

# Chemokine Receptor 7 (CCR7) Participates in the Migration of DCs and Has Impact on the Progression of Primary Pterygium

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## Research

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# Abstract

**Background:** The effect of chemokine receptors and their ligands for dendritic cells (DCs) plays a critical role in the immune response, but whether chemokine receptor 7 (CCR7) has an impact on DCs migrating in pterygia remains unclear. The aim of this article is to investigate the involvement of CCR7 signaling in pathogenesis of primary pterygium.

**Methods:** Slip lamp photographs of 85 pterygia patients were used to divide the pterygia into three groups, the width, extension, and area of pterygia were measured by computer software, the blood vessels of pterygium and general eye redness were quantitatively analyzed by a specific algorithm. Expression of CCR7 and its ligands C-C motif ligand 19 (CCL19) and C-C motif ligand 21 (CCL21) in normal conjunctivae and excised pterygia collected in surgery were analyzed by qRT-PCR and immunofluorescence staining. The phenotype of CCR7-expressing cells was identified by double-staining with major histocompatibility complex II (MHC II), CD11b and CD11c.

**Results:** The level of CCR7 was significantly increased with 9.6-fold in pterygium when compared with normal conjunctivae ( $p=0.008$ ). The higher expression of CCR7, the more blood vessels in pterygium ( $R^2=0.44$ ,  $p<0.001$ ) and more general ocular redness ( $R^2=0.25$ ,  $p<0.001$ ) pterygium patients had. CCR7 positive cells were co-located with D11b+, CD11c+ or MHC II in mature DCs. Immunofluorescence staining showed CCL21 co-staining with CCR7, which implied CCL21 may be a key ligand of CCR7 in pterygia, rather than CCL19. And CCR7 was significantly associated with the extension of pterygia ( $R^2=0.083$ ,  $p=0.047$ ,  $\beta=0.192$ ).

**Conclusions:** Our research revealed a possible involvement of CCR7 in the migration of mature DCs and influence the progression of primary pterygia. Targeting CCR7 may be considered as a potential therapeutic approach for the treatment of pterygium.

## Introduction

Pterygia is a multifactorial ocular surface disease caused by fibrovascular hyperplasia [1]. The common clinical symptoms of pterygia, which include irritation, foreign body sensation, dryness, decreased visual acuity and blindness, can have a debilitating impact on daily activities. The overall prevalence of pterygia in China is 9.9% (15–84 years old) [2]. Nowadays, there are more than 180 million pterygia patients and 20.1% of them are over 60 years old [3]. The reported rate of recurrence after simple excision without adjuvant treatment ranges from 24–89% [4]. It is a significant burden for society to manage pterygium. However, the pathogenesis of pterygia has not been elucidated yet. Recently, the important role of immunopathologic mechanisms have been mentioned in the pathogenesis of pterygia [2, 5].

CC chemokine receptor 7 (CCR7), a G protein-coupled receptor, is expressed in the mature dendritic cells (DCs). CCR7 has been reported to be the master mediator in the highly complex migratory process in which mature DCs migrate from the affected tissue to the lymph node paracortex in some ocular diseases such as dry eye disease[6, 7], ocular allergy [8], immune rejection [9]. However, there have not

been any papers studying CCR7 in pterygia yet. Ligands C-C motif ligand 19(CCL19) and ligands C-C motif ligand 21 (CCL21), the two important ligands of CCR7, are found in the paracortical regions of the lymph node, and CCL21 could also be expressed by lymphatic vessels in nonlymphoid tissue [10]. In the findings of our previous research [11], DCs which populate in the cornea of dry eye migrating to the lymphatic vessels could be affected by CCL21 and CCL19. Thus, the chemokine axis CCR7-CCL21/CCL19 may also accelerate migration of DCs to lymphatic vessels, which may represent the first stage of immune response in pterygia.

DCs capture antigens in the ocular surface and then migrate to the secondary lymph node. As an afferent pathway of immunological response, lymphatic vessels would transform DCs. Once antigens meet with naïve T cell in the Paracortical region of lymph node, the inflammatory cells and pathological T cells are generated to damage the ocular tissues through the transportation of blood vessels. In our previous study, we found that the more severity the pterygia had, the more lymphatic vessels increased in the pterygia. The high expression of vascular endothelial growth factor C (VEGF-C) may contribute to the occurrence of lymphangiogenesis [3]. What's more, both lymph-microvascular density and lymph-vascular area were correlated with the width and area of pterygia as well as lymph-vascular areas were also correlated with the extension of pterygia [12]. In addition, there are a huge number of immunocompetent cells and inflammatory factors in pterygia such as Mast cells, plasma cells, lymphocytes, DCs, CD4 + T cells, CD8 + T cells, macrophages, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), platelet-derived growth factor (PDGF), VEGF and so on [5, 13]. It suggested that cellular immune response may directly participate in the progression of pterygia.

This study presents the association between CCR7 and pterygia and focuses on the functional impact of CCR7 in pterygia. As far as we know, it is the first time to illuminate the effect of CCR7 in pterygia, so our findings will assist the further understanding on the immunological pathogenesis of pterygia.

