Multiple Chemokine Receptors, Including CCR6 and CXCR3, Regulate Antigen-Induced T Cell Homing to the Human Asthmatic Airway

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Multiple Chemokine Receptors, Including CCR6 and CXCR3, Regulate Antigen-Induced T Cell Homing to the Human Asthmatic Airway

Seddon Y. Thomas,* Aleena Banerji,* Benjamin D. Medoff,*† Craig M. Lilly,‡ and Andrew D. Luster2*

Human allergic asthma is a chronic inflammatory disease of the airways thought to be driven by allergen-specific Th2 cells, which are recruited into the lung in response to inhaled allergen. To identify chemotactic receptors that control this homing pattern, we used endobronchial segmental allergen challenge in human atopic asthmatics to define the pattern of chemotactic receptor expression on recruited T cells as well as the numbers of recruited CD1d-restricted NKT cells and levels of chemokines in the bronchoalveolar (BAL) fluid. CD1d-restricted NKT cells comprised only a small minority of BAL T cells before or after Ag challenge. BAL T cells were enriched in their expression of specific chemotactic receptors compared with peripheral blood T cells prechallenge, including CCR5, CCR6, CXCR3, CXCR4, and BLT1. Surprisingly, following segmental allergen challenge, no chemotactic receptor was specifically increased. However, CCR6 and CXCR3, which were expressed on virtually all CD4+ BAL T cells prechallenge, were markedly decreased on all recruited BAL T cells following Ag challenge, suggesting that these receptors were internalized following encounter with ligand in the airway. Our data therefore suggests a role for CCR6 and CXCR3, in conjunction with other chemotactic receptors, in the recruitment of inflammatory T cells into the BAL during the allergic asthmatic response. The Journal of Immunology, 2007, 179: 1901–1912.

Homeostatic and tissue-specific trafficking of T cells is required for effective immune surveillance. T lymphocytes are recruited to tissue- and inflammation-specific sites through the expression of specific chemotactic receptor and encounter with cognate lipid or peptide ligands. Certain chemokine receptors have been associated with T cell homing to tissue-specific sites, including CCR7 and CXCR5 for lymph node, CXCR4 and CCR10 for skin, and CCR9 for small intestine (7). No chemotactic receptor has been specifically linked with homing to the lung or air spaces. In addition to homeostatic and tissue-specific trafficking at baseline, T lymphocytes must respond to and be recruited into sites of Th1 or Th2 inflammation. With Th1-type inflammation, production of STAT1-inducible chemokines leads to recruitment of Th1 and effector CD8 T cells through the cognate Th1-associated chemokine receptors, CCR5, CXCR3, and CXCR6 (8). However, with Th2-type inflammation, production of STAT6-inducible chemokines leads to recruitment of Th2 cells through the cognate Th2-associated chemokine receptors, CCR3, CCR4, and CCR8 (9). Although these Th1- or Th2-associated receptors have been observed on polarized T cells in vitro, the chemotactic receptor expression pattern on Th1 or Th2 cells ex vivo is more complex.

To study human allergic asthma and the in vivo recruitment of T cells into the allergic airway, we have used a segmental allergen challenge protocol. Segmental allergen challenge allows pre- and postallergen challenge comparison of BAL T cells and other leukocytes within the same subject and mimics the human acute asthmatic response in a controlled setting. Each subject serves as its own control, providing specific information about the in vivo asthmatic response. The asthmatic response can be divided into an early and a late phase. The early phase (5–60 min) is typified by airway swelling and smooth muscle cell constriction and results from mast cell degranulation. The late phase (>4 h) is typified by lymphocyte and eosinophil recruitment to the airway (10). Because we were
interested in T lymphocyte recruitment following challenge, we characterized the percentage of CD1d-restricted NKT cells, chemotrafficking receptor profile, cytokine, and memory phenotype of peripheral blood (PB) and BAL T cells, and production of chemotrafficking ligands before and 24 h after segmental allergen challenge.

Materials and Methods

Segmental allergen challenge

Each subject provided written informed consent using forms approved by the Partners Healthcare Institutional Review Board. All subjects met the American Thoracic Society’s definition of asthma (11), had disease of mild severity based on spirometry and physician assessment (12), and had symptoms to cat or dust mite exposure with a corresponding positive skin prick test. Subjects based on spirometry and physician assessment (12), and had symptoms to cat asthma and loratadine for nasal symptoms, which were withheld 12 h before the bronchoscopy. The dosage of standardized allergen for bronchoscopic challenge was determined from quantitative skin testing (13). Segmental allergen challenge was performed at least 4 wk after measurements of atopy and any symptoms of infection as previously described (13). Briefly, bronchoscopy was performed under conscious sedation with a total of ~400 mg of topical lidocaine. The bronchoscope was advanced to airway orifice in the anterior segment of the left upper lobe, three 50-ml aliquots of warmed sterile saline were serially instilled, and prechallenge BAL fluid was recovered into Teflon containers by gentle aspiration. The bronchoscope was then moved to the right middle lobe, where 1 ml of standardized allergen solution (cat or dust mite) was delivered. The bronchoscope was then removed, and focal wheezing was confirmed to be present exclusively over the right middle lobe in all subjects by auscultation. Twenty-four hours later, Ag challenge BAL fluid was obtained from the right middle lobe in all subjects by bronchoscopy. The dosage of standardized allergen for bronchoscopic challenge was determined from quantitative skin testing (13).

Allergen extract

Standardized allergen extract for cat hair and Dermatophagoides pteronyssinus were purchased from Greer Laboratories. Standardized cat hair allergen extract contained <1 ng/ml endotoxin; standardized D. pteronyssinus allergen extract contained 9 ng/ml endotoxin.

