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ORIGINAL RESEARCH PAPER

Anti-angiogenic and anti-inflammatory effects of CD200–CD200R1 axis in oxygen-induced retinopathy mice model

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Abstract

Objective In this study, the expression changes and the potential effects of CD200 and its receptors during the process of retinal neovascularization (RNV) development had been detected, using a classic oxygen-induced retinopathy (OIR) mice model and CD200Fc (a CD200R1 agonist) intravitreal injection.

Materials and methods 7 day postnatal (P7) C57BL/6J mice were raised in hyperoxia incubators with 75±2% oxygen for 5 days, and returned to room air at P12. All animals were subdivided into three groups: normoxia control, OIR, and OIR+CD200Fc group. The mice of OIR+CD200Fc group were intravitreal injected with CD200Fc (2μg/μL, 0.5μL) at P12. Retinas and vitreous samples were harvested at P17. The expression and localization of CD200 and its receptors were analyzed by Western blot, quantitative real-time polymerase chain reaction (qRT-PCR), enzyme-linked immunosorbent assay (ELISA), and retinal whole-mount immunofluorescence. To investigate the effects of CD200Fc treatment, vascular endothelial growth factor (VEGF)-A, platelet-derived growth factor (PDGF)-BB, pro-inflammatory cytokines, NV area, and microglial activation were detected respectively.

Results In OIR group, both protein and RNA levels of CD200 and CD200R1 were significantly up-regulated. The increased CD200 and CD200R1 were co-localized with Alex594-labeled Griffonia simplicifolia isolectin B4 (IB4) on vascular endothelial cells in NV area of OIR samples, and CD200R1 was co-expressed with ionized calcium-bind adapter molecule 1 (iba1) on microglia in OIR samples at the same time. CD200Fc intravitreal injection could significantly reduce the release of VEGF-A, PDGF-BB, and pro-inflammatory cytokines; shrink the NV area; and inhibit the activation of microglia in OIR mice.

Conclusion These findings suggested that the up-regulation of CD200 and CD200R1 was closely related to RNV development, and the antiangiogenic effects of CD200Fc in OIR model might be realized by inhibition of inflammatory response and microglia activation. The results may provide a new therapeutic target for RNV diseases.

Keywords CD200–CD200R1 axis · Retinal neovascularization · Oxygen-induced retinopathy · Inflammatory response · Microglia

Introduction

Ischemic retinopathies (IRs), such as retinopathy of prematurity (ROP), diabetic retinopathy (DR) and retinal vein occlusion (RVO), have already been the main causes of visual impairment and blindness in each age group [1, 2]. Retinal neovascularization (RNV) is a characteristic process of IRs, which may lead to severe complications like vitreous hemorrhage (VH), proliferative vitreous retinopathy (PVR) and tractional retinal detachment (TRD) and finally result in irreversible visual loss. As we know, vascular endothelial growth factor (VEGF) is the foremost regulating agent of physiological and pathological angiogenesis. Mounting evidences indicate that in RNV diseases, VEGF also acts as a greatest promoter [3–5]. Thus, VEGF inhibitors have been the predominant treatment of RNV diseases [6]. However, the exorbitant prices of anti-VEGF agents brought great economic burden for patients and society, and their applications were limited in the clinical practice. Also, side effects and complications associated with anti-VEGF agents

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like endophthalmitis [7, 8], retinal atrophy [8], sustained intraocular pressure elevation [9, 10], and even cardio-cerebral vascular accidents [11–13] get more and more attentions. Considering these factors, exploring safer and more economical alternative treatments is necessary.

Clinical and basic researches have revealed a complex pathogenesis of RNV, in which inflammation caused by retinal hypoxia stands as a primary factor [14]. Strong evidence shows that inflammatory responses are tightly linked with pathological angiogenesis [15]. The constriction of retinal arteries and arterioles induces the secretion of various pro-inflammatory factors, such as interferon (IFN)-γ, interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α. [16–18]. These cytokines recruit inflammatory cells, disrupt the tight junction between endothelial cells, promote pericytes apoptosis, and destroy the blood-retinal barrier (BRB). The damage of BRB stimulates the secretion of various angiogenic cytokines, especially VEGF, and catalyzes the development of RNV [16].

CD200 (formerly known as OX2) is a type 1 glycoprotein of the immunoglobulin superfamily with two extracellular immunoglobulin domains and one short cytoplasmic region [19], and has two forms: membrane bound form and soluble form (sCD200). The membrane bound form CD200 is widely expressed on multiple cell types, including thymocytes, T and B lymphocytes, dendritic cells, bone marrow cells, endothelial cells, etc. [20] CD200 receptor (CD200R) is the specific receptor of CD200 and mainly expressed on lymphoid cells, NK and NKT cells, myeloid cells and microglial cells [20]. There are four members in CD200R family (CD200R1, CD200R2, CD200R3 and CD200R4), in which CD200R1 has the highest affinity with CD200 [21]. By binding with CD200R, CD200 acts as an immune suppressor [22]. The immunosuppressive signal transduced via CD200–CD200R axis is shown to play a significant role in various autoimmune diseases and neurodegenerative diseases, like rheumatoid arthritis, Parkinson's disease, Alzheimer's disease, etc. [23–25].

Recent researches have discovered that CD200 is expressed abundantly on retinal endothelial cells and ganglion cells in rat and human retinas [26]. Impairment of CD200–CD200R axis results in more serious retinal inflammation and participates in several retinal diseases. For example, in peripheral blood of patients with neovascular age-related macular degeneration (AMD), CD200 expression was significantly enhanced on the surface of CD11b+ monocytes [27]. In our previous study, we also found an increasing expression of sCD200 in the vitreous samples of proliferative diabetic retinopathy (PDR) patients and the level of sCD200 was statistically higher in patients with diabetic macular edema (DME) or tractional retinal detachment (TRD) compared with patients without these complications [28].

