

C-C chemokine receptor type 3 gene knockout alleviates inflammatory responses in allergic rhinitis model mice by regulating the expression of eosinophil granule proteins and immune factors

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Abstract. The present study aimed to investigate the effects of C-C chemokine receptor type 3 (CCR3) gene knockout on allergic rhinitis (AR) in mice, as well as the underlying molecular mechanisms. Ovalbumin was administrated to CCR3^{+/+} and CCR3^{-/-} BALB/c mice to establish an AR model. The mice were divided into four groups: i) Normal control (CG), ii) AR model (AR), iii) CCR3 knockout CG (CCR3^{-/-}CG) and iv) AR model with CCR3 knockout (CCR3^{-/-}AR). Histological sections of nasal mucosae were examined by hematoxylin and eosin staining, which revealed that CCR3 knockout suppressed the invasion of inflammatory cells and relieved the damage of nasal mucosae. Peripheral blood smear and nasal-washing smears were evaluated by Wright's staining. Eosinophil (EOS) numbers in nasal mucosae, peripheral blood, and nasal washings of the various groups were ranked in the order: AR>CCR3^{-/-}AR>CG>CCR3^{-/-}. mRNA expression levels of CCR3, EOS peroxidase (EPO), EOS cationic protein (ECP), and major basic protein (MBP) in the peripheral serum and nasal washings were detected by reverse transcription-polymerase chain reaction. Interferon- γ (IFN- γ), interleukin (IL)-4, IL-10, and immunoglobulin E (IgE) protein levels in the peripheral serum and nasal washings were investigated by ELISA. CCR3 mRNA expression was not detected in the CCR3^{-/-} and CCR3^{-/-}AR groups, whereas expression levels in the AR group were markedly higher compared with expression in

the CG group. Compared with the CG-associated groups (i.e., the CG and CCR3^{-/-}CG groups), the levels of EPO, ECP, MBP, IL-4, and IgE were significantly increased in the AR-associated groups (that is, R and CCR3^{-/-}AR). In addition, the CCR3^{-/-}AR group mice produced significantly lower levels of EPO, ECP, MBP, IL-4 and IgE compared with the AR group, whereas the expression levels of IFN- γ and IL-10 were increased. CCR3 gene knockout may alleviate EOS invasion and the inflammatory response in AR model mice by reducing the expression levels of EPO, ECP, MBP, IL-4, and IgE, and increasing the expression of IL-10 and IFN- γ .

Introduction

Allergic rhinitis (AR) is one of the common types of rhinitis; previous epidemiological studies revealed an average morbidity rate of 10-20% with an increasing annual tendency (1,2). Over the past few years, AR morbidity and severity have gradually increased in developed countries, and AR has become a serious problem that is damaging public health. AR may have a strong effect on the lives of people; apart from the influence on the daily lives and careers, AR may induce some complications, including nasosinusitis, bronchial asthma and Eustachian tube dysfunction; however, the development of treatments for AR has gained increasing attention. The pathogenesis of AR and the pathogenic factors involved are diverse and complex, but are not well understood. Current treatments for AR maintain the stage of symptom remission; however, complete healing is desired.

Previous studies have demonstrated that allergic diseases are not only localized but also may be systemic without the full elucidation of pathogenesis (3,4). Eosinophils (EOS) have remained the investigative focus in the pathogenesis of allergic diseases, and tissue invasion by numerous EOS is an important characteristic of this type of disease (5). Eotaxins are members of the C-C chemokine family that are able to induce the migration of EOS into inflammatory tissues by combining with C-C chemokine receptor 3 (CCR3) expressed in EOS (6). Different from most chemokines that function by binding to receptors, Eotaxin signals are specifically transduced via CCR3 (6). As a

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transmembrane G protein-coupled receptor, CCR3 is one of the main chemokine receptors, and CCR3 controls the recruitment of EOS into local inflammatory tissues (6). It was reported that Eotaxin also served an important role within *in situ* hematopoiesis of EOS in the inflammatory tissues by means of CCR3 (7).

CCR3 is a specific receptor for Eotaxin; therefore, CCR3 deficiency may lead to reduced binding of Eotaxin and subsequent inhibition of phosphatidylinositol-3-kinase (PI3K) signaling pathway activation and the inactivation of its subunit PI3K γ (8). Thus, various downstream signaling responses may be affected. The activation and migration of EOS and associated progenitor cells in the marrow might be suppressed and, consequently, the amount of mature EOS in the nasal mucosa may decrease, which may reduce degranulation and reduce AR morbidity rates (9). The morbidity of AR involving stimulation by allergens may activate the binding of Eotaxin to CCR3 and induce the key downstream PI3 K signaling pathway (8). This process may trigger the activation and migration of EOS in the marrow to the peripheral tissues and nasal mucosa (8). CCR3 knockout in the marrow cells may inhibit the activation and migration of EOS and subsequently prevent AR by inhibiting PI3K signaling (8,9). These previous results revealed that gene therapy may be used to regulate EOS and CCR3, which may provide novel insights into the treatment of AR.

The present study established an AR model mouse and examined the effects of CCR3 gene knockout on inflammation-associated damage and EOS numbers in nasal mucosa, peripheral blood, and nasal washings. In addition, alterations in the expression levels of EOS peroxidase (EPO), EOS cationic protein (ECP), major basic protein (MBP), interferon- γ (IFN- γ), interleukin (IL)-4, IL-10, and immunoglobulin E (IgE) were investigated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and ELISA.

Materials and methods

Materials. Aluminum hydroxide was purchased from Damao Chemical Reagent Factory (Tianjin, China). The hematoxylin and eosin (H&E) staining kit was obtained from Boster Biological Technology (cat. no. AR11800-1; Pleasanton, CA, USA). The Leukocyte Separation Medium kit for the separation of mouse peripheral blood was obtained from Yanjin Biological (WBC1092, Shanghai, China). Mouse interferon- γ (IFN- γ ; cat. no. SEA033Hu), interleukin (IL)-4 (cat. no. SEA077Mu), IL-10 (cat. no. SEA056Mu), and IgE (cat. no. SEA545Mu) ELISA kits were from Cloud-Clone Corp. (Wuhan, China). Ovalbumin (OVA; cat. no. P0003) was purchased from Beijing Solarbio Science & Technology Co. Ltd., (Beijing, China). Rapid Wright's Staining kit was obtained from Nanjing KeyGen Biotech Co., Ltd. (cat. no. KGA225; Jiangsu, China). TRIzol Reagent (cat. no. CW0580) and HiFi Script first strand cDNA synthesis kit (cat. no. CW2569) were obtained from CW Biotech (Beijing, China).

