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Original Article

CC-chemokine receptor 7 and its ligand CCL19 promote mitral valve interstitial cell migration and repair

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Abstract

The effect of CC-chemokine receptor 7 (CCR7) and CC-chemokine ligand 19 (CCL19) on rheumatic mitral stenosis is unknown. This study aimed to explore the roles of CCR7 and CCL19 in rheumatic mitral stenosis by measuring the expression of CCR7 and CCL19 in human mitral valves from rheumatic mitral stenosis patients. Additionally, we examined their effects on human mitral valve interstitial cells (hMVICs) proliferation, apoptosis and wound repair. CCR7 and CCL19 expression was measured in the mitral valves from rheumatic mitral stenosis patients ($n=10$) and compared to normal mitral valves ($n=5$). CCR7 was measured in cultured hMVICs from rheumatic mitral stenosis patients and normal donors by RT-PCR and immunofluorescence. The cells were also treated with exogenous CCL19, and the effects on wound healing, proliferation and apoptosis were assayed. In the rheumatic mitral valves, valve interstitial cells expressed CCR7, while mononuclear cells and the endothelium expressed CCL19. Healthy mitral valves did not stain positive for CCR7 or CCL19. CCR7 was also detected in cultured rheumatic hMVICs or in normal hMVICs treated with CCL19. In a wound healing experiment, wound closure rates of both rheumatic and normal hMVICs were significantly accelerated by CCL19. These effects were abrogated by a CCR7 neutralizing antibody. The CCR7/CCL19 axis did not influence the proliferation or apoptosis of hMVICs, indicating that wound healing was due to increased migration rates rather than increased proliferation. In conclusion, CCR7 and CCL19 were expressed in rheumatic mitral valves. The CCR7/CCL19 axis may regulate remodeling of rheumatic valve injury through promoting migratory ability of hMVICs.

Keywords: CC-chemokine receptor 7, CC-chemokine ligand 19, rheumatic mitral stenosis, migration, wound repair

Introduction

Although the prevalence of rheumatic fever has greatly decreased in developed countries, the late sequelae of rheumatic fever, rheumatic mitral stenosis, still results in high morbidity and mortality in developing countries[1]. While long-term penicillin treatment can effectively decrease the recurrence of rheumatic fever, it cannot prevent mitral valve pathology that results after rheumatic fever[2]. In response to rheumatic fever, an autoimmune response in the mitral valve damages the endothelial layer, which triggers a subsequent inflammatory response that involves neovascu-

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larization[3], stimulating lymphocyte infiltration into the valve. Even in aged calcified rheumatic valves, lymphocytes and pro-inflammatory cytokines, as well as increased endothelialization, are still present, indicating a persistent state of inflammation in the diseased valves[4-5].

CC-chemokine ligand 19 (CCL19) and its corresponding receptor, CC-chemokine receptor 7 (CCR7), regulate homing of T lymphocytes and dendritic cells[6]. In addition to a role in inflammatory cell trafficking, CCR7 has been implicated in metastatic breast cancer[7] and tissue repair[8]. CCR7 and CCL19 are expressed by mast cells, airway smooth muscle cells, myofibroblasts, and fibroblasts. Furthermore, CCR7 has been shown to mediate cell migration in asthma[9] and is expressed by several mesenchymal cell lines. Valve interstitial cells are the predominant mesenchymal cell type found in the 3 layers of the heart valve. In addition, valve interstitial cells are both fibroblasts and myofibroblasts, and are very important in control of heart valve homeostasis and diseased valve repair or remodeling[10-11]. Since significant lymphocyte infiltration occurs during all the stages of rheumatic mitral stenosis[12-13], we hypothesized that CCR7 and CCL19 may regulate the pathological behavior of human mitral valve interstitial cells (hMVICs), which are involved in chronic remodeling of stenotic mitral valves. In this study, we investigated the expression of CCR7 and CCL19 in rheumatic mitral stenosis mitral valves and whether the CRR7/CCL19 axis mediated hMVIC function.

Materials and methods

Ethics statement

This study was approved by the Ethics Committees of the authors' affiliated institution (Protocol No. 2011-SRFA-057) and conformed to the principles outlined in the Declaration of Helsinki. Written consent was obtained from all participants or their parents involved in this study.

Sample collection, cell isolation and culture

Rheumatic stenotic mitral valves were obtained from 10 patients who underwent surgical valve replacement for rheumatic mitral stenosis (3 males and 7 females with a mean age of 48 ± 7 years). Normal mitral valves were obtained from 5 healthy donor hearts following the donors' deaths in traffic accidents (2 males and 3 females with a mean age of 37 ± 6 years).

