4·7% of $CCR6^+$ cells *versus* 8·4 ± 1·7 of $CCR6^-$ cells; $n = 7$; $P = 0·01$).

CCR6 expression on $CD4^+$ cells derived from skin and lung

We next investigated whether CCR6 expression by $CD4^+$ T cells derived from the nasal mucosa was organ-specific or a property of cells from elsewhere in the respiratory tract or a completely separate organ. To address this, T cells were expanded from cutaneous punch biopsies from normal healthy skin and endobronchial biopsies taken at bronchoscopy from normal individuals. Matched cultured peripheral blood cells were used to compare CCR6 expression. We also tested freshly isolated unexpanded bronchoalveolar lavage $CD4^+$ T cells from healthy controls and compared data with CCR6 expression on freshly isolated peripheral blood $CD4^+$ T cells. In each case, CCR6 expression by tissue-derived $CD4^+$ cells was higher than in matched cultured or freshly isolated peripheral blood $CD4^+$ cells (Fig. 2b).

Nasal $CCR6^+$ T cells show enhanced responsiveness to CCL20

To determine if CCR6 on tissue-derived T cells represents a functional receptor, the ability of the CCR6 ligand CCL20 (macrophage inflammatory protein-3α) to induce chemotaxis and actin polymerization was assessed. T cells expanded from nasal biopsies and matched peripheral blood samples (from normal donors) were tested for chemotaxis in response to 10–1000 pg/ml of CCL20. A dose-dependent increase in chemotaxis of nasal but not blood T cells was observed that was highest at 1000 pg/ml of CCL20 (Fig. 3a). Within the $CCR6^+ CD4^+$ population, dose-dependent increases in actin polymerization were observed for both nasal and blood T cell lines, although changes were higher in the nasal T cell line (Fig. 3b). For both functional experiments, no statistical differences were observed between nasal and blood-derived cell lines ($n = 3$/group).

CCL20 but not CCR6 is increased in allergic responses

Although the proportions of T cells expressing CCR6 in nasal mucosal or peripheral blood T cell lines was not higher in subjects with active allergic rhinitis than normal controls, expression was also examined on freshly isolated $CD4^+$ and $CD8^+$ blood T cells. As with cell lines, no differences in $CCR6^+$ expression were seen between freshly isolated T cells of subjects with allergic rhinitis and normal controls. In addition, CCR6 expression was not increased from stimulation of T cells *in vitro* from atopic subjects with grass pollen allergen compared with control cultures (Fig. 4). To determine if CCL20 is up-regulated in allergic inflammation, concentrations were measured in bronchoalveolar lavage fluid of subjects with mild asthma before and after endobronchial segmental allergen challenge. CCL20 levels, as measured by

© 2008 The Author(s)