Animals. A total of 20 specific pathogen free (SPF) normal BALB/c mice (10 male and 10 female; age, 6-8 weeks; weight, 20-28 g) were obtained from Experimental Animal Center of Medical college of Nanchang University (Jiangxi, China). A total of 20 SPF CCR3^{-/-}BALB/c mice (10 male and 10 female; age, 6-8 weeks; weight, 20-28 g) were purchased from the Jackson Laboratory (Strain J005440; Bar Harbor, ME, USA).

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer (5'→3')
GAPDH	F: GCAAGTTCAACGGCACAG R: CGCCAGTAGACTCCACGAC
CCR3	F: TGCTGAGATGTCCCAATA R: GCCAGGTCCAGATGTTTA
MBP	F: ACCCACTATGGCTCCCTG R: CAATCCTCTTCCCTTTCCTT
ECP	F: AGGCGAAGCGTGAGTTGC R: GATCGGGAATGTTGGTGC
EPO	F: CGGGCGAAGACAAACAAAGG R: GGGCGGTAGAAGCCAAAGATC

MBP, major basic protein; CCR3, C-C chemokine receptor-3; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; F, forward; R, reverse.

The mice were raised with free access to water and food in a sterile environment of 18-29°C, 40-70% relative humidity and a 12/12 h light/dark cycle. The CCR3^{-/-} BALB/c mice were identified by gene analysis, including DNA extraction, PCR amplification, and agarose gel electrophoresis according to the instructions provided by Jackson Laboratory (Bar Harbor, ME, USA). B6 was the genomic DNA (wild-type), which served as negative control. Target fragments of wild-type and mutant were 120 and 280 bp, respectively. PCR template was not directly used for electrophoretic detection. It meant that template control was not set. The study protocol was reviewed and approved by The Institutional Animal Care and Use Committee, Nanchang University, [Jiangxi, China; approval number YanLinShen (2013) No. 16] and was conducted in accordance with the guidelines established by the Chinese Council of Animal Care (10).

AR model mouse establishment. A total of 20 SPF normal BALB/c mice and 20 SPF CCR3^{-/-}BALB/c mice were randomly divided into four groups (n=10/group): i) Normal CCR3^{+/+} control (CG); ii) CCR3^{+/+} AR model (AR); iii) CCR3^{-/-}CG; and iv) CCR3^{-/-}AR. Each mouse in the AR and CCR3^{-/-}AR groups was intraperitoneally injected with a mixture of 10 μ g OVA and 4 mg aluminum hydroxide twice per day, on day 1 and day 15 to induce sensitization. Subsequently, between days 21 and 27, 1 mg/ml OVA was dripped into the noses of these mice twice per day for provocation. An equal volume of normal saline was similarly administrated to the nasal cavities of mice in the CG and CCR3^{-/-}CG groups. Nasal washings were collected. At 24 h after the final administration of the OVA drip, all mice were anesthetized with 0.6% sodium pentobarbital (70 mg/kg) and euthanized. The upper palates of mice were dissected and the bilateral nasal septum mucosae were collected. The mucosae were fixed in 4% paraformaldehyde buffer at room temperature for 24 h for subsequent experiments. In addition, the chests of mice were opened and blood was extracted from the ventriculus dexter using injection syringes and into plain tubes, which were stored at 4°C for future analyses.

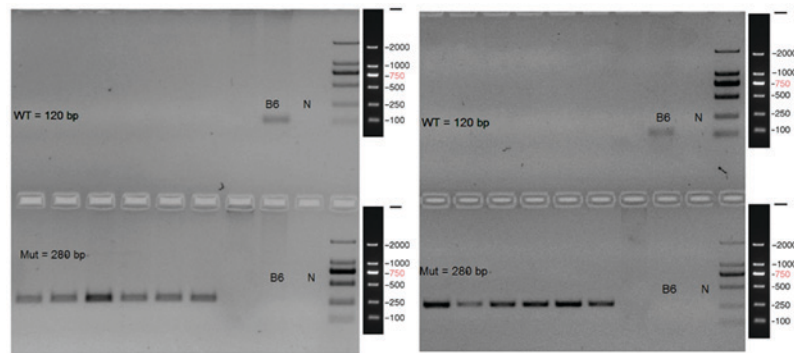


Figure 1. Identification of mice with CCR3 gene knockout by polymerase chain reaction. B6 was the genomic DNA (wild-type), which served as negative control. Target fragments of wild-type and mutant were 120 and 280 bp, respectively. N indicates the blank control. Template control was not set. The rightmost lane was the marker. There were no bands in the location of 120 bp in the left six lanes, but in the location of 280 bp Mut gene, notable bands were detected. Mut, mutant; WT, wild-type.

H&E staining. Fixed nasal mucosae were dehydrated in a gradient of 70, 80 and 90% ethanol, embedded in paraffin and sectioned (4 μ m). Sections were deparaffinized with dimethylbenzene at room temperature for 10 min, rehydrated in graded ethanol at room temperature for 5 min and stained with H&E at room temperature for 15 sec, according to the manufacturer's protocols. The sections were mounted with neutral resins and characterized under a conventional light microscope (XDZ-103; Lingcheng Biotech, Shanghai, China). EOS morphology was observed and EOS numbers were counted.

Wright's staining. Separation medium (5 ml, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to a 15 ml centrifuge tube. Blood (15 ml) was carefully sampled with a suction pipette and added to the surface of the separation medium. The mixture was centrifuged at 400-500 \times g and room temperature for 20-30 min. The ring-like milky layer of cells (one or two layers) was carefully collected with a pipette. Following the addition of 10 ml cleaning solution, the cells were mixed. The mixture was centrifuged at 250 \times g and room temperature for 10 min. The cell pellet was washed three times with cleaning solution to obtain the leukocytes. Nasal washings were centrifuged at 250 \times g and room temperature for 5 min. The pellet was resuspended in 0.3 ml phosphate buffer solution. Finally, blood leukocyte smears and nasal washings smears were prepared by smearing the suspension onto slides.

Following natural drying at room temperature for 30 min, smears were fixed in 4% paraformaldehyde at room temperature for 15 min. Solution A from the Wright's staining kit was applied to start staining for 1 min; solution B was added at double the volume of solution A, and the slides were gently agitated to mix the solutions. Staining was maintained at room temperature for 10 min. The staining solution was carefully washed away from one side of the slide with purified water; the surrounding water-drop was blotted with filter paper. Following natural drying at room temperature for 30 min, the slides were analyzed under a microscope (XDZ-103; Lingcheng Biotech). A total of 6 fields were examined per slide. EOS morphology was observed and EOS numbers were counted.

