Requirement for the Chemokine Receptor CCR6 in Allergic Pulmonary Inflammation

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Abstract

Allergic asthmatic responses in the airway are associated with airway hyperreactivity, eosinophil accumulation in the lung, and cytokine production by allergen-specific, T helper cell type 2 (Th2) lymphocytes. Here, we show that in a cockroach antigen (CA) model of allergic pulmonary inflammation, the chemokine macrophage inflammatory protein (MIP)-3α is expressed in the lung within hours of allergen challenge. To determine the biologic relevance of this expression, mice lacking CCR6, the only known receptor for MIP-3α, were studied for their response to CA. CCR6-deficient mice were immunized to the same extent as their wild-type counterparts, as judged by cytokine production in antigen-challenged lymphocytes. However, compared with CA-challenged wild-type mice, challenged CCR6-deficient mice had reduced airway resistance, fewer eosinophils around the airway, lower levels of interleukin 5 in the lung, and reduced serum levels of immunoglobulin E. Together, these data demonstrate that MIP-3α and CCR6 function in allergic pulmonary responses and suggest that these molecules might represent novel therapeutic targets for treatment of asthma.

Key words: CCR6 • MIP-3α • chemokine • asthma • lung

Introduction

Asthma is a chronic disease of the lung, characterized clinically by a reversible obstruction in airflow resulting from edema, mucus production, and hyperresponsiveness of the bronchi. Millions of people are affected by this disease, and its incidence is increasing, particularly in developed nations. Asthmatic responses to inhaled allergens are associated with IgE-mediated mast cell activation followed by the accumulation of leukocytes (1–3), especially eosinophils (1, 4), in and around the affected airway. Th2 lymphocytes, which are defined by their production of the proinflammatory cytokines IL-4, IL-5, and IL-13, have also been linked to the asthmatic condition and to the recruitment and activation of eosinophils (2, 5–9). Thus, Th2 lymphocytes and eosinophils are key effector cells of asthmatic responses.

CCR6 is a chemokine receptor that is expressed in immature dendritic cells (DCs) (10–12) as well as in B lymphocytes (13, 14) and memory T cells (13). The only known chemokine ligand for CCR6 is macrophage inflammatory protein (MIP)-3α (CCL20), although members of the β defensin family also bind CCR6 with a lower affinity (15). MIP-3α is constitutively expressed in the spleen and in the epithelium overlying the subepithelial dome (SED) of murine Peyer's patches (16, 17) and human tonsils (18). In gene-targeted mice lacking CCR6 (CCR6$^{-/-}$ mice), myeloid DCs are either absent or very underrepresented in the SED compared with wild-type mice (17). In addition, CCR6$^{-/-}$ mice have impaired mucosal immune responses to oral immunization with KLH and to enterovirus challenge.

Like the intestine, the airway is also covered with a mucosal surface that contacts inhaled allergens, suggesting that MIP-3α and CCR6 might also participate in the mucosal immune response to inhaled antigens. However, unlike the Peyer's patch, the lungs and lymph nodes of unchallenged mice do not constitutively express MIP-3α (16, 17), although its expression has been reported in lymph nodes during inflammatory conditions (12, 16). To determine whether MIP-3α and CCR6 participate in pulmonary allergic responses, we studied the response of CCR6$^{-/-}$ mice to cockroach antigen (CA). The diminished allergic responses seen in CCR6$^{-/-}$ mice support a

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role for CCR6 in allergic pulmonary inflammation and in Th2 immunity.

Materials and Methods

Mice. C57BL/6 mice were purchased from The Jackson Laboratory. CCR6$^{-/-}$ mice were obtained as described previously and subsequently backcrossed for eight generations onto the C57BL/6 background.

CA Challenge. C57BL/6 mice were immunized with 10 μg of CA (Bayer Pharmaceuticals) in incomplete Freund's adjuvant as described previously (19). For cytokine analysis, mice were challenged once with allergen on day 14. For airway hyperreactivity assessment, mice were challenged a second time, 48 h after primary challenge. For cytokine analysis of lymph node cells, animals were given a single intratracheal challenge, and the draining thoracic lymph nodes were harvested after 48 h. The single-cell suspension of lymph node cells was then restimulated in vitro with 5 μg of CA. Supernatants and cells were harvested for cytokine mRNA (Taqman) and protein (ELISA) analysis.