Normal valves and non-calcific rheumatic stenotic valves were used for cell isolation. hMVICs were isolated by collagenase digestion as previously described[14], and then were cultured in Dulbecco's modified eagle medium (DMEM; Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/mL penicillin, 100 mg/mL streptomycin, and 4 mmol/L L-glutamine. Primary hMVICs were phenotypically examined by immunostaining with primary antibodies against vimentin (Abcam, ab45939) and α-smooth muscle actin (Cy3 conjugated, Sigma, St Louis, MO, USA, C6198). Following incubation with the primary antibodies, sections were incubated with a secondary Dylight TM488-conjugated donkey anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratory, West Grove, PA, USA). Cells from passages 3 to 5 were used for all experiments.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed using the ImmunoCruz™ mouse ABC Staining System (Santa Cruz Biotechnology, Santa Cruz, CA, USA, sc-2017). The same leaflets of the mitral valve from the rheumatic mitral stenosis and normal groups were fixed in 4% paraformaldehyde for 17 hours and mounted in paraffin. Sections (4 μm) were pre-incubated with 10% hydrogen peroxide after re-hydration, and then treated with 10% normal goat serum after antigen retrieval (0.1% trypsin at 37°C for 30 minutes). The sections were incubated with antibodies against CCR7 (monoclonal mouse anti-CCR7 antibody; Santa Cruz Biotechnology, sc-73846) or CCL19 (monoclonal mouse anti-human CCL19 antibody; R&D Systems, Abingdon, UK, MAB361) for 90 minutes at 37°C. Secondary antibodies were HRP conjugated donkey anti-mouse IgG or donkey anti-goat IgG (Santa Cruz Biotechnology). The slides were rinsed in PBS (pH 7.4) 3 times after each incubation step. Finally, the slides were counterstained with hematoxylin, mounted, and observed under the light microscope.

For immunofluorescence, hMVICs were grown to confluence on glass coverslips, fixed in 4% formaldehyde for 10 minutes, lysed with 0.3% Triton X-100 (Promega) for 10 minutes at room temperature, and incubated with a mouse anti-CCR7 antibody overnight at 4 °C. The next day, hMVICs were incubated with a Dylight TM488-conjugated donkey anti-mouse IgG antibody (Jackson), and then counterstained with DAPI (Sigma). Staining was detected by fluorescent microscopy at same exposure time and CCR7 positive cell number and total cell number were calculated using Image Pro Plus 6.0 software.

Quantitative PCR

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed by formaldehyde agarose gel electrophoresis and was quantified spectrophotometrically. RNA was reverse transcribed using the Transcriptor Strand cDNA

Synthesis kit (Roche, Version 6.0). The following primers for CCR7 q-PCR were used: forward 5′-TGAGGTCACGGACGATTACAT-3′, reverse 5′-TGGAGGACAGTGAAGAAAACG-3′ and the amplicon length was 143 bp. Real-time PCR was performed using 2x SYBR Green®PCR Master Mix on an AB 7900 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Each sample was analyzed in triplicate, and target genes were normalized to the reference housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Fold differences were then calculated for each treatment group using normalized CT values for the control. PCR products were additionally assessed by agarose gel electrophoresis, and then analyzed spectrophotometrically.

Wound healing assays

hMVICs were grown in 6-well culture dishes. After reaching confluency, a flat cell scraper (width 1 mm) was used to create a linear wound across the confluent monolayer. The wounded monolayer was then washed 3 times with standard medium to remove cell debris. Immediately after wounding, cells were treated with 100 or 200 ng/mL CCL19 (R&D, 361-MI-025), with or without 15 μg/mL anti-CCR7 neutralizing antibody (R&D, MAB197). Cells were subsequently fixed with 4% paraformaldehyde 0, 24 or 48 hours after wounding and CCL19 treatment. The width between the base of each wound edge was calculated as previously described[15] for assessment of migration rate. In all conditions, experiments with 3 sets of cultured dishes for each treatment were conducted in at least triplicate.

Proliferation assay

Cell proliferation was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were seeded onto 3 microtiter plates at a density of 3,000 cells per 100 μL per well. After 24 hours of serum starvation, cells were incubated for 24, 48 or 72 hours with recombinant CCL19 (R&D, 361-MI-025) at various concentrations. DMEM alone served as the negative control. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) reagent (20 μL) was added to each well and incubated at 37 °C for 3 hours. Absorbance was recorded at 490 nm with a microplate reader (ELx800, Bioteke, Beijing, China). All experiments were repeated 3 times.

Apoptosis assay

Cells were seeded onto 6-cm culture dishes. After 24 hours of serum starvation, cells were incubated for 24 and 48 hours with CCL19 (R&D, 361-MI-025) at 100 ng/mL and 200 ng/mL. DMEM alone served as controls. Then, cells were respectively harvested using trypsin/EDTA, washed with PBS, resuspended in 1 mL binding buffer, and stained with 10 μL annexin V-FITC and 10 μL propidium iodide (PI) at room temperature for 15 minutes (Biovision, CA, USA). Fluorescence of FITC and propidium iodide was analyzed using flow cytometry as previously described[16].