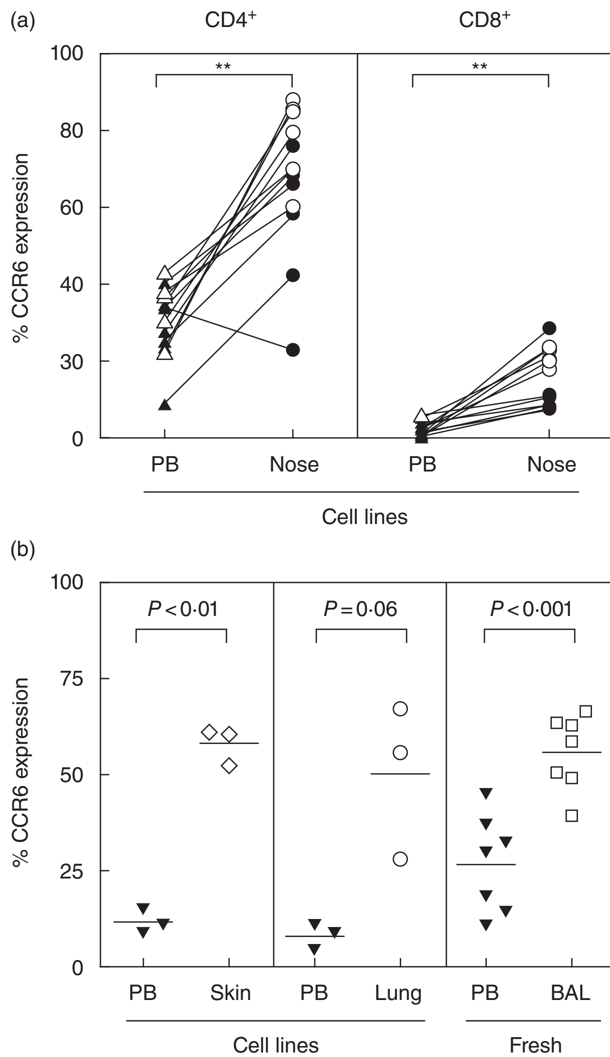

Fig. 2. CCR6 expression on nose, lung, skin and bronchoalveolar lavage T cells. (a) Short-term T cell lines derived from nasal tissue (nose) and blood (peripheral blood: PB) of atopic ($n = 7$, closed symbols) and normal ($n = 7$, open symbols) donors were stained with CCR6 and CD4 or CD8 and analysed by flow cytometry. The figure shows the percentage of CD4 or CD8 T cells co-staining for CCR6 for individual donors. **< 0·01, Student's paired *t*-test. (b) Short-term T cell lines were generated from lung and skin tissue and matched peripheral blood. T cells from freshly isolated bronchoalveolar lavage (BAL) or peripheral blood were also investigated. Cells were stained for CCR6 and CD4 and analysed by flow cytometry and the graphs show individual data for tissue or BAL compared with matched PB.

means of ELISA, were increased significantly in response to allergen exposure (pre-challenge, 0·61 ± 0·53 pg/ml; post-challenge, 5·0 ± 7·2 pg/ml, $P = 0{\cdot}03$; Fig. 5).

Discussion

Chemokine receptors play an important role in leucocyte homeostasis and inflammatory responses by directing the restricted recruitment of individual leucocyte populations. We report that functionally active CCR6 is expressed on a higher proportion of $CD4^+$ T cells from the nasal mucosa, lung and skin when compared with peripheral blood $CD4^+$ cells, whereas CCR6 up-regulation is not a feature of atopic allergic disease. Collectively, these data suggest that $CCR6^+$ $CD4^+$ T cells compartmentalize in diverse tissues even in the absence of inflammation or disease. The increased expression of the CCR6 ligand CCL20 following allergen provocation in allergic asthma suggests that the CCR6–CCL20 axis could potentially augment memory cell recruitment during allergic inflammation.

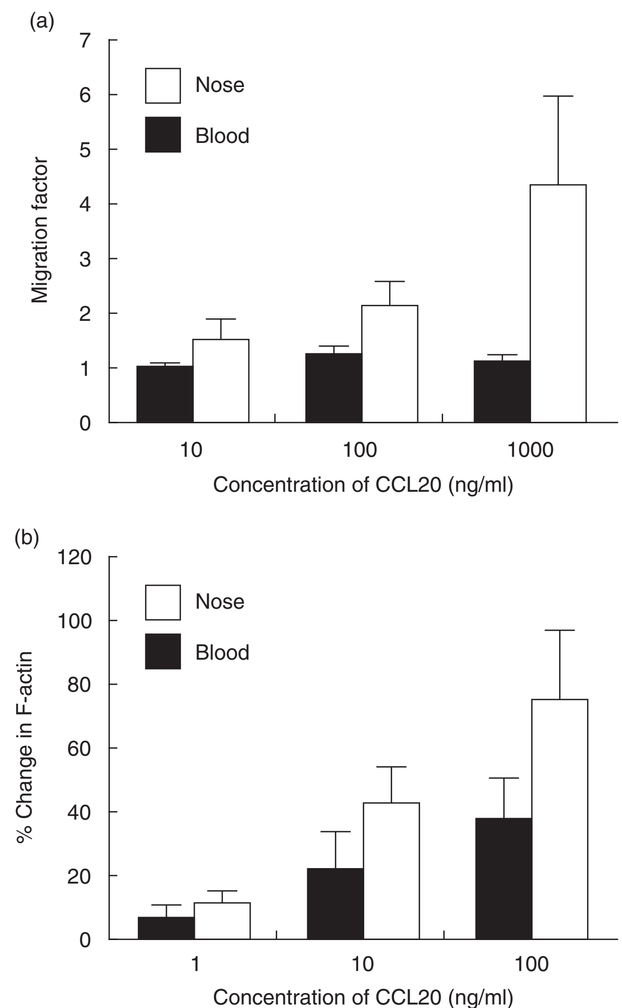

Fig. 3. Functional responses of nose and blood-derived T cells to CCL20. (a) Short-term cell lines derived from either nasal tissue or blood from normal donors ($n = 3$) were tested in migration assays to a dose range of CCL20. Data are shown as a fold-increase in migration compared with controls. (b) Nasal or blood-derived cells from normal donors ($n = 3$) were stained with CCR6 and CD4 and stimulated with a dose range of CCL20. Changes in F-actin polymerization on $CCR6^+$ cells were measured by flow cytometry and data are shown as mean ± standard error.