Measurement of Airway Hyperreactivity. Airway hyperreactivity in anesthetized mice was measured as described previously (20) with a mouse plethysmograph (Buxco) using a direct ventilation method specifically designed for low tidal volumes.

RNA Isolation and Analysis. Lungs were harvested at various times after challenge, and RNA was prepared from them using RNA-STAT 60 reagent (Tel-Test, Inc.) according to the manufacturer's specifications. Complementary (c)DNA was prepared from individual lungs using the Superscript RT kit (Life Technologies) with the addition of random sequence hexamers (Promega) to 10 ng/μl. cDNA was analyzed by quantitative, real-time PCR using a Taqman 7700 instrument (Applied Biosystems). Taqman PCR reactions consisted of 25 ng of cDNA, 0.9 μM each diagnostic primer, 0.25 μM diagnostic probe, 1× final of rRNA PDAR (predeveloped assay reagent) (Applied Biosystems), and 1× final of Taqman Universal PCR Mastermix (Applied Biosystems). The default 7700 thermocycler parameters were used. Spectral data from Taqman runs were analyzed using the Sequence Detection Systems software (version 1.6.3; Applied Biosystems). Raw data were normalized to the rRNA internal control standards.

ELISAs. Whole lungs were homogenized in 1 ml of high salt lysis buffer containing protease inhibitors. Debris-free supernatants were isolated and the cytokines measured by ELISA as described (21). Antibody pairs from R & D Systems were used for IL-5 and IL-4 analysis, and polyclonal antibodies made in our University of Michigan laboratory were used in the IL-13 ELISA. The sensitivity of the analyses was ~10 pg/ml. No cross-reactivity to any other chemokine or cytokine was detected.

Statistics. Statistical significance was determined by ANOVA. $P < 0.05$ was considered significant.

Results and Discussion

MIP-3α Is Induced by Allergen Challenge. We first investigated whether MIP-3α is expressed in the lungs of wild-type C57BL/6 mice after sensitization and challenge with CA. This allergen was used because of its clinical relevance and because it induces responses typical of asthma, including production of Th2 cytokines, eosinophil infiltration, and airway hyperreactivity. Quantitative PCR analysis (Taqman) was performed on cDNAs derived from total

lung RNA. In sensitized but unchallenged mice, only very low levels of MIP-3α–specific mRNA were detected. However, shortly after CA challenge, these levels increased dramatically, peaking at 8 h after challenge (Fig. 1). These data show that MIP-3α, the only known chemokine ligand for CCR6, is expressed in allergen–challenged lungs.

CCR6$^{-/-}$ Mice Have Reduced Airway Hyperreactivity after CA Challenge. To investigate whether the induction of MIP-3α in lungs and lymph nodes was of functional importance in pulmonary allergic responses, we investigated the physiologic response of CCR6$^{-/-}$ mice to CA challenge. As airway hyperreactivity is the feature considered most relevant to human asthma, we measured this parameter in the lungs of both wild-type and CCR6$^{-/-}$ mice. In both sets of mice, airway resistance peaked at ~8 h after challenge. However, the resistance in CCR6$^{-/-}$ mice was two- to threefold lower than that seen in wild-type mice (Fig. 2). 24 h after challenge, the resistance in both groups had declined from that seen at 8 h, but in CCR6$^{-/-}$ mice it remained significantly lower than in wild-type controls. Similarly, 48 h after challenge, resistance in CCR6$^{-/-}$ mice was lower than in wild-type mice, although the difference at this last time point did not reach statistical significance.