RT-qPCR. The blood was centrifuged at 2,000 \times g and room temperature for 20-30 min to obtain the serum. Total RNA was extracted from the serum (200 μ l) and nasal washings (200 μ l)

using TRIzol (Life Technologies; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Total RNA concentration and purity of the samples were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA was reverse transcribed to cDNA with the HiFi Script First Strand cDNA synthesis kit, according to the manufacturer's protocol. Target genes, including CCR3, EPO, ECP, and MBP were detected using a CFX Connect PCR machine (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using primers synthesized by Sangon Biotech Co., Ltd., (Shanghai, China); primer sequences are provided in Table I, and GAPDH served as an internal control. The PCR system (25 μ l) contained 9.5 μ l RNase free ddH₂O, 1 μ l cDNA, 1 μ l forward primer, 1 μ l reverse primer, and 12.5 μ l 2xUltraSYBR Mixture (CW0957; Beijing CWBIO, Beijing, China). The PCR thermocycling conditions were as follows: Pre-denaturation for 3 min at 95°C; followed by 40 cycles of denaturation for 10 sec at 95°C, annealing for 30 sec at 50°C (GAPDH, CCR3, and ECP) or 55°C (EPO and MBP), elongation for 30 sec at 72°C. Amplification products (5 μ l) were loaded onto a 1% agarose gel for electrophoresis, stained with ethidium bromide, and images were captured with a ChemiDoc XRS Gel Imaging System (Bio-Rad Laboratories, Inc.). The relative expression was calculated by the $2^{-\Delta\Delta C_q}$ method (11).

ELISA. Serum and nasal washing supernatant were used to determine the levels of IFN- γ (pg/ml), IL-4 (pg/ml), IL-10 (pg/ml), and IgE (ng/ml) expression by ELISA, following the manufacturer's protocols. Expression levels were determined using a PT-3502 G microplate reader (Potenov Tech, Beijing, China).

Statistical analysis. Each experiment was replicated three times. All data were expressed as the mean \pm standard deviation. Statistical analysis was performed by using one-way analysis of variance followed by a Tukey's post hoc test with SPSS software 11.5 (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Mice identification. The homozygous genotypes of the CCR3 gene knockout mice that were used in the following were confirmed by PCR analysis (Fig. 1). The left gel shows the PCR identification result of Nos. 1-6 CCR3 gene knockout

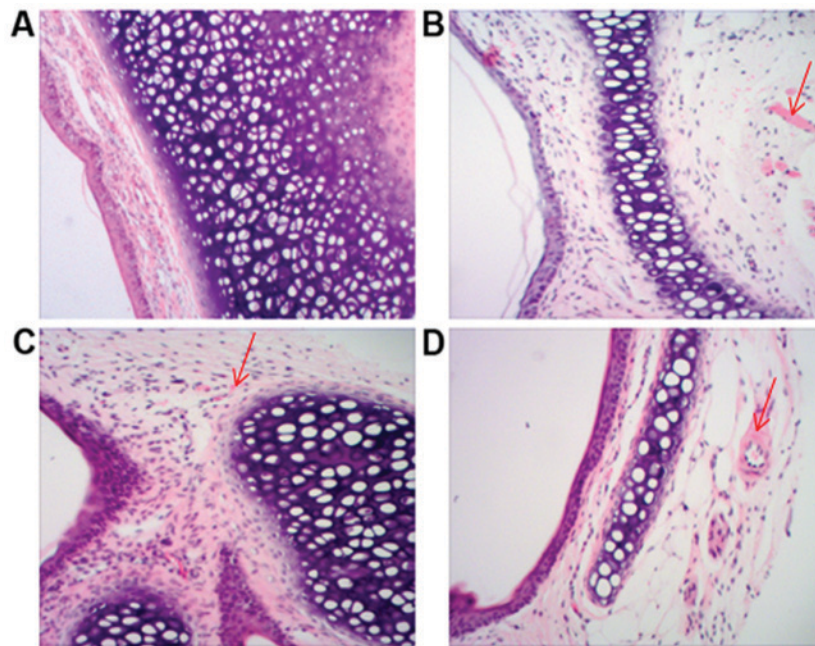


Figure 2. Histological examination of nasal mucosae. Sections of nasal mucosae from mice in the (A) CG, (B) CCR3^{-/-}CG, (C) AR and (D) CCR3^{-/-}AR groups were examined by hematoxylin and eosin staining; Detachment of epithelial cells with irregular arrangement, structural disorder of the mucosal layer and invasion of numerous inflammatory cells, including EOS, are indicated by arrows (magnification, $\times 100$). AR, allergic rhinitis model; CG, normal control; CCR3, C-C chemokine receptor 3; EOS, eosinophil.

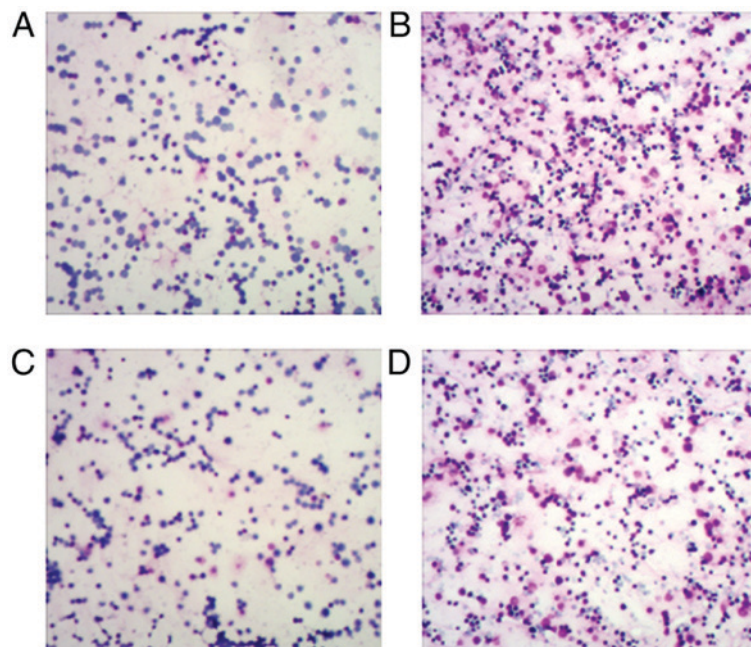


Figure 3. Analysis of peripheral blood smears. Blood smears from mice in the (A) CG, (B) AR, (C) CCR3^{-/-}CG and (D) CCR3^{-/-}AR groups were examined by Wright's staining; magnification, $\times 100$. CG, normal control; AR, allergic rhinitis model, CCR3^{-/-}CG, C-C chemokine receptor-3 knockout control; CCR3^{-/-}AR, AR model with CCR3 knockout.

mice. The right gel shows the PCR identification result of nos. 7-12 CCR3 gene knockout mice. Bands at 120 and 280 bp represent the wild-type and mutant, respectively. There were no bands in the location of 120 bp in the left six lanes, but in the location of 280 bp mutant gene, notable bands were observed. Bands at 280 bp showed that the CCR3 gene was completely knocked out. This suggested that these mice were homozygotes with CCR3 gene knockout.