Cytospin

Cell differential counts for prechallenge and postchallenge BAL were determined by enumerating alveolar macrophages, neutrophils, eosinophils, and lymphocytes on cytocentrifuge preparations with staining of Wright stain (EM Sciences) and Diff-Quick (Dade Behring).

Flow cytometry reagents

Abs to CCR1 (55304.111), CCR1X (42705.111), CCR2 (48607.121), CCR2X (48311.211), CCR3 (61828.111), CCR5 (51505.111), CCR6 (56811), CCR7 (150503), CCR9 (112509) were purchased from R&D Systems. CCR3 (1C6), CCR4 (1G1), CCR5 (3A9), CCR6 (11A9), CCR7 (2H4), CD3 (UCHT1), CD4 (SK3), CD8a (RPA-T8), CD19 (SI25C1), CD25 (M-251), CD27 (M-T271), CD45RA (H100), CD26L (Dreg 56), IFN-γ (B27), and IL-4 (MP4-2D2) were purchased from BD Pharmingen. V24 (C15) was purchased from Beckman Coulter. CXC3R1 (2A9-1) was purchased from MBL. BLT1 (2027B1) was purchased from Serotec. Abs used in these experiments were directly conjugated to their respective fluorochromes except for CXCR6 in which a primary Ab was used in combination with a fluorochrome-coupled secondary Ab.

Table I. Asthmatic subject characteristics, lung function tests, total serum IgE, and allergen dose used in segmental allergen challenge

<table>
<thead>
<tr>
<th>Subject</th>
<th>Symbol</th>
<th>Gender</th>
<th>Age</th>
<th>Race</th>
<th>FEV1 Predicted (%)</th>
<th>FEV1/FVC</th>
<th>Total IgE (kU/l)</th>
<th>Allergen</th>
<th>Challenge Dose</th>
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<td>0.79</td>
<td>158</td>
<td>DP</td>
<td>185, 20</td>
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<td>3</td>
<td>±</td>
<td>F</td>
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<td>0.72</td>
<td>331</td>
<td>C</td>
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<td>F</td>
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<td>0.83</td>
<td>220</td>
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<td>256</td>
<td>C</td>
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</tr>
</tbody>
</table>

Note: Symbols that represent a subject are listed here and are used throughout the article for total serum IgE are kilounits per liter. Units for allergen dose are measured either in bioequivalency allergy units per milliliter for cat hair allergen or in arbitrary units per milliliter for D. pteronyssinus allergen. Allergen dose in segmental allergen challenge was based on responsiveness to skin testing. In some instances, allergen challenge was performed on the same subject at a separate time and is indicated by a different symbol for the same subject. C, Cat hair; DP, D. pteronyssinus.

Flow cytometry staining

For extracellular epitopes, BAL and whole PB were blocked with 10% human serum and stained in PBS with 1% FCS. Whole PB was lysed following staining with FACS Lysing Solution (BD Biosciences). Cells were then fixed with 2% paraformaldehyde.

For intracellular cytokine staining, BAL and PB were incubated in RPMI 1640 with 10% FCS. Whole PB was lysed following staining with FACS Lysing Solution (BD Biosciences). Cells were then fixed with 2% paraformaldehyde.

Flow cytometry staining

For intracellular cytokine staining, BAL and PB were incubated in RPMI 1640 with 10% FCS in the absence of exogenous stimulation or in the presence of PMA and ionomycin. After 1 h, Golgaplug (BD Pharmingen) was added to block cytokine secretion, and cells were incubated for an additional 5 h before staining for extracellular epitopes (CD4 and CD8). Cells were then fixed and permeabilized with Fix & Perm (Caltag Laboratories) and then stained for intracellular IFN-γ and IL-4.

To determine total chemokine receptor levels (both extracellular and intracellular), BAL and PB were stained with CD3, CD4, and CD8 as described above, fixed, and permeabilized with Fix & Perm (Caltag Laboratories) and then stained for the chemokine receptor of interest.

Samples were run on a FACScalibur (BD Biosciences) cytometer and analyzed with either CellQuest or FlowJo. BAL and PB lymphocytes were specifically analyzed using forward and side scatter properties as previously described (14).

Cytometric bead array (CBA) and ELISA

Chemokines were measured by CBA or ELISA using either neat or 10-fold concentrated BAL supernatant. BAL was concentrated in Centriplus YM-3 centrifugal filter devices (Millipore). Levels of CXCL8, CXCL9, CXCL10, CCL2, and CCL5 in prechallenge and postchallenge BAL fluid were measured with the human chemokine CBA (BD Pharmingen) using a FACScalibur cytometer. Levels of CXCL11, CXCL16, CCL11, CCL17, CCL20, CCL22, and CCL26 were determined by ELISA (R&D Systems).

Magnetic bead separation of CD4 T cells from BAL and PB

PBMC were prepared by centrifugation through a density gradient on Histopaque-1077 (Sigma-Aldrich). To select for CD4 T cells, BAL cells and PBMC were initially depleted of monocytes by negative selection with anti-CD14 magnetic beads according to the manufacturer’s protocol (Miltenyi Biotec). The CD14-depleted BAL cells and PBMC were then positively selected with anti-CD4 magnetic beads. Purity was evaluated by quantitative PCR with primers for CD4, CD8, CD19, and CD14 to determine the contribution of unwanted cell subsets. Magnetically selected CD4 T cells from BAL were >92% pure and from PB were >96% pure (data not shown).

