CXCR1$^+$CD4$^+$ T Cells in Human Allergic Disease


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Chemokine receptors play an important role in the migration of leukocytes to sites of allergic inflammation in humans. In this study, we have identified increased expression of the chemokine receptor CXCR1 on CD4⁺ T lymphocytes derived from patients with atopic disease compared with normal donors. Enhanced expression of CXCR1 by atopic donors was identified on freshly isolated peripheral blood cells and on expanded cell populations derived from nasal mucosal biopsies and from the periphery. Identification of CXCR1 expression on CD4⁺ cells in the nasal mucosa was confirmed by double immunofluorescence. In addition, expression of CXCR1 was dramatically decreased in patients undergoing successful treatment of allergic rhinitis by specific immunotherapy. CXCR1 provided a functional receptor capable of regulating T cells in the context of allergic disease, since expression of CXC chemokine ligand 8 was up-regulated at the site of allergic inflammation and freshly isolated CXCR1⁺CD4⁺ cells from atopic donors showed an enhanced functional response to this ligand. CXCR1 expression on CD4⁺ T cells was increased in vitro in response to the pro-Th2 cytokine IL-4. Phenotypic analysis reveals that IFN-γ expression was lower in the CXCR1⁺CD4⁺ cells. The identification of CXCR1 as a marker of allergic rhinitis reveals a possible target for therapeutic intervention in atopic disease. The Journal of Immunology, 2004, 172: 268–273.

In allergic diseases such as asthma and rhinitis, inflammation is significantly dependent upon T cell recruitment, activation, and cytokine production at mucosal surfaces. IL-4, IL-13, and IL-5 produced by mucosal T cells drive local IgE production by B cells and promote the accumulation of eosinophils (1).

There is evidence of selective T cell chemokine receptor expression on specific subsets. Restricted patterns of chemokine (in tissues) and chemokine receptor expression (on T cells) may direct the recruitment of selected T cell subsets to the mucosa in allergic disease (2). Th2-type T cells in vitro preferentially express CCR3, CCR4, and CCR8 (3–5), although of these, it has become clear that CCR4 is not completely Th2 selective (6). Nonetheless, there is evidence for generation of ligands for CCR3 and CCR4 in human allergic inflammation at many sites, including the lung and skin (7, 8), and T cells infiltrating into allergen-challenged sites show expression of CCR4 and CCR8 (3, 7). Also, animal models suggest that CCR3 and CCR4 may be involved in pulmonary Th2 cell recruitment during acute and chronic allergen exposure, respectively (8, 9). However, the relative contribution of these chemokine receptors to human disease therefore remains uncertain, and we hypothesized that other receptors may contribute to trafficking of T cells relevant to human allergic disease.

In the present study, our aim was to identify new associations between chemokine receptor expression on T cells and the presence of active allergic disease. Initial experiments performed on peripheral blood identified increased numbers of CXCR1-expressing CD4⁺ T cells in allergic patients. CXCR1 is a relatively selective CXC chemokine receptor, binding CXC chemokine receptor ligand (CXCL)3 8 (IL-8) and CXCL6 (granulocyte chemotactic protein 2) only (10) and is strongly expressed by neutrophils (11) with weaker expression on monocytes (12) and dendritic cells (13). Based on these initial observations, we hypothesized that the CXCR1–IL-8 axis contributes to mucosal CD4⁺ T cell responses in human allergic disease. We have examined CXCR1 expression by peripheral blood T cells, T cells expanded from nasal mucosal tissue, and in vivo in nasal biopsies. Moreover, we have compared the activity of IL-8 on CXCR1⁺CD4⁺ T cells from atopic and control subjects and have investigated the effects of immunotherapy, a highly effective systemic immunomodulatory treatment, on CXCR1 expression. Finally, we have investigated the effects of proallergic cytokines such as IL-4 on CXCR1 expression and have examined the cell surface and cytokine-producing phenotype of CXCR1⁺CD4⁺ T cells.