## Methods

### Subject

After excluding conjunctivitis, keratitis, dry eye and some other ocular inflammatory diseases, eighty-five patients (37 males and 48 females; average age,  $46.3 \pm 9.1$  years) whose primary pterygia were located in nasal sides were included in the study. All patients underwent excision by the bare sclera technique and combined with conjunctival transplantation in the Department of Ophthalmology at the Third Affiliated Hospital of Sun Yat-sen University. The head, neck and a part of body (approximate 3mm from limbus) of the pterygia were collected as pterygium samples. Twenty-three nasal-side epibulbar conjunctival segments (patients average age,  $21 \pm 8.6$  years, 10 males and 13 females) excised during strabismus surgery near the limbus, were used as control tissues. Forty-eight pterygia and eight epibulbar conjunctival tissues were used for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Fourteen pterygia and six epibulbar conjunctival tissue were used for immunohistochemistry. And twenty-three pterygia and nine epibulbar conjunctival tissue were used for immunofluorescence.

## The grading and size of pterygia

Pterygia were defined as a radially oriented fibrovascular lesion crossing the nasal or temporal limbus [14, 15]. Grading of pterygia was performed as previous<sup>3,14</sup>. Briefly, Grade 1 denoted a pterygium in which episcleral vessels underlying the body of the pterygium were unobscured and with mild clinical signs of inflammation (conjunctival congestion and edema). Grade 2 was defined as vessels partially visible with moderate inflammation, and Grade 3 as vessels wholly obscured with severe signs of inflammation (Figure 1).

Total area was measured with computer software to circle the area of pterygia and circled automatically. The extent of two intersecting points between pterygia and cornea was measured as the width of pterygia. And the horizontal extension from pterygia to cornea limbus was recognized as the extension of pterygia (millimeters).

The quantitative evaluation of general redness and blood vessels of pterygium

The degree of visible blood vessels in the pterygium and general redness were quantitatively analyzed by an automatic recognition program. The algorithm mainly adopted a traditional image processing method. Binarization contour extractions of pterygium images were implemented for obtaining the edge information of blood vessels. The HSV color model was used to extract the red pixels to obtain the hyperaemic regions. The interference information was eliminated by morphological method which was usually used for picture processing. By counting the number of pixels in the characteristic area and obtaining the area (unit: pixel), the proportion of blood vessels in the pterygium and general redness could be calculated respectively (Microsoft Visual C++, version 14.1, USA). What's more, the composite result of general redness, and blood vessels of pterygium was considered as the average of those two proportion.

## Quantitative real-time PCR

TRIzol (Invitrogen, United States) was used for total RNA extraction and purification. Reverse transcription into cDNA was performed with a reverse transcriptase kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The primer sequences used for CCR7, C-C motif ligand 19 (CCL19), C-C motif ligand 21 (CCL21) and Human Tubulin Beta (TUBB) are listed in Table 1. The Quantitative real-time PCR was performed with the StepOne Real-Time PCR System (Applied Biosystems, Alameda, CA, USA) according to the manufacturer's instructions. For the qRT-PCR analysis, the parameters consisted of predenaturation at 95°C for 60 seconds, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing, and extension at 60°C for 30 seconds. Then, a melt curve analysis was conducted to assess amplification specificity. Threshold cycle (CT) values were calculated for each well using the software of the real-time thermocycler. Gene expression and the fold change in gene expression were calculated using the  $\Delta\Delta\text{Ct}$  method. The  $\Delta\text{Ct}$  data were calculated using GAPDH as the housekeeping gene, and the fold change was calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method. samples were used as controls and processed and analyzed separately using the same method.

## **Immunohistochemistry and Immunofluorescence**

After being fixed in 4% paraformaldehyde for 24h, samples were embedded in paraffin, serially sectioned at a thickness of 5µm, and rehydrated with graded ethanol-water mixtures. These excised segments were washed by distilled water. Endogenous peroxidase activity was blocked by incubating the sections with 30 mL/L hydrogen peroxidase for 30 min. For antigen retrieval, these tissue sections were autoclaved at 121°C in 10 mmol/L citrate buffer (pH 6.0) for 5 min. Then, these sections were allowed to cool at room temperature for 30 min.

For immunohistochemistry, each sample was cut into 10 sections and each section was eight-micrometer. These sections were incubated overnight at 4°C with a mouse anti-human CCR7 antibody (1:100, Abcam PLC, Cambridge, UK), and a goat anti-mouse IgG antibody (1:500, Abcam PLC, Cambridge, UK) was used as the secondary antibody. These slides were incubated with diaminobenzidine (DAB) and counterstained with hematoxylin. The color of CCR7+ cells was brown after staining. Next, the sections were analyzed using standard light microscopy (Carl Zeiss, Oberkochen, Germany) under 200X and 400X magnification.

