Downregulation of mouse CCR3 by lentiviral shRNA inhibits proliferation and induces apoptosis of mouse eosinophils

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Abstract. RNA interference has been considered as an effective gene silencing method in basic and preclinical investigations. The aims of the present study were to construct a lentiviral vector expressing a short hairpin RNA (shRNA) targeting the murine CC chemokine receptor 3 (mCCR3), and to investigate its effects on the proliferation and apoptosis of mouse eosinophils. A recombinant lentiviral vector expressing four fragments of mouse CCR3 shRNA (pLVX-mCCR3-1+2+3+4-shRNA) was constructed using subcloning techniques. This novel lentivirus was then packaged into 293T cells by co-transduction with plasmids, including Baculo p35, pCMV R8.2 and VSV. The interference effects of the vector were verified using polymerase chain reaction (PCR) and western blot analyses. The effects of the interference on the proliferation and apoptosis of mouse eosinophils were investigated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium and terminal deoxynucleotidyl transferase dUTP nick end labeling methods, respectively. The results of the PCR and western blot analyses confirmed that the novel recombinant vector, pLVX-mCCR3-1+2+3+4-shRNA, had high efficiency in inhibiting the mRNA and protein expression levels of mCCR3 in mouse eosinophils. The downregulation of mCCR3 significantly inhibited proliferation of the eosinophils. Furthermore, the present study found that the downregulation of mCCR3 significantly promoted apoptosis of the eosinophils. Therefore, the downregulation of mCCR3 led to the inhibition of proliferation and induction of apoptosis in mouse eosinophils. The predominant characteristics of allergic rhinitis are eosinophil infiltration and release of inflammatory mediators, which appear in a variety of clinical manifestations. The results of the present study indicate that mCCR3 silencing may serve as a putative approach for the treatment of allergic rhinitis.

Introduction

Allergic rhinitis (AR) is a common global health problem, which has a severe affect on daily life. The morbidity rate of AR has increased in previous decades and affects 10-20% of the population in western countries (1). A report from 2014 from Beijing Tongren Hospital on the prevalence of allergic rhinitis in China showed that the morbidity rate of AR in China is also increasing, as is the prevalence of a 'western'-type lifestyle (2).

AR has been identified as a chronic inflammatory disease of the nasal mucosa, which is characterized by symptoms, including sneezing, watery rhinorrhea, nasal obstruction and nasal itching. Eosinophils have long been considered to be the prominent effective cells in allergic inflammation, and eosinophilia has been suggested to favor the development of allergy (3-5). Although the mechanisms underlying the pathogenesis and regulation of AR have been thoroughly investigated, current treatments can only relieve its symptoms. There is currently no treatment method able to cure AR, therefore, additional approaches are required for AR treatment. Genetic therapy offers a promising approach in treating patients with AR.

In response to a variety of stimuli, eosinophils are released from bone marrow to inflammatory tissues through cell surface receptors (4). In particular, the CC chemokine receptor 3 (CCR3), which is a cell-surface guanosine-binding protein-coupled receptor containing a typical motif of seven hydrophobic regions, is primarily expressed on the cell surface of eosinophils. It has been reported that CCR3 is activated in response to eotaxin and contributes to G-protein-dependent intracellular signaling cascades, which leads to the migration of eosinophils (6,7). The importance of CCR3 signaling in allergy was demonstrated previously in studies involving CCR3-deficient mice, which exhibited reduced Th2 responses and an absence of eosinophilia upon allergen sensitization and challenge (8,9). In addition, previous studies have suggested that anti-CCR3 antibody inhibits eosinophil infiltration in animal models and human samples (10,11). Thus, the direct inhibition of CCR3 may serve as a novel approach to effectively alleviate eosinophilia in AR.

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The first case of RNA interference was reported in *Caenorhabditis elegans* as an endogenous defense mechanism by Fire *et al* in 1998 (12). RNA interference is an effective gene silencing method, achieved through the transduction of either small interfering RNA (siRNA) or short hairpin RNA (shRNA) (13). Using siRNA or shRNA, rather than oligonucleotide antisense and antibody inhibition, appears to be a more efficient and long-lasting approach to inhibit certain cellular functions due to its ability to target mRNA and affect protein expression in cells (14). Synthetic siRNAs can reduce gene expression, however, this is transient and dose-dependent. By contrast, shRNA can be continuously expressed in cells and then processed by Dicer into siRNA targeting desired genes (15). shRNA carried by a lentivirus can integrate into the host genome and silence gene expression permanently (16). In the present study, the shRNA and lentiviral delivery approach was used for the construction of a mouse CCR3-shRNA-expressing lentiviral vector. CCR3 gene silencing is able to reduce the proliferation of eosinophils and promote eosinophil apoptosis, thereby reducing eosinophil infiltration, and alleviating the symptoms of allergic rhinitis. Therefore, the present study evaluated the effects of this vector on the proliferation and apoptosis of eosinophils.

