

Full-length article

CCR3 monoclonal antibody inhibits airway eosinophilic inflammation and mucus overproduction in a mouse model of asthma¹Hua-hao SHEN², Feng XU², Gen-sheng ZHANG, Shao-bin WANG, Wei-hua XU*Department of Respiratory Diseases, the Second Affiliated Hospital, Zhejiang University College of Medicine, Hangzhou 310009, China***Key words**

asthma; CCR3 monoclonal antibody; eosinophilic inflammation; mucus overproduction; MUC5AC; epidermal growth factor receptor

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Abstract

Aim: To explore the effect of a rat anti-mouse CC-chemokine receptor-3 (CCR3) monoclonal antibody (CCR3 mAb) on airway eosinophilia and mucus overproduction in asthmatic mice. **Methods:** An asthma model was sensitized and challenged by ovalbumin (OVA) in male C57BL/6 mice. Asthmatic mice were given dual administration (intraperitoneal injection and aerosol inhalation) of CCR3 mAb or nonspecific rat IgG (ns-IgG). The number of total and differential inflammatory cells in the bronchial alveolar lavage fluid (BALF) was counted. Eosinophils number, the goblet cell percentage (GCP) and airway mucus index (AMI) were measured in the lung tissues. Interleukin (IL)-5 levels in the BALF were examined. The expression of MUC5AC and the epidermal growth factor receptor (EGFR) mRNA in the lung tissues was detected by semi-quantitative RT-PCR. The results were compared among the groups. **Results:** CCR3 mAb significantly suppressed the increased eosinophils in the BALF and lung tissues in OVA-challenged mice compared with ns-IgG-treated mice. IL-5 levels in the BALF in CCR3 mAb and ns-IgG administration mice exhibited no obvious changes relative to OVA-challenged asthmatic mice. CCR3 mAb reduced the increased GCP and AMI after OVA challenge and decreased the enhanced expression of MUC5AC and EGFR mRNA in lung tissues in asthmatic animals. **Conclusion:** CCR3 mAb can significantly inhibit airway eosinophilia and mucus overproduction in asthmatic mice. Blockage of CCR3 may represent a new strategy to asthma therapy.

Introduction

Allergic asthma is a chronic disease characterized by airway hyperresponsiveness (AHR), airway inflammation, and reversible airway obstruction. Since the mid-1980s, there has been a wealth of information documenting a potential pathogenic and destructive role for eosinophils in asthma^[1]. A line of mice devoid of eosinophils was recently created to confirm hypotheses that a causative link exists between eosinophils and asthma-related pathogenesis^[2]. Eotaxin is a potent eosinophil chemoattractant causing selective infiltration of these cells into the lungs. It binds to CC-chemokine receptor-3 (CCR3) that appears to be restricted to the eosinophil in mice^[3]. Therefore, the eotaxin/CCR3 pathway is a potentially important target for asthma therapy.

We explored the effect of a CCR3 monoclonal antibody

(CCR3 mAb) on airway eosinophilia and goblet cell hyperplasia (GCH)/mucus overproduction, the 2 most important hallmarks in asthma. The MUC5AC gene expression is thought to be a marker of GCH^[4], and the epidermal growth factor receptor (EGFR) pathway is involved in mucin production in the airways^[5]. In this study, we also examined the effect of CCR3 mAb on MUC5AC and EGFR gene expression in the lungs of asthmatic mice.

Materials and methods

Reagents Rat anti-mouse CCR3 mAb^[6] was received as a gift from Dr James J LEE (Mayo Clinic, Phonex, Arizona, USA). Nonspecific rat IgG (ns-IgG) was purchased from Chemicon Co (Temecula, CA, USA); chicken ovalbumin (OVA, grade V) was from Sigma Chemicals (St Louis, MO, USA);

inject alum was from Pierce Co (Rockford, IL, USA); IL-5 ELISA kit was from R&D Co (Minneapolis, MN, USA); Trizol for extracting RNA was from Invitrogen Co (Carlsbad, CA, USA).