### **Immunofluorescence**

For immunofluorescence, these sections were double-stained and the following antibodies were used: a rabbit anti-human CCR7 antibody, a mouse anti-human MHCII (major histocompatibility complex class II) antibody, a mouse anti-human CD11c antibody, a goat anti-human CD11b antibody (1:100, Abcam PLC, Cambridge, UK) and a goat anti-human CCL21 antibody. Then, an Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody (1:500, Abcam PLC, Cambridge, UK) was used to detect the anti-CCR7 antibody. An Alexa Fluor 555-conjugated donkey anti-goat IgG antibody was used as the secondary antibody for the anti-CD11b and an Alexa Fluor 555-conjugated goat anti-mouse IgG antibody was used to detect staining with the anti-MHCII and anti-CD11c antibodies. Epifluorescence microscopy (Carl Zeiss, Oberkochen, Germany) was used for examination. Pictures of the same location in one section were taken in two excitation wavelengths under 400X magnification. Next, the two pictures were merged and if the proteins were located, it would show yellow.

### **Statistical analysis**

Mann-Whitney and Bonferroni's test were used to analyze differences in the PCR, results between the grades of pterygia and normal conjunctiva. Spearman's analysis was used to analyze the association among CCR7, CCL19 and CCL21. Linear regression was used to analyze the association between CCR7 and the characters of pterygium, and between the proportion of blood vessels in the ocular surface and CCR7. Values are shown as the mean ± SD. All reported P-values are 2-tailed, and statistical significance was defined at the  $\alpha=0.05$  level. All analyses were performed using SPSS software (version 26.0; SPSS, Inc., Chicago, IL).

## **Results**

## 1. CCR7 more highly expressed in severe pterygia

We detected the CCR7 mRNA level of pterygia and normal conjunctivae using q-RT-PCR. The expression level of CCR7 in pterygia was about 9.6 times higher than normal conjunctivae ( $p=0.008$ ). With the use of immunohistochemistry staining, CCR7 positive cells were stained by brown and we could see that there were lots of CCR7 positive cells in the pterygia while few in the normal conjunctivae (Figure 2). After grading the severe of pterygia (grade 1: twelve, grade 2: twenty-two, grade 3: fourteen) by slit lamp, samples were used to detect the CCR7 expression by qRT-PCR. The CCR7 mRNA expression levels in grade 1, 2 and 3 were approximately 6.8 times ( $p=0.037$ ), 8.1 times ( $p=0.006$ ) and 14.3 times ( $p=0.002$ ) higher than normal conjunctiva (Figure 3D). However, there were not significantly difference among grade 1, grade 2 and grade 3 in CCR7 expression ( $p=0.079$ ).

In addition, the proportion of blood vessels in the pterygium and general redness which calculated by specific algorithm were associated with CCR7. The higher expression of CCR7, the more blood vessels in pterygium and more general ocular redness pterygium patients had. The detail results were illuminated in the table 2. The pictures which the computer recognized and outputted were showed in the Figure 3B and 3C.

## 2. CCR7 expressed in the mature DCs

MHCII, CD11c and CD11b are the marks of the mature DCs. Double-stained immunofluorescence was used to detect whether the CCR7 expressed in mature DCs in pterygia. We used excitation wavelength with 555nm to detect the expression of MHCII, CD11c or CD11b that were showed in red. The CCR7+ cells were displayed in green in excitation wavelength with 488nm. After merging two kind of pictures taken in the same view, some areas appeared yellow (Figure 4). It suggested that numbers of green signal colocalized with the red signal, which meant many cells expressed both CCR7 and MHCII / CD11c / CD11b.

## 3. CCR7 was correlated with the extension of pterygia

Characters of pterygia like the extension, width, and area were measure by the computer software in the photograph (Table 3). The extension ( $p=0.773$ ) and the width ( $p=0.792$ ) of pterygia increased with the grade, while the differences among grades were not significant. Then, we analyzed the association among CCR7, the extension, width and area of pterygia. The expression of CCR7 was significantly associated with the extension of pterygia ( $R^2=0.083$ ,  $p=0.047$ ,  $\beta=0.192$ , 95%CI 0.002-0.383). However, the co-relationship among the expression of CCR7, the width ( $p=0.098$ ) and area of pterygia ( $p=0.081$ ) were not statistically different.

## 4. CCR7 combined with CCL21 in pterygia

CCL19 and CCL21, as the two commonest ligands of CCR7, were detected in pterygia. Although the mRNA expression of CCL21 ( $p=0.785$ ) and CCL19 ( $p=0.056$ ) in pterygia were not higher than normal

conjunctivae (Figure 3E, 3F), after analyzing the relationship between CCR7 and its ligands in pterygium, it showed that CCR7 was significantly associated with CCL21 in pterygium ( $p=0.02$ ). Thus, we further investigated their association using double-stained immunofluorescence. Some CCR7 positive cells showed green co-located with red CCL21, which made the cells showed orange. The orange staining revealed that chemokine, CCL21, was combined with CCR7 positive cell which implied CCL21 may be a key ligand of CCR7 in pterygia, rather than CCL19 (Figure 5).

## Discussion

Because of the high prevalence and recurrence after surgery, pterygia always make ophthalmologists anxious. Although the techniques for pterygium surgery is developing, it still can't satisfy the increasing number of pterygia patients. Thus, the pathogenesis pterygium should be understood more clearly to assist the progress of treatment. Our previous study demonstrated that pterygia are greatly associated with lymphatic vessels which are the afferent pathway of immunological response [3, 12, 14, 16]. The first and crucial step of immune response is antigen transportation. However, for pterygium, we couldn't explain the specific way that how the antigens arrive at the lymph node and activate the naïve T cells to induce inflammatory cells and factors yet.