Materials and methods

Animals. Male BALB/c mice (5-6-week-old) were maintained on an ovalbumin-free diet under pathogen-free conditions in our animal experimental institute (the Medical Laboratory Animal Center of Nanchang University) at room temperature (22-24˚C) with a 12-h dark:light cycle. The study protocol was approved by the institutional Animal Care and Use Committees of Nanchang University School of Medicine (Nanchang, China). The present study was performed in accordance with the ethical guidelines of Directive 2010/63/EU (Comments on the European Directive 2010/63/EU for the Protection of Laboratory Animals - see http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm).

Culture of bone marrow-derived eosinophils. The eosinophils originating from bone marrow pluripotent hematopoietic stem cells were collected from the femurs and tibias of wild-type BALB/c mice (Laboratory Animal Centre of Nanchang University School of Medicine), as described previously (17). Briefly, the BALB/c mice were sacrificed by cervical dislocation. The separated femurs and tibias were soaked in 75% ethanol for 5 min, rinsed with 2X phosphate-buffered saline (PBS) and then the ends of the femurs and tibias were cut off. The bone marrow was flushed out with Dulbecco's modified Eagle's medium (DMEM) and collected in a plate. A single cell suspension of the bone marrow was obtained by filtering the bone marrow through a syringe with size 7 and size 4 needles. Red blood cell pyrolysis liquid was added to the single cell suspension to eliminate red blood cells, and the single cell suspension was centrifuged at 1,500 rev./min for 10 min. The supernatant was discarded. A cell layer containing eosinophils and eosinophil stem cells was cultured in eosinophil basic culture medium, RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), which was supplemented with 20% fetal

bovine serum (FBS; Hyclone; GE Healthcare Life Sciences), 2 mM L-glutamine (Hyclone; GE Healthcare Life Sciences), 50 μM β-mercaptoethanol (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10 μg/ml streptomycin, 100 IU/ml penicillin (Hyclone; GE Healthcare Life Sciences), 25 mM HEPES, 1 mM sodium pyruvate and 1X non-essential amino acids (Gibco; Thermo Fisher Scientific, Inc.). Following resuspension, the cells were cultured with the above medium pulsed with 100 ng/ml FMS-related tyrosine kinase 3 ligand (FLT3-L; PeproTech, Inc., Rocky Hill, NJ, USA) and 100 ng/ml stem-cell factor (SCF; PeproTech, Inc.) for 4 days. On day 4, 10 ng/ml recombinant mouse interleukin-5 (rmIL-5; PeproTech, Inc.) was added to replace the FLT3-L and SCF. The cells were then cultured in medium containing rmIL-5 for 10 days. All cell cultures (10^7/ml) were incubated at 37˚C in humidified air with 5% CO_2. The eosinophils were identified by hematoxylin and eosin staining for subsequent viral infection.

Construction of the murine CCR3 (mCCR3)-shRNA-expressing lentiviral vector. The mCCR3 sequence was obtained from GeneBank (accession no. NM_009914.4). Our previous studies revealed that the formed shuttle plasmid (pLVX-mCCR3-1+2+3+4-shRNA) had a more marked effect on silencing the CCR3 gene (18,19). In the present study, four short tandemly arranged fragments of CCR3 shRNA were subcloned into lentiviruses to form pLVX-mCCR3-1+2+3+4-shRNA (18). This lentiviral vector can deliver a substantial quantity of viral RNA into the DNA of the host cell, and this viral RNA can be then integrated into the DNA of host cells. According to the mCCR3 sequence, four pairs of primers of CCR3 shRNA were designed to amplify four CCR3 shRNA fragments by polymerase chain reaction (PCR). The primer sequences are summarized in Table I. The PCR reaction comprised (in a total volume of 50 μl): 5 μl 10X short hairpin (sh)DNA annealing buffer, 5 μl sense and antisense strands (100 μM) and 35 μl double distilled H_2O. The PCR cycling steps were 95˚C for 5 min; 85˚C for 5 min; 75˚C for 5 min and 70˚C for 5 min; and samples were stored at 4˚C.