CCR6$^{-/-}$ Mice Have Reduced Peribronchial Eosinophil Accumulation. Previous studies have demonstrated that airway hyperreactivity after multiple allergen challenges is closely associated with the level of peribronchial eosinophil accumulation (19). To determine whether the interaction of MIP-3α and CCR6 is required for the eosinophilic response to CA, lung sections were prepared and eosinophils counted with high-power microscopy. This analysis revealed that CA-challenged CCR6$^{-/-}$ mice had 10-fold fewer peribronchial eosinophils than challenged wild-type mice (Fig. 3). One possible explanation for this decrease is that MIP-3α directly mediates eosinophil recruitment. This hypothesis is supported by the observation that in humans, MIP-3α can directly induce eosinophil migration (22). However, in our experiments, eosinophils harvested from IL-5–overexpressing transgenic mice did
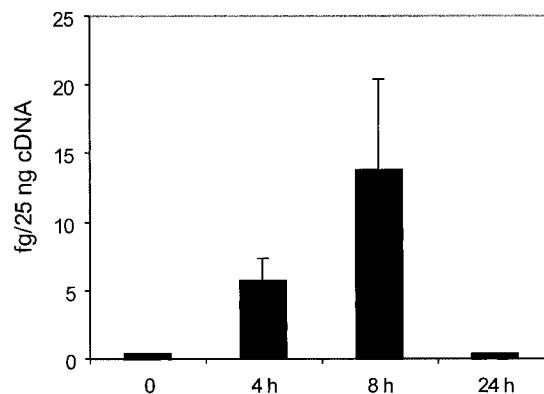

Figure 1. MIP-3α expression in allergen-challenged lungs. RNA was prepared from lungs harvested at the indicated times after challenge with CA. cDNA prepared from this RNA was used in a quantitative PCR (Taqman) assay. Values shown represent femtograms of MIP-3α cDNA per 25 ng total cDNA.
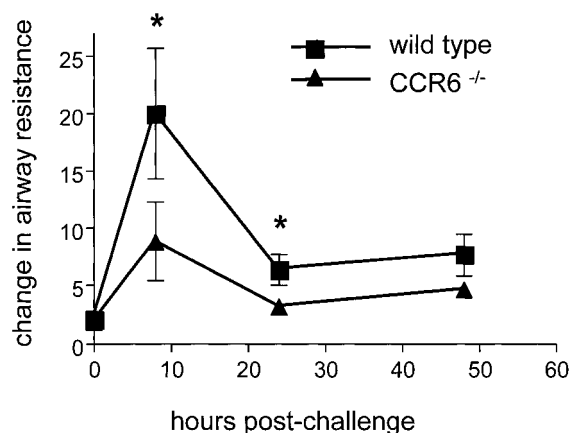
Figure 2. Lung function in CA-challenged mice. After intratracheal challenge with CA (10 μg), mice were ventilated and administered methacholine (100 μg/kg) intravenously. Values shown represent the change in airway resistance (cm/H$_2$O/ml/s), defined as peak airway resistance minus the background resistance. Mean values ± SE are shown. $n = 5$ animals per treatment group. *$P < 0.05$.

not migrate in response to murine MIP-3α under conditions in which murine eotaxin did induce migration (unpublished observation).

Analysis of Pulmonary Cytokines. One possible explanation for the decreased eosinophil recruitment into the airway of CCR6$^{-/-}$ mice is that their immune response, in particular their Th2 type immune response, is impaired. Previous experiments using blocking antibodies and gene-disrupted mice have shown that the Th2-associated cytokines IL-4, IL-5, and IL-13 function to maintain a proasthmatic environment in animal models of asthma (7, 23, 24). IL-4 can initiate IgE isotype switching and may induce selective vascular cell adhesion molecule 1 expression, IL-5 is important for eosinophil maturation and enhances accumulation in the lung, and IL-13 functions together with IL-4 to induce mucous production (8, 9). To determine whether the levels of these important cytokines are affected by the absence of CCR6, we performed ELISAs on lung
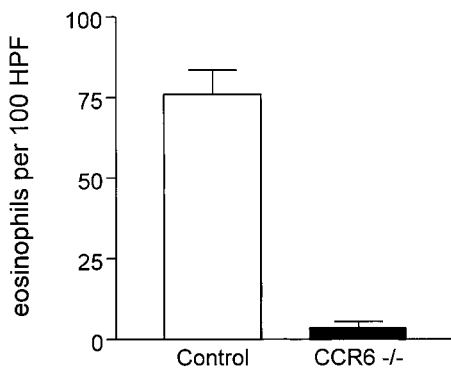

