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Retina

Pro-Fibrotic Role of Interleukin-4 in Influencing Idiopathic Epiretinal Membrane in Cataract Patients: Analysis From Clinical–Experimental Approaches

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Purpose: To evaluate the role of interleukin-4 in influencing idiopathic epiretinal membrane (iERM) formation and early progression post cataract surgery (PCS) from clinical and experimental perspectives.

Methods: We quantified levels of IL-4 in aqueous humor (AH) samples from 22 iERM patients and 31 control subjects collected before and 20 hours after cataract surgeries using ELISA. After a 3-month follow-up, the association between IL-4 levels and iERM progression measurements was identified. In addition, in vitro studies were conducted to investigate the effects of IL-4 on primary rat retinal Müller glia proliferation, migration, and glial–mesenchymal transition (GMT).

Results: Concentrations of IL-4 were significantly higher in preoperative AH samples from iERM patients versus controls ($P = 0.006$). Postoperatively, although IL-4 levels were elevated in both groups compared to their respective preoperative levels, they were even more obviously so in the iERM group ($P < 0.001$). Multivariate linear regression analyses revealed that, postoperatively, IL-4 level elevation was positively associated with macular volume and thickness increase (both $P < 0.05$) in iERM patients. However, no correlations were observed between IL-4 level (changes) and clinical characters in the controls. In vitro studies demonstrated that IL-4 promoted Müller glia proliferation and migration and increased the expression of GMT-related markers in a manner independent of transforming growth factor-β1 (TGF-β1).

Conclusions: IL-4 plays a crucial pro-fibrotic role in iERM formation and early progression 3 months PCS possibly by stimulating Müller glia proliferation, migration, and GMT in a TGF-β1–independent manner.

Translational Relevance: The current study suggests the potential of IL-4 as a novel therapeutic target for iERM.

Introduction

Epiretinal membrane (ERM), a relatively prevalent retinal fibrotic disorder, is characterized by pre-retinal fibrocellular growth, which can result in mild to severe visual acuity loss, metamorphopsia, and micropsia, thereby compromising the quality of life for patients.[1,2] Idiopathic ERM (iERM) is a ubiquitous variant of ERM that arises in the absence of any pre-existing ocular abnormalities, and its pathogenesis is still poorly understood.[3] ERM is histologically comprised of various cells, including Müller glia, hyalocytes, and fibroblasts, among others, along with extracellular matrix (ECM) components such as fibronectin and collagen.[4] The formation process typically involves cell proliferation, migration, epithelial–/glial–mesenchymal transition

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(EMT/GMT), and deposition of ECM.[5] ERM may be either stable or progressive, with cataract surgery often serving as a potential trigger for the progression and development of ERM post cataract surgery (PCS).[6–8] Given the escalating annual rate of cataract surgeries, strategies for the prevention of ERM progression PCS as early as possible are becoming increasingly important.

The mechanisms underlying ERM progression PCS are multifactorial, with cytokine mediation playing a significant role.[9,10] Aqueous cytokines with elevated levels PCS, such as interleukin-8 (IL-8) and transforming growth factor-β (TGF-β), are believed to contribute to postoperative ocular fibrotic changes, including the fibrotic transition of corneal endothelial cells, amplification of fibrotic scars, and accelerated ECM deposition.[9,11] Our prior study employing the Luminex assay has also demonstrated that the levels of many aqueous cytokines significantly increase PCS in iERM patients, with IL-4 being a prominent one.[12] Known as a pro-fibrotic cytokine, IL-4 plays a role in fibrotic diseases across various organs, such as the salivary glands, nose, lung, and heart.[13–16] Within ocular conditions, IL-4 has been implicated in the fibrotic activation of conjunctival cells and tissue fibrosis during the development of proliferative vitreoretinopathy (PVR).[17–19] Consequently, IL-4 is likely to be a critical regulator of fibrosis in iERM progression, a role that warrants further comprehensive studies.

Müller glia, providing essential structural support to the neuroretina, is an important cell type believed to play a central role in the pathogenesis of iERM.[5,20] Müller glia proliferation and migration, GMT, and the extracellular composition induced by profibrotic cytokines are of great importance during iERM development.[5,21,22] TGF-β is an acknowledged profibrotic cytokine involved in iERM development by activating Müller glia.[22,23] Despite its being a well-known profibrotic cytokine, research about the effect of IL-4 on Mu¨ller glia is remarkably scarce.

Therefore, in this study, we evaluated the role of IL-4 in iERM formation and early iERM progression PCS from both a clinical and experimental perspectives. Specifically, we investigated the expression of IL-4 in pre- and post-cataract-surgery aqueous humor (AH) samples from iERM and control subjects with enzyme-linked immunosorbent assays (ELISAs), and we analyzed the correlations between IL-4 and clinical characters in both patient groups. Then, we investigated the potential pro-fibrotic impacts of IL-4 on primary rat retinal Müller glia in vitro.