OVA sensitization and challenge Male C57BL/6 mice, weighing 18–23 g, were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). The mice were sensitized and challenged with chicken OVA as previously described^[7]. All the mice were sensitized by intraperitoneal injections (100 μ L) of 80 μ g OVA emulsified in 2 mg of Inject Alum on d 0 and d 14. The mice were exposed for 30 min to 1% OVA aerosol generated by an ultrasonic nebulizer on d 24–26. Blank control mice were sensitized and challenged with normal saline. In the preliminary experiment, we found that 6 μ g/kg-mice of CCR3 mAb intraperitoneal administration resulted in over 90% reduction of eosinophils in the peripheral blood and bronchial alveolar lavage fluid (BALF). In order to strengthen the inhibition effect of CCR3 mAb on eosinophils, the mice were injected intraperitoneally with 150 μ g of CCR3 mAb, then 50 μ g of CCR3 mAb was mixed with the OVA solution and administered with each of the 3 OVA challenges in our formal experiment. Negative control mice were administered the same amounts of rat ns-IgG by both routes.

Cell count in the BALF Mice were sacrificed for sample collection on d 28. The tracheas were cannulated, the right lungs were ligated with a suture thread, and the left lobes of lungs were lavaged 3 times with 0.3 mL of ice-cold phosphate buffered saline (PBS). BALF 10 μ L was used for the total cell counts and the residual BALF was centrifuged. The cell pellet was used to prepare cell slides by cytopspin and stained with methylene blue/eosin. Four hundred cells were counted for estimating the differential counts of various cell types afterwards. The supernatant was preserved at -70 °C for cytokine detection. The right lungs without lavage were stored at -70 °C for total RNA isolation.

Lung histology The left lobes of the lungs were fixed in 10% buffered formalin. After embedded in paraffin, the tissues were cut into 4 μ m thick sections. The lung sections were stained with hematoxylin/eosin (HE) and Alcian blue/periodic acid-Schiff (AB/PAS), respectively. Infiltrating lung eosinophils in parasagittal sections with HE staining were counted under microscopy. The goblet cell percentage (GCP) and airway mucus index (AMI) were measured as follows: GCP=the amount of goblet cells/total number of airway epithelial cells; AMI=the area of airway epithelium with AB/PAS staining/total area of the conducting airway epithelium. The analysis of the mucus content of the airway epithelium of mice from different groups was performed using the imag-

ing program of Leica Co (Wetzlar, Hessen, Germany).

Cytokine assay Interleukin (IL)-5 levels in the BALF were measured by ELISA method according to the manufacturer's protocol for users. The limit of IL-5 assay was 10 pg/mL.

Semi-quantitative RT-PCR The total RNA was extracted from about 0.2 mg of the lung tissues with Trizol reagents. For cDNA synthesis, 20 μ L RT mixture containing total RNA 2 μ g, dNTP 1 mmol/L, Olig(dt)₁₈ prime 0.2 μ g, RNasin 20 U, M-MLV reverse transcriptase 200 U was incubated at 42 °C for 60 min, then the reverse transcriptase was inactivated by heating the reaction mixture at 72 °C for 15 min. The expression of MUC5AC and EGFR mRNA in the lungs was measured by RT-PCR. Oligonucleotide primers specific for mouse MUC5AC, EGFR and glyceraldehyde phosphate dehydrogenase (GAPDH, an internal control) were synthesized according to published sequences: MUC5AC: sense: 5' CAG CCGAGAGGAGGGTTT GATCT 3', anti-sense: 5' AGTCTC TCT CCG CTC CTC TCAAT 3'^[4]; EGFR: sense: 5' GTG TGA AGA AGT GCC CCC GAA AC 3', anti-sense: 5' AAC GAC CGC CAA AGAAAA CTG ACC 3'^[8]; GAPDH: sense: 5' CTG GTG CTG AGT ATG TCG TG 3', anti-sense: 5' CAG TCTTCT GAG TGG CAG TG 3'^[4], with the product sizes 389 bp, 452 bp and 296 bp, respectively. PCR was performed as follows: 1 μ L of cDNA mixture was subjected to amplification in 50 μ L of final volume with MgCl₂ 1.5 mmol/L, dNTPs 0.2 mmol/L, 20 pmol of each primer, and 2 U of *Taq* DNA polymerase in the reaction buffer on a PE2400 cyclor (Perkin-Elmer, Foster City, CA, USA). The condition of PCR reactions was used below: 94 °C for 5 min; then 94 °C for 1 min; 56 °C for MUC5AC, 58 °C for EGFR and 60 °C for GAPDH for 1min; 72 °C for 45 s for 30 cycles (GAPDH) or 35 cycles (MUC5AC and EGFR) and 72 °C for 10 min to end the reaction. PCR products were electrophoresed by 1.5% agarose gel containing ethidium bromide. The density of each band was measured by Gel-pro analyzer 4.0 (Exon-Intron, Inc, Loganville, PA, USA). The results were normalized as ratios compared with GAPDH.