pGenesil1.1, pGenesil1.2, pGenesil1.3 and pGenesil1.4 were used as vectors for subcloning the four fragments described above. The restriction enzyme, *Bsa*I (New England BioLabs, Inc., Ipswich, MA, USA), was utilized to cut pGenesil1.1 (bp 2101-2799), pGenesil1.2 (bp 1853-2551), pGenesil1.3 (bp 2103-2801) and pGenesil1.4 (bp 1880-2578). The four fragments of CCR3 shRNA were ligated to the four cut pGenesil vectors using T4DNA ligase separately. The pGenesil vector containing the mCCR3 shRNA was then transformed into recombinant cells, replicated as the recombinant cells proliferated and was extracted from the recombinant cells using a DNA extraction kit. Sequence correct pGenesil-mCCR3-1-shRNA and pGenesil-mCCR3-2-shRNA were cut using *Hind*III and *Bam*H I enzymes. The large cut fragment of pGenesil-mCCR3-1-shRNA containing the promoter and mCCR3-1-shRNA was ligated with the small fragment of pGenesil-mCCR3-2-shRNA, (280 bp mCCR3-2-shRNA), to construct pGenesil-mCCR3-1+2-shRNA. Similarly, sequence correct pGenesil-mCCR3-3-shRNA and pGenesil-mCCR3-4-shRNA were cut using *Eco*RI and *Sal*I. The large cut fragment of pGenesil-mCCR3-3-shRNA containing

Table I. Primers for CCR3 short hairpin RNA amplification.

mCCR3-1F	5'-CACCGGTTGTGTTGATCCTCATAAATTCAAGAGATTTATGAGGATCAACACAACCTTTTTTG-3'
mCCR3-1R	5'-AGCTCAAAAAAGGTTGTGTTGATCCTCATAAATCTCTTGAATTTATGAGGATCAACACAACC-3'
mCCR3-2F	5'-TTTGGCTGACAATTGACAGATACCTTTCAAGAGAAGGTATCTGTCAATTGTCAGCTTTTTTG-3'
mCCR3-2R	5'-AGCTCAAAAAAGCTGACAATTGACAGATACCTTCTCTTGAAAGGTATCTGTCAATTGTCAGC-3'
mCCR3-3F	5'-CCTCGCAGCATTGCCTGAATTTATCTTCAAGAGAGATAAATTCAGGCAATGCTGCTTTTTTG-3'
mCCR3-3R	5'-AGCTCAAAAAAGCAGCATTGCCTGAATTTATCTCTCTTGAAGATAAATTCAGGCAATGCTGC-3'
mCCR3-4F	5'-TCCCGACCACACCCTATGAATATGATTCAAGAGATCATATTCATAGGGTGTGGTCTTTTTTG-3'
mCCR3-4R	5'-AGCTCAAAAAAGACCACACCCTATGAATATGATCTCTTGAATCATATTCATAGGGTGTGGTC-3'

mCCR3, murine CC chemokine receptor 3; F, forward; R, reverse.

the promoter and mCCR3-3-shRNA was ligated with the small cut fragment of pGenesil-mCCR3-4-shRNA (380 bp mCCR3-4-shRNA) to form pGenesil-mCCR3-3+4-shRNA. pGenesil-mCCR3-1+2-shRNA and pGenesil-mCCR3-3+4-shRNA were cut using *Bam*H I and *Sal*I. The large cut fragment of pGenesil-mCCR3-1+2-shRNA containing the promoter and mCCR3-1+2-shRNA was ligated with the small cut fragment of pGenesil-mCCR3-3+4-shRNA to form pGenesil-mCCR3-1+2+3+4-shRNA. To construct the lentiviral mCCR3-1+2+3+4-shRNA, a pLVX-shRNA2-m lentiviral vector was used. The pLVX-ShRNA2-m vector (Biowit Technologies Ltd., Quincy, MA, USA) was cut using *Mlu*I and *Xho*I enzymes. pGenesil-mCCR3-1+2+3+4-shRNA were cut using *Mul*I and *Sal*I enzymes. The large fragment of the cut pLVX-shRNA2-m containing a promotor was ligated with the small fragment of the cut pGenesil-mCCR3-1+2+3+4-shRNA, to form the target plasmid, pLVX-mCCR3-1+2+3+4-shRNA. Validation of the sequences of mCCR3-1+2+3+4-shRNA were confirmed using DNA sequencing and are shown in Table II.