Materials and Methods

Patients

A total of 53 patients with age-related cataract (ARC) were enrolled in this study, including 31 patients with ARC alone (31 eyes) and 22 patients with coexisting ARC and iERM (22 eyes). The research was conducted in the Ophthalmology Department of the Affiliated Hospital of Nantong University between July 2020 and May 2021. This study adhered to the tenets of the Declaration of Helsinki and was approved by the ethics committee of the hospital. Written informed consent was collected from all patients. Optical coherence tomography (OCT) examinations were utilized to diagnose ERM when irregular, hyperreflective lines were observed on the inner limiting membrane.[2] Exclusion criteria for this study included glaucoma, high myopia, systemic comorbidities (e.g., diabetes mellitus), uveitis, a history of surgery in either eye, or any other ocular abnormalities leading to secondary ERMs

Ophthalmologic Examinations

Comprehensive ophthalmologic assessments, including slit-lamp examination, visual acuity measurement, CIRRUS HD-OCT (Carl Zeiss Meditec, Dublin, CA), and other basic evaluations, were conducted meticulously pre- and postoperatively at 3-month intervals. Quantitative analysis of central foveal thickness (CFT), macular thickness (MT), and macular volume (MV) was performed based on OCT imaging with an Early Treatment Diabetic Retinopathy Study (ETDRS) grid. CFT was recorded as the mean retinal thickness within the central 1-mm area. The mean retinal thickness and volume within the central 6-mm area were referred to as MT and MV, respectively. The severity of ERM was evaluated and classified into four stages using a multistage grading method according to OCT imaging.[24] Stage 1 was characterized by the presence of mild ERMs with little morphological distortion, the preservation of foveal depression, and easily identified retinal layers. Stage 2 was defined by the presence of ERMs with lost foveal depression and a thicker outer nuclear layer but still with clearly identified retinal layers. Stage 3 was characterized by the presence of ERMs with absent foveal depression and continuous ectopic inner foveal layers (EIFLs) anomalously crossing through the fovea; the retinal layers on OCT were still clear. Stage 4 was defined by the presence of ERMs with EIFLs, completely destroyed

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anatomy, and lack of clear identification of retinal layers.

Collection of AH

Standard cataract phacoemulsification surgery and AH collection procedures were performed as we previously described.[12] Briefly, preoperative AH samples (100–150 µL) were acquired before making the first corneal incision with a 30-gauge needle during the surgery. Postoperative AH samples were acquired approximately 20 hours PCS by two experienced oculists. One operator identified the side port site and inserted a 30-gauge needle connected to an insulin syringe through the side incision under slit-lamp biomicroscopy. Then, the second operator withdrew 50 to 70 µL of AH samples, decided by the depth of anterior chamber. Immediately after collection, samples were stored at −80°C until final use. AH samples were all acquired by the same experienced surgeon.

ELISA of AH

Concentrations of IL-4 in all collected AH samples were examined using human IL-4 ELISA kits (R&D Systems, Minneapolis, MN) per the manufacturer's instructions.

Primary Rat Retinal Müller Glia Culture

All animal experiments were conducted following the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Primary rat retinal Müller glia cultures were prepared following previous procedures.[25] Briefly, retinas were isolated from postnatal 5-day Sprague Dawley rats and digested with Gibco 0.25% trypsin–EDTA (Thermo Fisher Scientific, Waltham, MA) for 5 minutes at 37°C. The resulting cell suspensions were cultured in Gibco Dulbecco's modified Eagle's medium (DMEM)/F12 (Thermo Fisher Scientific), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Thermo Fisher Scientific), at 37°C and 5% CO_2. The culture medium was refreshed every 48 hours. The third-generation Müller glia cultures, cultivated for at least 20 days, were utilized in the experiments. Cells from different culture batches were used for each independent experiment.

Immunofluorescent Staining

Immunofluorescent staining was performed following previous protocols.[26] Briefly, cultured Müller glia was fixed for 20 minutes (4% paraformaldehyde), blocked for 2 hours (5% goat serum and 0.5% Triton X-100), and then incubated with primary antibodies overnight at 4°C. Rabbit anti-GS (1:200; Abcam, Cambridge, UK), a Müller glial marker, was used to identify Müller glia. The following day, secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:200; Invitrogen, Carlsbad, CA) and 4′,6-diamidino-2-phenylindole (DAPI, 1:10000; Abcam) were used. Finally, a confocal microscope (SP8; Leica, Wetzlar, Germany) was employed to photograph and count glutamine synthetase $(GS)^+$ cells in six randomly chosen fields in each dish.

Müller Glia Proliferation Assay

Cell Counting Kit-8 (CCK-8; Dojindo, Kamimashiki-gun, Kumamoto, Japan) was utilized to evaluate cell proliferation. Briefly, 5000 cells/well (from the same culture batch) were plated into three 96-well plates, each for a different time. After incubating the cells with recombinant rat IL-4 (PeproTech, Rocky Hill, NJ) at 10 ng/mL or 100 ng/mL for 8, 16, and 24 hours in different plates at 37°C; 10% CCK-8 reagents were added and cultured for another 1.5 hours. Absorbance (450 nm) was measured with a microplate reader (ELx800, BioTek, Winooski, VT).