Figure 3. Peribronchial eosinophil accumulation in CCR6$^{-/-}$ mice. Morphometric enumeration of eosinophils was performed in a blinded fashion on five to six mice in each group. Lungs were harvested 24 h after challenge, processed, and sectioned. Bronchi-associated eosinophils were counted for 100 high-power fields (HPF). $P < 0.05$.
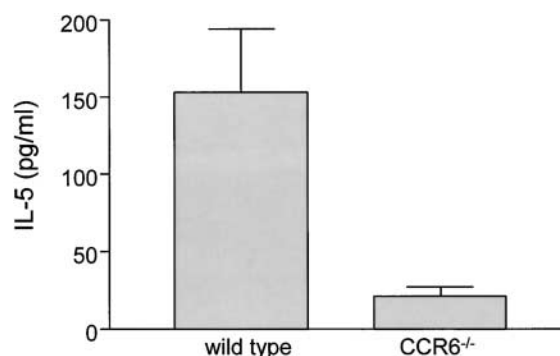

Figure 4. Pulmonary IL-5 levels in CCR6$^{-/-}$ mice. Whole lungs were harvested 24 h after challenge and examined for IL-5 protein by ELISA. Values shown represent the mean ± SEM of three separate experiments with six mice in each group. $P < 0.05$.

homogenates of challenged mice. A five- to eightfold decrease in IL-5 levels was seen in CCR6$^{-/-}$ compared with wild-type mice (Fig. 4), suggesting that the reduced number of eosinophils in lungs of the CCR6$^{-/-}$ mice might be due to decreased IL-5 production. Interestingly, levels of IL-4 and IL-13 were not significantly reduced in CCR6$^{-/-}$ mice compared with wild-type mice (data not shown). Thus, IL-5 but not other Th2 cytokines are reduced in the chronic phase of CA-challenged CCR6$^{-/-}$ mice.

IgE Production. Another hallmark of the asthmatic response is an increase in circulating levels of IgE. To determine whether the reduced IL-5 and airway hyperreactivity seen in the CCR6$^{-/-}$ mice was also associated with diminished IgE production, serum levels of this antibody isotype were assessed. This analysis revealed that CCR6$^{-/-}$ mice had considerably lower levels of IgE than wild-type mice (Fig. 5). This finding is consistent with previous results demonstrating an association between levels of IL-5 and IgE (25).

Immunization. To address the possibility that the reduced response in the lungs of CCR6$^{-/-}$ mice was related to a reduced capacity to be immunized, we prepared lymphocyte cultures from the lymph nodes of CA-immunized mice and measured cytokine production after CA stimula-
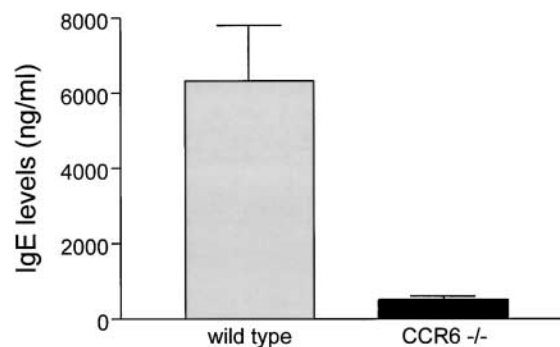

Figure 5. Serum IgE. Total IgE levels were measured in serum prepared from normal and allergic wild-type and CCR6$^{-/-}$ mice. IgE in unsensitized animals was below the limit of detection. Values shown represent the means ± SEM of six mice per group. $P < 0.05$.

tion in vitro. Compared with lymphocytes prepared from nonimmunized mice, large increases in IL-4 production were seen in both challenged wild-type (590 ± 11-fold) and challenged CCR6$^{-/-}$ mice (481 ± 7-fold). Similarly, IL-5 was increased in wild-type (6.2 ± 0.4-fold) and CCR6$^{-/-}$ mice (6.1 ± 0.2-fold). Thus, the extent of immunization was comparable in the two groups of challenged mice.

Taken together, the data from these studies support a role for CCR6 in allergic airway responses. In particular, CCR6 is required for normal allergen-induced eosinophil accumulation and for production of IL-5 and IgE. This blunted response in the lung mucosa is consistent with the diminished humoral response previously seen in the intestinal mucosa of CCR6$^{-/-}$ mice and suggests that CCR6 might have a general function in mucosal immune responses. The expression of CCR6 in DCs, memory T cells, and B lymphocytes suggests that the diminished allergic response in CCR6$^{-/-}$ mice might result from impaired function of one or more of these cell types. Thus, although the mechanism of CCR6 action in this model remains unclear, the data presented here identify CCR6 as a potential therapeutic target for individuals suffering from pathophysiologic responses to airway allergens.

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