Packaging of the pLVX-ShRNA2-m vector and the constructed pLVX-mCCR3-1+2+3+4-shRNA plasmid, and the culture of eosinophils with viral infection. To ensure the safety and the titer of the pLVX-ShRNA2-m vector and the constructed pLVX-mCCR3-1+2+3+4-shRNA, these two lentiviruses were separately co-transfected with the packaging plasmids, Baculo p35, pCMV R8.2 and VSV (quantities: 2 μg Baculo p35, 2 μg VSV plasmid, 4.7 μg pCMV R8.2 plasmid and 2.3 μg Lentiviral vector), into 293T cells in 100-mm tissue culture dishes of DMEM containing 10% FBS without antibiotics at 37°C. The medium was replaced 24 h later, and the virus-containing medium was harvested 48 h following transduction. The supernatants were filtered through a 0.22 μm syringe filter (EMD Millipore, Billerica, MA, USA). The eosinophil cultures on day 10 were infected with the supernatants at a multiplicity of infection of 50, and polybrene (Sigma-Aldrich; Thermo Fisher Scientific, Inc.) was added to a final concentration of 8 μg/ml. The harvested eosinophils were transfected with either a blank control (RPMI 1640 medium), empty vector (pLVX-shRNA2-m) or the constructed target plasmid (pLVX-mCCR3-1+2+3+4-shRNA), respectively. The culture medium was aspirated 48 h following transduction and the cells were washed with PBS for the subsequent quantitative

Table II. Sequences of four shRNAs for plasmid construction

mCCR3-1 shRNA	5'-GGTTGTGTTGATCCTCATAAA-3'
mCCR3-2 shRNA	5'-GCTGACAATTGACAGATACCT-3'
mCCR3-3 shRNA	5'-GCAGCATTGCCTGAATTTATC-3'
mCCR3-4 shRNA	5'- GACCACACCCTATGAATATGA-3

mCCR3, murine CC chemokine receptor 3; shRNA, short hairpin RNA.

(q)PCR and western blot analyses, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT) assays.

qPCR analysis. The eosinophils were suspended in TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and RNA was extracted according to the manufacturer's protocol. The RNA was converted into cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The cDNA was used with SYBR® Green qPCR SuperMix (Invitrogen; Thermo Fisher Scientific, Inc.). For the detection of CCR3, GAPDH was used as a control. The primers of CCR3 (ID: NM_009914.4) and GAPDH (ID: NM_017008.4) were designed as follows: CCR, forward 5'-CTG GCA CAC AGA CCC TAG AA-3' and reverse 5'-TTG AGT CTC TGA ACG CAT CA-3'; and GAPDH, forward 5'-GGC CTC CAA GGA GTA AGA AA-3' and reverse 5'-GCC CCT CCT GTT ATT ATG G-3'. The total reaction mixture was run on a 7500 Real-Time system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with relative quantitation according to the manufacturer's protocol. The following thermocycling steps were used: 95°C denaturation for 10 sec, one cycle; 95°C denaturation for 5 sec; 54°C annealing extension for 30 sec, a total of 40 cycles; 95°C for 1 min, one cycle, and 55°C for 30 sec, 41 cycles.