Statistical analysis

Statistical analysis was performed using SPSS 17.0. Data were expressed as mean ± SD. For continuous variables, differences of mean values between the RMS and the normal groups were tested using Student's *t*-test, and for categorical variables Chi-square test was used to compare proportions. One-way ANOVA was performed to compare non-treated and treated experiments, and $P<0.05$ was considered statistically significant.

Results

Baseline characteristics of study patients

Diagnosis of 10 enrolled rheumatic mitral stenosis patients met the updated Jones criteria[17]. The clinical characteristics of all patients are summarized in ***Table 1***. The rate of atrial fibrillation was 80%. The mean valve orifice area and mean left atrial diameter were 0.91 cm^2 ± 0.05 cm^2 and 48.3 mm ± 1.0 mm, respectively.

CCR7 and CCL19 expression in the rheumatic mitral valves

All valves from patients with rheumatic mitral stenosis demonstrated marked hypercellularity. CCR7 and CCL19 were not observed in explanted normal mitral valves (***Fig. 1A*** and ***C***). Valve interstitial cells within the rheumatic valves expressed CCR7 (***Fig. 1B***). CCL19 was also highly expressed in the rheumatic

Table 1 **Baseline characteristics of the rheumatic mitral stenosis subjects**

Variables	RMS (n=10)
Age (years)	48 ± 7
Gender (female/male)	7/3
History of RF (n)	10
Valve orifice area (cm^2)	0.91 ± 0.05
Left atrial diameter (mm)	48.3 ± 1.0
Atrial fibrillation (n)	8

NOTE: some data expressed as mean ± SD. RF: rheumatic fever; RMS: rheumatic mitral stenosis

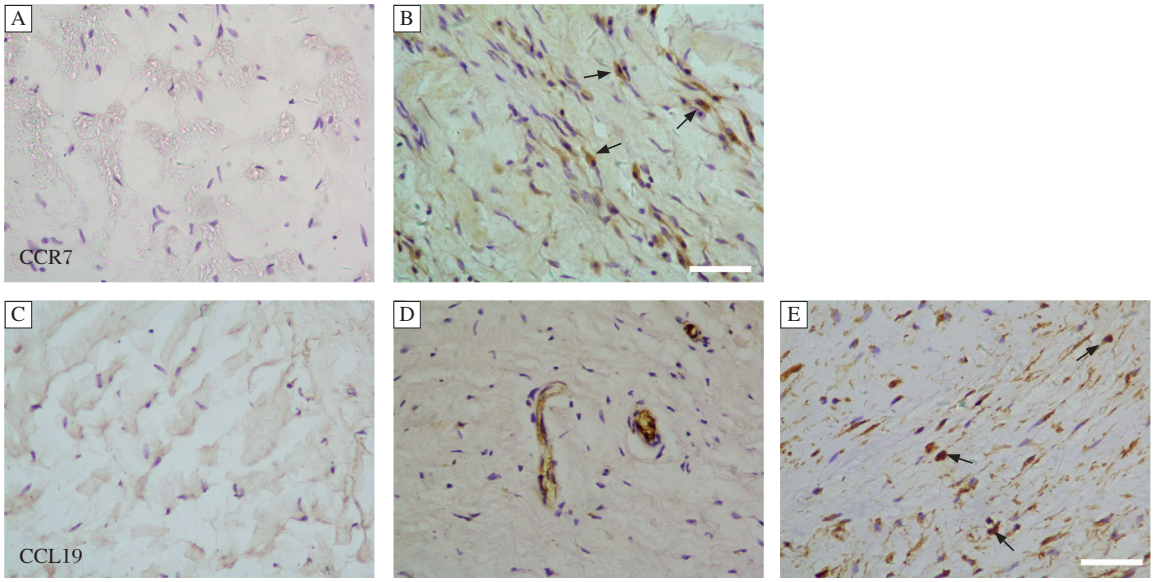

***Fig. 1* Representative photomicrographs of CCR7 and CCL19 expression in mitral valves.** (A) CCR7 was not found in normal mitral valves. (B) CCR7 was expressed by hMVICs in mitral valves of RMS (arrow). (C) CCL19 was not found in normal mitral valves. (D) CCL19 was also observed in the endothelium of vessels in mitral valves of RMS. (E) CCL19 was expressed by mononuclear cells in mitral valves of RMS (arrow). The scale bar is 50 μM.