Wound Healing Assay

Müller glia was seeded at 5×10^5/well in 12-well plates. When cells reached 80% to 90% confluence, they were starved for 1 day. Then, Müller glia monolayers were scratched using 200-µL plastic tips and incubated with 10 ng/mL or 100 ng/mL IL-4 for 24 hours. Wounded monolayers were photographed (THUNDER Imaging Systems; Leica) at 0 hour and 24 hours after scratching. For analysis, wound closure was measured in three randomly selected fields.

Quantitative Polymerase Chain Reaction

Following 1 day of starvation, Müller glia was incubated with 10 ng/mL or 100 ng/mL IL-4. Twenty-four hours later, total RNA was isolated from cultured Müller glia by TRIzol Reagent (Invitrogen) according to protocols. Quantitative polymerase chain reaction (qPCR) was performed as previously described.[27] The difference in cycle time between the target mRNA and an internal standard (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) was used to calculate relative expression. The data were analyzed with the $2^{-\Delta\Delta CT}$ method. Supplemental Table S1 shows the oligonucleotide primers used.

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Statistical Analysis

All quantitative data were described using mean ± SD. Data analysis was conducted with SPSS Statistics 25.0 (IBM, Chicago, IL) and Prism 8.0 (GraphPad, San Diego, CA). Best-corrected visual acuity (BCVA) was transformed to logMAR for analysis. Categorical variables were analyzed using the χ^2 test. When continuous variables were normally distributed, Student's *t*-test (for two groups) or one-/two-way ANOVA tests (for multiple comparisons) were conducted as appropriate. The nonparametric Wilcoxon or Mann–Whitney *U* test was used if the data failed normal distribution. To compare the postoperative IL-4 levels, we used analysis of covariance adjusting for baseline clinical characters and preoperative IL-4 levels. Correlations between cytokine concentrations and clinical characters were determined using Spearman correlation, as well as univariate and multivariate linear regression analyses. Independent variables with $P < 0.15$ in the univariate analysis were further analyzed in the multivariate analysis.[28] $P < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

Results

Clinical Characters of Patients

Totally, 53 patients with 53 eyes (22 iERM patients and 31 control subjects) were enrolled in this study. Baseline clinical characteristics, including age, sex, BCVA, CFT, MT, MV, phacoemulsification time and energy, and cumulative dissipated energy (CDE) were compared between the two groups, and no significant differences were observed (Table 1). Among the iERM patients, 13, five, and four patients were classified as stage 1, 2, or 3, respectively.

Postoperatively, all patients completed the 3-month follow-up visit, and no one reported discomfort or symptoms. Compared to the control group, the patients with iERM demonstrated significantly less improvement in BCVA and greater increase in CFT, MT, and

Table 1. Comparison of Clinical Characters Between ARC and IERM Groups

	ARC Group ($n = 31$)	IERM Group ($n = 22$)	*P*
Age (y), mean ± SD	70.71 ± 9.29	74.00 ± 6.04	0.143[a]
Sex (male/female), *n*	17/14	11/11	0.728[b]
BCVA (logMAR), mean ± SD			
Baseline	0.53 ± 0.34	0.49 ± 0.26	0.730[c]
Three months	0.02 ± 0.15	0.18 ± 0.20	0.002[c]
Δ	−0.51 ± 0.30	−0.31 ± 0.30	0.011[c]
CFT (μm), mean ± SD			
Baseline	253.77 ± 13.13	313.23 ± 96.76	0.065[c]
Three months	257.84 ± 14.46	336.73 ± 116.56	0.013[c]
Δ	4.06 ± 7.73	22.45 ± 27.51	0.011[c]
MV (mm^3), mean ± SD			
Baseline	10.07 ± 0.44	10.46 ± 1.07	0.072[a]
Three months	10.24 ± 0.48	10.99 ± 1.02	0.006[c]
Δ	0.16 ± 0.35	0.52 ± 0.46	0.003[c]
MT (μm), mean ± SD			
Baseline	276.65 ± 12.55	285.45 ± 31.24	0.151[a]
Three months	282.16 ± 15.15	300.23 ± 27.34	0.023[c]
Δ	5.52 ± 9.83	15.36 ± 14.90	0.010[c]
Phacoemulsification energy (%), mean ± SD	24.24 ± 20.89	25.44 ± 20.09	0.220[c]
Phacoemulsification time (s), mean ± SD	18.88 ± 13.66	23.67 ± 15.48	0.223[c]
CDE, mean ± SD	3.66 ± 3.30	4.00 ± 2.78	0.312[c]

$P < 0.05$ is considered statistically significant. Δ represents changes postoperation versus pre-operation.
[a] *P* value for independent *t*-test.
[b] *P* value for χ^2 test.
[c] *P* value for Mann–Whitney *U* test.