Lately, some researches showed that the interaction of CD200–CD200R has the potential to regulate microglia activation and limit immunogenic inflammatory damages in ocular nerve diseases [29, 30]. CD200Fc is a fusion protein composed of the extracellular domain of CD200 and a murine IgG2a Fc sequence. It can bind with CD200R and imitate the function of CD200 to act as a CD200R agonist [31]. Evidence showed that CD200Fc intravitreal injection could attenuate the apoptosis of retinal ganglion cells (RGCs) in optic nerve crush (ONC) rat model via inhibiting retinal glial cells hyper-activation [30].

All these evidences revealed that CD200–CD200R axis might paly a critical role in vitreous and retinal inflammatory diseases. Nevertheless, its expression and effects in RNV diseases remain unclear.

In the present study, we investigated the expression changes of CD200 and CD200R in the process of RNV using the oxygen-induced retinopathy (OIR) model in mice and then detected the effects of CD200Fc treatment on RNV developing and tried to deal with its mechanism. These findings may further explore the potential role of CD200–CD200R axis in RNV diseases and may provide a latent target to RNV treatment in the future.

Materials and methods

Animal model

C57BL/6J wild-type mice were obtained from the Laboratory Animal Center of Xi'an Jiaotong University (Xi'an, Shaanxi, China). OIR model was produced as previously described by Smith et al. [32]. 7 days postnatal (P7) mice and their nursing mother were raised in hyperoxia incubators with 75 ± 2% oxygen for 5 days, and return to room environment (21% oxygen) at P12. Normoxia controls were even-aged mice handled in the same environment but without hyperoxia.

Subgrouping and intravitreal injections

All animals were subdivided into three groups: normoxia control, OIR and OIR + CD200Fc groups. Each group contained 6 mice. The mice of OIR + CD200Fc group were anesthetized by 4.3% chloral hydrate intraperitoneal injection at P12, and CD200Fc (2 μg/μL, 0.5 μL) was administered intravitreally by 33G microsyringe (Hamilton Company, Reno, NV, USA). Because there was no previous study that could be referred to, the concentration of CD200Fc was chosen on the basis of its experimental experience in rats [30, 31].

Western blot analysis

For protein extraction and western blot analysis, mice were killed by neck dislocation at P17 and retinas from unfixed eyes were separated. Two retinas from one mouse were used for one sample. The retinal tissues were dissected and washed in cold phosphate-buffered saline (PBS) and then homogenized in RIPA lysis buffer (Pierce, Rockford, IL, USA) with a mixture of protease inhibitors and phosphatase inhibitors. Proteins were extracted from each sample in equal amounts (20 μg) and decomposed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidine difluoride filter (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membranes were incubated with primary antibodies (as shown in Table 1) overnight at 4 °C after 1 h blocking at ordinary temperature and incubated these membranes with appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000) at ordinary temperature for 2 h. Finally, relative protein quantities were analyzed by an enhanced chemiluminescence system (ECL, Millipore, Bedford, MA, USA).

Table 1 Primary antibodies used in western blot analysis of this study

Name	Type	Dilution	Company (Cat#)
CD200	Rabbit polyclonal	1:500	Abcam (ab203887)
CD200R1	Rabbit polyclonal	1:500	Abcam (ab92794)
CD200R2	Rabbit polyclonal	1:500	Abcam (ab203660)
CD200R3	Rabbit polyclonal	1:500	BioLegend (142205)
CD200R4	Rat monoclonal	1:500	Sino Biological (50639-RP01)
GADPH	Mouse monoclonal	1:1000	Abcam (ab8245)

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Harvest retinal tissues of all group mice at P17 as described above, and total RNA was extracted with TRIzol (Invitrogen Life Technologies Co., Carlsbad, CA, USA). The isolated RNA was transcribed into complementary DNA (cDNA) by a system for cDNA first-strand synthesis (Fermentas, Canada). Gene expression was determined in accordance with the supplier's protocol (Biotool, Houston) by qRT-PCR system with SYBR Green RT-PCR Master mix. Each sample was analyzed three times. β-Actin was employed as an internal control to allow normalization between samples. PCR results were quantified by $2^{-\Delta\Delta Cq}$ method [33]. The PCR primers were designed on the basis of the NCBI GeneBank database and are shown in Table 2. The mRNA of CD200, CD200R family, VEGF-A, PDGF-BB, and pro-inflammatory cytokines such as TNF-α, cyclooxygenase-2 (COX-2), monocyte chemoattractant protein (MCP)-1 and IL-6 has been detected.

Vitreous samples collection

OIR and control group mice were killed by hyper-anesthesia (0.2 mg/mL pentobarbital sodium intraperitoneal injection) at P17. Eyes were enucleated and vitreous humors were taken carefully in sterile tubes. Then, serving the samples on ice promptly and centrifuged (15,000*g*, 5 min) at 4 °C. The vitreous samples were kept in − 80 °C until the analysis was performed.