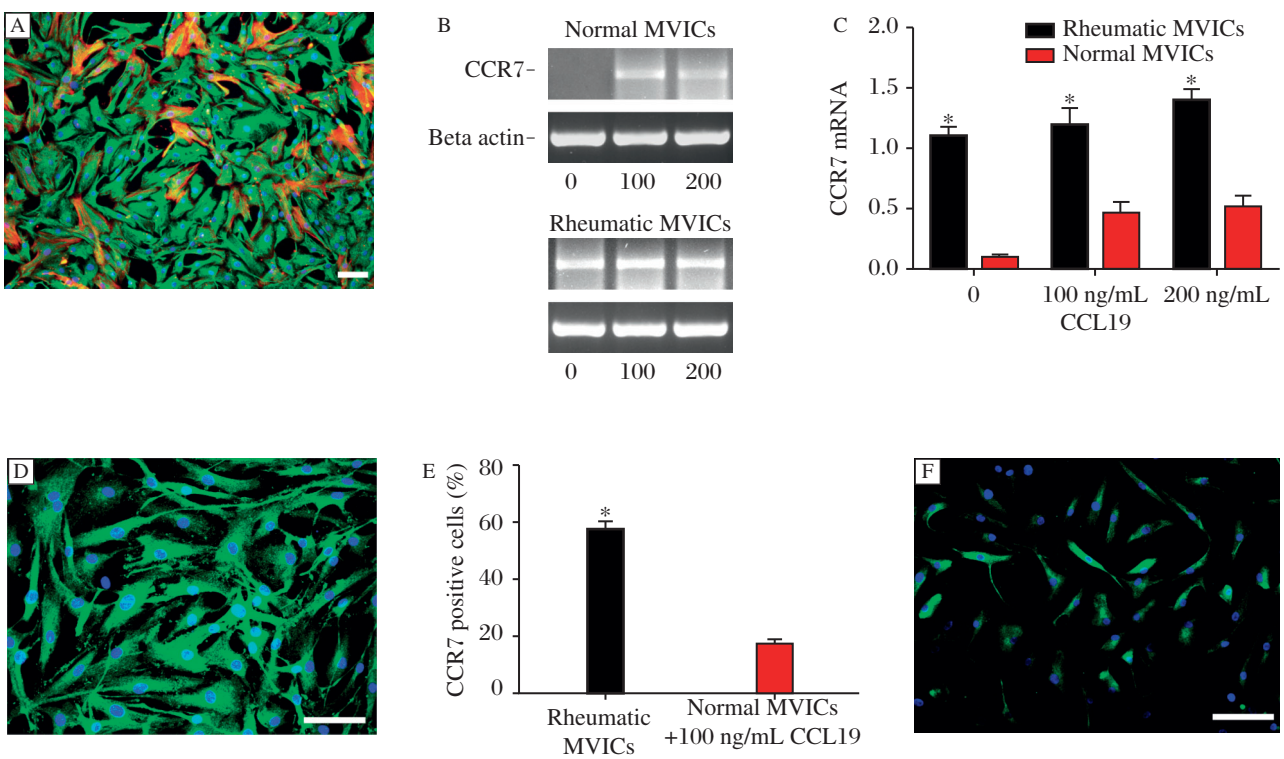

***Fig. 2* CCR7 expression in cultured hMVICs**. (A) Representative photomicrograph of rheumatic hMVICs. Immnofluorescent staining showed that hMVICs consisted of vimentin positive (green) fibroblastic cells and vimentin and a-SMA double positive (red) myofibroblasts. (B) CCL19 stimulation was required before CCR7 was observed in normal hMVICs, while rheumatic hMVICs showed positive CCR7 staining without additional CCL19 stimulation. (C) The CCR7 mRNA level of rheumatic hMVICs was higher than that of normal hMVICs, but the concentration of CCL19 did not alter the level of CCR7 expression. $*P < 0.05$. (D-F) CCR7 expression was confirmed by immunofluorescence in rheumatic hMVICs and 100 ng/ml CCL19 treated normal hMVICs. The more CCR7 positive cells were observed in rheumatic group even without CCL19 stimulation. $*P < 0.05$. The imaging photos are captured at the same exposure time. The scale bar is 100 μM.

valves, but was localized to the endothelium of vessels, infiltrated mononuclear cells, and the extracellular matrix (***Fig. 1D-E***).

CCR7 is expressed by cultured hMVICs *in vitro*

hMVICs isolated from the rheumatic mitral valves were similar to those from normal valves. Both groups of cells were positive for vimentin and α-SMA, consistent with the appearance of spindle and polygon fibroblast or myofibroblast cells (***Fig. 2A***). CCL19 was not expressed in cultured hMVICs from either the rheumatic or normal valves.

