

# Effects of chemokine receptor 3 gene silencing by RNA interference on eosinophils

YUEHUI LIU, XINHUA ZHU and HAO ZHANG

Department of Otorhinolaryngology-Head and Neck Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China

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**Abstract.** The present study aimed to use RNA interference (RNAi) to silence chemokine receptor 3 (CCR3) and observe the effects on eosinophils (EOS) in mice with allergic rhinitis (AR). CCR3 small interfering RNA (siRNA) lentiviral vectors were transduced into purified EOS cells cultured *in vitro*. Reverse transcription-polymerase chain reaction (RT-PCR) and western blot analyses were also used to detect the efficiency of silencing, and flow cytometry was used to detect the EOS apoptosis rates. Experimental mice were grouped for nasal administration, and the lentivirus was then dispensed to AR mice. RT-PCR and western blots were performed to detect the expression levels of CCR3 mRNA and protein in EOS extracted from bone marrow, peripheral blood and nasal mucosa. Furthermore, flow cytometry was performed to detect changes to CD34-positive (CD34<sup>+</sup>) cells in each group. The CCR3 siRNA lentiviral vector exhibited high efficiency in silencing CCR3 mRNA and protein expression, inhibited growth and promoted apoptosis of EOS. In addition, the expression of CCR3 mRNA and protein in the bone marrow, peripheral blood and nasal mucosa of mice in the CCR3 siRNA treatment group were lower than those in the control group ( $P < 0.05$ ), whereas the number of CD34<sup>+</sup> cells in the CCR3 siRNA treatment group was not significantly different compared with that in the control group ( $P > 0.05$ ). CCR3 RNAi could effectively silence the expression of CCR3 mRNA and protein both *in vitro* and *in vivo*, thus promoting apoptosis of EOS and inhibiting its growth, migration and invasion.

## Introduction

Allergic rhinitis (AR) is a chronic inflammatory disease of the nasal mucosa and is characterized by sneezing, runny nose, nasal congestion and nasal itching when patients with allergic diseases come into contact with specific allergens (1). AR is a common disease worldwide that affects the quality of peoples' daily lives (2). Studies have estimated that patients with AR form ~20% of the global population, and with the continuing destruction of peoples' living environments, the number of patients with AR will continue to increase (3-5).

AR is a multifactorial disease, which may involve local and systemic processes (6). To date there is no cure for AR, therefore, further research is required. Recent studies have demonstrated that eosinophils (EOS) are the primary effector cells in AR, and the localization and activation of a large number of EOS is an important feature of allergic diseases (7-10). Due to the signaling link between the nasal cavity and bone marrow, a large number of EOS are stimulated by allergens that infiltrate local tissues (11). Bone marrow releases EOS hematopoietic progenitor cells, namely CD34-positive (CD34<sup>+</sup>) cells, which are targeted to various tissues and organs that then differentiate and develop into mature EOS under the control of local growth factors (12). Mature EOS produce, store and rapidly secrete diverse mediators, including cationic proteins, cytokines, chemokines and growth factors, that are important in inflammation and immune regulatory responses (13). Moreover, chemokines are being increasingly studied due to their association with AR bone marrow hematopoiesis and hematopoietic cell specification *in situ* (14). The majority of chemokines interact with EOS through binding to the eotaxin receptor, chemokine receptor 3 (CCR3) (15). CCR3 is a transmembrane G protein-coupled receptor that is highly expressed in EOS (16). Furthermore, eotaxin belongs to the CC chemokine family, which activates G-protein-dependent intracellular signaling cascades that stimulate the migration of EOS (17-19).