Statistical analysis All data were presented as mean \pm SEM. One-way ANOVA was used for statistical analysis of the differences between the groups. A value of $P < 0.05$ was considered statistically significant.

Results

Effect of CCR3 mAb on airway eosinophilic inflammation Numerous eosinophils appeared around the bronchioles and blood vessels in asthmatic mice whereas smooth airway walls and regular epithelium without inflammatory cell infiltration were seen in the saline control group. CCR3

mAb treatment obviously attenuated eosinophil infiltration, however, no significant difference was observed between the ns-IgG-treated mice and OVA-challenged mice (Figure 1). Eosinophils significantly increased and became the predominant inflammatory cells in the BALF and lung tissues of the OVA-challenged mice. CCR3 mAb treatment significantly reduced the number of total cells and eosinophils in the BALF and eosinophils number in the lung parenchyma (Table 1), but the administration of CCR3 mAb had no obvious effect on other cell types including lymphocytes, neutrophils and macrophages (data not shown).

Effect of CCR3 mAb on GCH and mucus overproduction

The pathological results from the lung tissues showed that CCR3 mAb treatment significantly alleviated epithelial hypertrophy and mucus production in the airways of OVA-challenged mice compared with the ns-IgG treatment mice (Figure 2). However, GCP and AMI of the airway epithelium of CCR3 mAb-treated mice were still higher than those of the saline-challenged control mice (Table 2).

Effect of CCR3 mAb on IL-5 levels in the BALF The increased IL-5 levels were detected in the BALF of OVA-challenged mice vs the saline-treated mice. However, the IL-5 concentration in the BALF in CCR3 mAb and the ns-IgG-treated mice exhibited no obvious changes compared with