Quantitative PCR assays showed that CCR7 was expressed in rheumatic hMVICs, but not in normal hMVICs at baseline. Interestingly, normal hMIVCs expressed CCR7 after treatment with CCL19 (***Fig. 2B***). CCR7 mRNA level of rheumatic hMVICs was higher than that of normal hMVICs ($P<0.05$, ***Fig. 2C***). Immunostaining also confirmed the PCR results, as the fluorescent intensity of CCR7 in hMIVCs from the rheumatic valves was more intense than from normal valves ($P<0.05$, ***Fig. 2D-F***).

CCL19 promotes wound repair through CCR7 by stimulating migration

Wound healing response of hMVICs was significantly accelerated by treatment with recombinant CCL19. Both rheumatic and normal cells treated with CCL19 for 48 hours showed a significantly greater rate of wound closure than untreated cells (***Fig. 3A-I***). hMVICs treated with a neutralizing antibody against CCR7, regardless of the addition of CCL19, did not show a significant difference in the extent of wound

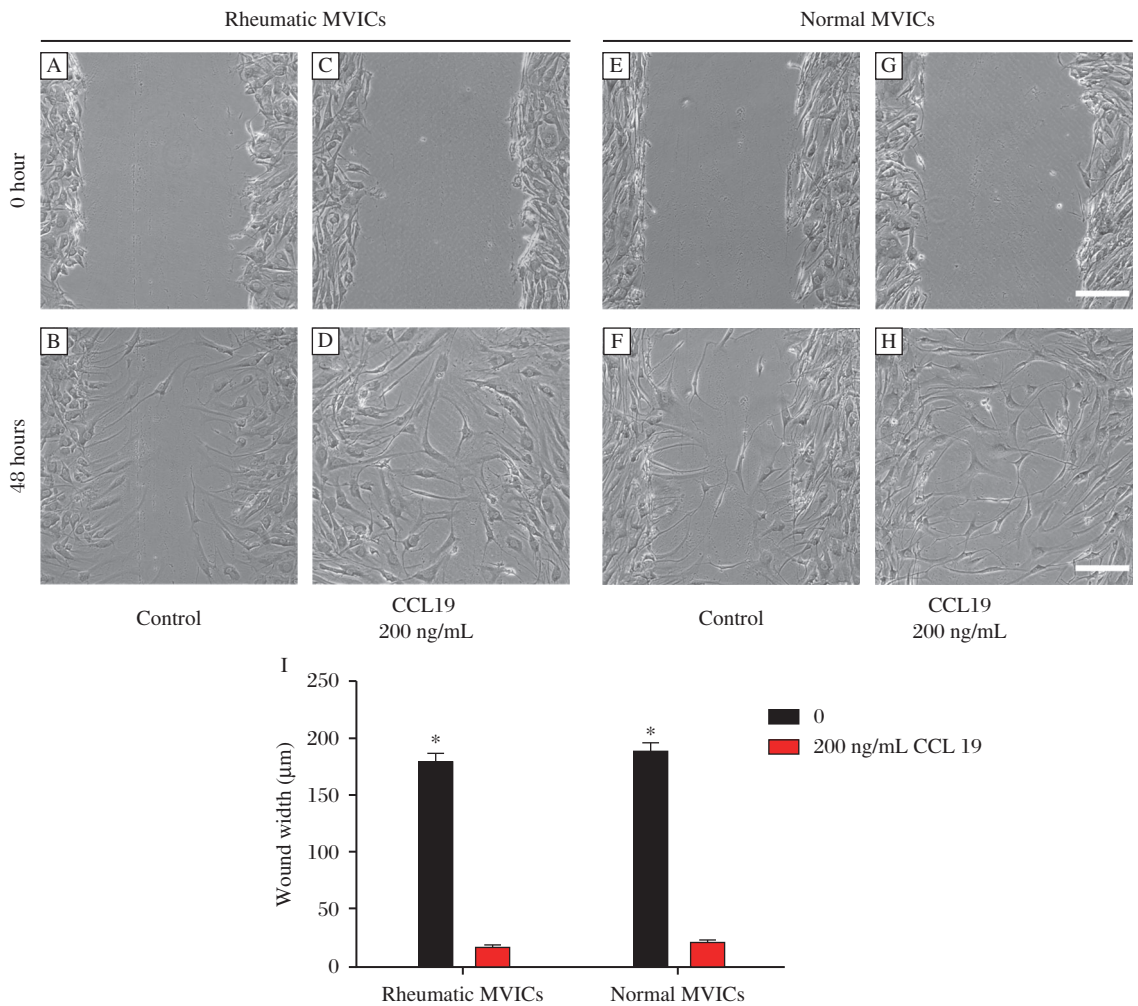

Fig. 3 **CCL19 accelerated wound closure of the wounded hMVICs monolayer.** (A-H) Representative photographs 48 h after wounding the hMVICs monolayer. (A-D, I) In rheumatic hMVICs, at 48 h after wounding, the wound remained unclosed in hMVICs without treatment, but the wound was sparsely closed and had no obvious gaps after 200 ng/ml CCL19 treatment. (E-H, I) In normal hMVICs, similar results with rheumatic hMVICs were observed. There were no significant differences between rheumatic and normal groups. *$P < 0.05$. The scale bar is 200 μM.