Western blot analysis. The eosinophils were homogenized in RIPA lysis buffer (Pierce Biotechnology, Inc., Rockford, IL, USA). After 20 min on ice, insoluble materials were removed by centrifugation at 4°C at 14,000 x *g*. The supernatants were mixed with SDS sample buffer and boiled for
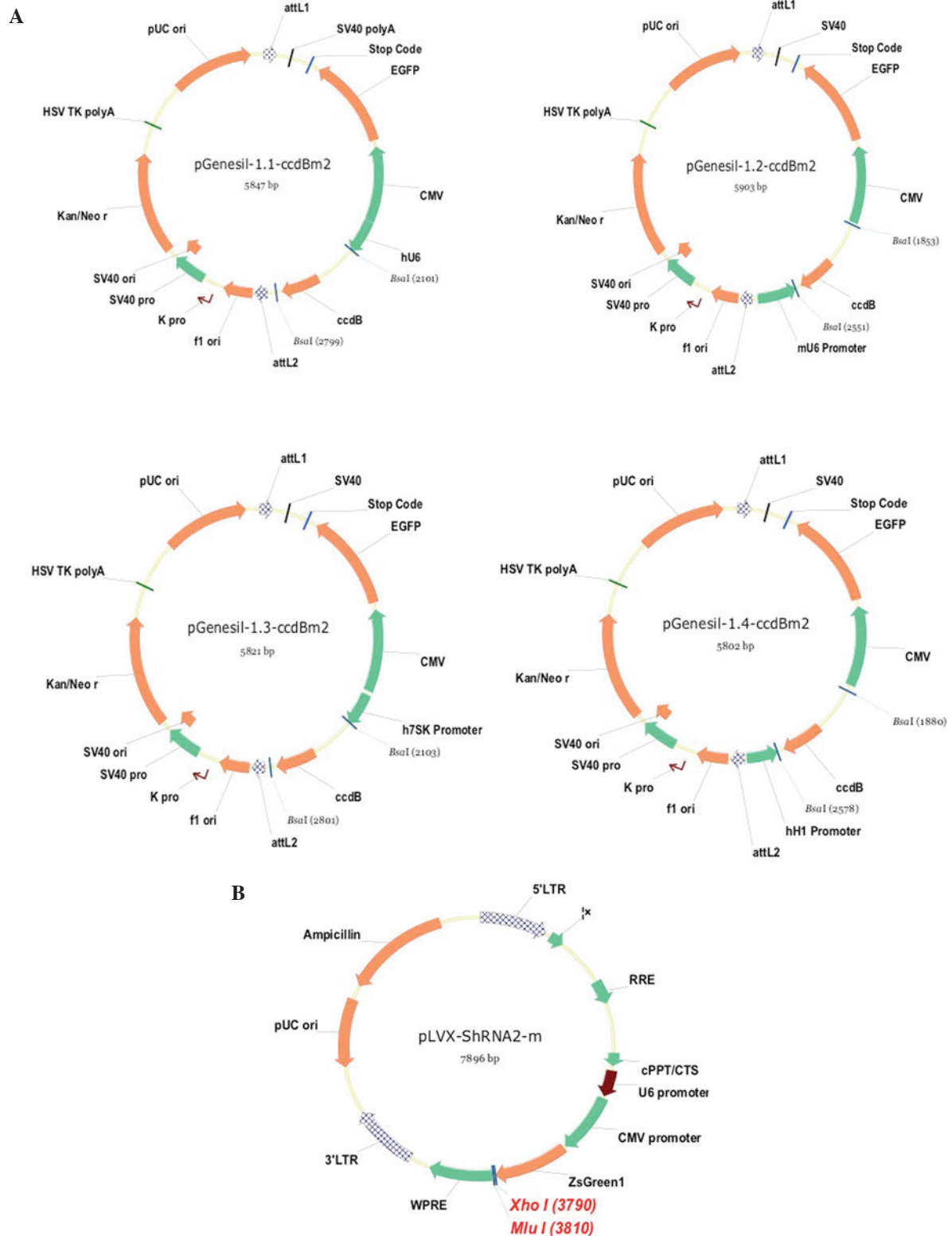
Figure 1. (A) Structures of pGenesil vectors. (B) Schematic structure for engineering of the pLVX lentiviral vector. A four-tandem-fragment of CCR3-1+2+3+4-shRNA was inserted between aa3790 and aa3810 to form the pLVX-CCR3-1+2+3+4-shRNA plasmid, which was ampicillin resistant and carried the green fluorescent protein gene. CCR3, murine CC chemokine receptor 3; shRNA, short hairpin RNA.

5 min. The proteins were separated on SDS-polyacrylamide (10%) gels, following which they were blotted onto PVDF membranes (EMD Millipore). Non-specific protein binding sites were blocked by incubation with 5% bovine serum albumin in TBST buffer (20 mM Tris-HCl, 137 mM NaCl and 0.05% Tween 20) at pH 7.6 for 1 h, followed by incubation with rabbit polyclonal anti-CCR3 primary antibody (cat. no. AJ1417a; 1:200 dilution; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The membranes were washed three times with TBST, followed by incubated with horseradish

peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. C1508; 1:20,000 dilution; SouthernBiotech, Burmingham, AL, USA) for 1 h at room temperature. The blots were visualized using an enhanced chemiluminescence system (GE Healthcare Life Sciences) according to the manufacturer's protocol. To normalize for protein content, the blots were stripped and stained with GAPDH antibody (cat. no. KC-5G5; 1:10,000 dilution; Abcam, Cambridge, MA, USA) overnight at 4˚C. The concentration of each target protein was normalized against that of GAPDH.