Quantification of sCD200 level in vitreous samples

The level of sCD200 in the supernatants of vitreous samples was quantified by an enzyme-linked immunosorbent assay (ELISA) kit (cat # 10886-H08HL, Sino Biological, Beijing, China). Each sample was diluted tenfold and 100 μL

Table 2 Primers for qRT-PCR

Gene	Forward	Reverse
CD200	TGTTCCGCTGATTGTTGGC	ATGGACACATTACGGTTGCC
CD200R1	AGGAGGATGAAATGCAGCCTTA	TGCCTCCACCTTAGTCACAGTATC
CD200R2	AGAGACTGCCCCACCTAAGGA	AGAGACTGCCCCACCTAAGGA
CD200R3	AGTGCCACAGGGAGAAAAACA	TCCAGTTATCAGTCAAATGGGAGAT
CD200R4	GACCACCCCCCGTTCCT	CCTACGTTAAGAAGAATAATCACCAAAA
β-Actin	CAACGAGCGGTTCCGATG	GCCACAGGATTCCATACCCA
COX-2	CCAGATGATATCTTTGGGGAGAC	CTTGCATTGATGGTGGCTG
IL-6	TGCATGACTTCAGCTTTACTCTTTG	GGGGAGATAGAGCTTCTCTTTCGTT
MCP-1	ACTGAAGCCAGCTCTCTCTTCCTC	TTCCTTCTTGGGGTCAGCACAGAC
TNF-α	CAGGGGCCACCACGCTCTTC	CTTGGGGCAGGGGCTCTTGAC
PDGF-BB	TGCTGAGTGACCACTCGATC	TCCAAGGGTCTCCTTCAGTG
VEGF-A	GGAGGGCAGAATCATCACG	TGGAAGATGTCCACCAGGG

diluted fluid was used for analysis. The results were read at wavelength of 450 nm in microplate reader (FLUOstar Omega-Microplate reader, BMG Labtech, Offenburg, Germany). Actual data of sample concentration, which could be obtained by multiplying the correction result by dilution factor, were calculated by four-parameter fit logistic (4-PL) curve equation.

Retinal whole-mount immunofluorescence

Mice were killed at P17 by method described above when RNV area got to the maximum [32]. Eyes were enucleated and fixed for 1 h at 4 °C with 4% paraformaldehyde (PFA). After cutting the eyeballs along the ora serrata, and removing cornea, lens, iris, vitreous and sclera, the intact retinas were carefully separated under microscope. The separated retinas were blocked and permeabilized with 5% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) and 0.5% Triton-X-100 in 1 × PBS overnight at 4 °C. To identify the expression of CD200 and CD200R1 in retinal tissues and the effects of CD200Fc, double immunofluorescent staining was performed. The retinas were incubated with primary antibodies for Alex594-labeled *Griffonia simplicifolia* isolectin B4 (IB4, endothelial cell marker, 1:50, Invitrogen Life Technologies Co., Carlsbad, CA, USA), ionized calcium-bind adapter molecule 1 (iba1, microglia marker, 1:200, Santa Cruz, CA, USA), CD200 (1:100, Abcam, Cambridge, MA, USA) and CD200R1 (1:100, Abcam, Cambridge, MA, USA) overnight at 4 °C, washed the retinas and incubated them with a cocktail of fluorescein isothiocyanate- and Cy3-conjugated secondary antibodies (1:500, Cell Signaling Technology, Beverly, CA, USA) for 2 h at ordinary temperature. DAPI was added to counterstained for 10 min. Washed the retinas by PBS again and radial incisions were made at four equal intervals of the retinas, and mounted the retinas on slides and analyzed by confocal microscopy (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

Data were analyzed by SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All samples were tested in triplicate and all results are expressed as mean ± SD. Comparison of two groups used Mann–Whitney *U* test. Differences were considered to be statistically significant at P value < 0.05.

Results

Changes in protein levels of CD200 and its receptors in retina after OIR

To investigate the association between protein expression of CD200 and its receptors and pathological RNV development, western blot was used to detect the protein levels of CD200, CD200R1, CD200R2, CD200R3 and CD200R4 in retinas from OIR mice and control mice at P17. The protein levels of CD200 and CD200R1 were remarkably elevated in OIR group ($***P < 0.001$). Meanwhile, the protein expression of CD200R2, CD200R3 and CD200R4 did not show any difference between OIR group and control group (Fig. 1).

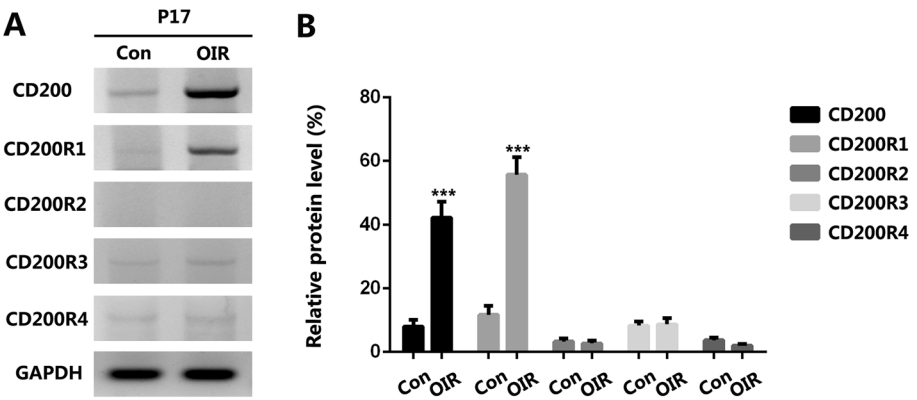

Fig. 1 Changes in protein levels of CD200 and CD200R family in retinas from OIR mice and control mice. Retinal tissues from OIR group and control group were harvested at P17. **a** Protein levels of CD200 and CD200R1–4 in retinas from different group were examined by western blot. GADPH was used to ensure equal loading. **b** Quantification graphs for CD200 and CD200R1–4. Optical density from each band was normalized by the relative GAPDH level. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus control group ($n = 6$). CD200 and CD200R1 protein levels increased remarkably at P17 in OIR group, and the protein expression of CD200R2–4 remained low in both OIR and control groups and showed no difference between two groups

Changes in mRNA levels of CD200 and its receptors in retina after OIR

The mRNA levels of CD200 and its receptors after OIR were detected via qRT-PCR analysis. At P17, the mRNA levels of CD200, CD200R1, CD200R2, CD200R3 and CD200R4 in OIR and control samples underwent a similar changing pattern as that of protein levels described above. The mRNA expressions of CD200 and CD200R1 increased significantly in OIR group (***$P<0.001$), and mRNA levels of CD200R2, CD200R3 and CD200R4 showed no difference between OIR group and control group (Fig. 2). These data indicated that the expressions of CD200 and CD200R1 were up-regulated substantially after OIR.