closure compared with non-treated cells. The addition of CCL19 to hMVICs treated with a CCR7 neutralizing antibody resulted in a statistically reduced extent of wound closure, as compared to cells treated with CCL19 alone (***Fig. 4A-H***). Interestingly, the addition of 200 ng/mL CCL19 accelerated wound closure at 24 hours compared to cells treated with 100 ng/mL CCL19 in normal cells (***Fig. 4I***).

Since wound closure involves both cell proliferation and cell migration, we measured cell proliferation to determine the impact of CCL19 on cell division. The number of cells, from both the rheumatic and normal valves, cultured for 24, 48 or 72 hours in the presence of CCL19 (1 ng/mL to 1,000 ng/mL) was not significantly different compared to cells cultured in media alone. This result was confirmed with a MTS proliferation assay (***Fig. 5A-B***). To exclude the impact of CCL19 associated apoptosis on cell growth and migration, we additionally measured cell apoptosis in the presence of CCL19 (100 ng/mL and 200 ng/mL). The ratio of apoptotic cells in the CCL19-treated groups slightly decreased at 48 hours, but showed no differences compared to the untreated groups (***Fig. 5C-D***). Collectively, these results indicate that CCL19 exerted effects on migration, rather than proliferation or apoptosis, in wound healing.

Discussion

In this study, we demonstrated that CCR7 and CCL19 were expressed in human rheumatic stenotic mitral valves, but not in normal valves. CCR7 was