Histological examination of nasal mucosae by H&E staining. Nasal mucosae were examined by H&E staining. In the CG and CCR3^{-/-}CG groups (Fig. 2A and B, respectively), the nasal mucosae were intact with explicit structure and without notable invasion of inflammatory cells. However, in the AR group (Fig. 2C), detachment of epithelial cells with irregular arrangement, structural disorder of mucosal layer and invasion of numerous inflammatory cells, including

Table II. EOS numbers in the nasal mucosae of mice in the CG, AR, CCR3^{-/-}CG, and CCR3^{-/-}AR groups.

Group ^a	EOS numbers (x10 ⁶)
CG	1.21±0.13
AR	18.23±1.21 ^b
CCR3 ^{-/-} CG	1.19±0.20
CCR3 ^{-/-} AR	8.32±1.27 ^{b,c}

^an=10/group. ^bP<0.05, AR group vs. the CG group and the CCR3^{-/-}AR group vs. the CCR3^{-/-}CG group. ^cP<0.05, CCR3^{-/-}AR group vs. the AR group. AR, allergic rhinitis model; CCR3, C-C chemokine receptor-3; CCR3^{-/-}AR, AR model with CCR3 knockout; CCR3^{-/-}CG, C-C chemokine receptor-3 knockout control; CG, normal control; EOS, eosinophil.

Table III. EOS numbers in the peripheral blood smears from mice in the CG, AR, CCR3^{-/-}CG, and CCR3^{-/-}AR groups.

Group ^a	EOS numbers (x10 ⁶)
CG	0.23±0.15
AR	2.51±0.21 ^b
CCR3 ^{-/-} CG	0.19±0.10
CCR3 ^{-/-} AR	1.22±0.24 ^{b,c}

^an=10/group. ^bP<0.05, AR group vs. the CG group and the CCR3^{-/-}AR group vs. the CCR3^{-/-}CG group. ^cP<0.05, CCR3^{-/-}AR group vs. the AR group. AR, allergic rhinitis model; CCR3, C-C chemokine receptor-3; CCR3^{-/-}AR, AR model with CCR3 knockout; CCR3^{-/-}CG, C-C chemokine receptor-3 knockout control; CG, normal control; EOS, eosinophil.

EOS, were observed. Moreover, local swelling of nasal mucosae was observed. Notably, in the CCR3^{-/-}AR group (Fig. 2D), the damage of mucosal epithelial cells and the detachment of epidermal cells were markedly alleviated as compared with the AR group; however, slight edema in epithelial cells and mild invasion of a few inflammatory cells were observed.

In addition, total EOS numbers in the nasal mucosae of the various groups were ranked in the following order, from highest to lowest (Table II): AR>CCR3^{-/-}AR>CG>CCR3^{-/-}CG. Compared with the CG group, the average EOS numbers were significantly increased in the AR group (P<0.05), and the average EOS numbers were also significantly higher in the CCR3^{-/-}AR group compared with the average EOS numbers in the CCR3^{-/-}CG group (P<0.05). Although the average EOS numbers in the CCR3^{-/-}CG group were lower compared with those in the CG group, no significant difference was identified. Compared with the AR group, EOS numbers in the CCR3^{-/-}AR group were significantly reduced (P<0.05), which suggested that EOS in these mice had reduced invasive ability. These data indicated that CCR3 gene deficiency may have suppressed the invasion of inflammatory cells and relieved the nasal mucosae damage in AR model mice.

Table IV. EOS numbers in the nasal washings smear in the CG, AR, CCR3^{-/-}CG and CCR3^{-/-}AR groups.

Group ^a	EOS numbers (x10 ⁶)
CG	1.31±0.14
AR	2.83±0.71 ^b
CCR3 ^{-/-} CG	1.19±0.20
CCR3 ^{-/-} AR	2.12±0.57 ^{b,c}

^an=10/group. ^bP<0.05, AR group vs. the CG group and the CCR3^{-/-}AR group vs. the CCR3^{-/-}CG group. ^cP<0.05, CCR3^{-/-}AR group vs. the AR group. AR, allergic rhinitis model; CCR3, C-C chemokine receptor-3; CCR3^{-/-}AR, AR model with CCR3 knockout; CCR3^{-/-}CG, C-C chemokine receptor-3 knockout control; CG, normal control; EOS, eosinophil.

Examination of blood smear and nasal washing smears by Wright's staining. Blood and nasal washing smears from mice in each group were examined by Wright's staining. Similar results were identified between the blood (Fig. 3; Table III) and nasal washings (Fig. 4; Table IV) samples. Compared with the CG group, the AR group had sharply increased EOS numbers (P<0.05), and the EOS numbers were also remarkably higher in the CCR3^{-/-}AR group compared with the EOS numbers in the CCR3^{-/-}CG group (P<0.05). Compared with the AR group, EOS numbers in the peripheral blood smear and the nasal washing smears were notably reduced in the CCR3^{-/-}AR group (P<0.05). Although average EOS numbers in the peripheral blood smear and nasal washing smears were decreased by 17.4 and 9.2%, respectively, in the CCR3^{-/-}CG group compared with in the CG group, there was no significant difference. It was suggested that CCR3 deficiency may be capable of weakening the invasion of inflammatory cells in AR model mice.

mRNA expression levels of CCR3, EPO, ECP, and MBP in the peripheral serum and nasal washings. mRNA expression levels of CCR3, EPO, ECP, and MBP were determined by RT-qPCR on the peripheral blood and nasal washings of mice following various treatments, and similar trends in mRNA expression levels were observed (Figs. 5 and 6, respectively). For example, CCR3 mRNA was not detected in the CCR3^{-/-}CG and CCR3^{-/-}AR groups, whereas expression levels in the AR group were significantly higher compared with CCR3 mRNA expression in the CG group (P<0.05). The mRNA expression levels EPO, ECP, and MBP exhibited similar trends among the different groups; compared with the CG group, mRNA expression levels were significantly increased in the AR group (P<0.05), and the expression levels were also significantly higher in the CCR3^{-/-}AR group compared with expression levels in the CCR3^{-/-}CG group (P<0.05). In addition, the CCR3^{-/-}AR group exhibited significantly lower EPO, ECP, and MBP mRNA expression levels compared with the respective expression levels in the AR group (P<0.05); however, no significant differences were identified between the CG and CCR3^{-/-}CG groups.

