

# Increased Circulating CCR3+ Type 2 Helper T Cells in House Dust Mite-Sensitive Chinese Patients with Allergic Diseases

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A characteristic feature of inflammation is the recruitment of effector leukocytes into affected tissues, which is a dynamic process that consists of multiple regulated steps involving both adhesion molecules and chemotactic cytokines.<sup>1</sup> Chemokines regulate the activation step of leukocyte-trafficking by inducing cell motility and by activation of adhesion molecules.<sup>1,2</sup> These molecules are also involved in a number of other physiological processes including lymphocyte development, modulation of angiogenesis and cell compartmentalization in lymphoid tissues.<sup>2-4</sup> Chemokine receptors are G-protein-coupled, seven-transmembrane receptors, whose expression is tightly regulated in accordance with the differentiation and activation stages of lymphocytes.<sup>4,5</sup> Analysis of chemokine receptor expression on leukocyte subsets has revealed important mechanisms of cell-trafficking regulations

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**SUMMARY** Chemokine receptor expression has been shown to be associated with the differentiation of T helper cells. The CCR3, CXCR4 and CCR5 expression on circulating T cells were studied in 30 house dust mite sensitive-patients with allergic diseases and in another 30 healthy controls. The expression was analyzed in CD4, CD8 and double negative (DN) T cells by triple fluorescence staining. In addition, intracellular cytokine staining was performed in the CCR3+ CD4+ T cells. Increased circulating portions of CCR3+ CD4+ T cells and CCR3+ DN T cells were found in these patients ( $p < 0.01$ ). There was no statistically significant difference in the expression of CXCR4 and CCR5 on T cells. The follow-up data of the patients did not show a statistically significant change in the CCR3 expression. IL-4 was expressed within CCR3+CD4+T cells upon activation. The IL-4 secreting CCR3+ type 2 T helper cells may play a pathogenetic role in immune responses of house dust mite-sensitive Chinese patients with allergic diseases.

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within the immune system.<sup>2,5</sup>

Type 1 and type 2 helper T (Th1 and Th2) cells mediate different types of immune responses by secreting different cytokines and interacting with different types of leukocytes.<sup>6</sup> Th1 cells produce IFN- $\gamma$  and associate with macrophages in delayed-type hypersensitivity lesions, while Th2 cells secrete IL-4 and co-localize with eosinophils and basophils at allergic inflammation

sites.<sup>7</sup> Differential recruitment of Th1 and Th2 cells is controlled by expressions of CXCR3/CCR5 and of CCR3/CCR4/CCR8, respectively.<sup>4,8</sup> Selective recruitment of certain lymphocyte subset by chemokines is

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mainly defined by the presence of their corresponding chemokine receptors.<sup>2</sup>

In the case of allergic inflammation, it is the Th2 cell, the eosinophil and the mast cell that characterize the tissue inflammatory response.<sup>9</sup> Production of Th2 cytokines such as IL-4 and IL-5 has been reported to correlate with disease severity in atopic patients.<sup>9,10</sup> In animal models for allergic asthma, the depletion of CD4+ cells or the neutralization of IL-4 or IL-5 results in impaired airway hyperresponsiveness.<sup>11,12</sup> Up-regulation of eotaxin and CCR3 in the bronchial mucosa and in skin with lesions has been found in patients with allergic asthma and atopic dermatitis, respectively.<sup>13,14</sup> Sequential production of Th2 cytokines may induce sequential production of chemokines which result in further recruitment of Th2 cells with production of more inflammatory mediators that may lead to the development of allergic reactions.

CCR3 is an eosinophil chemoattractant receptor for multiple inducible CC chemokines involved in modulating the movement of eosinophils to points of allergen challenges.<sup>1</sup> Consistent with this, CCR3 knockout mice do not recruit eosinophils to the skin after repeated epicutaneous sensitization with ovalbumin (OVA) and exhibit reduction in the eosinophil influx into the bronchoalveolar lavage after aerosolized OVA challenge.<sup>15</sup>

Most of the studies on human chemokine receptors in allergic diseases have focused on eosinophils and relative little is known about T cells especially about Th2 cells. In this study, we studied the expression

of chemokine receptors including CCR3, CXCR4 and CCR5 on circulating T cells of house dust mite-sensitive Chinese patients with allergic diseases. All of the subpopulations of T cells including CD4+, CD8+ and double negative (DN) cells were examined. We further studied the intracellular cytokine staining in CCR3+ CD4+ T cells.