Chemokine axes play important roles in the migration of antigen-presenting cells from peripheral tissues to lymphatic vessels and the following with the lymph fluid arrive at the lymph nodes [17]. CCR7, as one of the most important chemokine receptors, make effect in many diseases like ocular allergy [8, 18], immune rejection of allogeneic corneal transplants[19-21] and so on. To our knowledge, this is the first time to study CCR7 in the pterygia. Our research demonstrated that high level of CCR7 which expressed in the mature DCs had impacts on the development of pterygia, especially in the extension of pterygia to the cornea. CCL21, one of commonest ligands of CCR7, may contribute to the migration of mature DCs when combining with CCR7.

DCs are highly potent stimulators of the immune system, and their contribute to the pathogenesis of ocular surface inflammatory disease has been well established [22-23]. To identify the relationship between CCR7 and DCs in pterygia, we co-stained the markers of DC (MHCII, CD11c and CD11b) and CCR7 in pterygia. As we assumed, CCR7 expressed in mature DCs in pterygia. However, DC is not the only immune cell that migrates from the periphery to the lymph node with the assistance of CCR7. The effect of CCR7 chemokine axis takes T cells and mature DCs flow to the lymph node in which Naive T cells could meet cognate antigen presented by DCs [24, 25]. What's more, regulatory T (Treg) cells contribute to the immunological tolerance that can suppress pathogenic T cells and abnormal immune responses to maintain the tissue homeostasis. With the high expression of CCR7, Treg cells get access to lymph node from periphery circulation and achieve lymphoid tissues through high endothelial venules [26-28]. Our research only focused on DCs, thus, the influence of CCR7 for T cells in pterygium needs more researches.

The increasing of lymphatic vessels in pterygium, which is illustrated in our previous researches [12, 14], combined with the results of this study implied that CCR7 may activate DC migrating to lymphatic vessels, and accomplish the first step of immune response in pterygia. What's more, we measured the characters of the pterygium, including the horizontal extension onto the cornea from the limbus (the extension of pterygium), the width of the base at the limbus and the total area of pterygium. We found that the extension of pterygium was significantly associated with the expression of CCR7. It implied that CCR7 might make effects in the invasion of pterygia from limbus to the center of cornea. In addition, one of main innovative contributions of our research was that it is also the first time to use algorithm to quantitatively measure blood vessels of pterygium and general redness in pterygium patients. The grading for pterygium in previous studies [13, 14] is usually a manual classification and thus inherently subjective. We utilized a computer technique to calculate the proportion of blood vessel in the pterygium and general redness to analyze the association between them and the expression of CCR7. It may be more objective and make more sense for the clinical treatment of pterygium.

Although the mRNA levels of CCL19 and CCL21 did not significantly increase, CCL21 was correlated with CCR7 in pterygium. Double-stained immunofluorescence showed that some CCR7 positive cells combined with CCL21. Blood vessels and lymphatic vessels form the two arms of immunological reflex. When CCR7 expressed in the mature DC combines with CCL21, antigens captured by DCs tend to be more effective to migrate to lymphatic vessels [29], and the first step of immunological response was activated. Antigens following with lymphatic fluid arrive at the lymph nodes, and T cells are stimulated by antigens to produce numerous of inflammatory cells and factors. Subsequently, these inflammatory cells and factors return to the ocular surface through blood vessels, which cause the ocular damage (figure 6). Thus, CCR7 may accelerate the immune response in pterygium to some extent and affect the extension of pterygia to invade the cornea. However, our results also implied that there may be some other chemokines that would combine with CCR7 in pterygium, and the influence of CCL21 need further confirmation.

## Conclusions

We gave evidences for the first time that CCR7 combined with CCL21, as a significant chemokine axis, is involved in the progression of pterygia invading to the cornea and could accelerate the migration of mature DCs in pterygia. Our findings suggest a novel action of CCR7-CCL21 chemokine axis in pterygium, which may serve as a potential therapeutic target for treatment.

## Declarations

### Acknowledgements

Not applicable.

### Authors' contributions

Ting Wang and SQL contributed to the concept of the study. Tao Wang, LZ, XYL XHJ and TCB provided and collected the tissues. Ting Wang Tao Wang and LZ performed the bioinformatics. HQN, Ting Wang and JX contributed to the data analysis. Ting Wang wrote the original draft, while SQL and HTL reviewed and edited. The project was funded by HTL. All authors read and approved the final manuscript.

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## **Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request

## **Ethics approval and consent to participate**

All patients and controls have been informed of the experimental nature of this procedure and signed consents have been beforehand. All procedures were conducted according to the principles expressed in the Declaration of Helsinki and was approved by the IRB of the 3rd Affiliated Hospital of Sun Yat-Sen University (NO: [2018] 02-009-01).

## **Consent for publication**

Not application.

## **Competing interests**

The authors have no financial conflicts of interest.