Expression levels of IL-4, IgE, IFN-γ, and IL-10, in the peripheral serum and nasal washings. The levels of IL-4, IgE,

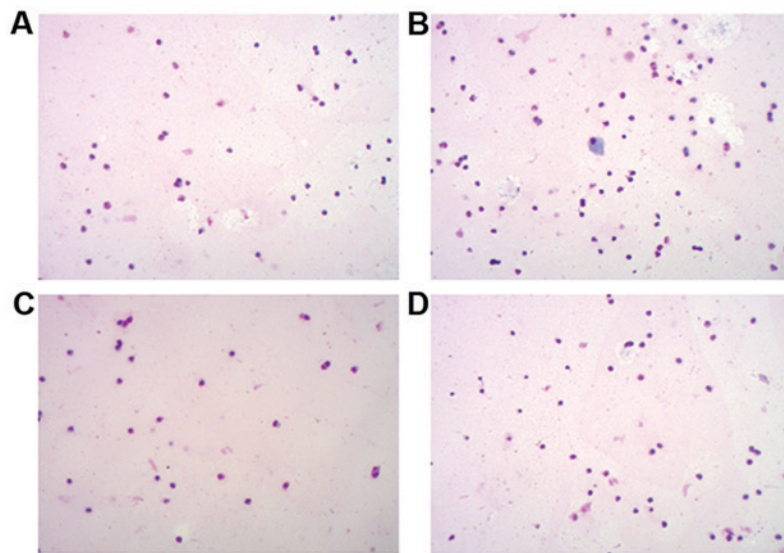


Figure 4. Images of the nasal washings smear in the (A) CG, (B) AR, (C) CCR3^{-/-}CG, and (D) CCR3^{-/-}AR groups which were examined by Wright's staining (magnification, x100). AR, allergic rhinitis model; CG, normal control; CCR3^{-/-}CG, C-C chemokine receptor-3 knockout control; CCR3^{-/-}AR, AR model with CCR3 knockout.

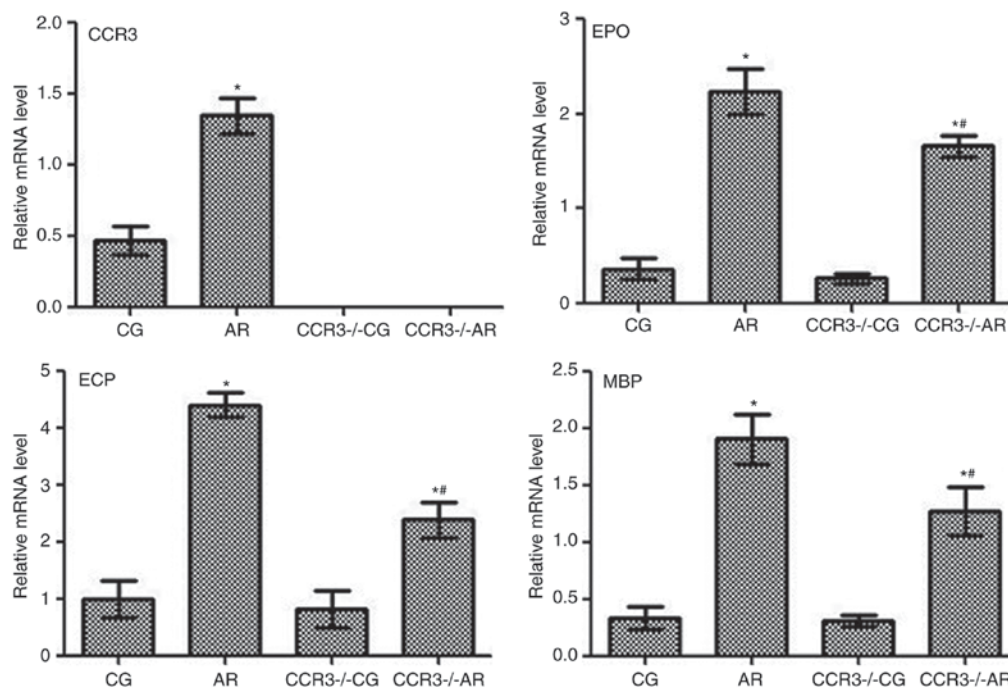


Figure 5. mRNA expression levels of CCR3, EPO, ECP, and MBP in the peripheral serum of mice in various groups were determined by reverse transcription-quantitative polymerase chain reaction. * $P < 0.05$, AR group vs. the CG group and the CCR3^{-/-}AR group vs. the CCR3^{-/-}CG group. # $P < 0.05$, CCR3^{-/-}AR group vs. the AR group. AR, allergic rhinitis model; CCR3, C-C chemokine receptor-3; CG, normal control; CCR3^{-/-}CG, CCR3 knockout control; CCR3^{-/-}AR, AR model with CCR3 knockout; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; MBP, major basic protein.

IFN- γ and IL-10 in the peripheral blood and nasal washings were evaluated by ELISA (Figs. 7 and 8). IL-4, IgE, IFN- γ , and IL-10 expression levels between the CG and CCR3^{-/-}CG groups were similar. The expression levels of IL-4 and IgE were significantly increased in mice in the AR group compared with CG group mice ($P < 0.05$); similar results were observed between the CCR3^{-/-}AR group compared with the CCR3^{-/-}CG group ($P < 0.05$). IL-4 and IgE expression levels in the peripheral blood were significantly decreased in the CCR3^{-/-}AR mice compared with the AR group mice ($P < 0.05$);

however, no significant difference was identified in the nasal washings between these two groups ($P > 0.05$).