TUNEL assay. Quantitative assessment of apoptosis in the eosinophils was performed using a TUNEL method according to the manufacturer's protocol (Promega, Madison, WI, USA). Briefly, the eosinophils were incubated with either the blank control (RPMI 1640 medium), empty vector (pLVX-shRNA2-m) or the constructed target plasmid (pLVX-mCCR3-1+2+3+4-shRNA), respectively, for 48 h. The cells were then trypsinized, fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton-X-100 in 0.1% sodium citrate. Following washing with PBS, the cells were incubated with the reaction mixture for 60 min at 37˚C. The stained cells were then analyzed using a FACScan cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell proliferation assay. The eosinophils were plated at a density of $1x10^5$ per well (100 μl) in 96-well plates and treated with either blank control (RPMI 1640 medium), empty vector (pLVX-shRNA2-m) or the constructed target plasmid (pLVX-mCCR3-1+2+3+4-shRNA) for different durations (0, 24, 48 and 72 h). The culture media were then removed and the cells were washed with PBS. An MTS assay was performed using a kit from Promega in accordance with the manufacturer's protocol. The absorbance was measured at a fixed wavelength of 490 nm on a microplate reader (VersaMax; Molecular Devices, LLC, Sunnyvale, CA, USA). Each data point was normalized to the value of their corresponding control samples.

Statistical analysis. The results obtained from the blank control, empty vector (pLVX-shRNA2-m) and constructed target plasmid (pLVX-mCCR3-1+2+3+4-shRNA) groups were analyzed using one-way analysis of variance using SPSS 18 software (SPSS, Inc., Chicago, IL, USA). Each experiment was repeated three times. Data are presented as the mean ± standard error of the mean of triplicate samples. P<0.05 was considered to indicate a statistically significant difference.

Results

Construction of the mCCR3-shRNA plasmid. As described in the previous section, mCCR3-1+2+3+4-shRNA, the sequence of which was confirmed using DNA sequencing, was successfully ligated using a sub-cloning technique with the pGenesil1 vectors, pGenesil1.1, pGenesil1.2, pGenesil1.3 and pGenesil1.4 (Fig. 1A). The recombinant fragment of the mCCR3-1+2+3+4-shRNA was successfully inserted into the pLVX-shRNA2-m lentiviral vector, to obtain the pLVX-CCR3-1+2+3+4-shRNA vector (Fig. 1B). This novel

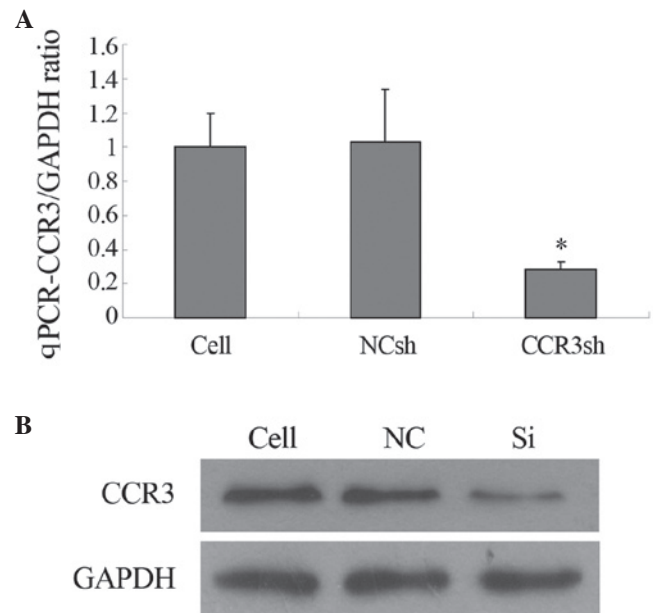
Figure 2. Expression of CCR3. (A) qPCR determination of cellular CCR3/GAPDH ratio. The expression of CCR3mRNA was significantly inhibited by pLVX-shRNA2-mCCR3-1+2+3+4shRNA, but not by the NCsh vector (*P<0.05). (B) Expression of CCR3, determined using western blot analysis. GAPDH, a house-keeping protein was used as a control. The expression of CCR3 was significantly inhibited by pLVX-shRNA2-mCCR3-1+2+3+4shRNA, but not by NC. CCR3, murine CC chemokine receptor 3; shRNA, short hairpin RNA; Cell, blank control (cells only); NCsh/NC, cells transfected with empty vector; CCR3sh/Si, cells transfected with pLVX-shRNA2-mCCR3-1+2+3+4shRNA; qPCR, quantitative polymerase chain reaction.

lentiviral vector was then packaged into 293T cells by co-transfection with the Baculo p35, pCMV R8.2 and VSV packaging plasmids. In a pilot experiment, the highest transduction efficiency of the pLVX-mCCR3-1+2+3+4-shRNA virus was observed at a multiplicity of infection of 10.