Materials and Methods

Reagents and Abs

Anti-human integrin αβ₂ (P4G9), CD11a (MHM24), CD44 (DF1485), CD29 (K20), CD103 (Ber-Act8), CD49f (M3511), CD4-RPE-Cy5, CD8-FITC, and secondary Abs anti-mouse IgG-FITC, IgG-PE, and IgG-RPE-Cy5 were obtained from DAKO Cytomation (Ely, U.K.). Anti-human CXCR1-PE mAb (5A12), unconjugated CXCR1 (5A12; also a kind gift from S. Qin, Millennium Pharmaceuticals, Cambridge, MA), anti-IFN-γ-FITC mAb, and anti IL-4-PE mAb were purchased from BD PharMingen (Cowley, U.K.). Recombinant human IL-2, IL-4, CXCL6, and CXCL8 were obtained from PeproTech (London, U.K.). Cell culture, Histopaque, and general laboratory reagents were purchased from Sigma-Aldrich (Poole, U.K.).
Patients

The study was approved by the Ethics Committee of the Royal Brompton and Harefield Hospitals National Health Service Trust and performed with the written consent of the donors. Atopy was defined by one or more positive skin prick tests (SPT) at least 3 mm greater than the negative diluent control to common aeroallergens (house dust mite), grass pollen, dog, cat, three trees (silver birch, alder, hazel), Asp fumigatus (Soluprick; ALK Abelló, Hørsholm, Denmark). Atopic donors (n = 9–12) were all sensitized to grass pollen, symptomatic at the time of venesection, with a clear history of perennial rhinitis symptoms, and had median (lower quartile, upper quartile) Radioallergosorbent test (RAST) scores of 67 IU/ml (23.88) to grass pollen, positive SPT to grass (8 mm (5.8)), and raised total IgE levels (234 IU/ml (90,526)). Immunotherapy (IT) subjects (n = 9–14) had received grass pollen immunotherapy for at least 1.5 years and all subjects had reported an improvement in hay fever symptoms since starting treatment (p < 0.01). IT donors were well matched to atopic donors and had positive RAST tests and SPT to grass pollen (RAST = 58 IU/ml (41,100) SPT = 8 mm (8,10) and had raised total IgE levels (178 IU/ml (76,568)). Normal, nonatopic subjects (n = 9–14) were all nonsymptomatic, had negative SPTs to the same allergen panel, and RAST tests to HDM and grass pollen were <0.35 IU/ml.

Culture of T cells from nasal mucosa and peripheral blood

As a source of mucosal tissue, 2.5-mm nasal biopsies were taken from the inferior nasal turbinate using Gerritsma forceps. Biopsies were placed in 24-well culture plates containing 2 ml of complete medium and supplemented with 10 ng/ml IL-2. Autologous PBMC were isolated from heparinized blood by centrifugation over Histopaque and resuspended at 1 × 10^6 cells/ml in the presence of IL-2 as described above. Following incubation for 5 days, biopsy tissue was removed from culture wells, and the remaining lymphocytes were restimulated with 1 × 10^6 cells/ml irradiated PBMC (3000 rad), 10 ng/ml IL-2, and 1 µg/ml PHA. Peripheral blood T cell lines were cultured in parallel under identical conditions. T cells were expanded for an additional 8 days, with fresh complete medium and cytokine added every 2–3 days.

Flow cytometry

Leukocytes were washed with staining buffer (PBS plus 0.1% BSA plus 0.09% azide) and incubated with anti-CXCR1 (10 µg/ml) or a control Ab (IgG2b) for 30 min. Staining was detected using goat anti-mouse RPE-conjugated F(ab')2 Abs. Unused secondary Ab binding sites were then neutralized by a blocking step with mouse IgG, before labeling with CD8 and CD4, and cells were finally resuspended in PBS containing To-Pro 3, 7M FITC-phalloidin, 0.125 mg/ml L-lysophosphatidylcholine, and 4.0% paraformaldehyde in PBS. Cells were analyzed by flow cytometry.