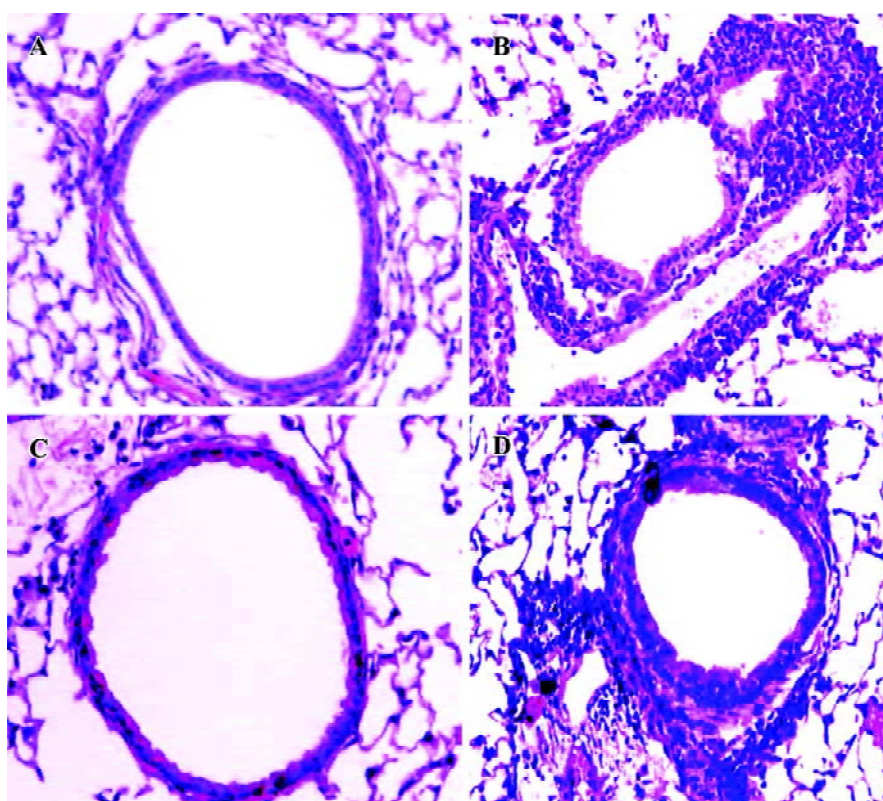


Figure 1. Effect of CCR3 mAb on eosinophil infiltration in the lung parenchyma (HE×200). A: saline-treated mice; B: OVA-treated mice; C: OVA/CCR3 mAb-treated mice; D: OVA/ns-IgG-treated mice.

Table 1. Inhibitory effect of CCR3 mAb on eosinophils in BALF and lung parenchyma in repeated OVA-challenged mice ($n=5-8$ animals/group). ^b $P<0.05$ vs saline-treated mice; ^c $P<0.05$ vs OVA-treated mice.

Group	$10^4 \times$ Total cells in BALF (mL ⁻¹)	$10^4 \times$ Eosinophils in BALF (mL ⁻¹)	Eosinophils in lung parenchyma (mm ⁻²)
Saline	11.67±1.80	0.09±0.02	5.4± 0.7
OVA	89.00±5.86 ^b	37.98±1.63 ^b	853.8±37.2 ^b
OVA/CCR3 mAb	43.80±3.44 ^{bc}	0.97±0.10 ^c	81.5±8.0 ^c
OVA/ns-IgG	79.83±5.22 ^b	33.40±3.63 ^b	780.2±51.7 ^b

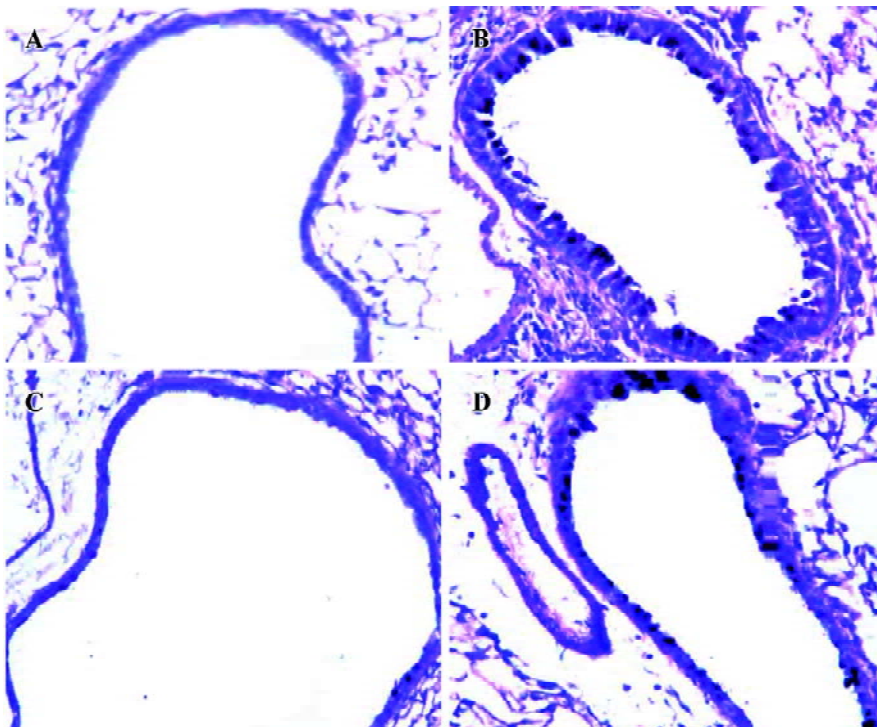


Figure 2. Effect of CCR3 mAb on goblet cell hyperplasia and mucus overproduction (AB/PAS×200). A: saline-treated mice; B: OVA-treated mice; C: OVA/CCR3 mAb-treated mice; D: OVA/ns-IgG-treated mice.

Table 2. Effect of CCR3 mAb on goblet cell percentage (GCP) and airway mucus index (AMI) of the airway epithelium in repeated OVA-challenged mice (n=5–8 animals/group). ^bP<0.05 vs saline-treated mice; ^cP<0.05 vs OVA-treated mice.