Previous studies have indicated that administration of low molecular weight CCR3 antagonists in mouse models of allergic airway inflammation could prevent airway hyperresponsiveness and remodeling (20). Compared with CCR3 antagonists exhibited significantly reduced EOS infiltration into local tissues (21,22) and higher levels of EOS survival factors [such as interleukin (IL)-5, granulocyte macrophage

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*Correspondence to:* Professor Yuehui Liu, Department of Otorhinolaryngology-Head and Neck Surgery, The Second Affiliated Hospital of Nanchang University, 1 Minde Road, Nanchang, Jiangxi 330006, P.R. China  
E-mail: yuehuliuvn@163.com

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colony-stimulating factor (GM-CSF) and IL-3] in local inflamed tissues, which prolonged EOS survival (23). However, other studies have suggested that EOS cultured *in vitro* in the absence of survival factors can partially survive for 72 h and be recruited to inflamed tissues, leading to persistent inflammation (24). This process was associated with the survival of EOS by eotaxin through the CCR3 receptor, which indicated that CCR3 was closely associated with the apoptosis of EOS (25,26).

RNA interference (RNAi) can specifically degrade target RNAs to inhibit and downregulate the expression of specific genes (27). A previous study (28) revealed that silencing CCR3 by lentiviral vector-mediated RNAi inhibited the degranulation of EOS, thereby inhibiting the release of granule proteins and potentially reducing inflammation in AR. Therefore, it can be argued that silencing CCR3 by RNAi affects EOS in AR. However, the mechanisms and processes that underlie this effect have not been fully elucidated yet (29). In the present study, lentiviral vectors that express short hairpin RNAs (shRNAs) to silence the CCR3 gene were transduced into EOS cultured *in vitro* in order to observe the effects of CCR3 silencing (mRNA and protein) on EOS apoptosis. In addition, using an established AR mouse model, RNAi oligos synthesized *in vitro* were used to specifically inhibit the expression of CCR3 in EOS and block signaling, via the eotaxin/CCR3 pathway, in order to observe changes in EOS of the bone marrow, peripheral blood and nasal mucosa. The objective of the present study was to understand the roles and effects of the CCR3 gene in EOS, and thus develop a further understanding of the pathogenesis of AR.

## Materials and methods

**Animals.** Male BALB/c mice that were 6–8 weeks old and 20–25 g (specific pathogen-free grade) were obtained from the Experimental Animal Center of Nanchang University (Nanchang, China). Mice were bred in a clean environment at 22–24°C under a 12-h light/dark cycle and fed with an ovalbumin (OVA)-free diet. The present study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee of Nanchang University.

**Animal grouping and allergization.** In total, 24 BALB/c mice were randomly divided into four groups (n=6 per group): Group I, no-treatment control (control); group II, PBS treatment control (PBS control); group III, scramble small interfering RNA (siRNA) treatment (off-target); and group IV, CCR3 siRNA treatment (pLVX-shRNA-mCCR3).

The mice in groups II to IV were intranasally administered with 8  $\mu$ l PBS, control siRNA or CCR3 siRNA, respectively, twice a day on days 0 and 14. Additionally, these mice were intraperitoneally injected with an OVA/aluminum hydroxide [Al(OH)<sub>3</sub>] mixture [containing 10  $\mu$ g OVA and 4 mg Al(OH)<sub>3</sub>] for allergization twice a day on days 2 and 16 and continuously intranasally administered with 600  $\mu$ g/ml OVA twice a day from day 21 to 27 for excitation. At 5 h before excitation, the mice were administered intranasal treatments as described

above. The control group was administered the same dose of saline. Samples of the bone marrow, peripheral blood and nasal mucosa were obtained 24 h after administration of the final treatment.

**Culture and purification of bone marrow-derived EOS.** BALB/c mice were sacrificed and their femurs were isolated. Femoral marrow was rinsed with RPMI 1640 medium (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), supplemented with streptomycin and penicillin (HyClone), 20% fetal bovine serum (HyClone), Fms-related tyrosine kinase 3 ligand (FLT3-L; PeproTech, Inc., Rocky Hill, NJ, USA), stem cell factor (SCF; PeproTech, Inc.) and recombinant mouse IL-5 (rmIL-5; PeproTech, Inc.). A total of 10<sup>7</sup> cells/ml were incubated at 37°C in 5% CO<sub>2</sub>.