Quantitative PCR

Quantitative PCR was performed on cDNA obtained from total RNA extracted from magnetically isolated CD4 T cells as previously described. Primers are published online at www.immunologynet.org.

Internalization

BAL or PB cells were placed in RPMI 1640 with 10% FCS at a concentration of 1 million cells/ml and allowed to equilibrate to 37°C in the
incubator for 30 min. In each tube, various concentrations of chemokine (or no chemokine control) were then added to each well and cells were allowed to incubate for 30 min. After this time, each well was divided in two for staining for either extracellular or total (both extracellular and intracellular) chemokine receptor expression. Cells were stained at 4°C to prevent further internalization or re-expression of the chemokine receptor of interest.

FIGURE 1. Chemoattractant receptor expression is increased on CD4⁺ and CD8⁺ BAL T cells of asthmatic subjects as compared with PB T cells at baseline. A, Flow cytometry staining for chemoattractant receptor expression (CD4 or CD8 on x-axis and chemoattractant receptor on y-axis) is shown for a representative asthmatic subject (square symbol) for CD4⁺ and CD8⁺ PB and BAL T cells. B, The percentage of CD4⁺ or CD8⁺ PB or BAL T cells that express a specific chemokine receptor at baseline is displayed. C, The MFI for chemoattractant receptors on CD4⁺ or CD8⁺ PB or BAL T cells at baseline is displayed. D, Copies of chemoattractant receptor per copy of GAPDH is shown for cDNA isolated from CD4⁺ PB or BAL T cells at baseline. Each symbol represents an individual subject with the black bar representing the mean (as shown in Table I).
Statistics
Samples were analyzed for statistically significant differences using the two-tailed paired Student \( t \) test with \( p < 0.05 \) considered statistically significant.

Results
Selection of allergic asthmatic subjects for segmental allergen challenge study
Asthmatic subjects were selected with known allergy to cat or dust mite (Table I). Lung function tests were performed on each subject to determine their percent of predicted forced expiratory volume in 1 s (FEV\(_1\)) and FEV\(_1\)/forced vital capacity (FVC) ratio, and total serum IgE levels were measured. Values were consistent with mild to moderate obstructive airway disease and a clinical diagnosis of asthma. Each subject reacted to allergen exposure with visible airway narrowing and focal wheezing exclusively over the challenged lung segment. Each subject served as their own control because comparisons were made between paired PB and BAL or paired pre- and postchallenge BAL.

Chemokine expression profile on PB and BAL T cells at baseline in asthmatic subjects
Initially, we examined the surface chemokine receptor profile on CD4\(^+\) and CD8\(^+\) PB and BAL T cells at baseline by flow cytometry (Fig. 1, A–C). We hypothesized that specific chemokine receptors would be important for homing to the airway at baseline and observed the percentage of CCR1, CCR5, CCR6, CCR9, CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, and BLT1 were significantly enriched on CD4\(^+\) BAL T cells as compared with CD4\(^+\) PB T cells. A smaller subset of these receptors, including CCR5, CCR6, and BLT1 were significantly enriched as a percentage on CD8\(^+\) BAL T cells as compared with CD8\(^+\) PB T cells (Fig. 1B). In addition, we also examined the mean fluorescence intensity (MFI), a measure of receptor density per cell. We found that the MFI for CXCR3, CXCR4, CCR5, and CCR6 was significantly increased on both CD4\(^+\) and CD8\(^+\) BAL T cells as compared with PB T cells (Fig. 1C).

To study receptor expression when mAbs were not available and to determine whether differences observed in surface chemokine receptor expression were reproducible at the RNA level, we isolated RNA from CD4\(^+\) PB and BAL T cells and used quantitative RT-PCR to compare the copies of each receptor per copy of GAPDH (Fig. 1D). CCR8 and CCR10 were only examined by quantitative PCR because Abs to these receptors were not commercially available. CCR8 was found at similar levels in CD4\(^+\) PB and BAL T cells, while CCR10 was significantly decreased in CD4\(^+\) BAL T cells as compared with PB (Fig. 1D). In addition, although CCR3, CCR4, CCR5, CCR7, CXCR5, and CXCR6 reflected a similar pattern of RNA levels and surface chemokine receptor expression in CD4\(^+\) PB and BAL T cells, the RNA levels of CCR6, CXCR4, and BLT1 were unchanged, variable, or even the opposite of what was observed for surface receptor expression. This suggests that the surface expression of specific receptors may be regulated at translational or posttranslational steps and that measuring RNA levels may not always provide an accurate reflection of surface expression for all chemokine receptors.

FIGURE 2. Lymphocytes and eosinophils accumulate in the BAL following segmental allergen challenge. A, Baseline and allergen challenge BAL total, lymphocytes, eosinophils, alveolar macrophages, and polymorphonuclear neutrophil (PMN) cell numbers. Each symbol represents an individual subject with the black bar representing the mean (as shown in Table I). B, Percentage of lymphocytes, eosinophils, alveolar macrophages, and polymorphonuclear neutrophils from baseline and allergen challenge BAL segments based on cytosin counts from five subjects.