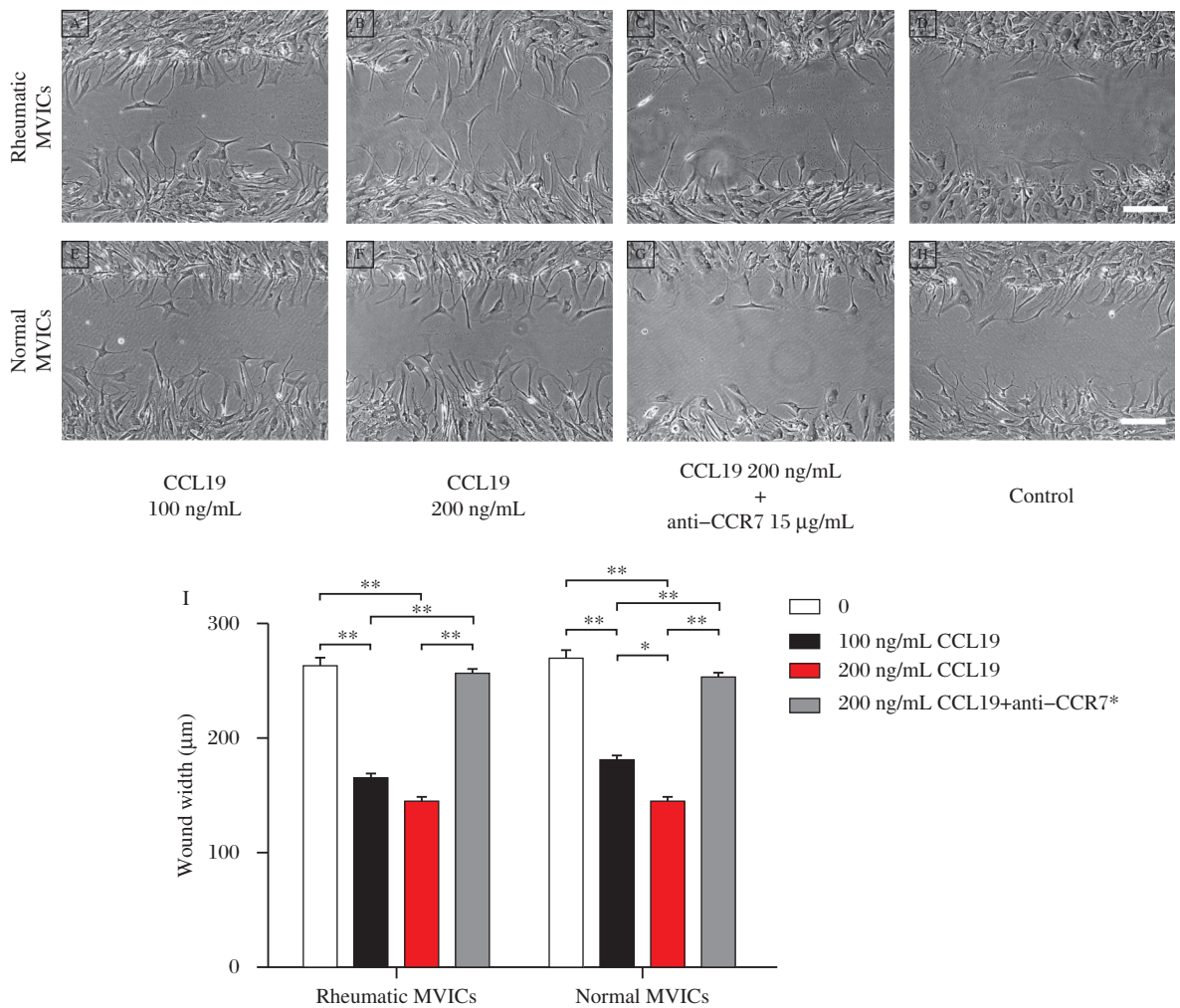

Fig. 4 **CCR7 dependent CCL19 promoted hMVICs migration.** (A-H) Representative photomicrographs of hMVICs 24 h after wounding of the hMVICs monolayer. (A-D, I) In rheumatic groups, the migration rate of CCL19-treated hMVICs significantly increased compared with hMVICs cultured in media alone. There were no statistical differences between the 100 ng/ml and 200 ng/ml CCL19 groups. (E-I) In normal groups, the migration rate of CCL19-treated hMVICs significantly increased when compared with hMVICs cultured in media alone; however, the migration rate of cells treated with 200 ng/ml CCL19 was longer than cells treated with 100 ng/ml CCL19, indicating a dose response. The effect of CCL19 on migration was blocked with an anti-CCR7 neutralizing antibody. *15ug/ml anti-CCR7, $*P < 0.05$, $**P < 0.01$. The scale bar is 200 μM.