Group	GCP (%)	AMI
Saline	1.35±0.23	0.44±0.06
OVA	43.61±2.84 ^b	27.28±2.06 ^b
CCR3 mAb/OVA	17.24±1.94 ^{bc}	7.20±0.76 ^{bc}
ns-IgG/OVA	38.67±3.80 ^b	23.03±2.15 ^b

the OVA-treated animals (Table 3).

MUC5AC and EGFR mRNA expression in the lungs of mice The upregulated MUC5AC and EGFR mRNA expression was found in the OVA-challenged mice. CCR3 mAb

Table 3. Effect of CCR3 mAb on IL-5 level in BALF (n=5–8 animals/group). ^bP<0.05 vs saline-treated mice.

Group	IL-5 (pg/mL)
Saline	17.0±2.3
OVA	33.2±2.8 ^b
OVA/CCR3 mAb	33.3±3.6 ^b
OVA/ns-IgG	35.6±3.7 ^b

treatment decreased the expression of MUC5AC and EGFR mRNA. No obvious difference of MUC5AC and EGFR mRNA expression was observed in the ns-IgG-treated mice compared with the OVA-challenged mice (Figures 3, 4).

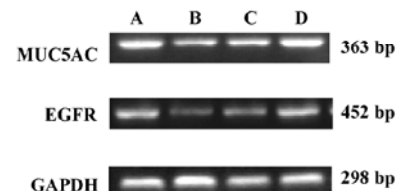


Figure 3. Representative RT-PCR analysis of MUC5AC and EGFR mRNA expression in lung tissues. A: OVA-treated mice; B: saline-treated mice; C: OVA/CCR3 mAb-treated mice; D: OVA/ns-IgG-treated mice.

Discussion

Although the key role of eosinophil in allergic pulmonary diseases is challenged by the clinical trials of the anti-IL-5 monoclonal antibody^[9], more and more data from both asthma patients and mouse models have demonstrated that airway eosinophilia infiltration is one of the most important characteristics of asthma. Human eosinophils mediate pathogenic effects from several independent mechanisms that al-

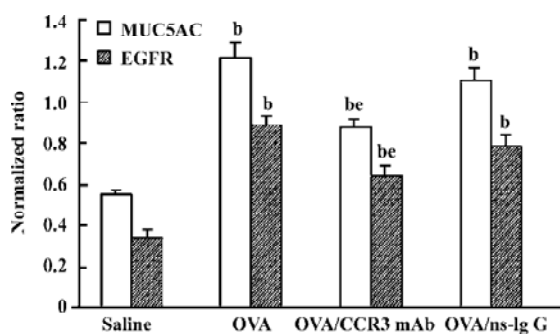


Figure 4. Effect of CCR3 mAb on MUC5AC and EGFR mRNA expression in the lungs of mice ($n=4-6$ animals/group). ^b $P<0.05$ vs saline-treated mice; ^e $P<0.05$ vs OVA-treated mice.

low the eosinophil to modulate the intensity of pulmonary inflammation, as well as elicit cell death and loss of structural integrity of the lung, leading to pulmonary dysfunction^[10]. Recently, a line of mice devoid of eosinophils was created to confirm hypotheses that a causative link exists between eosinophils and asthma-related pathogenesis such as airway epithelial pathology, mucus hypersecretion and AHR in Lee Laboratory (Mayo Clinic, Phonex, Arizona, USA)^[2,7]. Therefore, blocking the onset and progression of asthma by specifically depleting eosinophils is effective in asthma therapy.

The chemokine receptor CCR3 seems to play a major role in allergic diseases which is supported by the detection of this receptor on human eosinophils, Th2 cells, mast cells and basophils^[11-13]. However, Grimaldi *et al* revealed that mouse CCR3 was expressed on eosinophils, but not on stem cells, dendritic cells or cells from the thymus, lymph node, or spleen of normal mice by flow cytometry. Unlike human Th2 cells, mouse Th2 cells did not express detectable levels of CCR3 nor did they give a measurable response to eotaxin^[3]. The reactivity of CCR3 mAb in our experiment was proven to be restricted to eosinophils^[3,6].