FIGURE 3. NKT cells are enriched in BAL as compared with PB but are not the predominant lymphocyte population in the BAL. A, Flow cytometry staining for NKT cells (V\(\alpha\)24\(^+\) 6B11\(^+\)) is shown for a representative asthmatic subject (square symbol) from PB and BAL before allergen challenge and BAL following segmental allergen challenge at 24 h. B, The percentage of NKT cells in the lymphocyte gate is shown for each subject for prechallenge PB and pre- and postchallenge BAL. Each symbol represents an individual subject with the black bar representing the mean (as shown in Table I).
Cellular recruitment into BAL following segmental allergen challenge

To observe the recruitment of cells into the human asthmatic airway in vivo, we used a segmental allergen challenge protocol. We tested the skin allergen responsiveness of each subject and used this to determine the allergen dose for segmental allergen challenge. At baseline, prechallenge BAL was procured from the left upper lobe by bronchoscopy and then allergen instilled in the right middle lobe of the lung. After 24 h, postchallenge BAL was taken from the challenged lung segment. PB was drawn at baseline and at 24 h following segmental allergen challenge. Pre- and postchallenge PB T cells were similar in terms of NKT cell populations.

**FIGURE 4.** Chemoattractant receptor expression is decreased for CCR6 and CXCR3 on CD4⁺ and CD8⁺ BAL T cells following segmental allergen challenge. A, Flow cytometry staining for chemoattractant receptor expression (CD4 or CD8 on x-axis and chemoattractant receptor on y-axis) is shown for a representative asthmatic subject (square symbol) for CD4⁺ and CD8⁺ BAL T cells before and 24 h following segmental allergen challenge. B, The percentage of CD4⁺ or CD8⁺ BAL T cells that express a specific chemokine receptor before and after allergen challenge is displayed. C, The MFI for chemoattractant receptors on CD4⁺ or CD8⁺ BAL T cells before and after allergen challenge is displayed. D, Copies of chemoattractant receptor per copy of GAPDH is shown for cDNA isolated from CD4⁺ BAL T cells before and 24 h following segmental allergen challenge. Each symbol represents an individual subject with the black bar representing the mean (as shown in Table I).
chemokine receptor expression profiles, intracellular cytokine staining, and memory markers, demonstrating that the segmental allergen challenge induced tissue-specific but not systemic T cell responses to allergen challenge (data not shown).

To compare the recruitment of cells to the BAL pre- and post-challenge, the total number of cells recovered was enumerated and differential cell counts determined by cytospin (Fig. 2A). The total number of cells recruited into the BAL varied from subject to subject but on average increased 7-fold from baseline. In terms of differential cell counts, lymphocytes increased an average of 15-fold, eosinophils increased 250-fold, and alveolar macrophages increased only 3-fold. Neutrophils increased 80-fold on average but were not consistently increased as shown by the distribution on the graph. The percentage of lymphocytes and eosinophils in the BAL increased significantly following allergen challenge indicative of an allergic response, while the percentage of alveolar macrophages decreased significantly following challenge (Fig. 2B).

NKT cells are enriched in BAL as compared with PB but are not the predominant lymphocyte in asthmatic lung

A recent report suggested that NKT cells account for >60% of T cells in the BAL from asthmatic subjects but not sarcoid or healthy controls (3). Because Ag-specific Th2 cells have been thought to be the predominant lymphocyte that promotes the asthmatic response, we examined PB and BAL from asthmatic subjects for the presence of NKT cells before and after segmental allergen challenge. CD1d-restricted NKT cells can be identified based on either staining with CD1d tetramer loaded with a surrogate ligand for NKT cells, α-GalCer, or by double staining with the Abs, 6B11 and Vα24. 6B11 recognizes the invariant Vα24Jβ11 rearrangement on the TCR Vα24 chain, but 6B11 staining in the absence of staining for the Vα24 chain may actually include B or non-CD1d-restricted T cells. Staining with CD1d tetramer loaded with α-GalCer or double staining with 6B11 and Vα24 Abs has been shown to recognize similar numbers of CD1d-restricted NKT cells from human PB (15).
In this study, we identified CD1d-restricted NKT cells through costaining with 6B11 and Vα/H24 Abs (Fig. 3). CD1d-restricted NKT cells were found at low levels in prechallenge PB (0.0099–0.26% of lymphocyte gate) but were significantly increased in prechallenge BAL (0.39–2.16%) from the asthmatic subjects, as previously described (14). Interestingly, the percentage of BAL CD1d-restricted NKT cells following segmental allergen challenge was similar to levels found in prechallenge BAL. This suggests that CD1d-restricted NKT cells were recruited into the asthmatic lung following segmental allergen challenge as part of an increase in total lymphocytes but were not preferentially recruited in comparison to other lymphocyte subsets. These data also demonstrate that NKT cells are not the major CD4/H11001 T lymphocyte in the asthmatic BAL.

Chemotactant receptor expression profiles from BAL T cells before and after segmental allergen challenge

To determine the significance of specific chemotactant receptors in recruitment of T cells to the asthmatic lung, we examined the chemotactant expression profile for CD4/H11001 and CD8/H11001 BAL T cells before and 24 h following segmental allergen challenge (Fig. 4, A–C). Although we predicted that we would see an increase in Th2-associated chemotactant receptor expression (CCR3, CCR4, CCR8) on BAL T cells following segmental allergen challenge, no receptor was significantly increased in comparison to levels found in prechallenge BAL T cells as a percentage or by MFI (Fig. 4, B and C). Surprisingly, we found that CCR6 and CXCR3 were significantly decreased both as a percentage and as mean fluorescence intensity on CD4/H11001 and CD8/H11001 BAL T cells following segmental allergen challenge (Fig. 4, B and C). This trend was also true for CD4+/H4 BAL T cells, although the findings do not reach statistical significance. We would suggest that the higher percentage of T cells expressing CCR7 implies that a higher percentage of T cells have lymph node homing potential.