Immunohistochemistry

Six-µm acetone-fixed cryostat sections from nasal biopsies taken from hay fever sufferers during the pollen season were washed in TBS and then blocked with normal horse serum (Vector Laboratories, Burlingame, CA). Serum was replaced with anti-CXCR1 or an isotype control. The sections were washed in TBS and incubated with biotinylated secondary Ab (Vector Laboratories). This was followed by Vector ABC-AP (avidin-biotin complex linked to alkaline phosphatase) and its substrate Fast Blue. Sections were then washed in TBS and blocked with normal mouse serum. Following further washes the sections were incubated overnight with CD4-FITC. Sections were washed and mounted with Vectashield (Vector Laboratories).

ELISA

Bronchoalveolar lavage (BAL) samples were obtained from allergen-sensitive asthmatics before and after segmental allergen challenge (1–4). Concentrations of CXCL8 and CXCL6 in BAL samples were measured in duplicate by ELISA using BD Pharmingen-matched Ab pairs and human recombinant cytokines as standards (PeproTech). The limits of assay detection were ~4 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 (15).

Intracellular actin polymerization

Freshly isolated PBMC were labeled with CXCR1-PE and CD4-Cy5 as described. Cells at 5 × 10^6 cells/ml were incubated for 10 min at 37°C in RPMI 1640. Then, CXCL5 or CXCL6 at 100 ng/ml was added to the cell suspension and every 15 s, 0.5 × 10^6 cells were removed and mixed with 400 µl of 10^{-7} M FITC-phalloidin, 0.125 mg/ml t-α-lysophosphatidylcholine, and 4.0% paraformaldehyde in PBS. Cells were analyzed by flow cytometry.

Statistics

Groups of data were analyzed by Wilcoxon’s matched paired test or Mann-Whitney U test using the GraphPad Instat program (GraphPad Software, San Diego, CA). Data in the text are shown as mean ± SE.

Results

Association of CXCR1^+ CD4^- lymphocytes and atopic disease

Pilot studies detected an increase in CXCR1 expression on T cells from atopic donors. Fig. 1 shows that in further well-characterized subject groups, we found increased T cell CXCR1 expression in atopy. An average of 9.4 ± 1.4% of CD4^- lymphocytes expressed CXCR1 in the atopic group compared with 4.3 ± 0.8% in the normal group (p = 0.018). No difference in the expression of CXCR1^+ CD8^- cells was observed (atopic, 43.9 ± 8.1%; normal, 46.22 ± 5.4%, p = 0.666). Also, expression of the closely related receptor, CXCR2, was similar on CD4^- cells from atopic and normal groups (atopic, 21.4 ± 3.7; normal,16.3 ± 3.4, p = 0.345). To determine whether CXCR1 provided a likely mechanism for T cell recruitment in atopy, we investigated the expression of CXCR1 on CD4^- T cells derived from nasal tissue. Nasal biopsies (2.5 mm) were taken from the inferior nasal turbinate of normal and atopic donors and T cell lines were generated in vitro from this tissue. For comparison, PBMC-derived cell lines were also generated from the same donors. These results showed that a significant increase in the number of CXCR1^+ CD4^- lymphocytes was observed in nasal- and blood-derived cell lines in atopic donors compared with normal donors (nasal cell lines, p = 0.021; blood-derived cell lines, p = 0.015). Again, no difference in the expression of CXCR2 by CD4^- T cells was observed between atopic and normal groups (nasal cell lines: atopic, 35.0 ± 10.7; normal, 17.9 ± 3.55, p = 0.455; blood cell lines: atopic, 29.94 ± 6.52; normal, 31.32 ± 6.27, p = 0.927).