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Table 2. Comparison of IL-4 Levels in AH Between ARC and IERM Groups

	ELISA Groups			Luminex Assay Groups		
IL-4 Levels	ARC ($n = 31$)	IERM ($n = 22$)	*P*	ARC ($n = 25$)	IERM ($n = 25$)	*P*
Preoperative	0.48 ± 0.08	0.57 ± 0.13	0.006[a]	0.17 ± 0.10	0.28 ± 0.12	0.008[a]
Postoperative	0.58 ± 0.10	0.84 ± 0.23	<0.001[a] <0.001[b]	0.29 ± 0.08	0.56 ± 0.16	<0.001[a] <0.001[b]

$P < 0.05$ is considered statistically significant.
[a]*P* value for Mann–Whitney *U* test.
[b]*P* value for analysis of covariance adjusting for baseline clinical characters and preoperative IL-4 levels.

Table 3. Association Analysis of ΔMV With Clinical Characteristics in IERM Eyes

	Univariate		Multivariate	
	Beta Coefficient (95% CI)	*P*	Beta Coefficient (95% CI)	*P*
Age	0.005 (−0.031 to 0.041)	0.774	—	—
Sex	0.082 (−0.337 to 0.501)	0.688	—	—
BCVA	0.463 (−0.362 to 1.287)	0.256	—	—
CFT	0.000 (−0.003 to 0.002)	0.688	—	—
MV	−0.141 (−0.330 to 0.049)	0.137	−0.195 (−0.774 to 0.384)	0.488
MT	−0.005 (−0.011 to 0.002)	0.127	0.004 (−0.017 to 0.024)	0.689
Phacoemulsification energy	0.001 (−0.010 to 0.012)	0.854	—	—
Phacoemulsification time	0.001 (−0.013 to 0.015)	0.921	—	—
CDE	0.020 (−0.057 to 0.097)	0.599	—	—
Preoperative IL-4 levels	−0.587 (−2.285 to 1.112)	0.480	—	—
ΔIL-4 level	0.954 (0.243 to 1.666)	0.011	0.911 (0.112 to 1.710)	0.028

Only variables with $P < 0.150$ in the univariate model were entered in the multivariate model.

MV (all $P < 0.05$) (Table 1). However, no changes were observed regarding the ERM stage.

IL-4 Levels in AH Samples

Levels of IL-4 were measured using ELISA in all pre- and postoperative AH samples. Preoperatively, concentrations of IL-4 were significantly higher in the iERM group than in the controls ($P = 0.006$) (Table 2). To better elucidate the role of IL-4 in iERM eyes PCS, we compared postoperative IL-4 levels between iERM and control subjects using analysis of covariance adjusting for baseline clinical characters and preoperative IL-4 levels. Although postoperative IL-4 levels were elevated in both groups compared to their respective preoperative levels, they were even more obviously elevated in the iERM group ($P < 0.001$) (Table 2). These findings were all consistent with the previous Luminex assay results (Table 2).

Correlations of IL-4 and Clinical Characters

To determine whether there was a correlation between IL-4 and clinical character changes PCS, we performed a correlation analysis. Our results demonstrated that the ΔIL-4 level (post- vs. pre-operation) was positively correlated with 3-month ΔMV ($r = 0.571$, $P = 0.005$) and ΔMT ($r = 0.645$, $P = 0.001$). However, no obvious correlations were observed between IL-4 level (changes) and clinical characters in the control group.

Furthermore, correlations between ΔMV/MT and other characters were calculated to identify other factors that may affect iERM anatomical changes in the iERM group (Tables 3, 4). Univariate analysis revealed that the ΔIL-4 level was significantly linked to ΔMV ($B = 0.954$, $P = 0.011$) and ΔMT ($B = 32.139$, $P = 0.008$), whereas baseline MT was negatively correlated with ΔMT ($B = -0.203$, $P = 0.048$). In multivariate analysis, ΔIL-4 level was found to be a significant factor affecting both ΔMV ($B = 0.911$, $P = 0.028$) and ΔMT ($B = 24.991$, $P = 0.047$).

IL-4 Promotes the Fibrogenic Activity of Müller Glia

To explore the pathogenic role of IL-4 in iERM, we studied the pro-fibrotic effect of IL-4 on primary

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Table 4. Association Analysis of ΔMT With Clinical Characteristics in IERM Eyes

	Univariate		Multivariate	
	Beta Coefficient (95% CI)	*P*	Beta Coefficient (95% CI)	*P*
Age	−0.256 (−1.401 to 0.889)	0.646	—	—
Sex	−1.091 (−14.663 to 12.481)	0.869	—	—
BCVA	8.654 (−18.560 to 35.869)	0.515	—	—
CFT	−0.012 (−0.084 to 0.060)	0.730	—	—
MV	−4.660 (−10.754 to 1.435)	0.126	5.681 (−12.171 to 23.533)	0.512
MT	−0.203 (−0.405 to −0.002)	0.048	−0.319 (−0.950 to 0.312)	0.302
Phacoemulsification energy	0.176 (−0.160 to 0.512)	0.287	—	—
Phacoemulsification time	−0.074 (−0.522 to 0.373)	0.733	—	—
CDE	0.827 (−1.644 to 3.298)	0.493	—	—
Preoperative IL-4 levels	5.452 (−50.009 to 60.914)	0.840	—	—
ΔIL-4 level	32.139 (9.598 to 54.681)	0.008	24.991 (0.349 to 49.634)	0.047

Only variables with $P < 0.150$ in the univariate model were entered in the multivariate model.