We also compared the total number of chemotactant receptor-positive CD4/H11001 and CD8/H11001 BAL T cells before and after segmental allergen challenge and found that the number of CD4/H11001 or CD8/H11001 T cells expressing each chemotactant receptor examined was increased except for CXCR5+/H CD4/H11001 T cells, which were found at lower levels postchallenge (data not shown). In addition, RNA levels of CCR8 or CCR10 in CD4/H11001 BAL T cells were unchanged following segmental allergen challenge (Fig. 4D).

To examine whether the decrease in chemotactant receptor expression was regulated at the RNA level or reflected a posttranscriptional alteration in surface expression, we isolated RNA from CD4/H11001 BAL T cells before and 24 h following segmental allergen challenge (Fig. 4D). There were no statistically significant changes in RNA of any chemotactant receptor. Although CCR6 and CXCR5 surface protein levels were significantly decreased following segmental allergen challenge, CCR6 and CXCR5 RNA levels were similar pre- and postchallenge. This suggests that the decrease in CCR6 and CXCR5 surface expression was regulated posttranscriptionally.

BAL T cells from asthmatic subjects are skewed toward IL-4 production in the absence of exogenous stimulation but retain ability to produce IFN-γ

Because specific chemotactant receptors have been associated with Th1 or Th2 cytokine production, we determined the composition of IFN-γ and IL-4 producing CD4/H11001 and CD8/H11001 T cells in the PB and BAL pre- and postchallenge (Fig. 5, A–C). In the absence of exogenous stimulation, we found that the percentage of IL-4-producing CD4/H11001 BAL T cells was higher than the percentage of IFN-γ-producing CD4/H11001 BAL T cells (Fig. 5B). However, the
addition of PMA and ionomycin altered this pattern of cytokine production to a more Th1/Th1-type profile (Fig. 5C). With stimulation, the percentage of IFN-γ-producing PB and BAL T cells was increased dramatically, whereas the percentage of IL-4-producing PB and BAL T cells increased modestly or was unchanged. This suggests that a subset of BAL T cells may be activated to produce IL-4 or IFN-γ at baseline and the total number of cytokine-producing BAL T cells increase following challenge. With the addition of exogenous T cell stimulation, the BAL T cells display increased skewing toward a Th1 profile, consistent with a higher percentage of T cells with IFN-γ-producing potential. The addition of PMA and ionomycin induces signaling downstream of the TCR and is likely to reveal T cell cytokine potential rather than actual cytokine production following allergenic encounter. Because Th2 cytokines are
critical to the asthmatic response, IL-4 production observed in the absence of exogenous stimulation may be associated with increased baseline Th2 activity in asthmatics.

**Memory markers on prechallenge PB and BAL and postchallenge BAL**

Although proliferation of pre-existing lymphocytes in the asthmatic lung may have occurred following segmental allergen challenge at 24 h, most of the lymphocytes would have to be recruited from blood and other tissue sites to account for the >15-fold average increase in lymphocytes, as demonstrated in murine studies (16). To understand what combination of naive and memory T cells were recruited, we phenotyped CD4+ and CD8+ PB and BAL T cells pre- and postchallenge using the markers CD45RA and CCR7 (Fig. 5, D−F). CD45RA was used to distinguish naive vs memory T cells as studies have shown that loss of CD45RA expression occurs as a T cell transitions from naive to memory (17). In addition, we used CCR7 as a marker of lymph node homing potential as recent studies have shown that CCR7 defines T cells with the capability of homing to lymph nodes from the tissue via afferent lymphatics (18, 19). Thus, CD45RA and CCR7 provide important information about memory phenotype and lymph node homing potential.

CD4+ T cells were divided into subsets: CD45RA+CCR7+ (naive), CD45RA−CCR7+ (CCR7+ memory), and CD45RA−CCR7− (CCR7− memory) (Fig. 5, D and F). Fewer naive and more CCR7− memory CD4+ T cells were present in prechallenge BAL (as a percentage) as compared with either prechallenge PB or postchallenge BAL, while the CCR7+ memory CD4+ T cell population was relatively unchanged between prechallenge PB or postchallenge BAL, while the CCR7− memory CD4+ T cell population was relatively unchanged between prechallenge PB and postchallenge BAL. CD8+ T cells were divided into similar subsets as the CD4+ T cells: CD45RA+CCR7+ (naive), CD45RA−CCR7+ (CCR7+ memory), CD45RA−CCR7− (CD45RA−CCR7− memory), and CD45RA−CCR7− (CD45RA re-expressing CCR7− memory) (Fig. 5, E and G). Fewer naive, more CCR7− memory, and CD45RA−CCR7− memory CD8+ T cells were present in prechallenge BAL as compared with prechallenge PB or postchallenge BAL, while the CD45RA re-expressing CCR7− memory CD8+ T cell population was relatively unchanged. Because the distribution of CD4+ and CD8+ memory T cell populations were most similar between the prechallenge PB and postchallenge BAL samples, these data suggest that T cells are recruited uniformly from PB regardless of memory phenotype and that both naive and memory populations are recruited to the asthmatic lung.