Changes of sCD200 level in vitreous humor after OIR

The level of sCD200 in vitreous humor from control and OIR mice was detected at P17 by ELISA kit. The result showed that there was a prominent elevation of sCD200 level in OIR group compared with the control group ($P<0.005$) (Fig. 3).

Localization of CD200 in retina after OIR

To determine the cellular localization of CD200 in retina after OIR, retinal whole-mount double-labeling immunofluorescence with antibodies specific to CD200 and IB4 (retinal endothelial cells marker) was used. Strong staining of CD200 (green) was observed in OIR samples at P17, and the staining of CD200 was co-localized with IB4 signal (red) on retinal endothelial cells in NV areas, while other cell types showed no CD200 staining. Besides, extremely weak CD200 signal could be detected in control samples (Fig. 4). These

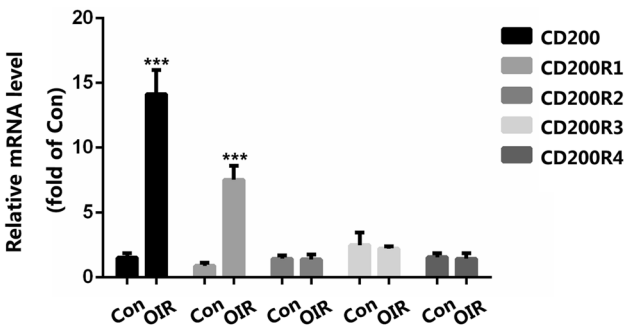

Fig. 2 Changes in mRNA levels of CD200 and CD200R family in retinas from OIR mice and control mice. mRNA levels of CD200 and CD200R1–4 in retinas from different group were examined by qRT-PCR. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus control group ($n=6$). mRNA levels of CD200 and CD200R1 in OIR group were significantly higher than that in control group. But there was no difference in CD200R2–4 mRNA levels between OIR and control groups

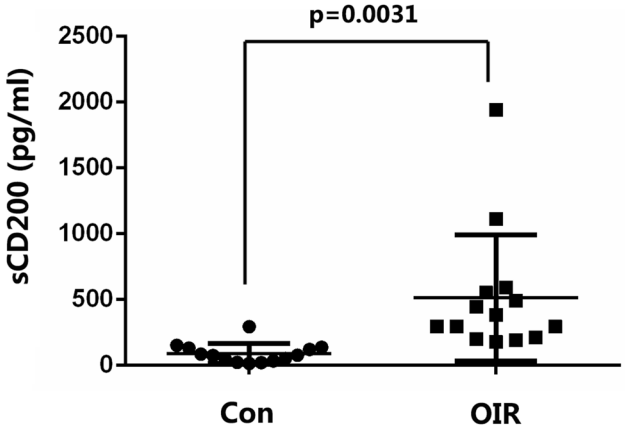

Fig. 3 Changes of sCD200 level in vitreous humor from OIR mice and control mice. The level of sCD200 in vitreous humor from different group was detected by ELISA kit. Compared with control group, sCD200 level was elevated remarkably in OIR group ($n=6$, $P<0.005$)

results indicated that CD200 up-regulated predominantly in retinal endothelial cells after OIR.

Localization of CD200R1 in retina after OIR

Retinal whole-mount double-labeling immunofluorescence with antibodies specific to CD200R1, IB4 and iba1 was used to identify the distribution of CD200R1 in retina at P17 after OIR. Compared with the control group, fluorescence signal of CD200R1 was significantly enhanced in OIR samples at P17. Double-labeling immunofluorescence showed that CD200R1 was co-expressed with IB4 on retinal endothelial cells in NV areas and also co-expressed with iba1 on microglial cells at the same time (Fig. 5b, c). Similar with that of CD200, CD200R1 signal was extremely low in control samples (Fig. 5a). These results implied that CD200R1 was expressed predominantly on retinal endothelial cells and microglia after OIR.

CD200Fc attenuated pathological RNV in OIR mice

To explore the effects of CD200Fc on RNV, we used retinal whole-mount immunofluorescence with IB4 to show the changes of NV area in OIR group and OIR + CD200Fc group (Fig. 6). As shown in retinas of OIR group (Fig. 6a1, a2), multiple NV tufts were generated at the edge of avascular area, while in OIR + CD200Fc group (Fig. 6b1, b2), the NV tufts and NV area shrank remarkably and the shape of vascular branches became more regular. The ratio between NV area and total retinal area was dramatically reduced in OIR + CD200Fc group compared to that in OIR group (Fig. 6c).