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## Tables

<b>Table 1 Primer sequences for qRT-PCR</b>			
Gene	Primer sequences (5'-3')	Product length(bp)	Tm (°C )
CCR7	F: GTGGTGGCTCTCCTTGTCATTT	160	60
NM_001838.3	R: ACATGATAGGGAGGAACCAGGC		
CCL21	F: TGAGCCTCCTTATCCTGGTTCTG	221	60
NM_002989.3	R: CTCCTTTGGGTCTGCACATAGCT		
CCL19	F: GGCACCAATGATGCTGAAGACT	191	60
NM_006274.2	R: CTCTGGATGATGCGTTCTACCC		
TUBB	F: CTGACCACACCAACCTACGG	158	60
NM_178014.2	R: CCAGGCATAAAGAAATGGAGAC		

<b>Table 2 the relationship between CCR7 and characters evaluated by computer</b>					
proportion	average (%)	SD (%)	R <sup>2</sup>	p*	β
blood vessels of pterygium	12.92	5.98	0.44	<0.001	0.66
general redness	29.64	23.27	0.25	<0.001	0.5
the composite result of two factors	21.28	13.14	0.35	<0.001	0.59
*Linear regression was used to test					

<b>Table 3 Extension, width and area of pterygia</b>			
	Extension of pterygia(mm)	Width of pterygia(mm)	Area of pterygia(mm <sup>2</sup> )
	Mean±SD	Mean±SD	Mean±SD
Total	1.65±0.53	2.21±0.58	8.91±3.66
Grade 1	1.49±0.47	2.10±0.60	7.89±3.85
Grade 2	1.61±0.33	2.23±0.44	8.62±2.47
Grade 3	1.86±0.76	2.27±0.77	10.23±4.81

# Figures



Figure 1

Slit-lamp photos for different grades of pterygium. Normal: normal conjunctiva; T1: grade 1 pterygium; T2: grade 2 pterygium; T3: grade 3 pterygium.