© 2008 The Author(s)
Journal compilation © 2008 British Society for Immunology,

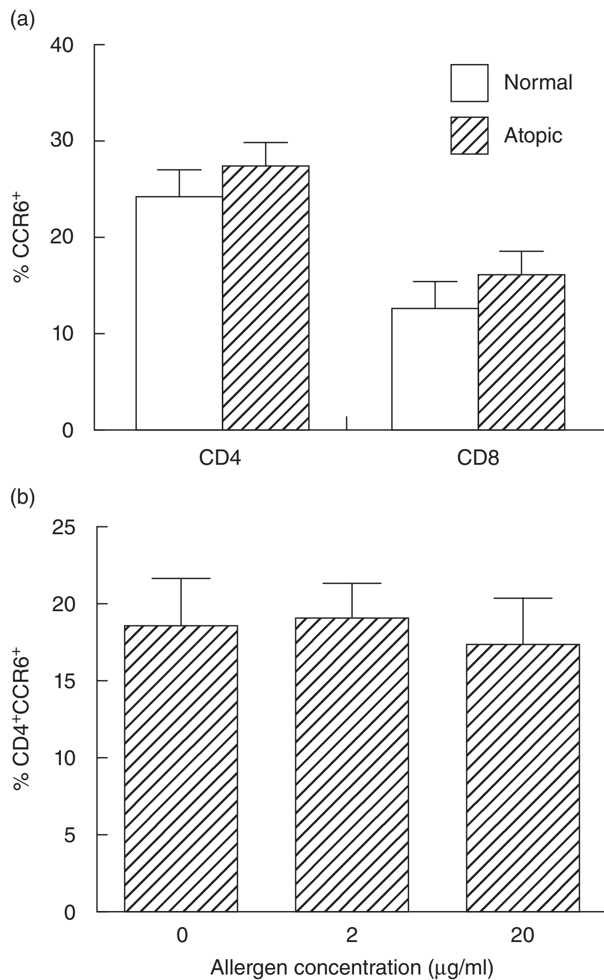

Fig. 4. CCR6 is not associated with allergic status or allergen-responsiveness of T cells. (a) Freshly isolated peripheral blood mononuclear cells (PBMC) from normal ($n = 7$) or atopic ($n = 7$) donors were stained for expression of CCR6 and CD4 or CD8 and analysed by flow cytometry. (b) Freshly isolated PBMC from atopic donors ($n = 7$) were stimulated with grass pollen allergen for 6 days and CCR6 expression on $CD4^+$ cells was assessed. Data are shown as mean ± standard error.

Our data support the findings of a recent study showing expression of CCR6 on bronchoalveolar lavage T cells and increases in lavage CCL20 following segmental allergen challenge [24]. Bronchoalveolar lavage represents the only practical means of harvesting significant numbers of T cells from a 'tissue' compartment for direct *ex vivo* analysis by flow cytometry. The novelty of our findings lies in the data showing that CCR6 is expressed additionally by tissue T cells from nasal mucosa, bronchial mucosa and skin (albeit following a short-term expansion step), suggesting that CCR6 expression is a general feature of tissue T cells, in both allergic and non-allergic states. Thomas and colleagues also provided evidence that CCR6 is internalized on CCL20 exposure. This observation is almost certainly significant in the context of allergen challenge, where the decrease in bronchoalveolar T cell CCR6 expression was accompanied by a marked rise in local CCL20 levels. However, we believe it is unlikely that this mechanism could explain the similar levels of CCR6 on nasal-derived T cells from allergic rhinitics and normal controls (i.e. by reducing surface CCR6 in rhinitics to the same levels seen in controls). Many of our experiments required lymphocytes to be eluted and expanded from biopsy material by short-term culture followed by a single stimulation step to generate short-term T cell lines, during which time any biopsy-derived CCL20 is likely to have dissipated. In spite of this short-term expansion step, CCR6 expression on freshly isolated $CD4^+$ T cells (25·8 ± 7·7%) was similar to that of blood-derived cell lines (30·1 ± 8·9%) and the observation that CCR6 expression is stable and relatively uninducible despite mitogenic activation has been reported previously [25]. However, other evidence suggests that activation of naive cells in the mixed lymphocyte reaction leads to induction of CCR6 expression [26]. In further support of the validity of our observations, bronchoalveolar lavage $CD4^+$ lymphocytes were analysed for CCR6 expression without culture or activation. CCR6 expression by these cells was not only higher than on $CD4^+$ lymphocytes compared with paired fresh blood specimens, but was similar to that seen in cells cultured from bronchial biopsies and consistent with previous findings [18,27,28]. Also consistent with other reports,