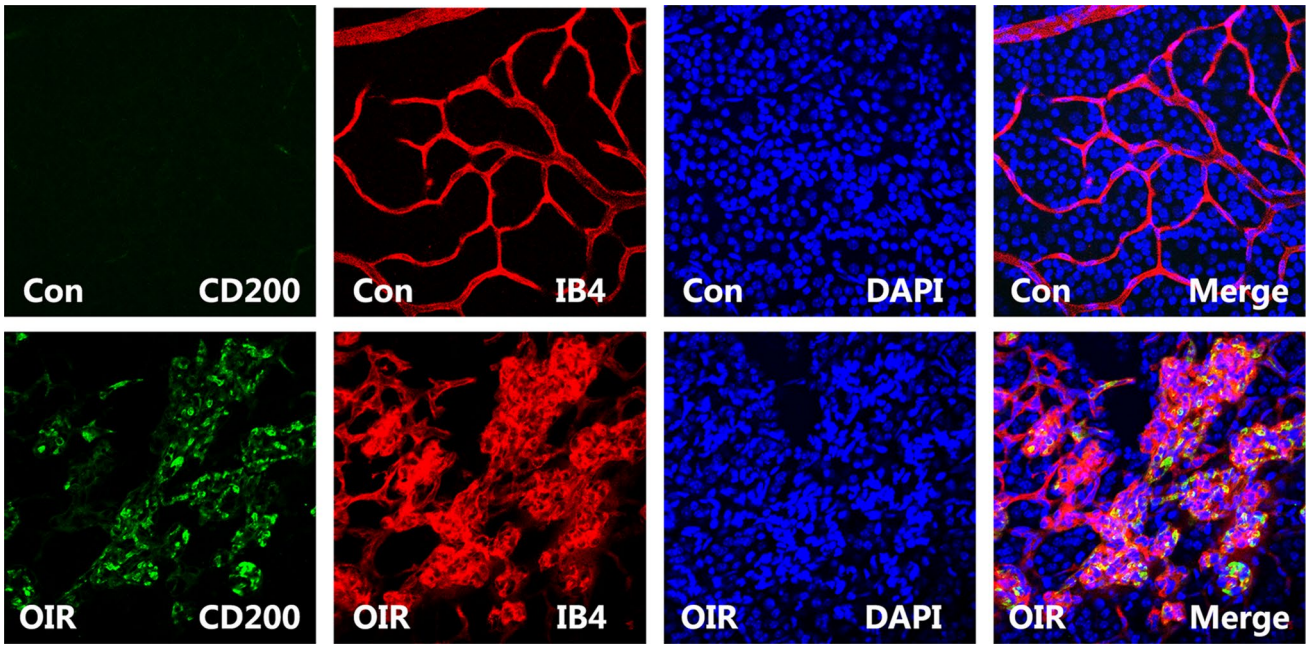

Fig. 4 Expression and localization of CD200 in retinas from OIR mice and control mice. Retinal mounts from different groups were prepared at P17 and stained with antibodies specific to CD200 (green), IB4 (red) and DAPI (blue). 40X magnification. The signal of CD200 was strongly positive on retinal endothelial cells which marked by IB4 in neovascular area of OIR samples, while extremely weak CD200 signal could be detected in control samples (Color figure online)

CD200Fc down-regulated VEGF-A, PDGF-BB and pro-inflammatory cytokines expression in retinas of OIR mice

At P17, qRT-PCR analysis was implemented on the retinas from OIR group and OIR + CD200Fc group, and the expression changes of VEGF-A, PDGF-BB and pro-inflammatory cytokines (COX-2, IL-6, MCP-1 and TNF-α) after CD200Fc treatment had been tested. The results showed that the mRNA level of VEGF-A, PDGF-BB, COX-2, IL-6, MCP-1 and TNF-α was extremely lowered in OIR + CD200Fc group (Fig. 7, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$). These results suggested that CD200Fc intravitreal injection could down-regulate the secretion of some representative angiogenic and pro-inflammatory factors.

CD200Fc inhibited microglia proliferation and activation in retinas of OIR mice

Retinas of OIR group and OIR + CD200Fc group were subjected to whole-mount immunofluorescent staining with IB4 and iba1 at P17. The results showed that at the NV areas of retinas in OIR mice, plenty of microglia had been hyperactivated and turned to ameboid phenotype (Fig. 8a1–a3), and these ameboid microglial cells were concentrated near the neovascular tufts. After CD200Fc treatment, the quantity of microglial cells, especially the ameboid microglia, was down-regulated significantly (Fig. 8b1–b3, c, $^{***}P < 0.001$), and the number of nonactivated microglia (dendritic phenotype) also showed a modest decline (Fig. 8c, $^{*}P < 0.05$). These results indicated that in retinas of OIR mice, CD200Fc intravitreal injection attenuated the proliferation and activation of microglia which may be associated with hypoxia-induced retinal microangiopathy.

Discussion

To the best of our knowledge, this study is the first to explore the expression changes and potential roles of CD200 and CD200R1 in OIR mice model. We have detected that the expression levels of CD200 and CD200R1 were statistically enhanced in retinal samples of OIR group, and the increased CD200 and CD200R1 were localized mainly on retinal endothelial cells in NV area, while CD200R1 was also expressed on microglia at the same time. CD200Fc is a specific agonist of CD200R1. In our study, we first investigated that CD200Fc intravitreal injection could shrink the pathological NV area, down-regulate the secretion of VEGF-A, PDGF-BB and pro-inflammatory cytokines (COX-2, IL-6, MCP-1 and TNF-α), and inhibit the proliferation and activation of microglia in OIR mice.