## MATERIALS AND METHODS

### Patients

From February 2000 to July 2002, thirty house dust mite-sensitive patients with allergic diseases (allergic type asthma, allergic rhinitis, allergic conjunctivitis and/or atopic dermatitis) were recruited from the Section of Allergy and Immunology and Section of Rheumatology, Department of Internal Medicine, National Cheng Kung University Hospital, Tainan, Taiwan. The study group consisted of 20 females and 10 males with a mean age of  $30.8 \pm 8.9$  (from 16 to 45). Another 30 sex-and-age matched healthy individuals were recruited as control group. All of the patients were sensitive to house dust mite, in terms of a hypersensitivity history, high levels of mite-specific IgE antibodies and, in 20 selected cases, positive skin tests.<sup>16</sup> Venous blood samples were drawn from the patients and the healthy controls. The follow-up blood samples were obtained from 12 patients at an interval of more than 3 months. Informed consents were obtained from the patients before enrollment.

### Determination of specific IgE antibodies to house dust mite

Commercial multiple antigen simultaneous tests (MAST)

(Mountain View, CA) were used to determine house dust mite-specific IgE antibodies in serum levels of *Dermatophagoides pteronyssinus* (Der p) and *Dermatophagoides farinae* (Der f). The results were assayed by chemiluminescence system and were categorized into class 0 to 4. Class 3 or 4 with anti-Der p and/or Der f concentrations of more than 1.90 net mVolt were considered having a high level of antibody response to house dust mites.

### Flow cytometry analysis

Peripheral mononuclear cells (MNCs) were purified from heparin-anticoagulated venous samples by Histopaque (Sigma Diagnostics, St. Louis, MO) gradient centrifugation. Triple fluorescence staining was used for the surface phenotype analysis. The monoclonal antibodies (mAbs) against human antigens including anti-CD3 (clone UCHT1), anti-CD4 (clone PRA-T4), anti-CD8 (clone PRA-T8), anti-CCR5 (clone 2D7), and anti-CXCR4 (clone 12G5) were purchased from PharMingen. Anti-CCR3 (clone 61828.111) was purchased from R&D System. Purified human MNCs were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3, anti-CD4 or anti-CD8, and phycoerythrin (PE)-conjugated anti-CCR3, anti-CXCR4 or anti-CCR5. For analysis of CCR3 expression on double negative (DN) T cells, the MNCs were stained with Cy-chrome-conjugated anti-CD3, FITC-conjugated anti-CD4 and anti-CD8, and PE-conjugated anti-CCR3 mAbs. The cells were incubated with these mAbs for 30 minutes on ice in the dark. After washing, the stained cells were analyzed with FACSsort (Becton Dickinson, Mountain View, CA) and CellQuest software programs (Bec-



ton Dickinson). The DN T cells were gated on the CD3+ and CD4- CD8- portion.<sup>17</sup> Control isotype mAb (PharMingen) stainings were included for each sample.

### Intracellular cytokine staining

The MNCs were stimulated with PMA (25 ng/ml) and ionomycin (1 µg/ml) for 4 hours with the addition of Brefeldin A (Sigma) in the culture. Cells were harvested and stained with Cy-Chrome-conjugated anti-CD4 (clone PRA-T4, PharMingen) and PE-conjugated anti-CCR3. After fixation with 1% paraformaldehyde (Sigma) for 20 minutes, these MNCs were stained with FITC-conjugated anti-IL-4 mAb (clone MP4-25D2, PharMingen) or with FITC-conjugated anti-IFN-γ (clone 4S.B3, PharMingen) in a staining buffer containing 0.1% saponin (Sigma). These cells were further subjected to flow cytometry analysis.

### Statistical methods

The statistical analyses were carried out using non-parametric tests, including the Mann-Whitney rank sum test for comparisons between allergic patients and healthy controls. The Wilcoxon signed ranks test was used for comparisons be-

tween data obtained from different periods of follow-up from these patients. A *p* value less than 0.05 was considered statistically significant.

## RESULTS

The expression of CCR3 percentages on CD3+ T cells and their subpopulations including CD4+, CD8+ and DN portions were shown on Table 1. Higher percentages of CCR3 expression were shown on the CD4+ T cells and DN T cells but not on the CD8+ T cells (*p* < 0.01). Representative figures of the expression of CCR3 on CD4+ T cells from an allergic patient and a healthy control are shown in Fig. 1. The follow-up data of CCR3 expression on CD4+ T cells and DN T cells from 12 patients after an interval of more than 3 months showed no statistical difference (for CD4, 2.90 ± 1.88 % vs 2.65 ± 1.51%; for DN, 2.45 ± 1.05% vs 2.23 ± 0.74%).