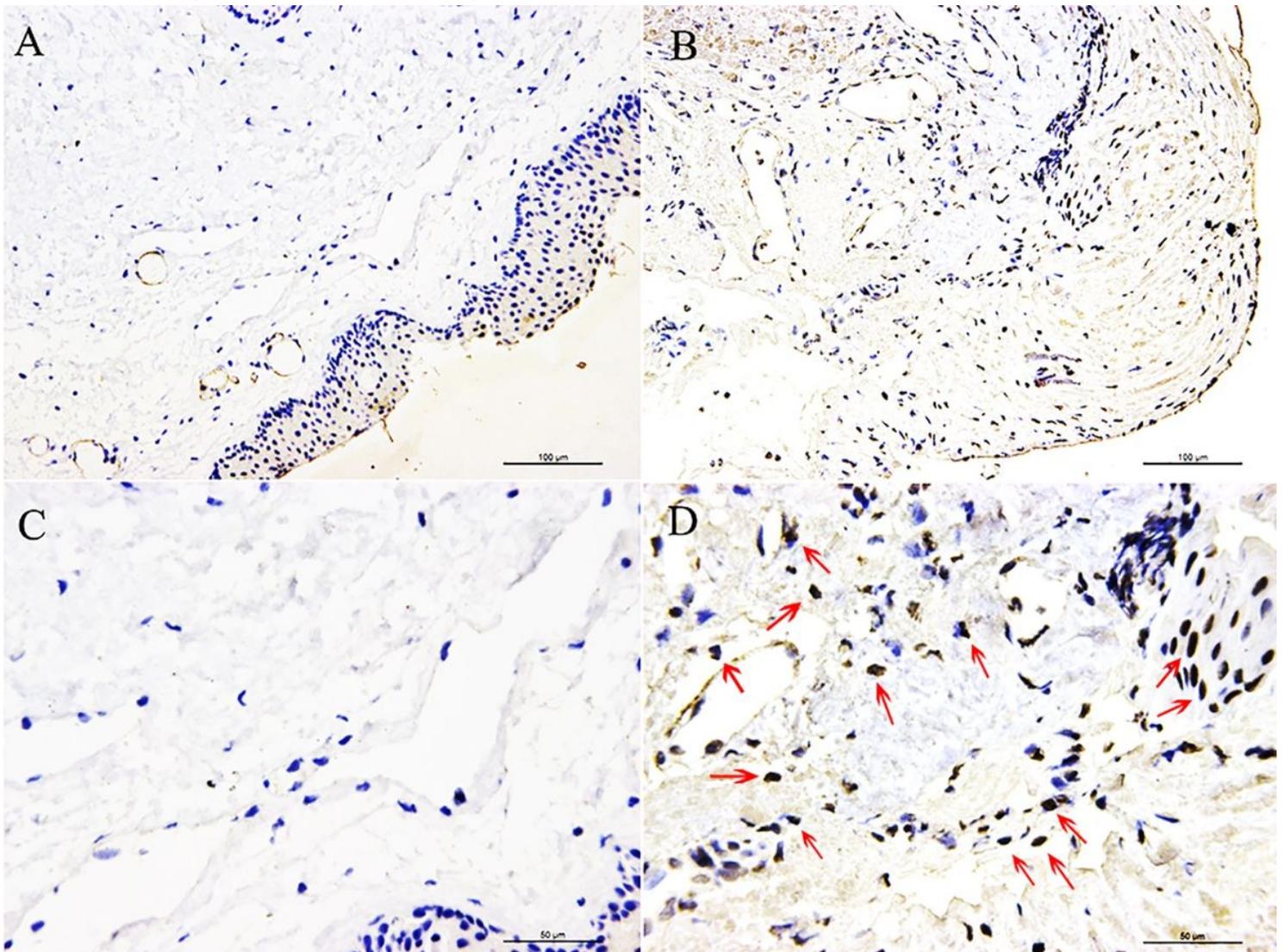
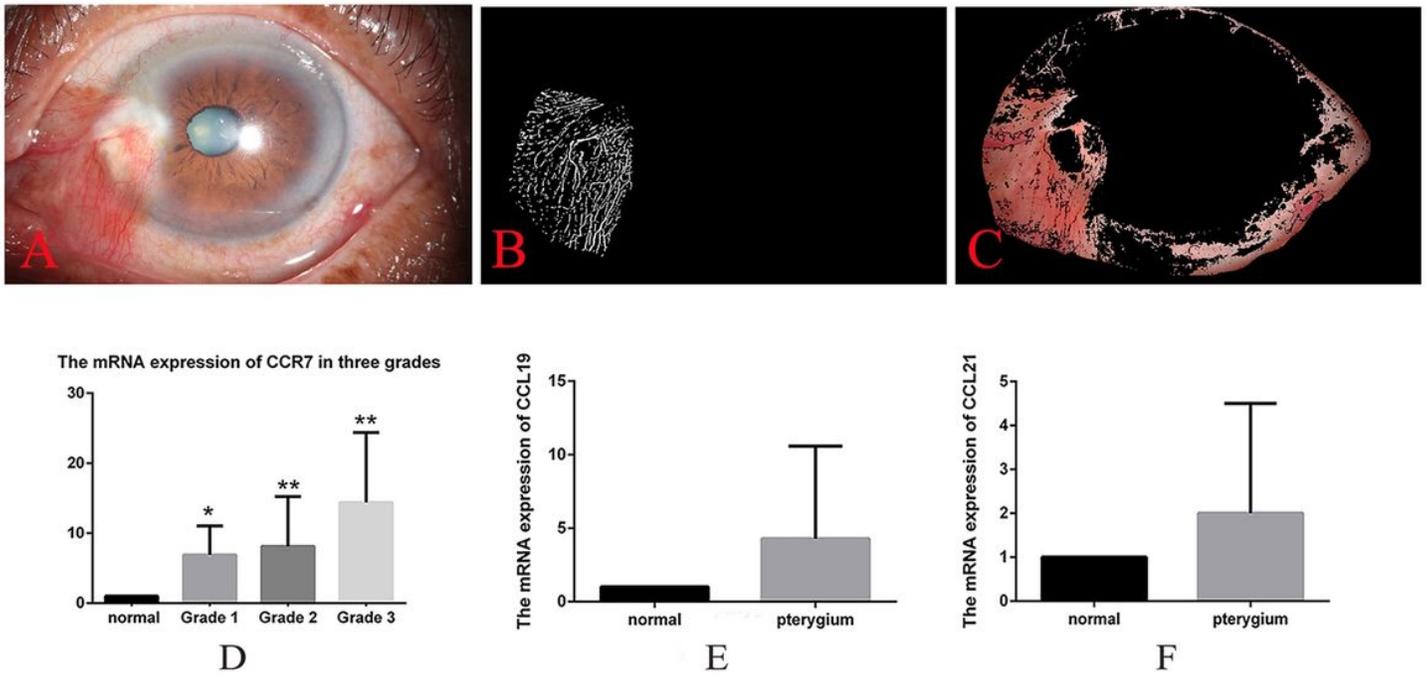