**Identification of EOS.** Untreated cells (1x10<sup>6</sup>) were collected from culture wells and rinsed with PBS, and the supernatant was discarded. Anti-IL-5 receptor (alpha) PE antibody (eBioscience, Inc., San Diego, CA, USA) and anti-CD34 fluorescein isothiocyanate (FITC) antibodies (eBioscience, Inc.) were added to untreated cells, single cell suspensions were prepared and double-immunopositive cells were sorted by flow cytometry.

**Lentivirus transduction.** Lentivirus transduction was performed on the tenth day of EOS culture. One day before transduction, EOS were seeded at a density of 5x10<sup>5</sup> cells/well into a 12-well culture plate, which were divided into three groups when performing transductions. Group I was treated with shRNA-mCCR3 virus mixed with medium to 100  $\mu$ l (with MOI=10); group II was treated with the same amount of control virus; and group III was treated with 0.1% PBS-medium mixture. Cells were cultured for 48 h after transduction; subsequently, the medium was aspirated, cells were washed with 0.1% PBS and then treated according to the following methods.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis of CCR3 expression in lentivirus-transduced EOS.** EOS were resuspended in TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China), and RNA was extracted according to the manufacturer's instructions. RNA was converted to cDNA using a reverse transcription kit (Takara Biotechnology Co., Ltd.), and primers were designed for CCR3 (IDNM\_009914.4) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ID: NM\_017008.4, as the control, synthesized by Oligo). Reverse transcription-polymerase chain reaction RT-PCR reactions used to amplify cDNA were set up in accordance with the appropriate annealing temperature and cycles. Furthermore, the expression of mRNA for each group was detected by electrophoresis. Samples of the bone marrow, peripheral blood and nasal mucosa were tested according to the procedures described above.

**Western blot analysis of CCR3 expression in lentivirus-transduced EOS.** EOS were homogenized in radioimmunoprecipitation assay lysis buffer (Pierce Protein Biology; Thermo Fisher Scientific, Inc., Rockford, IL, USA). The homogenate was cooled on ice for 20 min and centrifuged at 4°C and 14,000 rpm to

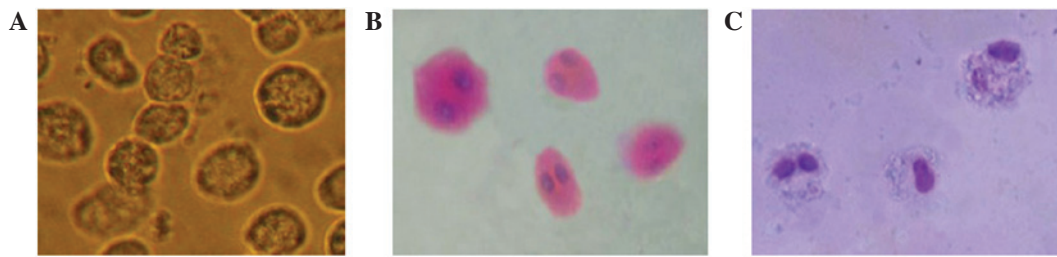


Figure 1. Identification of EOS. (A) Light microscopic image (magnification, x80): EOS had a larger volume, round or oval shape and were filled with granular materials within the cytoplasm. (B) Hematoxylin and eosin staining light microscopic image (magnification, x80) showing red eosinophilic particles filling the cytoplasm of the EOS. Nuclei with two lobes are exhibited in purple. (C) Wright staining light microscopic image (magnification, x80). Light purple particles filled the cytoplasm of EOS, and nuclei with two lobes were exhibited in blue-purple. EOS, eosinophils.