To examine CXCR1 expression in the context of disease activity, we investigated its expression on cells from patients undergoing grass pollen IT. Specific IT has been shown to alleviate symptoms and reduce medication use by allergic subjects (16). Subjects participating in this study reported a significant increase in their overall assessment scores relating to general allergic symptoms (p < 0.01). Fig. 1 demonstrates that patients undergoing specific IT had reduced numbers of CXCR1^+ CD4^- cells in the peripheral blood compared with matched atopic controls (p = 0.004). Moreover, both nasal and blood cell lines from these patients showed a significant decrease in CXCR1^+ CD4^- T cells (atopic vs IT; p = 0.01 and 0.005, respectively).

Analysis of CXCR1 expression by immunohistochemistry

To investigate the contribution of CXCR1 to T cell recruitment in vivo, we examined its expression in nasal biopsies from atopic subjects. CXCR1 cells exhibited discretel membrane staining on individual cells and were present in both the lamina propria and in
the epithelial layer (Fig. 2). A proportion of these cells also costained for CD4.

**CXCR1 is functionally active in atopic donors**

To investigate CXCR1 function on CD4+ lymphocytes, we measured the ability of CXCR1 ligands to induce actin polymerization, an early postreceptor signaling event indicative of T cell activation. Freshly isolated cells were stained for CXCR1 and CD4 and subsequently stimulated with CXCL8 or CXCL6. CXCR1+CD4+ cells were sensitive to triggering by CXCL8 (Fig. 3), whereas CXCR− cells did not respond to the chemokine. In addition, atopic donors showed an exaggerated response to the ligand compared with normal donors (p = 0.03). Similar results were obtained when cells were stimulated with CXCL6 (data not shown). In contrast, examination of CXCR1−CD4+ cells reveals that while these cells respond to ligand stimulation there were no significant differences in F-actin polymerization as measured by flow cytometry between normal and atopic donors (p = 0.205).

**FIGURE 1.** Increased expression of CXCR1+CD4+ cells by atopic donors. A, Freshly isolated PBMC (n = 9) from normal controls (NC), atopic rhinitic (AR), and IT donors were stained with CXCR1 and CD4. Cells were analyzed immediately by flow cytometry and subsequent analysis was performed on the lymphocyte-gated population. The graph represents the percentage of CXCR1-positive cells gating on CD4+ cells. B, Cell lines (n = 8) were derived from nasal biopsies and expression of CXCR1 on CD4+ lymphocytes was determined by flow cytometry. C, Cell lines were generated from PBMC (n = 10–13) and lymphocytes were stained for CXCR1 and CD4. Representative plots are shown for each cell source and quadrants were set according to the staining of control Abs.

**FIGURE 2.** Immunohistochemical analysis of CXCR1 and CD4 expression on fresh nasal tissue. A 3-mm nasal biopsy derived from an atopic donor was stained with CXCR1 and CD4-FITC and analyzed by microscopy.

**FIGURE 3.** Functional analysis of CXCR1+CD4+ cells by actin polymerization. Freshly isolated PBMC from atopic and normal donors (n = 4) were stimulated with 100 ng/ml CXCL8 and at 15-s intervals cells were removed and stained for intracellular F-actin. Cells were analyzed by flow cytometry. This figure represents the percentage increase of F-actin fluorescence on CD4+CXCR1+ gated lymphocytes compared with baseline measurements. No increase in F-actin from CD4−CXCR1− cells was detected. This figure is representative of two identical experiments.
CXCL8 production is increased after allergen challenge

We next investigated the effect of CXCR1 on the expression of CXCL8. BAL fluid obtained from 14 atopic patients before (pre) and after (post) segmental allergen challenge was tested for the presence of CXCL8 and CXCL6 by ELISA.