CD200–CD200R axis was identified as an immunoregulatory signaling pathway widely expressed on immune

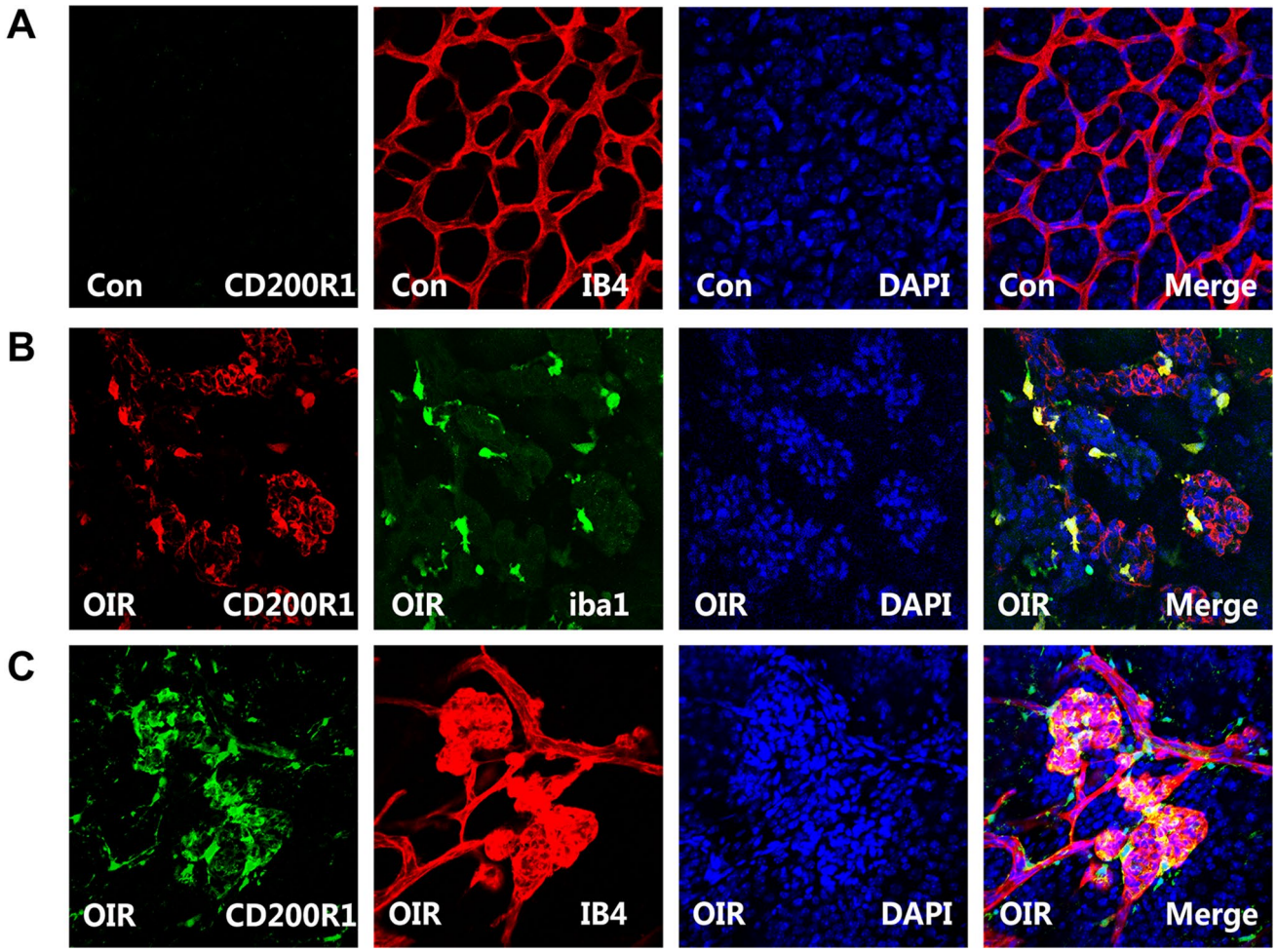

Fig. 5 Expression and localization of CD200R1 in retinas from OIR mice and control mice. Confocal images of retinal mounts from different groups showed the co-localization of CD200R1 (**b**, red) and iba1 (**b**, green) on microglia, and the co-localization of CD200R1 (**c**, green) and IB4 (**c**, red) on retinal endothelial cells after OIR. ×40 magnification. DAPI was shown as blue fluorescence. The fluorescence of CD200R1 was remarkably elevated on microglia marked by iba1 and retinal endothelial cells marked by IB4 in neovascular area of OIR samples. On the contrary, in control samples, extremely low signal of CD200R1 could be detected (**a**) (Color figure online)

cells and neurons, and mainly studied in the fields of autoimmune diseases, neurodegenerative diseases and human malignancies [34–36]. CD200R1 is the most important member in CD200R family and has the highest affinity with CD200 [21]. Our result is in accord with this finding. The protein and mRNA of CD200R1 were remarkably overexpressed in OIR group at P17, coupled with the high level of CD200 expression, while the expression of CD200R2–4 did not show significant differences between OIR group and control group.

Dick and his coworkers [26] first described the expression of CD200 and CD200R in normal and inflamed retinas from rat, mouse and human. They first observed an up-regulation of CD200 in an experimental autoimmune (EAU) mice model [26]. In the present study, we also found that the levels of CD200 and CD200R1 in retinal tissues and the level of sCD200 in vitreous samples were all elevated significantly in OIR group.