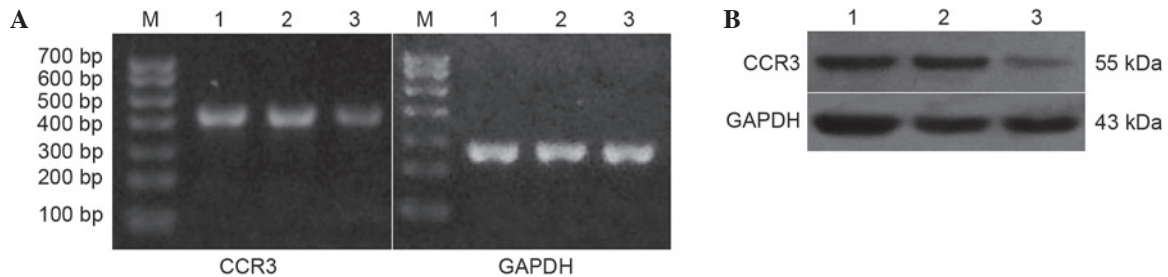


Figure 2. Identification of eosinophils by (A) reverse transcription-polymerase chain reaction and (B) western blot analyses. Lane 1, cell; lane 2, NCsh and lane 3, CCR3sh group. CCR3, chemokine receptor 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

remove the insoluble material. The supernatant was mixed with 4% SDS sample buffer, boiled for 5 min, and resolved using 10% SDS-PAGE (Ready Gel J; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Electrophoresed proteins were transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA), blocked with 5% skimmed milk diluted in 1X Tris-buffered saline with Tween 20 (TBST) buffer for 2 h, and incubated with the primary antibody (Abcam, Cambridge, UK) overnight at 4°C. The membranes were rinsed with TBST three times, incubated with the secondary antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd.; OriGene Technologies, Inc., Beijing, China) for 2 h, and washed again with TBST three times. An anti-GAPDH antibody was used as an internal reference protein to normalize protein loading (Anbo Biotechnology Co., Ltd., Nanjing, China) and concentrations of each target protein were normalized against GAPDH. The membranes were evenly mixed with chemiluminescence reagent, incubated in the dark at room temperature for 3-5 min, then exposed and developed using X-ray films. The intensity levels of bands from the electrophoretic gels and photographic films were measured using a dedicated image analysis software (Band Leader 3.0; <http://en.bio-soft.net/chip/BandLeader.html>). Samples of the bone marrow, peripheral blood and nasal mucosa were tested according to the procedures described above.

**Apoptosis detection.** Cells were treated with lentivirus for 48 h and control cells were incubated with antibodies provided in an apoptosis kit (PeproTech, Inc.), and single cell suspensions were prepared with PBS following the manufacturer's instructions. Apoptosis was detected with flow cytometry.

**Detection of CD34<sup>+</sup> cells.** Samples of the bone marrow and peripheral blood were collected, and cells were isolated with

lymphocyte isolation liquid (GE Healthcare Life Sciences) to prepare the single cell suspension. The cells were labeled with anti-CD34 FITC primary antibody and detected with flow cytometry following resuspension.

**Statistical analysis.** SPSS software, version 18 (SPSS, Inc., Chicago, IL, USA) was used for analysis of variance in order to determine intergroup differences, which were expressed as the mean  $\pm$  standard deviation. Each experiment was repeated three times. The error bars indicate the standard error of the samples.  $P < 0.05$  was used to indicate a statistically significant difference.

## Results

**Hematoxylin and eosin and Wright staining.** Under the induction of the growth factors, FLT3-L and SCF, the majority of the primary bone marrow cells differentiated into mature EOS. Due to the effect of rmIL-5, EOS survived and apoptosis was delayed. Moreover, an optical microscope was used to observe cell morphology after incubation with the indicated factors. HE and Wright staining of EOS are shown in Fig. 1.

**RT-PCR and western blot analyses of lentivirus-transduced EOS.** As shown in Fig. 2, the mRNA expression of CCR3 in the control that did not receive any treatment and the control siRNA treatment group was not significantly different (Fig. 2A). However, the mRNA expression of CCR3 in the CCR3 siRNA treatment group was significantly lower than that in the other two groups ( $P < 0.05$ ) (Table I).

As shown in Fig. 2B, the protein expression of CCR3 in the control group that did not receive any treatment and

Table I. CCR3 mRNA expression gray value.

Group	Ratio grayscale value ( $x\pm s$ )
Cell	0.720 $\pm$ 0.078
NCsh	0.750 $\pm$ 0.082
CCR3sh	0.230 $\pm$ 0.053 <sup>a</sup>

CCR3, chemokine receptor 3; NCsh, normal control short hairpin RNA. <sup>a</sup>P<0.05 vs. Cell group.