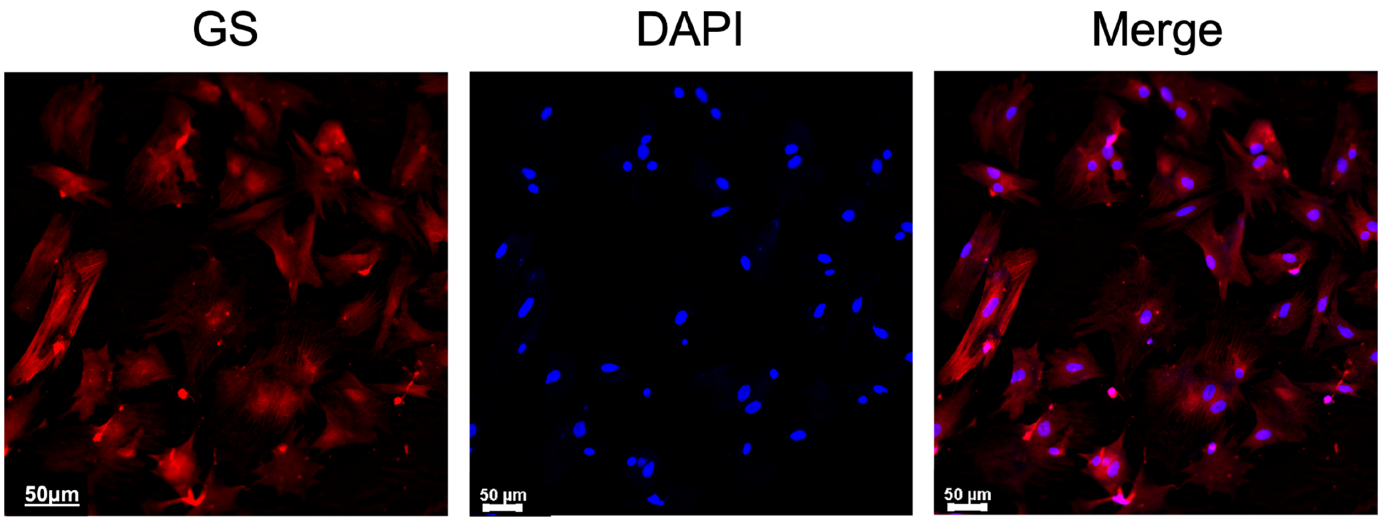

Figure 1. Primary rat retinal Müller glia identification. Representative images of third-generation primary rat retinal Müller glia cultures stained with glutamine synthetase (GS) (*red*) and DAPI (*blue*).

rat retinal Müller glia in vitro. Müller glia has been identified as a critical cell component of iERM in previous studies.[5,22,29] Immunofluorescent analysis was conducted to examine the purity of the third-generation primary rat Müller glia, which revealed that Müller glia cultures were comprised of approximately 93.1% ± 2.9% GS^+ cells (Fig. 1). These cultured cells were used for subsequent experiments.

Exposure of Müller glia to 10 ng/mL or 100 ng/mL IL-4 significantly promoted cell proliferation in a dose- and time-dependent manner versus controls (Figs. 2A, 2B). In addition, the scratch assay revealed an enhanced ability of migration in Müller glia after 10-ng/mL IL-4 treatment for 24 hours, which was even more pronounced after 100 ng/mL IL-4 treatment (Figs. 2D, 2E).

To evaluate the GMT process in Müller glia, we measured the expression of fibrotic markers by quantitative reverse transcription polymerase chain reaction (qRT-PCR), including *COL1A1*, which encodes for type I collagen and is an ECM marker; fibronectin, another ECM marker; α-smooth muscle actin (α-SMA), a myofibroblast marker; and glial fibrillary acidic protein (GFAP), a glial cell activation marker.[5] *CCND1* (encoding for cyclin D1 protein) was also detected to evaluate the proliferation results of Müller glia.[5] TGF-β1, a mediator of the fibrotic process and mediated by IL-4, was also measured to further determine how IL-4 functions.[30] Increased expression of *COL1A1*, fibronectin and α-SMA and decreased expression of GFAP are indicative of the GMT process in Müller glia.[5,31] Our results showed that IL-4 induced