**Chemokine expression in BAL is increased following segmental allergen challenge**

To understand which ligands might recruit CD4+ and CD8+ T cells to the asthmatic lung, BAL fluid was examined before and 24 h following segmental allergen challenge for production of specific chemokine ligands (Fig. 6). It has been previously demonstrated that human BAL fluid is diluted ~100-fold during bronchoscopy, so we would predict that actual chemokine levels would be ~100-fold higher in vivo than the values measured (20). CCL20 (CCR6 ligand) and CXCL8 (CXCR1/2 ligand) were significantly increased following allergen challenge. In addition, CXCL9, CXCL10, and CXCL11 (CXCR3 ligands) were significantly increased following allergen challenge. In addition, CCL17 and CCL22 (CCR4 ligands) and CCL2 (CCR2 ligand) were increased following segmental allergen challenge but did not reach statistical significance. CCL11 and CCL26 (CCR3 ligands), CCL5 (CCR1, 3, 5 ligand), and CXCL16 (CXCR6 ligand) were relatively unchanged following allergen challenge. The increase in ligand production following segmental allergen challenge suggests that CCR2, CCR4, CCR6, CXCR1/2, and CXCR3 may play important roles in recruitment of cells to the asthmatic lung. In addition, the decreased expression of CCR6 and CXCR3 on CD4+ and CD8+ BAL T cells following segmental allergen challenge may be due to internalization of these receptors through engagement with cognate ligand (Fig. 6).

**CCR6 and CXCR3 on CD4+ BAL T cells are internalized following encounter with cognate ligand in vitro**

To determine whether CCR6, CXCR3, and CCR4 ligands could induce internalization and whether this affected CCR6, CXCR3, and CCR4 surface levels, prechallenge CD4+ BAL T cells were used in an in vitro internalization assay (Fig. 7). CD4+ BAL T cells were incubated for 30 min with no ligand or cognate ligand at various concentrations and stained for CCR6, CXCR3, or CCR4, respectively. Extracellular CCR6 was down-regulated, but total CCR6 levels stayed the same following incubation with increasing levels of its cognate ligand, CCL20. Similarly, extracellular CXCR3 was down-regulated when incubated with either CXCL10 or CXCL11, but total CXCR3 was not altered following incubation with ligand. CXCL11 was a more potent inducer of CCR3 internalization than CXCL10, similar to our previously published data (21). For comparison, we examined extracellular CCR4 internalization and found that CCL22 was dominant over CCL17 in inducing internalization as previously shown (22) but induced smaller levels of internalization with no significant changes in total CCR4 levels. In addition, it is interesting to note that CCR4 is found at similar surface and total levels, while surface and total CCR6 and CXCR3 are discordant, suggesting different mechanisms of surface receptor regulation in vivo. These data argue that CCR6 and CXCR3 ligands in the BAL could induce internalization and be responsible for observed decreases in BAL T cell CCR6 and CXCR3 expression following segmental allergen challenge, while CCR4 ligands in the BAL induce more subtle changes in BAL T cell CCR4 expression.

**Discussion**

In our study, we examined the phenotype and recruitment of human T cells to the allergic lung and revealed novel biology for understanding the human asthmatic response. At baseline, we found that specific chemokine receptors, including CCR1, CCR5, CCR6, CCR9, CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, and BLT1 were significantly increased on CD4+ BAL T cells as compared with CD4+ PB T cells. CCR5, CCR6, and BLT1 were significantly increased on CD8+ BAL T cells as compared with CD8+ PB T cells. In addition, we examined for the first time the chemotactic receptor profile on both CD4+ and CD8+ BAL T cells before and 24 h following segmental allergen challenge. Although we predicted that we would observe an increase in IL-4-producing T cells and Th2-associated chemotactic receptors (CCR3, CCR4, and CCR8) following airway challenge, we did not observe a preferential enrichment of Th2 cells or Th2-associated receptors. Surprisingly, we found that no receptors were significantly enriched but rather that CCR6 and CXCR3 were significantly decreased on both CD4+ and CD8+ BAL T cells following challenge at 24 h. We did not rule out the significance of other chemotactic receptors in BAL T cell recruitment but focused on understanding why CCR6 and CXCR3 were down-regulated following challenge. Our data suggest that encounter with increased cognate ligand following challenge, rather than alterations in Th1/Th2 cytokine balance or memory phenotype, account for the decrease in CCR6 and CXCR3 on BAL T cells and that CCR6 and CXCR3 play an important role in propagating the human allergic asthmatic response.
Segmental allergen challenge is useful because it allows direct observation of the responses to allergen that occur in the human airway. In our studies, allergen-induced airway narrowing was followed by significant increases in the recovery of both lymphocytes and eosinophils, as is known to occur in the course of an asthma exacerbation. Although neutrophils were recruited into the BAL of some allergen challenged subjects, the increase varied widely from subject to subject and did not reach statistical significance. Recent studies examining the time course of leukocyte infiltration following segmental allergen challenge demonstrated that lymphocyte and neutrophil recruitment peaked at 18 h postchallenge, whereas eosinophil and alveolar macrophage recruitment peaked at 42 h (23). This suggests that the recruitment of lymphocytes may have reached its apex at 24 h postchallenge, while eosinophil numbers are likely to accumulate beyond this time point.