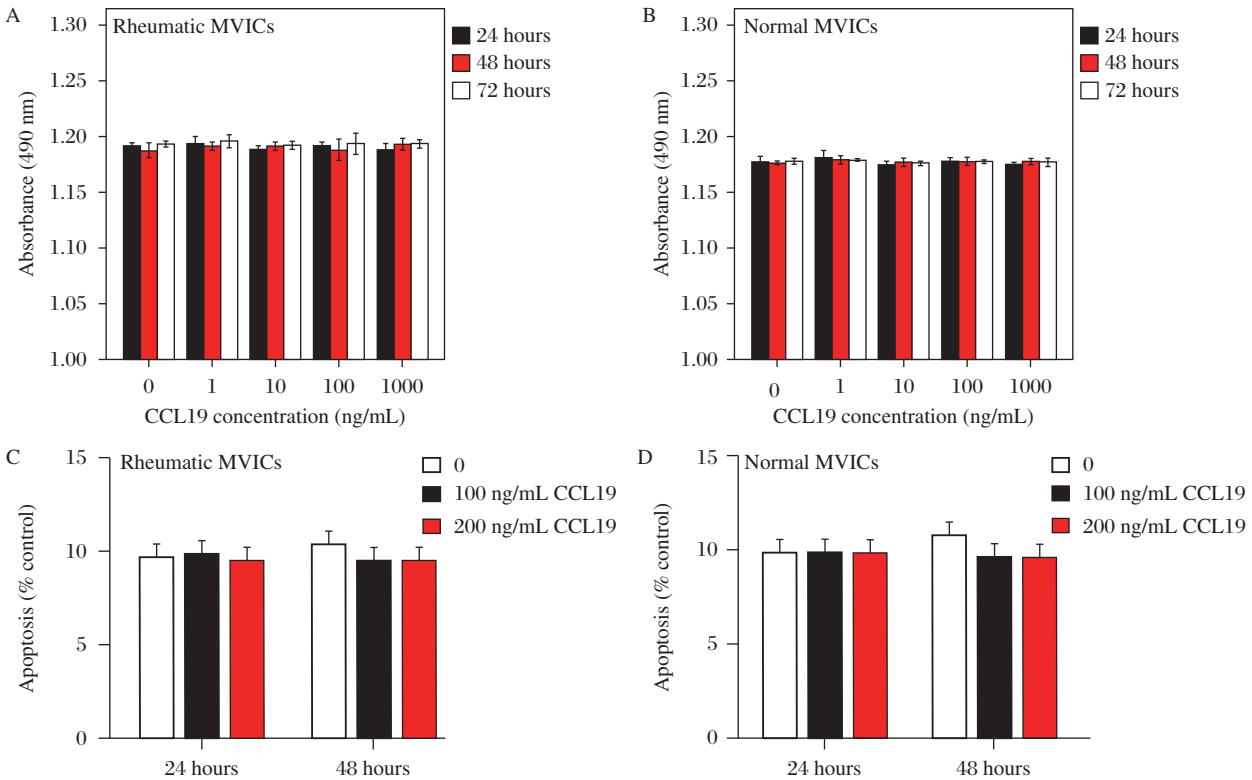

***Fig. 5* CCL19 did not affect hMVICs proliferation or apoptosis.** A-B) Both in the rheumatic and normal groups, the proliferation of hMVICs showed no change in the presence of CCL19 from 1 ng/ml to 1000 ng/ml after 24, 48 and 72 h. C-D) No significant difference was found at 24h, 48h after CCL19 treatment with respect to cell apoptosis both in rheumatic and normal groups.

mainly expressed in hMVICs *in vivo*, while mononuclear cells and the endothelium within the rheumatic mitral valves were the major source of CCL19. hMVICs treated with CCL19 showed enhanced wound healing rates, as a result of enhanced migration. Taken together, our results indicated that CCR7 and CCL19 may be therapeutic targets to prevent mitral valve remodeling following rheumatic fever.

CCR7 and its ligand CCL19 play important roles in lymphocyte homing to lymph nodes and balancing immunity and peripheral tolerance[6]. Several investigations have also shown that CCR7 is expressed by malignant cells involved in cancer metastasis[7,18-19] and by peripheral blood fibrocytes in response to wound healing[8,20]. This indicates that the chemotactic effects of CCR7 can be exerted on non-inflammatory cells. CCL19, but not CCL21, effectively stimulates CCR7 phosphorylation and internalization[21], leading to receptor desensitization. This suggests that CCR7-mediated cell responses to CCL19 may have a shorter time span, and CCL19-induced migration can be terminated[22]. The understanding of biology and pathophysiology of valve interstitial cells (VICs) remains poor. Previous studies showed cultured VICs maintained their molecular phenotype up to eight passages, it is accepted that cultured VICs of 3 to 5 passage were capable for *in vitro* study[15,23-24]. In our study, CCR7 was highly expressed in cultured rheumatic hMVICs, but not in normal hMVICs indicating the changed gene expression profiling of diseased MVICs. CCR7 was also not found in normal hMVICs *in vivo*, which may be due to the selection of relatively healthy valves. Interestingly, in cultured normal hMVICs, CCR7 was expressed significantly after treatment with CCL19. There has been no report of CCL19 directly promoting the expression of CCR7 in hMVICs. CCL19-induced up-regulation of CCR7 can be stimulated through NF-κB[18] or SOCS1[25].

Migration is an essential process in wound repair. Mesenchymal cells migrate into the wound from surrounding tissue and become activated to transform into myofibroblasts. hMVICs are involved in many aspects of wound healing, including migration, proliferation, apoptosis, and ECM remodeling[26]. Injury to the valvular endothelium may initiate the cascade of wound repair events that stimulate inflammation, eliciting a response in the underlying hMVICs to promote the accumulation of cells and ECM to promote valvular calcification[10,27]. The release of growth factors and cytokines occurs immediately after injury. Tissue

growth factor-β (TGF-β) and fibroblast growth factor-2 (FGF-2) have been reported to regulate the migration and proliferation of valve interstitial cells following injury *in vitro*[15,28].

The role of chemokines in valve repair has not been fully elucidated. In our study of the rheumatic mitral valves, CCR7 was mainly expressed in diseased hMVICs *in vivo* and *in vitro*. CCL19 and CCL21 are the sole ligands of CCR7. Therefore, secreted CCL19 expressed by mononuclear cells and endothelial cells may not only contribute to the homing of lymphocytes and dendritic cells, but also regulate the cellular behavior of CCR7-expressing hMVICs. In our study, the longer migration distance and accelerated closure of hMVICs from both the rheumatic and normal valves treated with CCL19 supported this hypothesis and suggest a novel role of the CCR7/CCL19 axis in rheumatic valve remodeling.

In addition to CCR7, CCL19 also binds with high affinity to the hepta-helical surface protein, termed the CC-X-chemokine receptor (CCX-CKR)[29]. Our findings demonstrated that the effect of CCL19 was completely abrogated by CCR7 neutralization, suggesting a dominant role of the CCR7 pathway. Concentration dependent migration was only observed for normal hMVICs treated with CCL19, suggesting that hMVICs in the rheumatic valves were already stimulated.

In conclusion, our findings support the concept that activation of CCR7 by CCL19 regulates inflammatory cells, endothelial cells and hMVICs in rheumatic valve disease. Our results highlight a novel tissue remodeling mechanism mediated by the CCR7/CCL19 axis that may provide a clinical target for the treatment of chronic rheumatic mitral valve disease.

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