CXCL8 production is increased after allergen challenge

To investigate whether CXCR1 expression is influenced by Th2-type cytokines, we generated PMBC-derived cell lines grown in the presence or absence of IL-4. Measurements of CXCL8 and CXCL6 levels were made in bronchoalveolar samples obtained from atopic subjects (n = 14) before and after segmental allergen challenge (14). CXCL8 levels were significantly elevated 48 h after allergen challenge (Fig. 4, p = 0.007). In contrast, levels of CXCL6 in the patients’ serum did not alter (prechallenge, 45 ± 69 pg/ml; postchallenge, 42 ± 71 pg/ml) and CXCL6 levels did not change after exposure to allergen (Fig. 4).

CXCR1+ CD4+ cell numbers are increased by culture under Th2 conditions

To investigate whether CXCR1 expression was influenced by Th2-type cytokines, we generated PMBC-derived cell lines grown in the presence or absence of IL-4. Results shown in Fig. 5 demonstrate that cell lines grown in IL-4 showed significantly increased numbers of CXCR1+ CD4+ T cells (p = 0.005).

Phenotypical characterization of CXCR1+ CD4+ T cells

Intracellular cytokine analysis of T cells derived from freshly isolated atopic blood (n = 6) show that CXCR1+ CD4+ cells produce similar amounts of IL-4 compared with the CXCR1+ CD4+ population (Fig. 6). In contrast, production of IFN-γ is decreased in CXCR1+ CD4+ cells (p < 0.031). Very few double-positive cytokine-staining cells were observed in either group. Thus, the ratio of Th1:Th2 cytokines is skewed toward a Th2 phenotype in CXCR1+ CD4+ cells.

The expression of a variety of adhesion molecules (integrin α4β1, CD11a, CD29, CD44, CD49f, CD103) were studied on T cells and CXCR1+ CD4+ cells. The expression of CXCR1+ CD4+ T cells was similar to the control group, with no significant differences between these two cell populations (data not shown).

Discussion

Chemokine receptors direct inflammatory responses through the recruitment of specific leukocyte subpopulations. This study is the first report of an association between CXCR1+ CD4+ T cells and human allergic disease. Subjects with allergic disease were found to have higher numbers of CXCR1+ CD4+ T cells in peripheral blood and in T cell populations expanded from nasal mucosal tissue. CXCR1 colocalized to CD4+ T cells by fluorescence immunohistochemical analysis of nasal biopsy specimens from symptomatic hay fever patients, and up-regulation of the CXCR1 ligand CXCL8 was demonstrated in BAL fluid after endobronchial allergen provocation. Moreover, peripheral blood CXCR1+ CD4+ T cells responded functionally to CXCL8, with polymerization of intracellular actin. These data are consistent with reports that human T cells are sensitive to CXCL8 in chemotactic assays and Ca2+ flux studies (17). We observed that cells derived from atopic donors induced a significantly greater intracellular actin polymerization response compared with normal controls. This difference was apparent even within the CXCR1+ CD4+ subset but could not be explained on the basis of per cell expression of CXCR1 expression.
since mean fluorescence intensity of CXCR1 was equivalent in atopics and control subjects. Therefore, not only are CXCR1 receptors up-regulated in allergic disease but they appear to be more effectively coupled to intracellular signaling in these individuals.

Next, we report that IL-4, a proallergic cytokine that promotes differentiation of Th2 cells and B cell IgE production, increased CXCR1 expression by CD4⁺ T cells undergoing activation and expansion. Also, we observed that immunotherapy, a highly effective systemic immunomodulatory treatment, was associated with significant reductions in CXCR1⁺ CD4⁺ T cells.