A recent report has suggested that CD1d-restricted NKT cells comprise the majority (>60%) of T cells in the asthmatic BAL as compared with healthy controls or subjects with sarcoidosis (3). We found that although CD1d-restricted NKT cells were significantly enriched in the BAL as compared with PB, they made up only <2.5% of the lymphocyte gate. Furthermore, we noted that following segmental allergen challenge the percentage of BAL CD1d-restricted NKT cells was unchanged from prechallenge levels. We believe that the discrepancy observed between our data and the findings of Akbari et al. (3) is a result of nonspecific staining from alveolar macrophages in the BAL (14). Akbari et al. (3) gated on CD3, which we believe led to nonspecific background staining and artificially inflated numbers of BAL CD1d-restricted NKT cells. This interpretation was supported by Vijayanand et al. (24) who demonstrated that improper gating can lead to artificially high numbers of CD1d-restricted NKT cells in the BAL. Our data describing low numbers of BAL NKT cells was recently confirmed by studies showing similar low numbers of BAL NKT cells in persons with mild, moderate, and severe refractory asthma and in healthy controls (24–26). Further, Vijayanand et al. (24) found that NKT cells were not preferentially enriched in the asthmatic airway as they found the same low number of CD1d-restricted NKT cells in airways of normal controls and subjects with chronic obstructive pulmonary disease as they did in subjects with asthma (24). Thus, while we found that CD1d-restricted NKT cells are enriched 10-fold in the BAL as compared with PB, this appears not to be specific to the asthmatic airway but more a reflection of a propensity for NKT cells to reside in the tissue. This is consistent with what we found following Ag challenge, in which the percentage of NKT cells in the lymphocyte gate was not increased. These data also argue against preferential recruitment of CD1d-restricted NKT cells into the asthmatic lung. We have previously demonstrated that CCR6 and CXCR3 are highly expressed on CD1d-restricted NKT cells and could allow for recruitment into tissue sites such as the lung, but these receptors would not be expected to support the >1000-fold enrichment in the BAL suggested by Akbari et al. (3). Although CD1d-restricted NKT cells may contribute to the asthmatic response through secretion of Th2 cytokines, conventional T cells make up the predominant T cell population in the asthmatic BAL and likely play the predominant role in promoting the asthmatic response.

It has been shown that similar chemokine receptor expression profiles are found on BAL T cells from healthy controls and asthmatic subjects (27–31), so the increased expression of specific chemokine receptors on BAL T cells as compared with PB T cells is likely to be related to both an increase in memory T cells in the BAL as compared with PB and to the requirement for specific chemotaxtractant receptors in trafficking to the BAL and other tissue sites. In addition to the skin-associated (CCR4 and CCR10) and gut-associated (CCR9) chemoattractant receptors, certain receptors are enriched on tissue-localized T cells as compared with PB T cells, including but not limited to CXCR3, CXCR6, CCR5, and CCR6. For example, CXCR3, CXCR6, and CCR5 are highly enriched on liver T cells, whereas CCR6 and CXCR6, but not CXCR3 or CCR5, are enriched on normal skin T cells (32, 33). These receptors may play a role in homing to tissue sites or be associated with memory T cells, which are enriched in the tissue. Because healthy controls and asthmatic subjects express similar chemotaxtractant receptors on BAL T cells before allergen challenge, we focused on differences in chemotaxtractant receptor expression on BAL T cells and chemokine production following segmental allergen challenge.

Another recent segmental allergen challenge study found that CCR4 and CCR7 were increased and CXCR3 decreased as a percentage on bulk BAL T cells 42 h following allergen challenge but only when selecting for the three subjects with highest eosinophilia (28). The difficulty in comparing our study with this study (28) is that specific chemokine receptors are expressed differentially on CD4+ and CD8+ T cells, including but not limited to CXCR3, CCR4, CCR5, CCR6, CXCR6, and CCR7. For instance, because CCR4 is expressed almost exclusively on CD4+ T cells, increases in the percentage of CCR4 on total BAL T cells may actually mark an increase in CD4+ T cells as compared with CD8+ T cells. This implies that grouping all CD3+ T cells together may mask (or amplify) alterations in the chemokine receptor profile due to differences in the CD4/CD8 ratio in the BAL and PB.

Panina-Bordignon et al. (34) found a significant increase in the total number of CCR4+ and CCR8+ T cells in bronchial biopsies from allergen-challenged, as compared with sham-challenged, asthmatics. We also found an increase in total CCR4+CD4+ T cells (like most chemotaxtractant receptor positive CD4+ or CD8+ T cells) but noted that the percentage of CCR4+ or CD8+ T cells of expressing CCR4 was unchanged following challenge. Differences between our findings and those of Panina-Bordignon et al. (34) may result from differences in staining technique (immunohistochemistry vs flow cytometry) and cell isolation from different tissue compartments (BAL vs bronchial biopsy).

Although Th2 cytokines play a critical role in setting up the asthmatic response, it has been shown that Th2 cells are not the predominant T cell present in the asthmatic BAL through intraacellular cytokine staining (29, 30, 35). In addition to determining the percentage of Th2 cells present before and after segmental allergen challenge, we determined the percentage of Th1 cells present in the asthmatic airway because IFN-γ may play an important role in either increasing or decreasing the severity of the asthmatic response (5, 6). After stimulating cells with PMA and ionomycin, we observed that the percentage of IL-4-producing BAL T cells was similar before and after segmental allergen challenge, while the percentage of IFN-γ-producing BAL T cells was somewhat decreased following segmental allergen challenge, similar to a previous study (29, 36). We also noted that in the absence of exogenous stimulation with PMA and ionomycin that the percentage of IL-4-producing BAL T cells was unchanged, but that the percentage of IFN-γ-producing cells was decreased significantly as compared with stimulated BAL T cells. Because the percentage of IL-4-producing BAL T cells was unchanged following segmental allergen challenge, it was not surprising that the percentage of Th2-associated receptors on BAL T cells was also unchanged.

Increases in some or all BAL Th2 cytokines have been measured following segmental allergen challenge (23, 37–40). Because IL-4, IL-5, and IL-13 can be produced by other leukocytes, including eosinophils, basophils, and mast cells (41, 42), increases...
in BAL Th2 cytokines may result from both T cell and non-T cell-derived sources. Although we did not observe an increase in the percentage of IL-4-producing Th2 cells, the total number of IL-4-producing Th2 cells was still increased following challenge and could result in increased total IL-4 production.