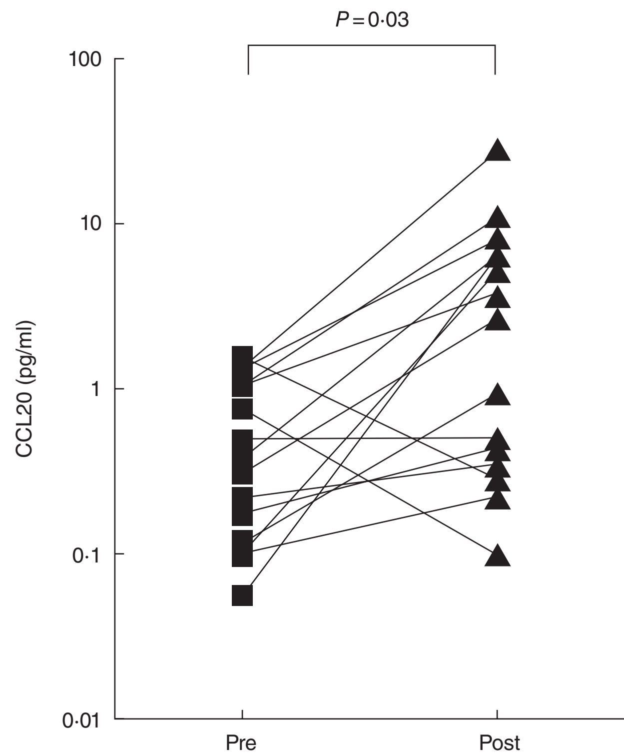

Fig. 5. CCL20 is increased after allergen challenge. Bronchoalveolar lavage fluid obtained from 15 atopic patients before (pre) and after (post) segmental allergen challenge [22] was tested for the presence of CCL20 by means of enzyme-linked immunosorbent assay.

© 2008 The Author(s)

CCR6 was expressed more strongly on CD4$^+$ than CD8$^+$ T cells [19,23,27,29].

As evidence for functionality of the CCR6 receptor, in a limited number of subjects we were able to show that CD4$^+$ CCR6$^+$ nasal mucosa lymphocytes demonstrated functional activity *in vitro* to recombinant CCL20, as shown by chemotaxis and actin polymerization. In addition, nasal CCR6$^+$ CD4$^+$ T cells were more responsive to CCL20 than CCR6$^-$ CD4$^+$ blood T cells. These differences could be attributed to differential CD4$^+$ expression by blood and nasal cell lines, as CCR6 is expressed preferentially by CD4$^+$ cells. However, in these experiments, while CD4 expression was higher in nasal cell lines, more than 40% of blood cell lines were CD4$^+$. Therefore, not only are CCR6 receptors expressed more highly by tissue lymphocytes, but they appear to have greater activity on individual cells, perhaps as a result of modulation of intracellular signalling pathways. Similar findings were reported by Homey and colleagues [30] when comparing responses of 'skin-homing' CLA$^+$ T cells to CCL20, with markedly higher chemotactic responses being observed with psoriatic than normal subjects, despite modest differences in cell surface CCR6 expression measured by flow cytometry.

The mechanism by which CCR6 is expressed selectively on lymphocytes in tissue is unknown. The simplest explanation is that CCR6 is induced on a subset of lymphocytes targeted for recruitment to tissue, but with tissue-specific signals being provided by additional other chemokine receptors and adhesion molecules. In support of this, CCR6$^+$ peripheral blood CD4$^+$ lymphocytes expressing CLA (skin homing) and $\alpha 4\beta 7$ integrin (gut homing) have been identified, suggesting that CCR6 expression precedes recruitment [25]. Furthermore, CCR6 is also expressed by epithelial dendritic cell subsets and appears to be required for the initiation of T cell responses in response to enteric pathogens [31]. Therefore, it is possible that tissue expression of CCL20 could contribute to homeostatic recruitment of CCR6$^+$ T cells, but additionally might play a role in T cell–dendritic cell interaction, possibly through co-localizing these cells to areas of CCL20 expression.