As of now, chronic inflammation has been proved to play a central role during ocular neovascularization; the mechanisms include the secretion of numerous pro-inflammatory factors, recruitment of leukocytes, and microglial activation, etc. [15–18, 37]. The relationship between CD200–CD200R axis and ocular neovascularization had been preliminarily observed in previous studies. In CD200R-deficient mice, laser-induced choroidal neovascularization (CNV) area was enhanced, while giving a CD200R agonist mAb could change macrophage phenotype, inhibit pro-angiogenic gene expression, and finally suppress the CNV development [38]. CD11b+CD200+ monocytes were significantly higher in venous blood of patients suffered from neovascular AMD [27] and

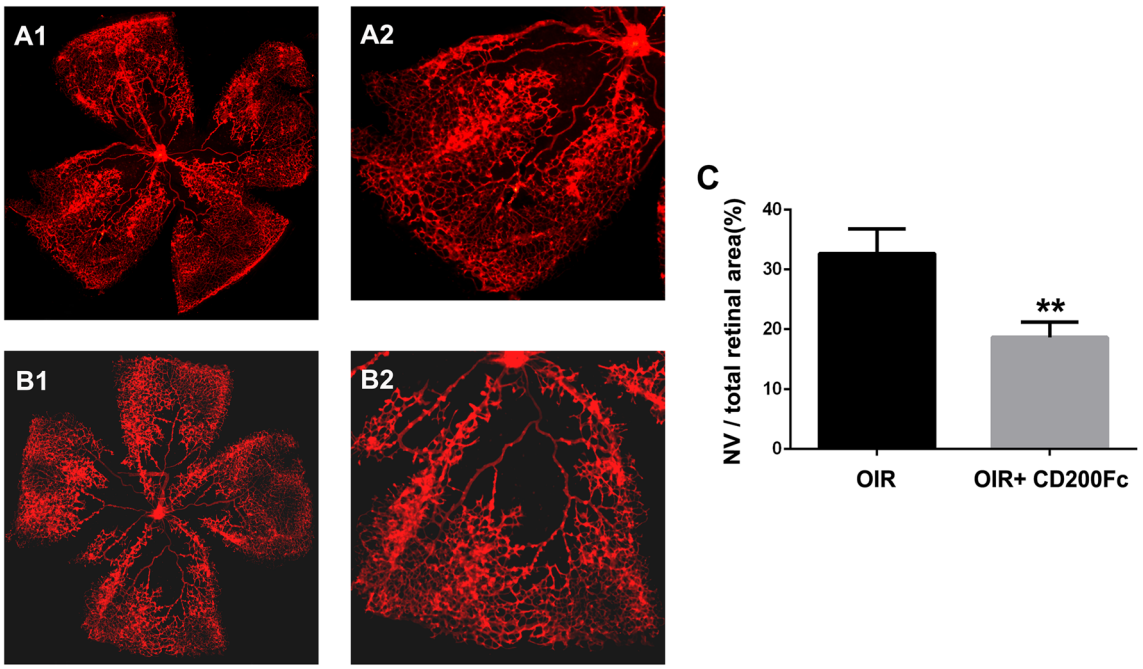

Fig. 6 The effect of CD200Fc on pathological RNV in OIR mice. Whole-mount retinal immunostaining with IB4 of OIR group (**a1**, **a2**) and OIR + CD200Fc group (**b1**, **b2**) was shown above. **a2** and **b2** were the partial enlarged detail of **a1** and **b1**. Retinas were harvested at P17. Compared with OIR group, the NV area was extremely shrunk after CD200Fc treatment (**c**, $n = 6$, $^{**}P < 0.01$)

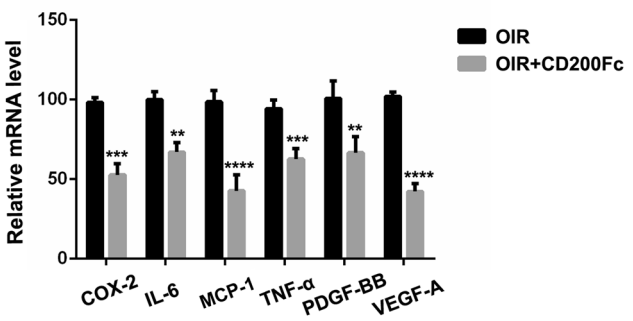

Fig. 7 The effect of CD200Fc on the secretion of VEGF-A, PDGF-BB and several pro-inflammatory cytokines in OIR mice. The mRNA levels of VEGF-A, PDGF-BB, COX-2, IL-6, MCP-1 and TNF-α in retinas from OIR group and OIR + CD200Fc group were examined by qRT-PCR at P17. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus OIR group ($n = 6$). In the retinas of OIR + CD200Fc group, the mRNA levels of VEGF-A, PDGF-BB, COX-2, IL-6, MCP-1 and TNF-α were reduced dramatically compared to OIR group

polypoidal choroidal vasculopathy (PCV) with branching vascular network (BVN) (type 1) [39]. The expression changes of CD200 and CD200R1 in OIR mice we had observed were novel findings to provide more evidences to prove the link between CD200–CD200R1 axis and RNV, but the regulatory effects and mechanisms remained unclear. Therefore, we further investigated the functions of CD200Fc in OIR mice and tried to analyze its mechanism. The results indicated that CD200Fc treatment could dramatically decrease the secretion of several important angiogenic and pro-inflammatory cytokines, inhibit the proliferation and activation of microglia, and narrow the NV area.

Microglial cells are resident immune surveillance cells in central nervous system (CNS), as well as in ocular nerve and retina [40]. The relationship between CD200–CD200R1 axis and neuro-inflammation mediated by microglial activities has already been confirmed in vitro and in vivo [41–43]. Besides the potential for neurodegenerative diseases, the function of microglia in neovascularization causes more concern and discussion. It has been demonstrated that local microglial cells take part in physiologic vascular development in retina [44]. But in pathological condition, like hypoxia, the proliferated and activated microglia would release excessive inflammatory factors, aggravate the micro-environment of retina and lead to pathological neovascularization [15, 45, 46]. Microglia activation and infiltration could be detected in an experimental branch retinal vein occlusion (BRVO) model [45]. The activated microglia could alter RMECs phenotype, stimulate the expression of VEGF-A and PDGF-BB, facilitate migration, proliferation, and permeability of RMECs, and finally promote angiogenesis [47]. Our results showed a plenty of ameboid microglial cells concentrated near the NV tufts in OIR mice and the quantity of these hyperactivated microglia was extremely decreased by CD200Fc treatment. On the basis of researches above, it is reasonable for us to believe