The levels of CXCR1 expression that we describe in this study are consistent with previous reports of freshly isolated blood cells (18). Another group has examined chemokine receptor expression on polarized Th1 and Th2 cell lines (5) but reported that CXCR1 was not detectable at the mRNA level, demonstrating the importance in these studies of comparing CXCR1 expression on both fresh blood and expanded T cell populations rather than in vitro-differentiated T cell clones. The abnormal expression of CXCR1 by T cells in patients with allergic disease was confirmed to the CD4⁺ subset. Other chemokine receptors associated with Th2 responses (CCR3, CCR4, and CCR8) are also generally associated with restricted expression to CD4⁺ lymphocytes (3, 7, 19). The numbers of CXCR1⁺ CD4⁺ T cells observed is such that this population is unlikely to comprise only allergen-specific T cells, but our data showing expansion of CXCR1⁺ T cells during activation in the presence of IL-4 again links these cells to the allergic phenotype. IL-4 is produced by Th2 cells, mast cells, basophils, and eosinophils. All of these cell types undergo activation at mucosal surfaces during allergic responses and T cells may be additionally activated in draining lymphoid tissue. Thus, microenvironmental IL-4 has the potential to act on local T cells, allergen specific or otherwise, to up-regulate CXCR1.

The observation that allergen-specific Th2 cell specific with reduced CXCR1 expression by CD4⁺ T cells provides further evidence that this T cell subset may play a functional role in human allergic disease. Since reduced numbers of CXCR1⁺ T cells are observed in both blood and nasal cells, it seems that it acts systemically to decrease the expression of this chemokine receptor. To our knowledge, this represents the first report that chemokine receptor expression on any leukocyte type can be modified by IT. The mechanism behind CXCR1 down-regulation is unknown, but could reflect reductions in local production of IL-4. Alternatively, the possibility that regulatory “alloergic” cytokines such as IL-10, TGF-β, or IL-12 might act directly on T cells to suppress expression of chemokine receptors requires further evaluation.

Results presented here reveal significant differences in CXCR1 expression between atopic and normal donors on CD4⁺ cells. However, it is noteworthy that significant numbers of CD8⁺ cells also express CXCR1, although we found no relation to allergic disease in this population. Therefore, it is also possible that CXCR1 plays a role in CD8 recruitment. In support of this notion, analysis of the CXCR1⁺ CD4⁺ lymphocyte population (which are mainly CD8⁺) reveals that these cells show increases in F-actin after ligand (CXCL8) stimulation. However, there were no significant differences between normal and atopic donors (p = 0.205). Thus, it is interesting that normals will have CXCL8 responses that act on CD8 cells and that the responses atopics will act on CD4 and CD8 cells, resulting potentially in a different pattern of T cell recruitment and significant effects on disease. To characterize the CXCR1⁺ CD4⁺ population, cellular phenotype was evaluated in terms of cytokine production and expression of cell surface markers. IFN-γ production was higher in CXCR1⁺ CD4⁺ than in CXCR1⁻ CD4⁺ cells in all six atopic patients examined (p < 0.05) while IL-4 production remained similar. The concept of an imbalance in production of IL-4 and IFN-γ is well established in atopic disease. Therefore, these data are consistent with the hypothesis that CXCR1 expression may be broadly associated with a population of proallergic T cells. We also assessed the expression of homing molecules (CD29, CD49f, CD11a, and CD44) related to specific locations in the nasal-associated mucosal tissue. Although we did not detect differences in cell surface markers between CXCR1⁺ and CXCR1⁻ CD4⁺ T cells from PBMC, levels of expression were similar to those reported elsewhere (20). CXCR1⁺ cells were found in both the naive and memory compartments, although CXCR1⁻ CD4⁺ cells showed a tendency toward the memory phenotype.

These studies highlight the potential importance of CXCR1 in the regulation of T cell recruitment to sites of allergic inflammation. Mice constitutively lack CXCR1 (21), emphasizing the importance of studies such as this, which focus on human disease. CXCR1 provides a novel bridge between innate immunity (e.g., regulating recruitment of neutrophils) and adaptive immunity through the regulation of subset-specific T cell recruitment. CXCR1⁺ T cells may represent a novel marker amenable to targeting in the treatment of human allergic disease.

References