Even with a higher expression of CCR3 (a Th2 marker) on CD4+ T cells, no difference was found in the expression of CCR5 (a Th1 marker) on these cells as shown in Fig. 2 (for CCR5, controls vs patients, 12.79 ± 3.67% vs 11.09 ± 4.59%). There was no statistical difference in the expression of CXCR4

on CD4+ T cells between control individuals and allergic patients (95.51 ± 3.54% vs 95.16 ± 4.11%).

For the results of intracellular staining, IL-4 was detected on the CCR3+ portion of CD4+ T cells by stimulation with PMA/ionomycin. IFN-γ was not detected on this portion. Samples were obtained from 6 patients with a high expression of CCR3. It was found that 12.71 ± 4.65% of CCR3+ CD4+ T cells showed positive IL-4 staining upon activation.

## DISCUSSION

Human studies examining the expression of chemokines and chemokine receptors support the emerging concept that CCR3, CCR4 and CCR8 and their ligands play important roles in the pathogenesis of allergic diseases.<sup>18</sup> Human CCR3 distribution on eosinophils, basophils and Th2 cells is compatible with the role in allergic inflammation.<sup>19</sup> CCR3 activities on human cells *in vitro* include eosinophil and Th2 cell chemotaxis, and degranulation of eosinophils and basophils.<sup>20,21</sup> Ligands for human CCR3 include eotaxin, eotaxin-2, eotaxin-3, monocyte chemoattractant protein-3 (MCP-3), MCP-4, MCP-5, which are regulated upon activation,

**Table 1** Positive CCR3 percentages on peripheral T cells including CD3+, CD4+, CD8+ and DN portions from allergic patients and healthy controls

Group	Numbers	% CCR3- CD3+	% CCR3- CD4+	% CCR3- CD8+	% CCR3- DN
Controls	30	0.84 ± 0.34 (NS)	0.88 ± 0.37 ( <i>p</i> < 0.01)	0.81 ± 0.38 (NS)	0.80 ± 0.32 ( <i>p</i> < 0.01)
Patients	30	1.23 ± 0.69	1.86 ± 1.50	0.91 ± 0.59	1.62 ± 0.97

Data represented by mean ± standard deviation  
DN, double negative  
NS, not statistically significant

normal T expressed and secreted (RANTES).<sup>22</sup> Targeted disruption of the eotaxin reduced the number of OVA-induced eosinophils in the bronchoalveolar fluid in a murine model of asthma.<sup>23</sup> Anti-eotaxin antibodies reduced bronchial hyper-reactivity and lung eosinophilia after each antigen exposure.<sup>24</sup> Eotaxin appears to be the most potent and selective ligand.

In patients with atopic asthma, recent studies have shown an up-regulation of eotaxin expression on epithelial cells and endothelial cells and increased CCR3 expressing mucosa-infiltrating cells in the bronchial mucosa.<sup>13</sup> Eotaxin produced by skin-infiltrating MNCs has been shown to play an important role in attracting CCR3+ T cells to skin with lesions from patients with atopic dermatitis.<sup>14</sup> A nasal allergen challenge of allergic rhinitis patients induced the expression of eotaxin from the nasal mucosa and the release of this protein into nasal secretions.<sup>25</sup> Anti-CCR3 antibody has been shown to inhibit chemotaxis induced by tear samples from patients with ocular allergy.<sup>26</sup> An increase in expression of eotaxin has been found in the conjunctiva of patients with vernal keratoconjunctivitis as compared to control subjects.<sup>27</sup> These data suggest a crucial role for the interaction of CCR3 with eotaxin in the immune responses of patients with allergic diseases.