Table II. CCR3 protein expression value.

Groups	Ratio grayscale value ( $x\pm s$ )
Cell	0.950 $\pm$ 0.158
NCsh	0.930 $\pm$ 0.087
CCR3sh	0.250 $\pm$ 0.042 <sup>a</sup>

CCR3, chemokine receptor 3; NCsh, normal control short hairpin RNA. <sup>a</sup>P<0.05 vs. Cell group.

the control siRNA treatment group was not significantly different, whereas the protein expression of CCR3 in the CCR3 siRNA treatment group was significantly lower than that in the other two groups (P<0.05). Furthermore, the experiment was repeated three times.

*Identification results of EOS.* EOS express both CD34 and IL-5; therefore, it is possible to identify EOS by the specific labeling of these proteins (Fig. 3) using anti-IL-5 PE and CD34 FITC antibodies. The CD34 epitope could be detected in early-differentiated EOS, whereas its expression was not detected significantly in apoptotic EOS. Moreover, the IL-5 epitope could be detected during both stages of EOS.

Using flow cytometry, the percentage of cultured cells labeled with the anti-IL-5 PE and CD34 FITC antibodies were found to be 65.7 $\pm$ 3.25 and 17.5 $\pm$ 2.27%, respectively, which were also significantly different (P<0.05). These percentages can be used to estimate the percentage of EOS in the cultured cells.

*Results of apoptosis detection.* The apoptotic rates of EOS were measured for the following groups: 24.52 $\pm$ 4.56% for the control that did not receive any treatment, 20.7 $\pm$ 2.32% for the control siRNA and 67.5 $\pm$ 5.88% for the CCR3 siRNA groups. The apoptotic rate of the CCR3 siRNA group was significantly higher than the apoptotic rates of the other two groups (P<0.05). However, there was no significant difference between the control that did not receive any treatment and the control siRNA groups (P>0.05; Fig. 4).

*RT-PCR and western blot analyses of animal specimens.* The mRNA expression levels of CCR3 in the PBS and control siRNA groups were not significantly different, whereas the mRNA expression of CCR3 in the CCR3 siRNA group was significantly lower than that in the other two groups (P<0.05; Fig. 5A). In addition, the experiment was repeated three times.

Moreover, the protein expression of CCR3 in the PBS and the control siRNA group was not significantly different, whereas the protein expression of CCR3 in the CCR3 siRNA group was significantly lower than that in the other two groups (P<0.05) (Table II and Fig. 5B). The experiment was repeated three times.

*Histological staining of the nasal mucosa.* Edema, inflammatory cell infiltration, epithelial shedding and quantity of exudates and EOS in the nasal mucosa were worse in the PBS

and control siRNA groups compared with the control group, indicating that the histological results of these animal models met the pathological characteristics of AR. Moreover, in the CCR3 siRNA group IV, the number of EOS was decreased and mucosal edema was alleviated, indicating that silencing of CCR3 reduced the pathology of AR (Fig. 6).

*Detection of CD34<sup>+</sup> cells.* No significant difference was detected in the number of CD34<sup>+</sup> cells from the bone marrow, peripheral blood and CCR3 siRNA groups (P>0.05; Fig. 7). Moreover, the experiment was repeated three times.

## Discussion

EOS are important effector cells in the induction of inflammation. The recruitment and activation of a large number of EOS in local tissues are characteristic of allergic diseases and T Helper 2 (Th2)-type immune responses (30). EOS release cytotoxic granules that contain biomolecules, including major allergy-related basic proteins, eosinophil peroxidase, eosinophil-derived neurotoxin and eosinophil cationic proteins through degranulation or cytolysis in local tissues (31). Conditioned media of degranulated EOS can reproduce pathological features of allergic diseases, including tissue damage, vascular leakage, mucus secretion and airway contraction (32,33).