Detection of the expression levels of mCCR3 in eosinophils. The preliminary experiment demonstrated that the pLVX-mCCR3-1+2+3+4-shRNA, which contained four different interfering shRNAs against mCCR3, had higher gene silencing efficiency, compared with any single shRNA of mCCR3 in the eosinophils, determined using qPCR. As shown in Fig. 2A, the mRNA level of mCCR3 was significantly inhibited only by transduction with pLVX-mCCR3-1+2+3+4-shRNA. The mRNA levels of mCCR3 were not affected by transduction with the negative control shRNA vector (Fig. 2A). In addition, the protein level of mCCR3 was markedly inhibited by transduction with pLVX-mCCR3-1+2+3+4-shRNA, as determined using western blot analysis. Similarly, the protein expression of mCCR3 was not altered by transduction with the negative control shRNA vector (Fig. 2B).

Silencing of mCCR3 with lentiviral shRNA promotes apoptosis of eosinophils. To investigate whether the downregulation of mCCR3 with lentiviral shRNA can induce apoptosis of eosinophils, the TUNEL method was used. The results showed that <8% of the eosinophils showed apoptosis in the blank control- and empty vector-transduced cells. However, 45% of the pLVX-mCCR3-1+2+3+4shRNA-transduced eosinophils
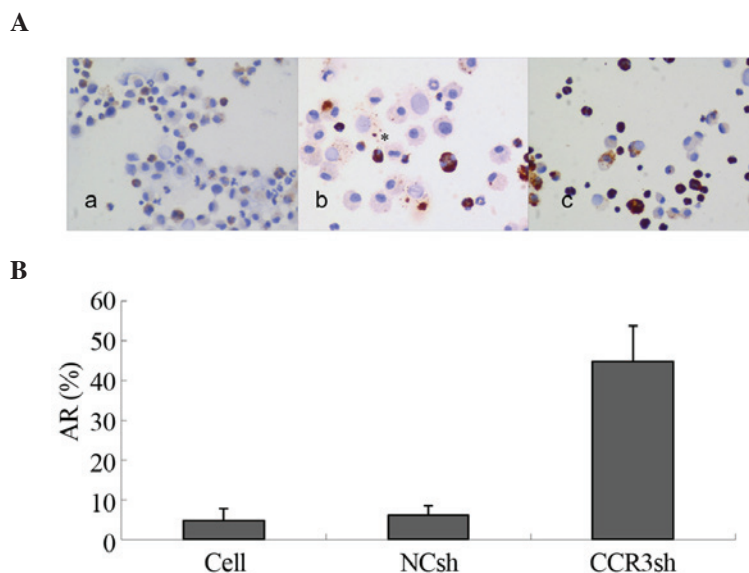
A

B

Figure 3. (A) Terminal deoxynucleotidyl transferase dUTP nick end labeling assay of eosinophil apoptosis. Cells with brown nuclei are apoptotic. (a) blank control cells; (b) cells with empty vector; (c) cells with pLVX-shRNA2-mCCR3-1+2+3+4shRNA. (B) CCR3shRNA promoted cell apoptosis *in vitro*. pLVX-shRNA2-mCCR3-1+2+3+4shRNA induced ~45% apoptosis of cells. CCR3, murine CC chemokine receptor 3; shRNA, short hairpin RNA; AR, apoptotic rate; Cell, blank control (cells only); NCsh/NC, cells transfected with empty vector; CCR3sh/Si, cells transfected with pLVX-shRNA2-mCCR3-1+2+3+4shRNA.
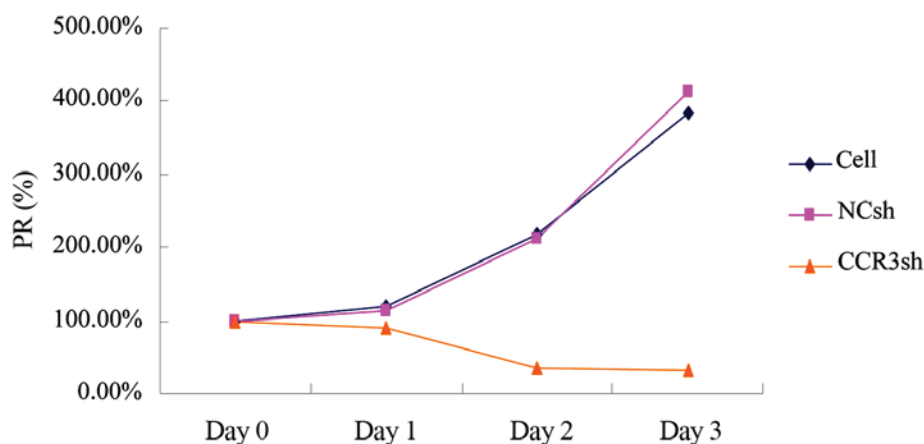