Our study showed that dual administration (intraperitoneal injection and aerosol inhalation) of CCR3 mAb can effectively ablate eosinophils in the BALF and significantly reduce lung eosinophilia relative to OVA-challenged mice. In addition, CCR3 mAb was found to have no observable effect on the number of other cell types (lymphocytes, macrophages and neutrophils) in the BALF and lung tissues, indicating the specificity of mouse CCR3 mAb treatment for only eosinophils. Using the CCR3-deficient mouse model, Humbles *et al* found that CCR3 depletion significantly abrogated eosinophil recruitment to the lung after allergen challenge, with the majority of the eosinophils being arrested in the subendothelial space^[14]. Similarly, a CCR3 monoclonal

antibody and small molecule CCR3 antagonists were demonstrated to be capable of reducing pulmonary eosinophilic inflammation in mouse allergenic models of airway inflammation^[15]. Taken together, CCR3 antagonism has the potential to influence eosinophil infiltration into the lungs of asthmatics and is therefore a promising therapeutic target.

Shi and colleagues pointed out that eosinophils were able to express major histocompatibility complex II complex and costimulatory molecules and function as antigen-presenting cells (APC)^[16]. Eosinophils have the potential to activate lung Th2 cells to release disease-modulating cytokines^[17], but the investigation from van Rijt *et al* did not support any role for airway eosinophils as APC to naive T cells, despite their migration to the draining lymph nodes at times of allergen exposure^[18]. In our study, Th2 cytokine (IL-5) levels in the BALF from CCR3 mAb-treated animals were unaffected compared with the OVA-treated mice. Justice *et al* also proved that the anti-CCR3-mediated loss of eosinophils from the lung did not have a demonstrable effect on the activities of T and B lymphocytes^[6]. So we think that eosinophils function as APC in the modulation of T cell activities which may be relatively limited and can be compensated by other APC (dendritic cells, macrophages and B lymphocytes) in the absence of eosinophils because dendritic cells are clearly superior in activating T cells in the draining lymph nodes of the lung^[18].

Globlet cell metaplasia (GCM), GCH and mucus hypersecretion are prominent features of allergic asthma^[19]. Numerous inflammatory mediators produced in asthmatic airways, together with neural mechanisms, can theoretically increase mucin secretion, induce plasma exudation, upregulate MUC gene expression (MUC5AC gene expression is thought to be a marker of GCH^[5]), increase mucin synthesis and cause GCH^[20,21]. Most of the above pathways operate through the EGFR and its tyrosine kinase intracellular signaling cascade^[22]. EGFR and MUC5AC expression at both gene and protein levels is upregulated in goblet cells in bronchial biopsies from asthmatic patients compared with non-asthmatic persons^[23]. Burgel *et al* demonstrated that activated human eosinophils induced mucin synthesis in human airway epithelial cells via EGFR activation^[24]. We analyzed MUC5AC and EGFR gene expression in the lungs of asthmatic mice. Over-expression of MUC5AC and EGFR mRNA in the lungs was found in the OVA-treated mice compared with the saline-treated animals, and CCR3 mAb treatment significantly reduced MUC5AC and EGFR mRNA levels in the lungs of OVA-challenged mice by the ablation of eosinophils relative to ns-IgG-treated mice. Therefore, we deduce that CCR3 mAb may downregulate MUC5AC gene expression, at least

partly, through the EGFR pathway.

Our results show that CCR3 mAb-mediated ablation of eosinophils in OVA-treated mice can significantly reduce GCM/GCH and mucus overproduction compared with ns-IgG-treated mice, indicating that the products of eosinophils are capable of stimulating an increase in goblet-cell number and mucus secretion. However, the GCP and AMI of CCR3 mAb-treated mice were still higher than those of the normal saline-treated mice, suggesting the existence of both eosinophil-independent and eosinophil-dependent pathways leading to allergen-mediated goblet cell metaplasia/mucus production^[6].

In summary, CCR3 mAb can effectively ablate lung eosinophilia and significantly inhibit mucus overproduction in a mouse model of asthma. Interfering with the CCR3 receptor may represent a potential new therapy for the treatment of asthma.

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