We did not observe increased CCR6 expression on CD4$^+$ T cells of patients with symptomatic seasonal allergic rhinitis compared with normal controls, regardless of whether cells were cultured from nasal mucosal biopsies (i.e. site of the disease) or analysed immediately after isolation from peripheral blood. Our previous studies have highlighted increased expression of a number of chemokine receptors on CD4$^+$ lymphocytes of such patients, including CCR3 (fresh blood) [5], CXCR1 (nose and blood) [6] and fractalkine (blood) [7]. While the high levels of constitutive CCR6 expression on tissue CD4$^+$ cells are compatible with a role in memory cell homeostasis, we do not exclude the possibility that the CCR6–CCL20 axis contributes to inflammatory responses. Also, we did not examine CCR6 expression by T cells in lymphoid tissue and it is possible that mobilization and recruitment of CCR6$^+$ cells from these sites may occur during allergic responses. In an experimental model of cockroach allergen-induced asthma, CCR6$^{-/-}$ mice were characterized by reduced airway inflammation and recruitment of pulmonary CD4$^+$ T cells, and also defects in CCR6$^{-/-}$ dendritic cell-induced activation of CCR6$^{+/+}$ T cells in cell mixing experiments [15]. We evaluated concentrations of CCL20 in BAL fluid collected 24 h after endobronchial allergen challenge [22]. The marked increases in CCL20 suggest that the CCR6–CCL20 axis may amplify recruitment of effector T cells during allergic late responses. However, the CCR6–CCL20 axis appears not to be specific for allergic responses, as expression of CCL20 and/or recruitment of CCR6$^+$ cells has been described in diverse inflammatory conditions including rheumatoid arthritis, psoriasis, inflammatory bowel disease and experimental autoimmune encephalitis.

In summary, our data support CCR6 as a general tissue-homing receptor for T cells during normal homeostasis. Up-regulation of CCR6 expression *per se* is not a specific feature of allergic diseases in the skin, nose or lung. However, our finding of locally enhanced CCL20 in BAL fluid after allergen provocation implies that the CCL20–CCR6 axis may be involved in the augmentation of bronchial inflammation in allergic asthma.

Acknowledgements

This study was supported by the Medical Research Council and GlaxoSmithKline in partnership with Imperial College Trust (academic Drug Discovery Initiative). I. S. is supported by an MRC Senior Clinical Fellowship (G116/170) and C. M. L. is a Wellcome Senior Research Fellow. S. J. T. holds a Clinician Scientist Fellowship supported by The Health Foundation.

References

1 Sallusto F, Mackay CR, Lanzavecchia A. Selective expression of the eotaxin receptor CCR3 by human T helper 2 cells. Science 1997; **277**:2005–7.

2 Bonecchi R, Bianchi G, Bordignon PP *et al.* Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med 1998; **187**:129–34.

3 D'Ambrosio D, Iellem A, Bonecchi R *et al.* Selective up-regulation of chemokine receptors CCR4 and CCR8 upon activation of polarized human type 2 Th cells. J Immunol 1998; **161**:5111–15.

4 Qin S, Rottman JB, Myers P *et al.* The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest 1998; **101**:746–54.

5 Francis JN, Lloyd CM, Sabroe I, Durham SR, Till SJ. T lymphocytes expressing CCR3 are increased in allergic rhinitis compared with non-allergic controls and following allergen immunotherapy. Allergy 2007; **62**:59–65.

6 Francis JN, Jacobson MR, Lloyd CM, Sabroe I, Durham SR, Till SJ. CXCR1+CD4+ T cells in human allergic disease. J Immunol 2004; **172**:268–73.