Conversely, IFN- γ and IL-10 expression levels in the peripheral blood and the nasal washings were significantly reduced in the AR group compared with the CG group, as well as in the CCR3^{-/-}AR group compared with the CCR3^{-/-}CG (both $P < 0.05$). In the peripheral blood, IFN- γ and IL-10 expression levels in the CCR3^{-/-}AR group were significantly upregulated compared with levels in the AR group ($P < 0.05$); however, in the nasal washings, no significant differences were

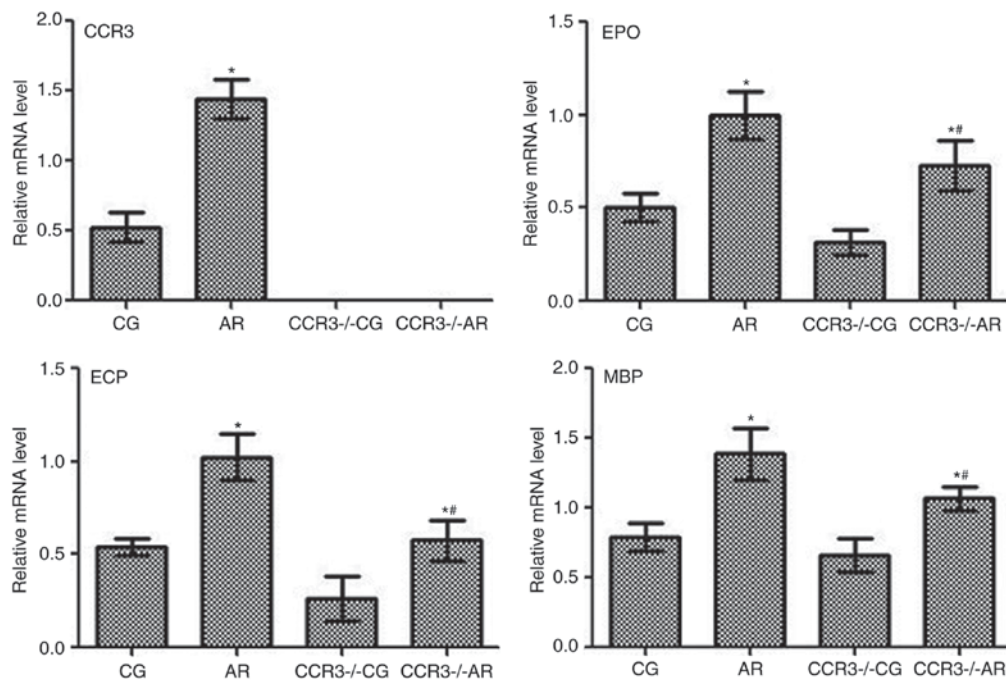


Figure 6. mRNA expression levels of CCR3, EPO, ECP, and MBP in the nasal washings of mice in various groups were determined by reverse transcription-quantitative polymerase chain reaction. *P<0.05, AR group vs. the CG group and the CCR3^{-/-}AR group vs. the CCR3^{-/-}CG group. #P<0.05, CCR3^{-/-}AR group vs. the AR group. AR, allergic rhinitis model; CCR3, C-C chemokine receptor-3; CG, normal control; CCR3^{-/-}CG, CCR3 knockout control; CCR3^{-/-}AR, AR model with CCR3 knockout; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; MBP, major basic protein.

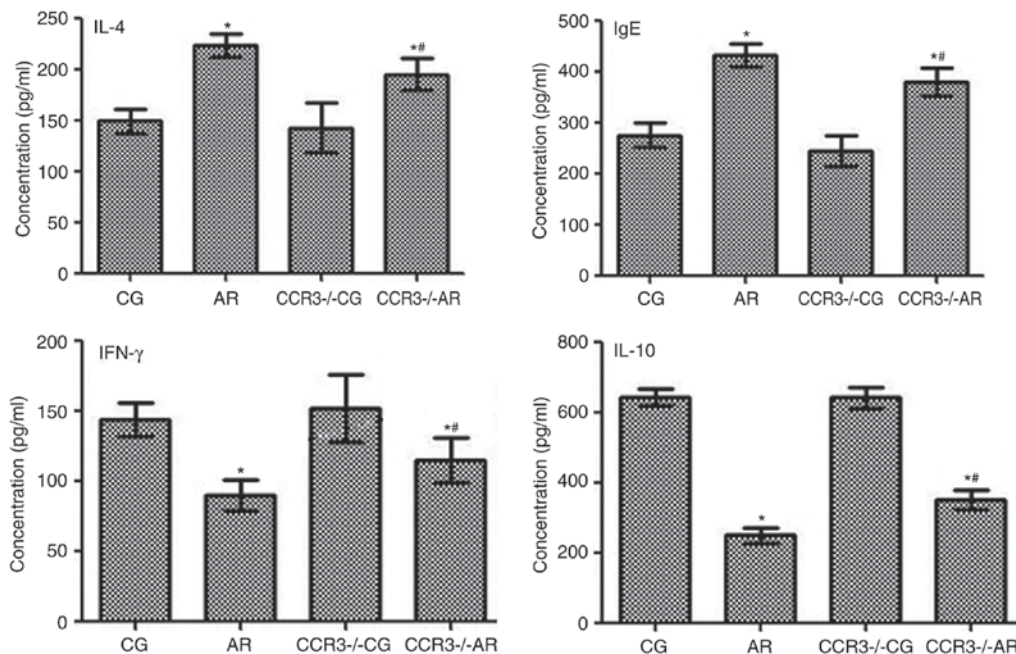


Figure 7. Expression levels of IFN-γ, IL-4, IL-10, and IgE in the peripheral serum of mice were evaluated by ELISA. *P<0.05, AR vs. CG and CCR3^{-/-}AR vs. CCR3^{-/-}CG; #P<0.05, CCR3^{-/-}AR vs. AR. AR, allergic rhinitis model; CCR3, C-C chemokine receptor-3; CCR3^{-/-}AR, AR model with CCR3 knockout; CCR3^{-/-}CG, CCR3 knockout control; CG, normal control; IFN-γ, interferon-γ; IgE, immunoglobulin E; IL-10, interleukin-10.

identified for the IFN-γ and IL-10 expression levels between the AR and the CCR3^{-/-}AR groups (P>0.05).

Discussion

The main effector cells of AR are EOS, which are a type of leukocyte. EOS are featured with 2-3 segments of nucleus and

are full of acidophil granules in the cytoplasm. The matured EOS contains primary and secondary granules. The secondary granules contain numerous proteins, including ECP, MBP, EPO, and EOS-derived neurotoxin (12). The life cycle of EOS is divided into three stages: In the marrow, peripheral blood, and tissues. EOS mainly exist in the epithelial tissues of hollow organs, including the intestinal epithelium and respiratory