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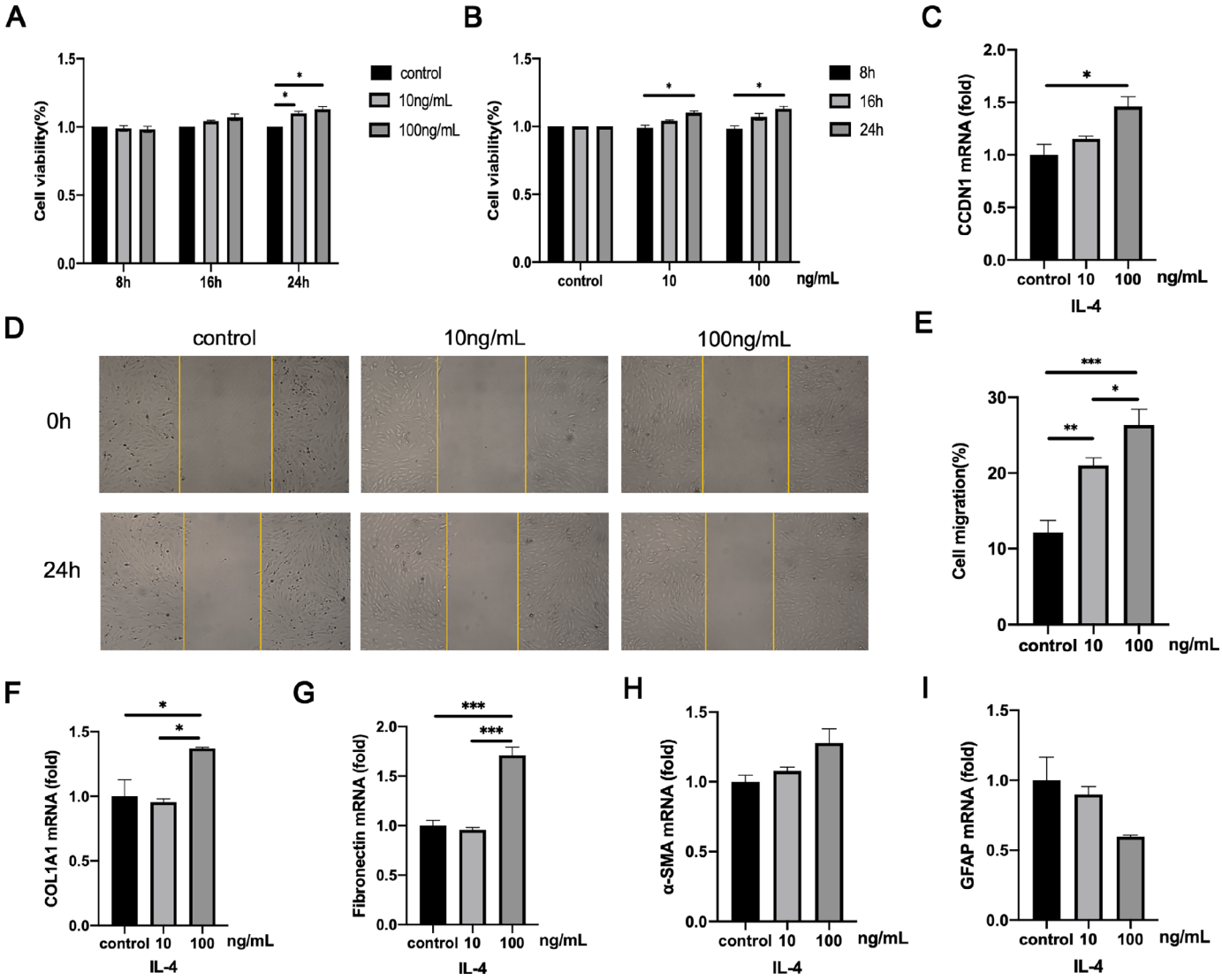

Figure 2. IL-4 promoted Müller glia proliferation, migration, and GMT. (**A**, **B**) Measurement of cell proliferation by the CCK-8 assay in Müller glia treated with 10 ng/mL and 100 ng/mL IL-4 for 8, 16, and 24 hours. Cell viability = $(OD_{EG} - OD_{BG})/(OD_{CG} - OD_{BG}) \times 100\%$, where OD_{EG}, OD_{CG}, and OD_{BG} are the optical densities of the experimental group, control group, and blank group, respectively. (**C**) Measurement of cell proliferation by qRT-PCR in Müller glia treated with 10 ng/mL and 100 ng/mL IL-4 for 24 hours. (**D**) Measurement of cell migration by scratch assay in Müller glia treated with 10 ng/mL and 100 ng/mL IL-4 for 24 hours. (**E**) Quantification of migrating Müller glia 24 hours after wound scratch. Migration rate = $(W_0 - W_{24})/W_0 \times 100\%$, where W_0 and W_{24} are the scratch widths at 0 and 24 hours, respectively. (**F–I**) Measurement of mRNA expression of *COL1A1*, fibronectin, α-SMA, and GFAP by qRT-PCR in Müller glia treated with 10 ng/mL and 100 ng/mL IL-4 for 24 hours. The control group received no treatment. Graphs represent the mean ± SD. Statistical analysis was performed using two-way or one-way ANOVA, followed by Tukey's post hoc. ***$P < 0.0001$, **$P < 0.005$, *$P < 0.05$.

an increased expression of *COL1A1*, fibronectin, and CCND1 in Müller glia (Figs. 2C, 2F, 2G). Additionally, compared to the controls, the expression of α-SMA and GFAP after 100-ng/mL IL-4 treatment marginally increased ($P = 0.060$) and decreased ($P = 0.067$), respectively (Figs. 2H, 2I). However, the expression of TGF-β1 showed no significant differences among all groups. (control vs. 10 ng/mL, $P = 0.448$; control vs. 100 ng/mL, $P = 0.084$; 10 ng/mL vs. 100 ng/mL, $P = 0.416$).