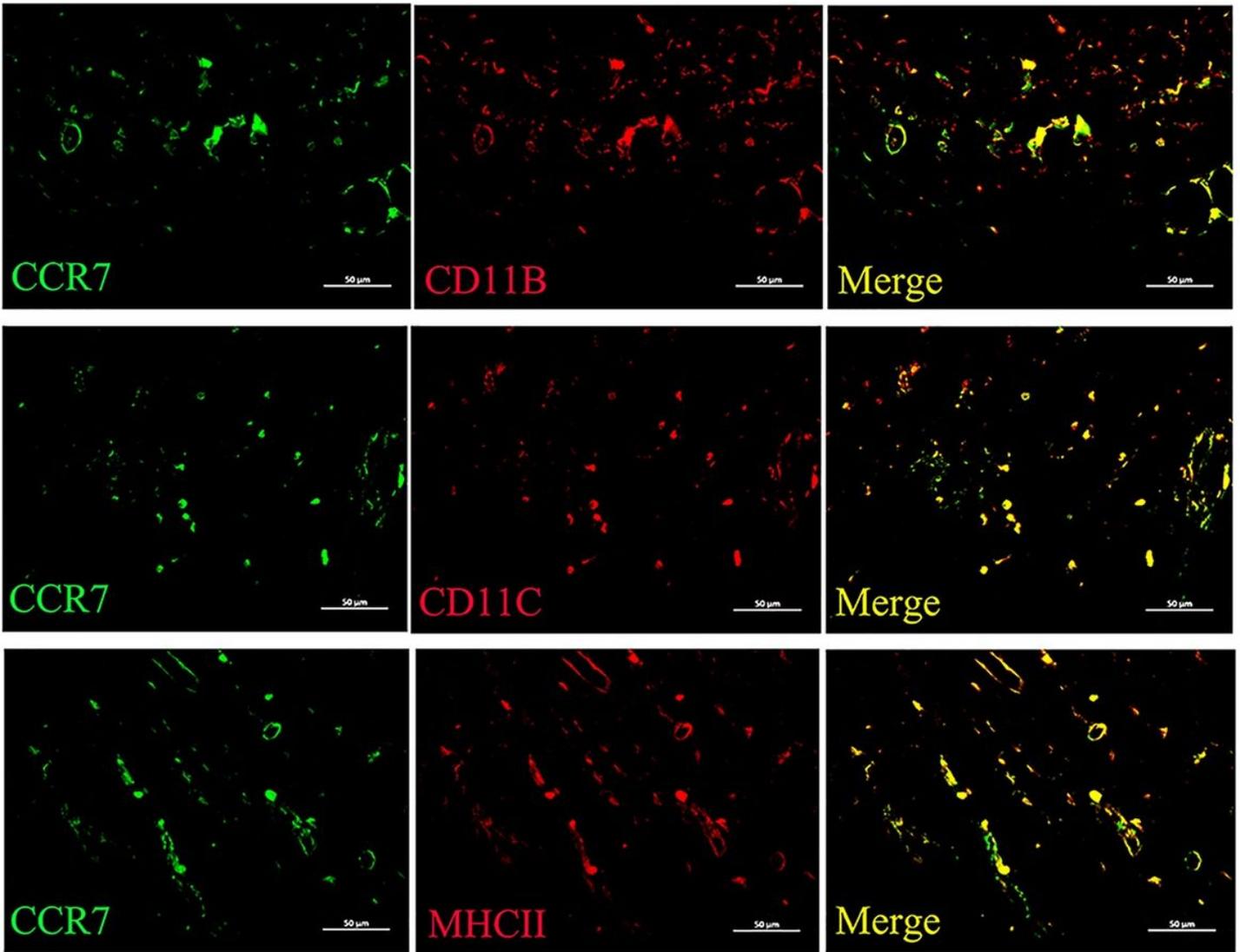
Figure 2

Immunohistochemistry for CCR7 of pterygium and normal conjunctiva. CCR7 positive cells express in the pterygium tissue but not in normal conjunctiva. Red arrows point to CCR7 positive cells. A and C: normal conjunctiva; B and D: pterygia. Original magnification of A and B: 200X; Original magnification of C and D: 400X.



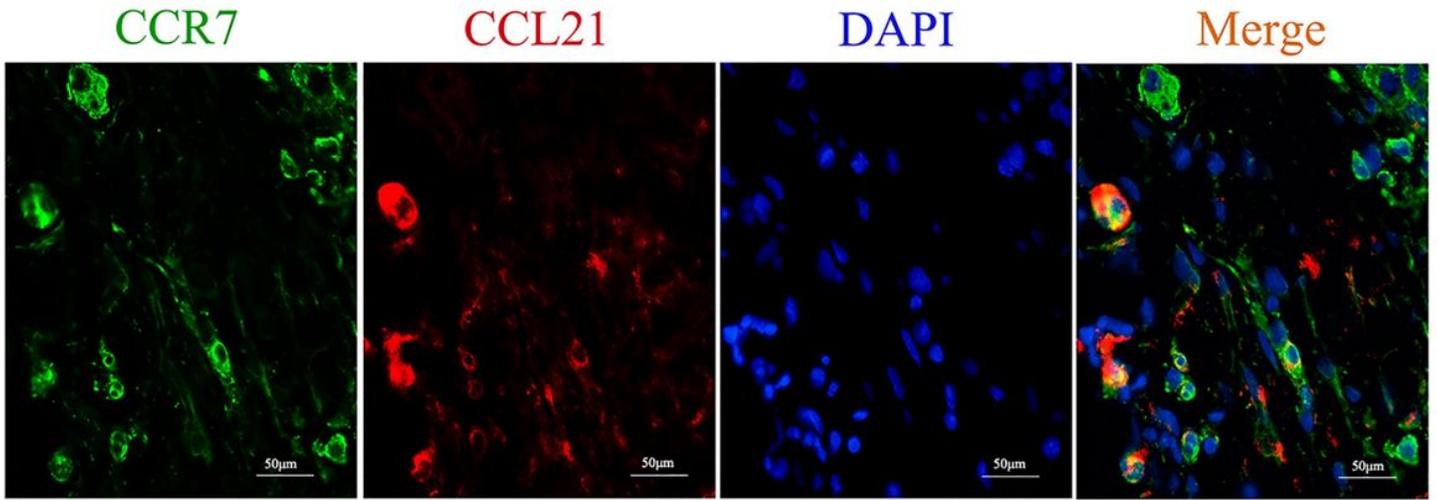
**Figure 3**

Character of pterygium recognized by computer and the mRNA expression of CCR7 and its ligands A is a slit lamp picture for pterygium. B and C are the pictures that computer outputted. B reveals the blood vessels in the pterygium and C shows the general redness. D, E and F showed the mRNA level of CCR7 in the three grades of pterygium were significantly higher than normal conjunctiva. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .



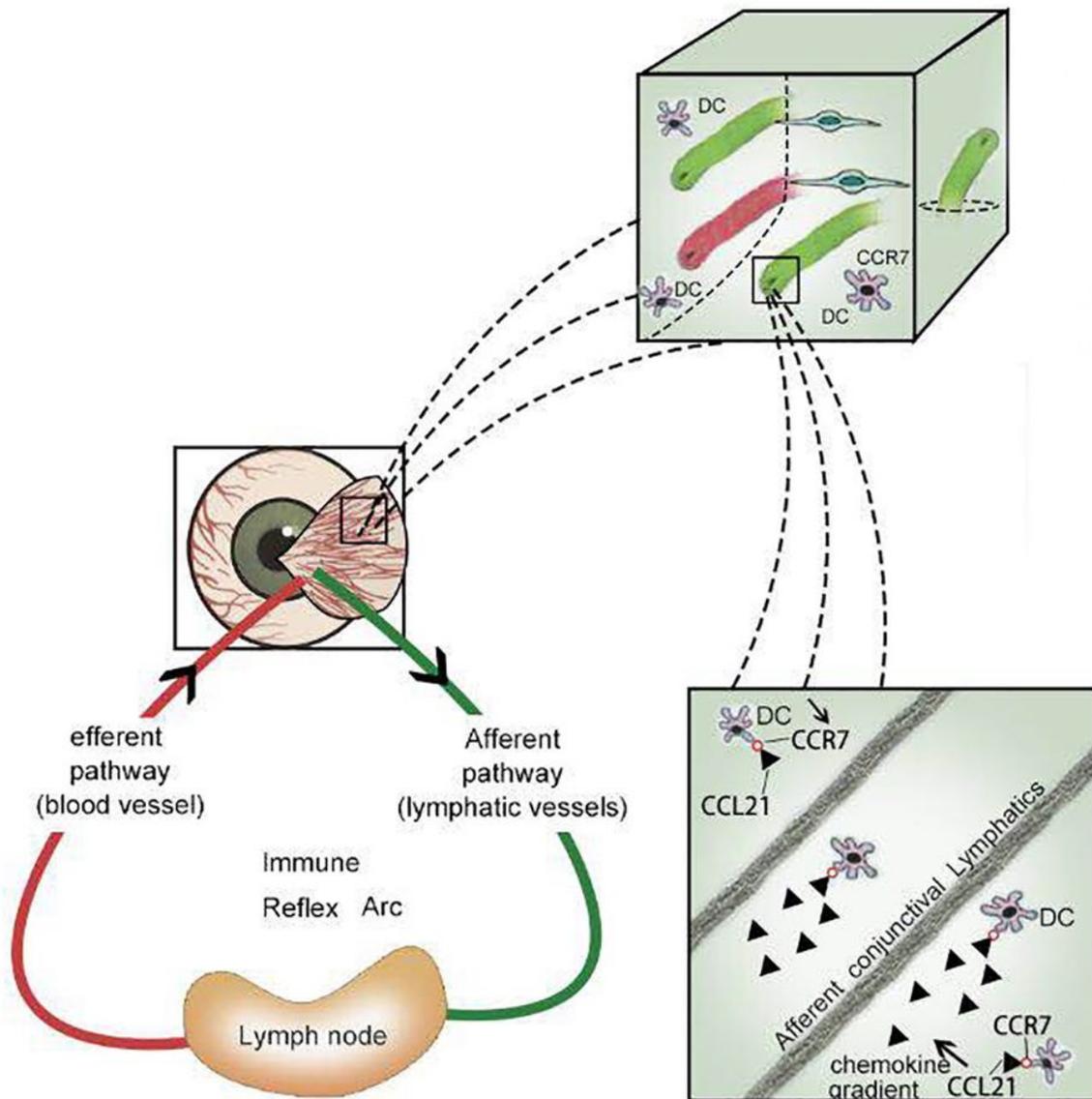
**Figure 4**

Immunofluorescence for CCR7 and makers of DCs in pterygia. Most of CCR7 positive cells co-stain with CD11c, CD11b or MHCII in pterygia The first column: CCR7 positive cells; the middle column: CD11c, CD11b and MHCII positive cells in first, middle and last line respectively; the last column: merge pictures of CCR7 and CD11c/CD11b/ MHCII positive cells. Yellow means CCR7 and CD11c/CD11b/ MHCII express in the same cell. Original magnification: 400X.



**Figure 5**

CCR7 combines with CCL21 in pterygium. In the merge figure, when green CCR7 combines with red CCL21, it shows yellow. Original magnification: 400X.



**Figure 6**

CCR7-CCL21 regulates the migration of DCs to lymphatic vessels. (1) Lymphatic vessels (green) increase in pterygium, (2) CCR7 on DC specifically are bound to CCL21 secreted by lymphatic endothelial cells, and migrate to lymphatic vessels under the direction of CCL21 concentration gradient. Lymphatic vessels and blood vessels (red) constitute the afferent and efferent channels of the "reflex arc" of the immune response. (1): an enlargement of "□" in the pterygium; (2): an enlargement of "□" in the picture (1).