As important processes in the development of allergic diseases, promoting the apoptosis of EOS and reducing the infiltration of EOS into local tissues could potentially relieve local inflammation in allergic diseases (7). IL-3, IL-5 and GM-CSF can promote the survival of EOS (23). Furthermore, IL-5 receptors quickly activate tyrosine kinase cascades that are transduced through the Janus kinase 2/signal transducer and transcription activating protein pathway (34). However, previous results have demonstrated that the treatment of patients with allergic diseases with anti-IL-5 monoclonal antibodies could only partially reduce the number of infiltrating EOS in airways, while EOS numbers in the peripheral circulation and bone marrow remained at a high level (22).

CCR3 is a transmembrane G protein-coupled receptor, and its ligand eotaxin belongs to the CC chemokine family (16). Furthermore, the binding of eotaxin to CCR3 induces EOS to migrate to specific tissues. CCR3 receptors are primarily expressed in EOS, while Th2 cells, basophils and mast cells may also constitutively or temporarily express CCR3 (35). In addition, the downregulation of CCR3 inhibits the delay of EOS apoptosis by IL-5, thus inducing EOS apoptosis and reducing local tissue invasion by EOS (23).

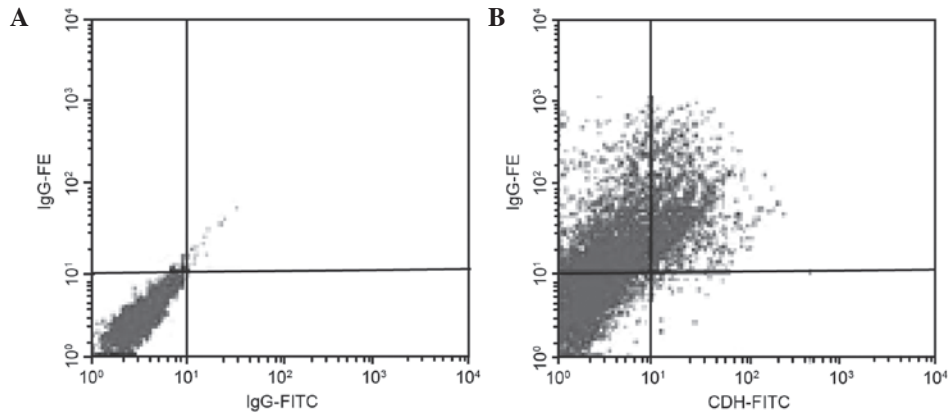


Figure 3. Identification of EOS by flow cytometry. (A) IgG blank control. (B) EOS incubated with IL-5 PE and CD34 FITC antibodies. Near X-axis, IL-5 PE positive expression; Near Y-axis, CD34 FITC-positive expression. IgG, immunoglobulin; FE, fractional excretion; FITC, fluorescein isothiocyanate; EOS, eosinophils; IL, interleukin.

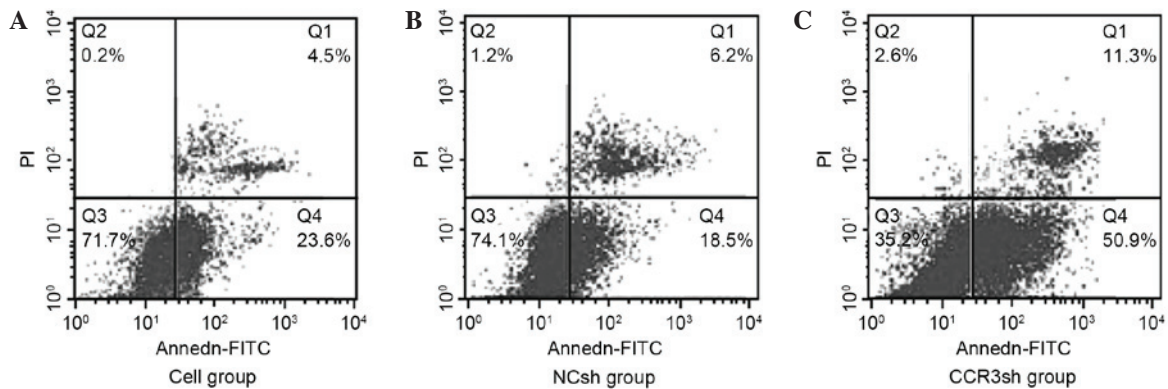


Figure 4. Apoptosis detection of eosinophils by flow cytometry for the (A) cell, (B) NCsh and (C) CCR3sh groups. The scattered points in the first and second quadrant represented the number of early and late apoptotic cells, respectively. PI, propidium iodide; FITC, fluorescein isothiocyanate.