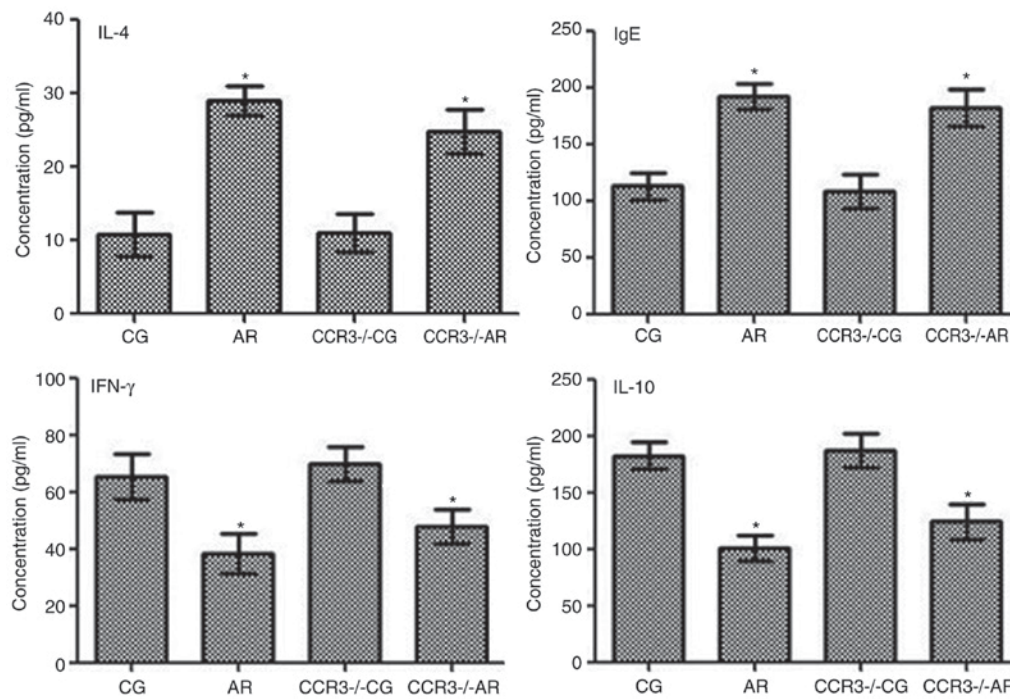


Figure 8. Expression levels of IFN- γ , IL-4, IL-10, and IgE in the nasal washings of mice in various groups were evaluated by ELISA. * $P < 0.05$, AR vs. CG and CCR3^{-/-}AR vs. CCR3^{-/-}CG. AR, allergic rhinitis model; CCR3, C-C chemokine receptor-3; CCR3^{-/-}AR, AR model with CCR3 knockout; CCR3^{-/-}CG, CCR3 knockout control; CG, normal control; IFN- γ , interferon- γ ; IgE, immunoglobulin E; IL-10, interleukin-10.

epithelium. In the present study, the peripheral blood and the nasal mucosae of AR model mice were revealed to contain numerous EOS.

In the AR mice, local swelling of nasal mucosae was observed, which may have been caused by histamine- and kinin-induced angiotelectasis and the subsequent enhancement of vascular wall permeability by stimulation of the receptors located on sensory nerves and blood vessel (13). Histamine is also known to augment glandular secretion and plasma effusion (13). Plasma effusion may have resulted in the nasal mucosal edema and swelling (14). Retention of a large number of exudates within connective tissues may oppress the superficial blood vessels, which ultimately led to pallor of the mucosae (14). The aforementioned pathological alterations occurred repeatedly, generating mucosal epithelial hyperplasia and mucosae thickening in consequence. Additionally, under the influence of the aforementioned factors, parasympathetic nerves may release acetylcholine, which may have maintained high levels of glandular secretion and induced the presence of abundant watery rhinorrhea (15). Mastocytes and basophils may release inflammatory mediators to aggravate the inflammatory reaction; however, they may secrete EOS chemotactic factors (15). These processes induced the aggregation of numerous EOS around the glands and blood vessels, and some EOS may enter into the secretions (15). Additionally, numerous EOS locally invaded into tissues, which is an important pathological feature of AR (16). These data agreed with the present study results, which demonstrated that EOS numbers in the nasal mucosae, peripheral blood, and nasal washings of AR model mice were significantly higher compared with the CG mice.

Basic pathological alterations of AR include angiotelectasis, enhancement of vascular wall permeability, augment of glandular secretion, and invasion of EOS (17). Following nasal

provocation, the expression of numerous factors, including histamine, kinins, leukotriene B₄, and EOS-derived factors (such as ECP, MBP, EPO, and EOS-derived neurotoxin) were upregulated in nasal secretions, (18,19). Similarly, the present study results demonstrated that EOS invasion and mRNA expression levels of EPO, ECP, and MBP were significantly increased in the peripheral blood and nasal washings of AR model mice.

The chemokine receptor CCR3 is an ~350 amino-acid-long transmembrane protein on the surface of immature eosinophil progenitor cells and mature EOS that exhibits homology with other C-C chemokine receptors (20). The combination of CCR3 and Eotaxin [also known as C-C motif chemokine 11 (CCL11)] may activate the G protein-dependent intracellular signaling pathways and further facilitate the release of EOS progenitor cells and mature EOS from the marrow into the AR affected tissues (20), which may serve an important role in the invasion of EOS into target sites (21,22). The binding of CCR3 and ligands was previously reported to amplify downstream signaling cascade effects (23). Its mechanism was as follows: The binding of the G α subunit and guanosine triphosphate activated the downstream factors, including phospholipase C, phosphatidylinositol and calcium ion via dissociation of G $\beta\gamma$ dimer subunit (23). In addition, its signal may mediate inflammatory reaction cascades and induce the degranulation of inflammatory cells by activating the mitogen activated protein kinase pathway (23). The ligands of CCR3 include Rantes (also known as CCL5), Eotaxin, Eotaxin-2 (also known as CCL24), Eotaxin-3 (also known as CCL26), monocyte chemotactic protein 2 (MCP-2; also known as CCL8), MCP-3 (also known as CCL7), and MCP-5 (also known as CCL12). The affinity of CCR3 to different chemokines varies; CCL11, CCL24, and CCL26 transduce signals only via CCR3, but not other

receptors. CCR3 serves a crucial role in the accumulation of EOS in inflammatory tissues, which indicates that CCR3 is closely associated with numerous type-I allergic diseases, including AR (24).

In the present study, EOS numbers were significantly reduced in CCR3^{-/-} mice bearing AR. Expression levels of the secondary EOS granule proteins MBP, ECP, and EOP were not significantly different between the CG and CCR3^{-/-}CG mice; however, compared with the AR group, the levels of MBP, ECP, and EOP were significantly reduced in the mice of CCR3^{-/-}AR group. This may have resulted from the deficiency of CCR3; as the CCR3^{-/-} mice did not produce CCR3, there was no CCR3 to bind to the ligands. CCR3 is a gene related to EOS. In AR mice, EOS numbers were elevated, but after CCR3 knockout, the EOS numbers were reduced. Therefore, there was a difference between CCR3 knockout and non-knockout in the AR model. However, there was no increase in the EOS numbers in the CG mice, so there was no significant relationship between CCR3 knockout and non-knockout in the CG mice. Consequently, the downstream signaling cascade effects of amplification may not be induced, which downregulated the expression of the secondary granules of EOS. Therefore, CCR3 knockout relieved the inflammatory reaction in the genesis and development of AR by downregulating the expression of EPO, ECP, and MBP in the present study. These findings were in accord with a previous study (25).