7 Rimaniol AC, Till SJ, Garcia G *et al.* The CX3C chemokine

© 2008 The Author(s)
Journal compilation © 2008 British Society for Immunology,

fractalkine in allergic asthma and rhinitis. J Allergy Clin Immunol 2003; **112**:1139–46.
8 Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999; **401**:708–12.
9 Sato K, Kawasaki H, Nagayama H *et al.* Chemokine receptor expressions and responsiveness of cord blood T cells. J Immunol 2001; **166**:1659–66.
10 Kepley CL, Taghavi S, Mackay G *et al.* Co-aggregation of FcgammaRII with FcepsilonRI on human mast cells inhibits antigen-induced secretion and involves SHIP-Grb2-Dok complexes. J Biol Chem 2004; **279**:35139–49.
11 Baba M, Imai T, Nishimura M *et al.* Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. J Biol Chem 1997; **272**:14893–8.
12 Vanbervliet B, Homey B, Durand I *et al.* Sequential involvement of CCR2 and CCR6 ligands for immature dendritic cell recruitment: possible role at inflamed epithelial surfaces. Eur J Immunol 2002; **32**:231–42.
13 Fitzhugh DJ, Naik S, Gonzalez E, Caughman SW, Hwang ST. CC chemokine receptor 6 (CCR6) is a marker for memory T cells that arrest on activated human dermal microvascular endothelium under shear stress. J Invest Dermatol 2000; **115**:332.
14 Cook DN, Prosser DM, Forster R *et al.* CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. Immunity 2000; **12**:495–503.
15 Lundy SK, Lira SA, Smit JJ, Cook DN, Berlin AA, Lukacs NW. Attenuation of allergen-induced responses in CCR6-/- mice is dependent upon altered pulmonary T lymphocyte activation. J Immunol 2005; **174**:2054–60.
16 Varona R, Cadenas V, Gomez L, Martinez A, Marquez G. CCR6 regulates CD4+ T-cell-mediated acute graft-versus-host disease responses. Blood 2005; **106**:18–26.
17 Varona R, Cadenas V, Flores J, Martinez A, Marquez G. CCR6 has a non-redundant role in the development of inflammatory bowel disease. Eur J Immunol 2003; **33**:2937–46.
18 Kallinich T, Schmidt S, Hamelmann E *et al.* Chemokine-receptor expression on T cells in lung compartments of challenged asthmatic patients. Clin Exp Allergy 2005; **35**:26–33.
19 Homey B, Dieu-Nosjean MC, Wiesenborn A *et al.* Up-regulation of macrophage inflammatory protein-3 alpha/CCL20 and CC chemokine receptor 6 in psoriasis. J Immunol 2000; **164**:6621–32.
20 Humbert M, Durham SR, Ying S *et al.* IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and non-atopic asthma: evidence against 'intrinsic' asthma being a distinct immunopathologic entity. Am J Respir Crit Care Med 1996; **51**:664–9.
21 Till SJ, Jopling LA, Wachholz PA *et al.* T cell phenotypes of the normal nasal mucosa: induction of Th2 cytokines and CCR3 expression by IL-4. J Immunol 2001; **166**:2303–10.
22 Till SJ, Durham SR, Rajakulasingam K *et al.* Allergen-induced proliferation and interleukin-5 production by bronchoalveolar lavage and blood T cells after segmental allergen challenge. Am J Respir Crit Care Med 1998; **158**:404–11.
23 Rennard SI, Basset G, Lecossier D *et al.* Estimation of volume of epithelial lining fluid recovered by lavage using urea as marker of dilution. J Appl Physiol 1986; **60**:532–8.
24 Thomas SY, Banerji A, Medof BD, Lilly CM, Luster AD. Multiple chemokine receptors, including CCR6 and CXCR3, regulate antigen-induced T cell homing to the human asthmatic airway. J Immunol 2007; **179**:1901–12.
25 Liao F, Rabin RL, Smith CS, Sharma G, Nutman TB, Farber JM. CC-chemokine receptor 6 is expressed on diverse memory subsets of T cells and determines responsiveness to macrophage inflammatory protein 3 alpha. J Immunol 1999; **162**:186–94.
26 Ebert LM, McColl SR. Up-regulation of CCR5 and CCR6 on distinct subpopulations of antigen-activated CD4+ T lymphocytes. J Immunol 2002; **168**:65–72.
27 Campbell JJ, Brightling CE, Symon FA *et al.* Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. J Immunol 2001; **166**:2842–8.
28 Morgan AJ, Guillen C, Symon FA *et al.* Expression of CXCR6 and its ligand CXCL16 in the lung in health and disease. Clin Exp Allergy 2005; **35**:1572–80.
29 Kondo T, Takata H, Takiguchi M. Functional expression of chemokine receptor CCR6 on human effector memory CD8+ T cells. Eur J Immunol 2007; **37**:54–65.
30 Homey B, Alenius H, Muller A *et al.* CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. Nat Med 2002; **8**:157–65.
31 Salazar-Gonzalez RM, Niess JH, Zammit DJ *et al.* CCR6-mediated dendritic cell activation of pathogen-specific T cells in Peyer's patches. Immunity 2006; **24**:623–32.

© 2008 The Author(s)