Chemokine receptor expression can be associated with specific subsets of memory T cells as defined by CD45RA and CCR7. Surprisingly, we found that both naive and memory T cells were recruited from PB to BAL. The recruitment of both naive and memory T cells to the BAL, in conjunction with an increase in chemokine ligands following allergen challenge, suggests that a number of chemokine receptors may be responsible for recruiting T cells to the asthmatic airway.

Following segmental allergen challenge, we surprisingly found that no specific chemokine receptor was enriched on CD4+ or CD8+ BAL T cells but rather that CCR6 and CXCR3 were dramatically and significantly decreased as a percentage of CD4+ and CD8+ BAL T cells. We focused on the change in percentage of receptor expression rather than total cell number because most chemokine receptors were increased as measured by total cell number. Although we believe that a number of chemokine receptor ligands play a role in recruitment to the asthmatic BAL based on increased chemokine production, the finding that CCR6 and CXCR3 were significantly decreased on BAL T cells following challenge was intriguing. This suggests that either CCR6low/CXCR3low− T cells were preferentially recruited to the asthmatic BAL or that these receptors were specifically down-regulated on BAL T cells following allergen challenge.

We believe a likely explanation for the decrease in CCR6 and CXCR3 following segmental allergen challenge is that these receptors are internalized following encounter with cognate ligand. CCR6 and CXCR3 expression following challenge is decreased on a population-wide scale with the highest MFI population absent rather than as discrete populations of high and low expressors. Both CCR6 and CXCR3 ligands are increased in the BAL fluid at 24 h postchallenge, and their respective ligands can induce internalization of the receptors in vitro with total (extracellular and intracellular) levels constant. It is theoretically possible that CCR6lowρ−/− and CXCR3lowρ− CD4+ and CD8+ T cells were specifically recruited following challenge and that pre-existing CCR6high and CXCR3high T cells selectively did not proliferate and/or underwent apoptosis but we believe this is unlikely. The down-regulation of CCR6 and CXCR3 following challenge could aid in retention of recruited T cells or allow for recruitment to other sites (i.e., draining lymph node) through other chemokine receptor-ligand pairs, such as CCR7 (18).

In addition to CCR6 and CXCR3 ligands, we also noted that CCR2 (CCL2), CCR4 (CCL17 and CCL22), and CCR1/2 (CXCL8) ligands were increased 24 h following segmental allergen challenge, while the CCR3 (CCL11 and CCL26), CCR6 (CXCL16), and CCR1/3/5 (CCL5) ligands were relatively unchanged. Specific chemokine ligands, including CCL2, CCL3, CCL5, and CCL11, are up-regulated following segmental allergen challenge at 4 h but return to baseline by 24 h, so the peak of chemokine production may be missed at the 24 h time point (43, 44). CCL2 and CXCL8 are likely to play important roles in recruiting monocytes and neutrophils, respectively, with minimal activity for recruitment of T cells. The eosinophil chemotactants, CCL11 and CCL26, were not increased at 24 h postallergen challenge. CCL11 is likely to peak early (~4 h), while CCL26 peaks closer to the 24 h time point but CCL26 may be predominantly associated with the bronchial mucosa, rather than the BAL fluid (45, 46). In terms of T cell chemotactants, CCR4 ligands (CCL17 and CCL22) are increased following challenge but do not seem to induce any alterations in CCR4 expression on CD4+ or CD8+ BAL T cells. CCL22 has been shown to be a potent inducer of CCR4 internalization as opposed to CCL17, so it is not clear why CCR4 levels were not also down-regulated on CD4+ BAL T cells following allergen challenge (22). This may be related to lower concentrations of CCR4 ligands found in the BAL as compared with other chemokine receptors or to rapid recycling of CCR4 to the surface following internalization as opposed to CCR6 and CXCR3.

Our results, in addition to other published literature, suggest that recruitment of T cells following allergen encounter is controlled sequentially by several distinct chemokine receptor-mediated pathways. During the earliest phase of allergen encounter, mast cells degranulate and release lipid and protein chemokine antagonists, such as BLT1 and CCR5 ligands, initiating recruitment of Th2 cells along with other effector T cell populations (47, 48). Following this early phase, CCR4 ligands are produced following IL-4 and IL-13 induction of the STAT6 pathway and lead to amplification of Th2 recruitment and the asthmatic response (9).

In addition to these chemokine receptors, our data suggest that CCR6 and CXCR3 play a critical role in propagating the asthmatic response. Data in mouse models of asthma suggest that increases in chemokine ligands following allergen challenge may increase the severity of the asthmatic response (49) and that CCR6 is required for the asthmatic response (50). Recently, Acosta-Rodriguez et al. (51) identified CCR6 as a marker of Th17 cells. Although we did not evaluate IL-17 our study, our finding that CCR6 is highly expressed on BAL T cells and down modulated after antigen challenge, suggests that Th17 cells may play a role in human asthma. CCR6 and CXCR3 ligands are produced in response to direct TLR and/or cytokine activation and induce recruitment of inflammatory T cells that can amplify the asthmatic response (52, 53). TLR stimulation during the asthmatic response can occur through TLR agonists present in aeroallergens, such as in animal dander or dust mites, or through TLR agonists present in respiratory viruses, which are known to exacerbate asthma (54, 55). In conclusion, our data provide support for additional chemokine receptor ligands in the human asthmatic response and suggest that CCR6 and CXCR3 play an underappreciated role in T cell recruitment to the asthmatic lung.

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Disclosures
The authors have no financial conflict of interest.

References