Figure 4. 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay of eosinophil proliferation. CCR3, murine CC chemokine receptor 3; shRNA, short hairpin RNA; PR, proliferation rate; Cell, blank control, cells only; NCsh: cells transfected with empty vector; CCR3sh: cells transfected with pLVX-shRNA2-mCCR3-1+2+3+4shRNA.

exhibited apoptotic characteristics (Fig. 3A and B). This result suggested that expression of mCCR3 was critical to the survival of the eosinophils.

Silencing of mCCR3 with lentiviral shRNA reduces the proliferation of eosinophils. The present study used an MTS assay to investigate the effect of mCCR3-shRNA on the proliferation of eosinophils. The cells were incubated with MTS reagents 0, 24, 48 and 72 post-transduction with virons carrying pLVX-mCCR3-1+2+3+4-shRNA. As shown in Fig. 4, transduction with pLVX-mCCR3-1+2+3+4-shRNA started to inhibit cell proliferation at 24 h (80%), and the cell proliferation rate decreased to 20% at 48 h and to 19% at 72 h (Fig. 4). By contrast, cell proliferation rates in the blank control group and empty vector control group were increased, reaching 200% at 48 h and 400% at 72 h (Fig. 4).

Discussion

Eosinophils are considered to be a critical factor in the induction of inflammation and allergy by releasing reactive oxygen species and cytotoxic molecules, including major basic protein, eosinophilic peroxidase, eosinophil-derived neurotoxin and eosinophil cationic protein (5). Eosinophils develop from CD34^{+} hematopoietic progenitor cells within the bone marrow under the stimulation of cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5 (20). IL-5 is predominantly expressed in white blood cells and is a key modulatory cytokine, which is important in regulating the proliferation, differentiation and activation of eosinophils (21). Allergic IL-5-deficiency in mice leads to reduced numbers of eosinophilia in the bone marrow and blood, and eosinophils are recruited to the tissue in reduced numbers in response to

allergen exposure. However, treating patients with anti-IL-5 monoclonal antibody only partially reduced eosinophilia in airway tissues and bone marrow (22,23), suggesting that other factors contribute to eosinophil survival in these tissues. With the exception of IL-5, GM-CSF and IL-3 have also been known to have growth factor effects on eosinophils (20). The present study revealed that knockdown of CCR3 by specific shRNA efficiently inhibited eosinophil proliferation and promoted eosinophil apoptosis. Although the mechanism underlying these effects were not investigated, there are several possible mechanisms. The FBS used in the culture medium contains the eosinophil-associated growth-factors IL-5, IL-3 and GM-CSF. As CCR3 protein was expressed in the eosinophils in control group, IL-5-, IL-3- and GM-CSF-induced eosinophil growth may require CCR3 in its growth signaling pathway. When CCR3 was silenced by CCR3 shRNA, the growth pathway involving IL-5, IL-3 and GM-CSF was not active, therefore, the proliferation rate of the eosinophils declined rapidly. In terms of why silencing CCR3 resulted in apoptosis, CCR3 may be associated with factors involved in the apoptotic signaling pathway, including p53, p73, B cell lymphoma-2-associated X protein (BAX), phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) and p53 upregulated modulator of apoptosis (PUMA). The present study hypothesized that CCR3 inhibits the above factors and inhibits apoptosis, and when CCR3 was silenced by CCR3 shRNA, the above factors in the apoptotic signaling pathway were activated, causing eosinophils to undergo apoptosis.

In conclusion, using the techniques described, pLVX-mCCR3-1+2+3+4shRNA was successfully constructed in the present study. The results demonstrated that virions pLVX-mCCR3-1+2+3+4shRNA significantly reduced the mRNA and protein expression levels of CCR3, promoted eosinophil apoptosis and inhibited eosinophil proliferation. This may have contributed to the inhibition of eosinophil infiltration in the airway. However, the mechanism underlying the eosinophil apoptosis and proliferation inhibition induced by CCR3 silencing requires further investigation. An understanding of the fundamental causes of regulating eosinophil apoptosis may lead to novel strategies for the treatment of allergic inflammation. Subsequent investigations aim to use a single shRNA-expressing lentiviral vector targeting IL-5 and CCR3 to affect eosinophil infiltration in the airway tissues *in vitro* and *in vivo*.

Acknowledgements

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