Discussion

In the present study, we explored the impact of IL-4 on iERM formation and early progression PCS from both clinical and experimental viewpoints. First, we investigated the expression of IL-4 in pre- and post-cataract surgery AH samples with ELISA and found that concentrations of IL-4 were significantly higher in iERM patients than in the controls both

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before and after cataract surgeries, which is consistent with previous Luminex assay results. Following this, multivariate regression analyses identified ΔIL-4 level as a significant factor affecting both postoperative macular volume and thickness progression in patients with iERM. In contrast, no correlations were found between IL-4 level (changes) and clinical characters in the control group. Additionally, we preliminarily investigated the pro-fibrotic effects of IL-4 on primary rat retinal Müller glia in vitro, and the findings suggest a role for IL-4 in promoting Müller glia proliferation, migration, and GMT in a way independent of TGF-β1.

In this study, we collected AH samples both preoperatively and 20 hours postoperatively, as done in our previous study,[12] to investigate the influence of IL-4 on iERM formation and early progression PCS and to verify our previous Luminex assay results. AH is a crucial ocular fluid revealing retinal conditions and sharing similar cytokine profiles with vitreous humor (VH).[32] Furthermore, AH is a safer, more practical, and more repeatable source for acquiring samples compared to VH.[33] Because cataract surgery can induce cytokine changes from anterior to posterior segments of the eye and thus influence the retina,[9,10,28] analyzing cytokine alterations PCS is more accurate and important for understanding the postoperative changes in the retina. Moreover, cytokine changes 1 day postoperatively have been reported to partially reflect the long-term alterations.[9,11,12] It should be noted that acquiring postoperative AH samples 18 to ~24 hours and 1 to ~17 months PCS has been successfully and safely executed without performing a second anterior chamber therapeutic operation beyond the primary cataract surgery.[34–36] Thus, we believe this procedure can be performed safely with appropriate precautions in sample acquisition and patient care.

Cytokine-mediated fibrosis is a prominent feature of iERM formation and progression. Our prior Luminex assay study demonstrated that many aqueous cytokines, including IL-4, showed significant increases in their levels PCS in patients with iERM.[12] IL-4, a cytokine typically involved in maintaining a type 2 immune profile and eosinophil migration, has been reported to play a role in various fibrotic diseases.[13] For example, in glandular fibrosis disease, IL-4 contributes to the activation of fibroblasts both in vitro and in vivo, ultimately leading to fibrotic development.[14] Additionally, IL-4 is overexpressed in nasal polyps, promoting differentiation of myofibroblasts and accumulation of ECM.[13] Fibrotic disorders in the lung and heart have also been linked to IL-4.[15,16] With regard to ocular diseases, although IL-4 is commonly associated with neuroprotective effects,[37] it can also intensify fibrotic development in conjunctival cells.[17,18,38] Notably, IL-4 levels were significantly higher in VH samples from patients with iERM than in the controls.[3] Consistent with these findings, our study revealed a significant increase in preoperative IL-4 levels in AH samples from patients with iERM compared to controls. This suggests a potential role for the pro-fibrotic cytokine IL-4 in the formation of iERM.

Although there has been extensive debate about the relationship between cataract surgery and ERM, definitive conclusions have yet to be reached. Fong et al.[6] demonstrated that the 3-year PCS incidence of ERM onset or progression was 11.2% and 43%, respectively, based on fundus photography examination. Kwon et al.,[8] who used an OCT-based multistage grading method, found that the 27-month PCS incidence of ERM onset or progression was 7.3% and 6%, respectively. However, unlike the study by Kwon et al.,[8] we observed no worsening of the ERM stage on OCT images at the 3-month PCS follow-up. This discrepancy may be due to the different focuses of the studies. Kwon et al.[8] aimed to determine the rate of ERM stage progression PCS over several years and identify associated risk factors. Our study, however, had a shorter follow-up period of 3 months and aimed to investigate the cytokines involved in early ERM progression PCS and the potential early intervention, such as during cataract surgery, for it. Our results also demonstrated that patients with coexisting cataracts and iERM tend to have worse anatomical and functional recovery outcomes PCS. A recent report may help explain this phenomenon, as that study showed that glial cell proliferation has a vital role in ERM progression and can result in retinal morphological changes and visual deterioration.[39] Glial cell proliferation, which is observed under the microscope, commonly begins before retinal morphological changes are detectable on OCT images.[39] As such, early iERM progression may occur before OCT-based ERM stage worsening and lead to poor recovery outcomes due to the glial cell proliferation in the first 3 months PCS.