Increased circulating CCR3+ CD4+ and DN T cells in house dust mite sensitive-patients with allergic diseases were demonstrated in this study. IL-4 has been reported to be produced by CCR3+CD4+T cells upon activation.<sup>19</sup> We also detected IL-4 on the CCR3+ portion of CD4+

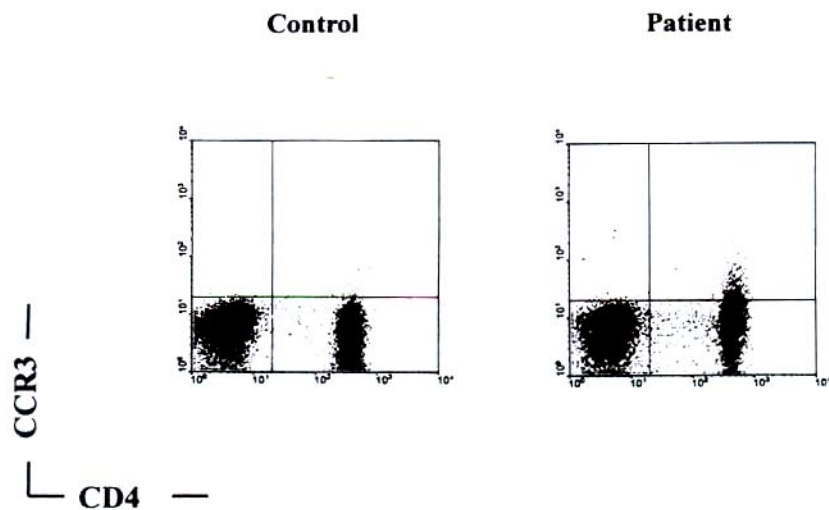


Fig. 1 Representative figures of CD4 (horizontal axis) and CCR3 (vertical axis) expression on circulating lymphocytes from a healthy control (left) and a house dust mite-sensitive patient (right).

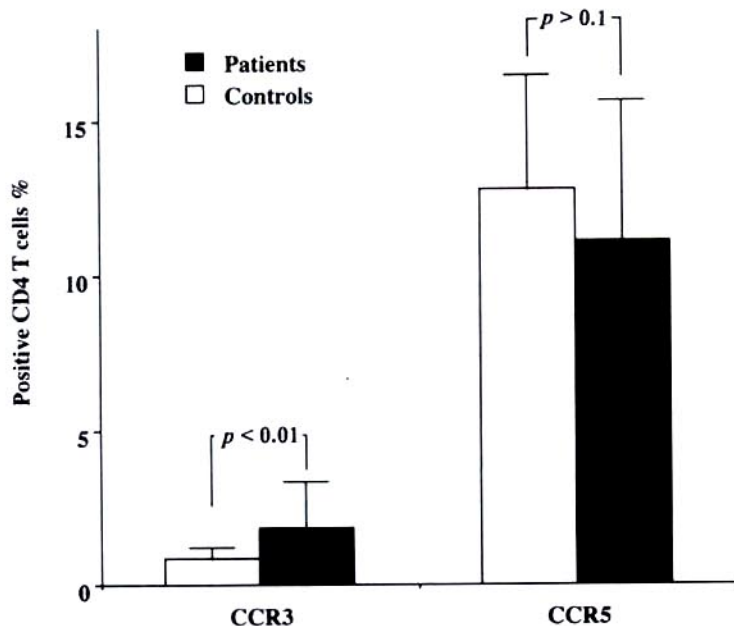


Fig. 2 Percentages of CCR3 and CCR5 expression on circulating CD4+ T cells from healthy controls and allergic patients. Bar heights represent mean values and brackets indicate standard deviation.



T cells by PMA/ionomycin stimulation. Upon activation *in vitro*, circulating human DN T cells have been reported to produce Th2 cytokines such as IL-4.<sup>28</sup> No difference was found in the follow-up data of CCR3+ CD4+ and DN T cell percentages from house dust mite sensitive-patients. Taken together, these results seem to suggest that CCR3+ Th2 cells play a pathogenetic role in these patients. Although an attractive hypothesis suggests the Th1/Th2 balance of immune responses in some human diseases, down-regulation in the CCR5 expression on CD4+ T cells was not observed in the present study.

Recent trials using inhibitory antibodies and receptor antagonists represent a new approach to immunotherapy in allergic diseases.<sup>29</sup> The interest in chemokine receptors as therapeutic targets has focused on the disclosure of several small molecule non-peptide inhibitors interfering with the signaling between chemokines and chemokine receptors.<sup>30</sup> The small molecule CCR3 antagonists blocking the eotaxin-induced chemotaxis *in vitro* are potential candidates for the prevention of eosinophil, basophil and Th2 cell infiltration in human allergic diseases.

In conclusion, increased circulating CCR3+ CD4+ T cells and CCR3+ DN T cells were found in house dust mite-sensitive Chinese patients with allergic diseases. The IL-4 secreting CCR3+ Th2 cells may play a pathogenetic role in the immune responses of these patients.

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