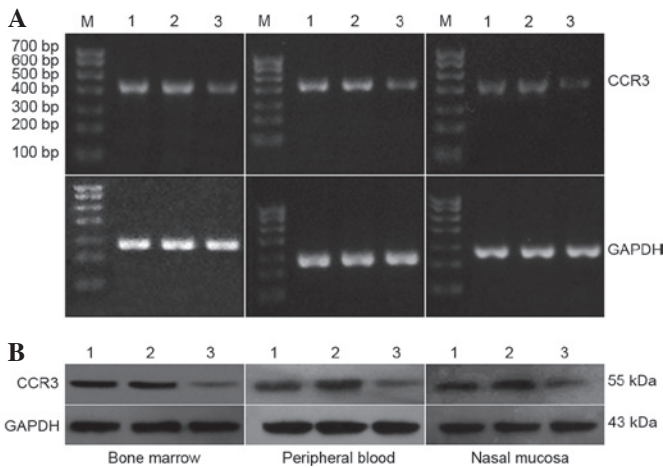


Figure 5. Results of (A) reverse transcription-polymerase chain reaction and (B) western blot analyses. Lane 1, phosphate-buffered saline group; Lane 2, siRNA group; Lane 3, CCR3 siRNA group. CCR3, chemokine receptor 3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; siRNA, small interfering RNA.

EOS is derived from CD34<sup>+</sup> hematopoietic progenitor cells under the stimulation of GM-CSF, IL-3 and IL-5 and other cytokines in the bone marrow (36). Ben *et al* (37) has performed external intervention with anti-CCR3 monoclonal

antibody in an allergic mouse model. Furthermore, anti-CCR3 monoclonal antibodies downregulated CCR3, inhibited chemokine-mediated migration of bone marrow CD34<sup>+</sup> progenitor cells and inhibited the IL-5/eotaxin pathway thus significantly reducing allergen-induced infiltration of EOS and CD34<sup>+</sup> progenitor cells. As a result, airway hyperresponsiveness was maintained at a lower level and the production of mucus was reduced (38).

RNAi robustly inhibits expression, has high *in vitro* transfection efficiency and has high target specificity (39). Moreover, RNAi can be potentially used for long-term treatments by silencing target genes in specific tissues or cells using specific tissue or cell promoters, which would potentially prevent damage in other tissues or cells and reduce complications by other factors. In addition, siRNA plasmids can be administered by liposomes and directly through the mouse mucosa, which makes it feasible to directly treat and improve AR with the transnasal mucosal administration of RNAi (40).

In the present study, EOS from mice were cultured and purified *in vitro* for transduction by CCR3 siRNA-recombinant lentiviral vectors. By measuring the expression of CCR3 at the mRNA and protein levels and measuring the apoptosis rates of EOS, the present study revealed that lentiviral vectors were effective in silencing and inhibiting the effects