IL-10 is an anti-inflammatory cytokine, and its major effect cells are antigen-presenting cells and T lymphocytes (26). During the different stages of the immune response process, IL-10 reverses the hyper-responsiveness of the body to allergens, which leads to immune tolerance (27). Furthermore, IL-10 exhibits other various immunomodulatory effects, including immunosuppression, immune anergy, and anti-inflammation. The major roles of the immunosuppressive effects of IL-10 are to suppress the secretion of cytokines from certain cells, including monocytes and natural killer cells, to reduce the generation of IgE and to protect the body from allergen-induced airway inflammation (28). During the inhibition of inflammatory response, inflammatory mediators derived from dendritic cells and macrophages may be partially inhibited by IL-10. In addition, previous studies using AR model mice revealed that IL-10 may partially alleviate asthmatic airway inflammation (29-32). In the present study, AR model mice (with and without CCR3 deficiency) expressed lower levels of IL-10 compared with control mice. Notably, CCR3^{-/-}AR mice exhibited a significant increase in IL-10 expression levels in the serum compared with CCR3^{+/+}AR mice, which suggested that CCR3 knockout was capable of relieving the inflammatory reaction in the genesis and development of AR by upregulating IL-10 expression. CCR3 knockout may upregulate IL-10 expression by influencing the expression of TH2 cytokines and Eotaxin (33).

IL-4 is a differentiation factor and growth factor of B cells, and it may serve an important role in the responses of B cells to antigenic stimulation (34). Wherein, the responses include the proliferation of B cells and antibody secretion from B cells (34). The allergen of pathological specimens collected from patients with AR from symptomatic to asymptomatic status was exposed *in vitro* (35). mRNA level of IL-4 was

identified to be markedly increased and same class-switch of B cells may produce IgE, but in non-AR, the phenomenon of same class-switch from B cells to IgE did not exist (35). Furthermore, IL-4 is able to induce the class-switch of B cells from producing other immune globulins to producing IgE (35). It is noted in particular that IL-4 promotes a strong transition of IgG to IgE (36). The silencing of the IL-4 may significantly suppress the occurrence of T helper (Th)-2 cell response (37). The synergistic effects of IL-4 and IL-3 restrain the secretion of IFN- γ , which is significant in the genesis of hypersensitivity diseases such as AR. Conversely, IL-4 is able to raise the expression of vascular cell adhesion molecule-1 and substance P, leading to the upregulation of major histocompatibility complex expression and the subsequent strengthening of immune response, followed by the final aggravation of EOS accumulation and invasion (37). IgE was produced by B cells in the lymphatic tissues of respiratory tract and intestinal tract (38). When the body is exposed to an allergen, the antigen will bind to IgE on the surface of basophilic granulocytes, mastocytes, and vascular endothelial cells in tissues, leading to the release of numerous active factors, including leukotriene, prostaglandin, endothelin, thromboxane A₂, and histamine and the subsequent attack of type-I allergic responses. This is in accordance with the results of the present study. It was identified that the expression of IL-4 and IgE was upregulated in AR mice and CCR3 knockout was able to alleviate the inflammation response in the genesis and development of AR by downregulating the expression of IL-4 and IgE. CCR3 knockout may downregulate IL-4 expression by influencing the expression of TH2 cytokines and Eotaxin as well (39). Furthermore, IL-4 affected B lymphocytes, thereby downregulating the IgE expression (39).

IFN is a type of active protein mainly produced by the monocytes and lymphocytes (40). IFN is able to enhance the activity of T lymphocyte, NK cells, and macrophages, thereby elevating the immunomodulatory effects of these cells and the antiviral capability of the bodies (40). There are three types of common IFN, and IFN- γ is an example of a Type II IFN. The immunomodulatory effect is the major biological activity of IFN- γ (40). In addition, IFN- γ exhibits antiviral and anti-proliferative activities to a certain extent (40). As a Th1-associated cytokine, IFN- γ was reported to affect the microenvironment of CD⁺ T cell differentiation, induce the shift of Th1/Th2 balance and cause the transformation CD⁺ T to Th1 type, which accordingly interdicts the differentiation of Th2 cytokines and the production of IL-4 (41,42). Additionally, IFN- γ may reduce the production of Th2 type cytokines by inhibiting the generation of Th2 cell-specific transcription factor and Th2 type cells (42). IFN- γ and IL-4 are important regulatory factors for synthesizing IgE (42). A previous report revealed that intranasal inhalation of IFN- γ may effectively inhibit the synthesis of IL-4 and IL-5 in AR model rats, thus reducing the level of OVA-specific IgE (43). In the present study, IFN- γ expression levels were significantly reduced in the AR model mice (AR and CCR3^{-/-}AR) compared with the control mice (CG and CCR3^{-/-}CG). However, in the CCR^{-/-}AR mice, IFN- γ expression levels were significantly increased following compared with CCR^{+/+}AR model mice, which indicated that the CCR3 knockout may alleviate the inflammatory response

in the genesis and development of AR by upregulating IFN- γ expression.

In conclusion, CCR3 gene knockout was demonstrated to reduce the number of invading EOS and the inflammatory response in the AR mice by downregulating the expression of EPO, ECP, MBP, IL-4, and IgE and upregulating the expression of IL-10 and IFN- γ . Regulators and modulators of CCR3, eosinophil granule proteins, and immune factors may potentially be developed as drugs to treat AR in clinic. These results may serve as guidelines for the design and development of promising drugs and strategies to treat allergic diseases such as AR. It should be noted that the results of the present study may require further validation in larger animals with a large-scale investigation in the future.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

XZ, KL, JW and YL designed the study and wrote the manuscript. HP, QP, SW and YJ collected and analyzed the data. All authors performed the experiments.

Ethics approval and consent to participate

The study protocol was reviewed and approved by The Institutional Animal Care and Use Committee, Nanchang University, [Jiangxi, China; approval number YanLinShen (2013) No. 16] and was conducted in accordance with the guidelines established by the Chinese Council of Animal Care (10).

Patient consent for publication

Not applicable.

Competing interests

The authors declare they have no competing interests.

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