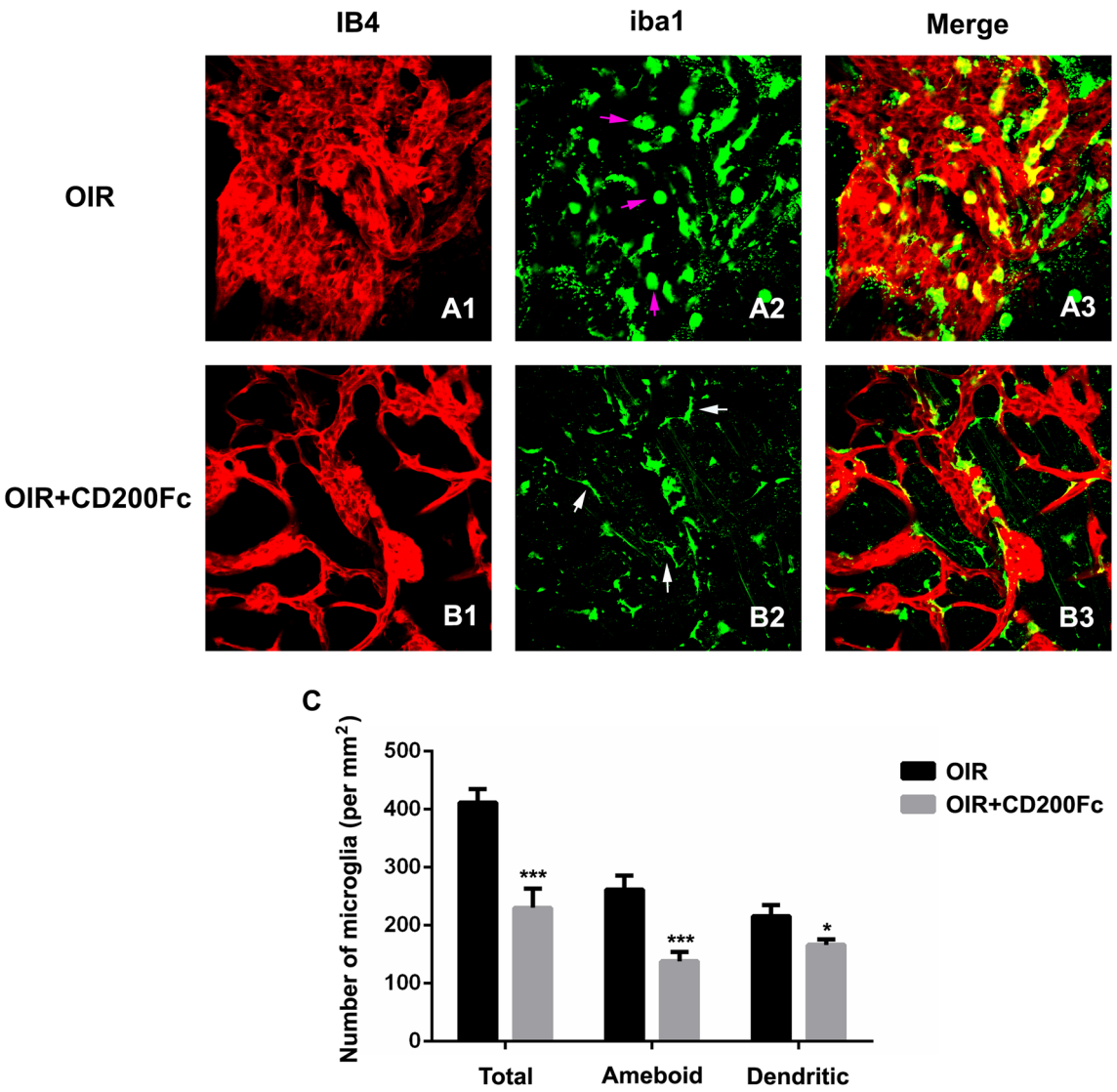

Fig. 8 The effect of CD200Fc on microglia proliferation and activation in OIR mice. Whole-mount retinal immunostaining with IB4 (red) and iba1 (green) were performed on retinas from OIR group (**a1**–**a3**) and OIR + CD200Fc group (**b1**–**b3**) at P17. ×40 magnification. A mass of activated ameboid microglia (**a2**, purple arrows) gathering around the neovascular area (**a3**), while in OIR + CD200Fc group, the ratio of ameboid microglia decreased dramatically and most microglial cells stayed in nonactivated dendritic phenotype (**b2**, white arrows). The quantity of total microglia and ameboid microglia was down-regulated significantly after CD200Fc treatment, while the number of nonactivated microglia (dendritic phenotype) also showed a modest decline at the same time (**c**). $n = 6$, $^{*}P < 0.05$, $^{***}P < 0.001$ (Color figure online)

that CD200–CD200R1 axis has the potential to attenuated pathological RNV and the mechanism might involve microglia inhibition.

Taking together, our study reported the expression profiles of CD200 and its receptors, and further explored their potential roles during the process of RNV formation for the first time. The results provided evidences that the expression changes of CD200–CD200R1 axis were highly correlated with neovascularization in retina. Activation of this axis showed strong anti-inflammatory and anti-angiogenic effects in OIR model, which revealed a new potential therapeutic target for RNV treatment. Further studies are needed to explore the exact functions and mechanisms of CD200–CD200R1 axis in RNV.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

Ethical approval All animals involved in experiments were carried out in accordance with the US National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals published by the US National Academy of Sciences and approved by the Ethics Committee of Xi'an Jiaotong University.

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