To further investigate the role of IL-4 in iERM progression PCS, we compared postoperative IL-4 levels between the two groups and found a specific significant elevation in the iERM group PCS, which implies an important role for IL-4 in this process. Then, univariate and multivariate regression analyses were conducted which revealed that IL-4 may support early progression of iERM by promoting retinal anatomical progression. This finding aligns with prior research indicating that IL-4 may contribute to the onset of ectopic inner foveal layers, which serve as anatomical evidence of iERM progression.[40] Chen et al.[19] also demonstrated that IL-4 is involved in fibrotic devel-

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opment in patients with PVR, and the elimination of ERM in a mouse model of PVR was associated with reduced IL-4 levels in the retina. Thus collectively, this study and others suggest that IL-4 plays a crucial role in early iERM progression PCS. If proven true, IL-4 may present a promising new therapeutic target for early intervention in iERM progression in the future.

Müller glia is believed to be pivotal during iERM formation and progression.[5,22] Cytokines, such as TGF-β, have been reported to impact Müller glia by inducing cell proliferation, migration, and GMT, which is in line with Müller glia behavior during iERM progression.[5,41] Like EMT, GMT is a transdifferentiation process represented by the upregulation of pro-fibrotic myofibroblast markers and downregulation of Müller glia markers.[5,23,41] To our knowledge, this research is the first to investigate the pro-fibrotic effect of IL-4 on Müller glia. Our results demonstrated that IL-4 stimulated Müller glia proliferation and migration and upregulated the fibrotic markers *COL1A1* and fibronectin, as well as the proliferation marker CCND1. This suggests that IL-4 has a functional role in activating pro-fibrotic behaviors of Müller glia. These findings echo previous reports showing that IL-4 could lead to cell proliferation and excessive ECM deposition in conjunctival cells.[17,18,38] Our results also support the speculation that glial cell proliferation occurs before ERM stage worsening is observed by OCT images and promotes ERM progression due to the stimulation of IL-4. However, in this study, expression of the myofibroblast marker α-SMA and Müller glia marker GFAP exhibited an insignificant but marginal increase and decrease, respectively. This could be explained by the concept of "partial GMT."[5] GMT is a transitional process where cells shift their status from glial to mesenchymal, undergoing multiple yet uncharacterized metastable intermediary phases, which are known as partial GMT.[5] Therefore, partial GMT represents the intermediate hybrid glial and mesenchymal phenotypes, which may not exhibit complete marker changes as seen in fully mesenchymal status. Recently, a strong association between partial EMT and wound healing, fibrosis, and cancer progression has been identified, which suggests the importance of studying partial GMT in similar contexts.[42] Consequently, our findings suggest that IL-4 may contribute to iERM formation and early progression PCS by inducing Müller glia proliferation, migration, and GMT. This provides a theoretical basis for more targeted therapy research for iERM and improves the potential for clinical translation of these findings.

Previous studies have proposed that IL-4 partially mediates fibrotic process by upregulating the expression of TGF-β, which can induce GMT in Müller glia via the TGF-β–SNAIL axis.[23,43,44] However, in our study, we did not observe an induction of TGF-β1 in Müller glia stimulated with IL-4. This result is similar to previous research demonstrating that IL-4 mediates cardiac fibrosis in hypertension independent of TGF-β1.[16] Thus, IL-4 may also induce pro-fibrotic activation in Müller glia in a manner independent of TGF-β1. In addition to the TGF-β1 pathway, other signaling pathways might play significant roles in the pro-fibrotic activities of IL-4 in Müller glia. For example, IL-4 promotes fibrosis in immunoglobulin G4 (IgG4)-related sialadenitis via the reactive oxygen species (ROS)/p38 mitogen-activated protein kinase (MAPK)–p16 pathway.[14] The Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, activated by IL-4, can lead to fibrosis in the skin, heart, and liver.[16,30,45] Furthermore, long non-coding RNA modulated by IL-4 has also been implicated in skin fibrosis.[46] Therefore, further investigations are warranted to elucidate the precise mechanism by which IL-4 activates pro-fibrotic behaviors in Müller glia.

Our study does have several limitations that should be acknowledged. First, the use of AH instead of the more representative VH might limit the generalizability of our findings. Nonetheless, AH is a safer and more practical alternative for pre- and postoperative collection for determining IL-4 alterations. Second, a larger sample size would have enhanced the robustness of our results. Third, IL-4 levels at 20 hours PCS may not accurately depict the levels at 3 months PCS. However, due to the invasive nature of the operation, repeated sampling from the same individuals postoperatively may not be ethical. Fourth, additional experiments, such as more experimental groups of positive controls and different IL-4 concentrations, as well as examination of IL-4-related mediators in iERM samples from patients with and without cataract surgeries, are required to elucidate the detailed pro-fibrotic role of IL-4 and the pathogenesis of IL-4–mediated GMT in Müller glia, which could provide new directions for early targeted treatment research for iERM.

In conclusion, our results suggest that patients with coexisting cataracts and iERM may undergo early iERM progression 3 months PCS without obvious OCT-based ERM stage worsening. Furthermore, IL-4 plays a crucial role in iERM formation and early iERM progression PCS possibly through the induction of Müller glia proliferation, migration, and GMT in a manner independent of TGF-β1. Given the lack of information on the molecular mechanisms of GMT, further investigations are warranted to provide more insight for future early targeted therapy research on iERM.

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