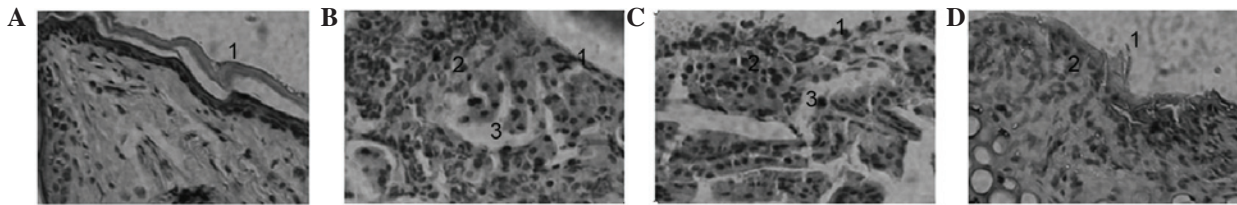


Figure 6. Hematoxylin and eosin staining of nasal mucosa. (A) Group I, nasal mucosal epithelium was intact, without inflammatory cell infiltration (magnification, x200); (B) Group II, mucosa swelled, epithelium shed, there existed submucosal edema, vascular dilatation and congestion, as well as the infiltration of a large number of plasma cells, lymphocytes and EOS (magnification, x200); (C) Group III, submucosal vessels dilated, with mucosal edema and EOS infiltration (magnification, x200); (D) Group IV, nasal mucociliary layer on mucosal surface was more complete, without thickening of mucous layer, while submucosal vasocongestion and edema were not obvious (magnification, x200). EOS, eosinophils.

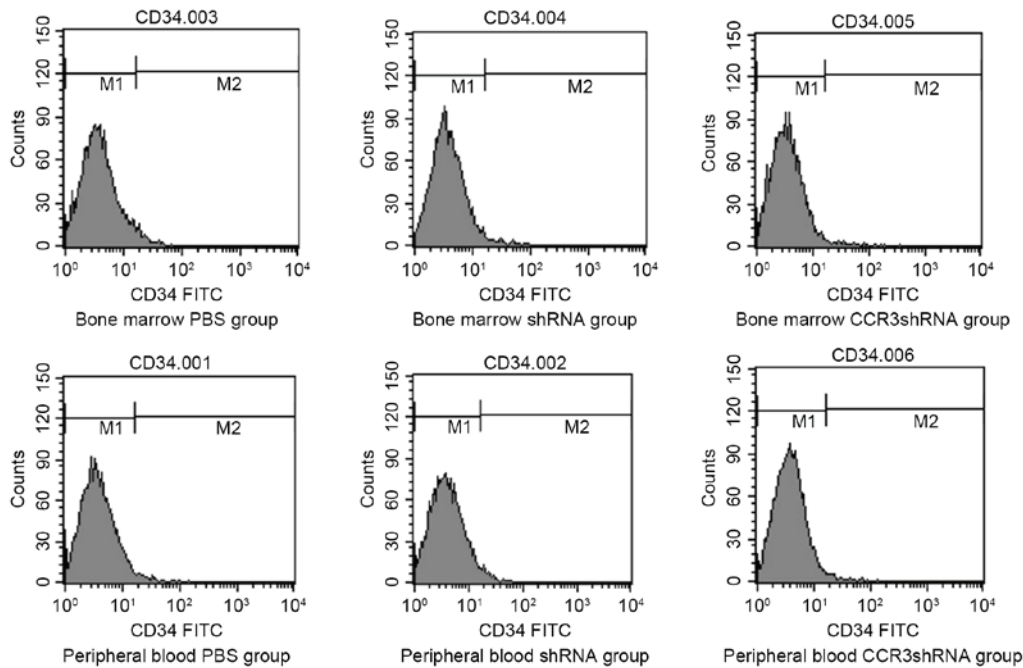


Figure 7. Detection of CD34<sup>+</sup> cells in EOS by flow cytometry. FITC, fluorescein isothiocyanate; EOS, eosinophils; PBS, phosphate-buffered saline; shRNA, short hairpin RNA.

of CCR3, which could significantly promote the apoptosis of EOS. Furthermore, CCR3 siRNA lentiviral vectors were used as an intervention in mice *in vivo*. The measurement of the expression of CCR3 at the mRNA and protein levels and measurement of EOS infiltration in local tissues revealed that direct transnasal administration could effectively silence the expression of CCR3 and could ameliorate pathological changes of the nasal mucosa in mice, including reductions in the migration and invasion of EOS and in nasal inflammation in mice with AR. Moreover, in the present study RNAi was effective in silencing the expression of the CCR3 gene in EOS, thus providing new directions to discover effective targets for the treatment of AR through gene therapy.

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