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Mesenchymal Stromal Cells from Human Wharton's Jelly Modulate the Intraocular Immune Response in a Glucocorticoid Hypertension Model: An Exploratory Analysis

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Keywords
Glaucoma · Mesenchymal stromal cells · Cytokine · Human Wharton's jelly · Immune response

Abstract

Introduction: Glaucoma is a neurodegenerative disease characterized by the loss of retinal ganglion cells. Recent research suggests immunological changes such as cytokine imbalance may affect its pathophysiology. This implies that immunomodulation, like that of mesenchymal cells, could be a potential therapeutic avenue for this disease. However, the effects of intravitreal injections of human Wharton's jelly-derived mesenchymal stromal cells (hWJ-MSCs) on intraocular immune response have not been assessed in ocular hypertension (OH) models. ***Methods:*** We explored this by measuring cytokine levels and expression of other markers, such as glial fibrillary acidic protein (GFAP) and T cells, in 15 randomly divided New Zealand rabbits: G1: OH, G2: hWJ-MSCs, and G3: OH+hWJ-MSCs. We analyzed the aqueous humor (IL-6, IL-8, and TNF-α) and vitreous humor (IFN-γ, IL-10, and TGF-β) using ELISA and flow cytometry (cell populations), as well as $TCD3^+$, $TCD3^+/TCD4^+$, and $TCD3^+/TCD8^+$ lymphocytes, and GFAP in the retina and optic nerve through immunohistochemistry. ***Results:*** We found a decrease in TNF-α, IL-6, IFN-γ, IL-10, and IL-8 in G3 compared to G1 and an increase in TGF-β in both G2 and G3. $TCD3^+$ retinal infiltration in all groups was primarily $TCD8^+$ rather than $TCD4^+$ cells, and strong GFAP expression was observed in both the retina and optic nerves in all groups. ***Conclusion:*** Our results suggest that cellular and humoral immune responses may play a role in glaucomatous optic neuropathy and that intravitreal hWJ-MSCs can induce an immunosuppressive environment by inhibiting proinflammatory cytokines and enhancing regulatory cytokines.

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Introduction

Glaucoma is a chronic neurodegenerative disease of multifactorial origin characterized by the death of retinal ganglion cells and progressive visual field loss. Studies in the serum and aqueous humor of patients with glaucoma indicate upregulation of specific cytokines, such as IL-1, IL-8, IL-6, TGF-β, and TNF-α. These changes in cytokine expression suggest a potential association between the immune response and a chronic inflammation in the pathogenesis of glaucoma [1, 2].

Currently, no therapies are available to reverse glaucomatous neuronal degeneration [3], making the search for neuroprotective therapies a relevant field of study. Ocular stem cell research is particularly attractive because the eye is considered an immune-privileged organ, developing various molecular and cellular mechanisms that limit immune responses to preserve vision [4]. The ocular anatomy offers distinct advantages for cell-based therapy, such as the accessibility of the eye that facilitates the delivery of treatments. Additionally, the capacity for direct monitoring allows for immediate assessment and adjustment of therapeutic strategies, potentially improving treatment outcomes. Moreover, the localized nature of ocular interventions substantially reduces the probability of systemic side effects [5, 6].

Mesenchymal stem cell (MSCs) transplantation is emerging as a tool for neural regeneration [7] due to its anti-inflammatory and immunomodulatory properties related to its paracrine or endocrine effect [8]. These properties are based on mechanisms that inhibit immune cell proliferation and decrease the Th1 and Th17 response, inhibiting the production of proinflammatory cytokines, antibodies, and cytotoxic mediators [9–11], which could be related to glaucoma. MSCs exhibit limited antigenicity and secrete trophic factors, further highlighting their potential therapeutic applications [3, 12, 13].

MSCs isolated from extraembryonic tissues, such as human Wharton jelly-derived mesenchymal stromal cells (hWJ-MSCs), have some advantages compared to those derived from adult tissues, such as better proliferative capacity, lifespan, a more primitive cellular character without tumorigenicity, and low immunogenicity, with a lower probability of allogeneic and xenogeneic transplant rejection [3, 14]. Thus, MSCs are capable of going unnoticed by the immune system since the host does not generate any response to them, which allows the transplantation of these cells between species other than those from which the cells come from [15]. Studies provide evidence of the successful integration of xenogeneic cells in animal models to treat various human ocular diseases such as retinal holes and age-related macular degeneration [16, 17]. Immunological rejection related to the use of different species has not been documented; on the contrary, studies reported that the cells derived from humans did not provoke an immune response at the application site, which may be due to the immunosuppressive properties of the cells [18].

In addition, hWJ-MSCs can produce anti-inflammatory and neurotrophic factors that exert immunosuppressive activity. However, to exert this immunosuppressive effect, MSCs must be activated in an inflammatory environment; otherwise, they remain quiescent [19]. The use of hWJ-MSCs in an immunosuppressive environment as in the case of ocular hypertension (OH) caused by glucocorticoids (GCs) has not been studied previously.

The pathogenesis of glaucoma involves the infiltration of T cells into the retina, a process not yet fully understood in terms of the specific roles of different T-cell subsets. Some of these cells may initiate neurodegeneration characteristic of glaucoma, while others may act as effector cells in the disease process [20]. Given this complexity, MSCs offer a promising therapeutic strategy. Their ability to induce cell division arrest can potentially mitigate the impact of T-cell activity in the retina, addressing a key aspect of glaucomatous neurodegeneration [3, 19]. This study aimed to evaluate the effect of intravitreal application of hWJ-MSCs on the intraocular inmmune response of a glucocorticoid-induced OH model by measuring cytokine levels in the aqueous humor (IL-6, IL-8, and TNF-α) and vitreous humor (IFN-γ, IL-10, and TGF-β), as well as the T cell responses in the retina and optic nerve (ON).

Materials and Methods

The study included 15 New Zealand White male rabbits aged approximately 4 months and weighing 2–3 kg (online suppl. Fig. 1; for all online suppl. material, see https://doi.org/10.1159/000538183). The animals were housed according to NIH guidelines (online suppl. Fig. 2). All experimental and animal care procedures were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Animal Ethics Committee of Escuela Superior de Oftalmología Instituto Barraquer de América (CEIA-ESO-IBA), under reference C-20190226-1. In addition, all animals underwent a complete ocular examination to rule out the presence of ocular diseases, such as chronic keratitis or conjunctivitis of any etiology, dry eye syndrome, lens opacity, and systemic inflammatory disorders, before inclusion in this study. Ophthalmic examinations included IOP measurement using

applanation tonometry (Tono-Pen AVIA Vet™/Reichert, Depew, NY, USA), slit-lamp biomicroscopy of the anterior segment (SL-15/Kowa, Tokyo, Japan), direct ophthalmoscopy (Welch Allyn, Skaneateles Falls, WA, USA), and indirect ophthalmoscopy (Vantage Plus binocular, Keeler, UK). Three intervention groups with 5 animals each were assigned as follows: group 1: OH; group 2: hWJ-MSCs; and group 3: OH+hWJ-MSCs. The untreated left eye was used as the control in all cases.

Anesthesia

For intravitreal injection of hWJ-MSCs, aqueous and vitreous humor collection, the animals were anesthetized with an intramuscular injection of ketamine (Ketafine®, Brouwer, Argentina) (35 mg/kg) and xylazine (Xilacina 2%, Erma, Colombia) (8 mg/kg). Anesthetic eye drops based on 0.5% proparacaine (Alcaine®, Alcon, Barcelona) were used before the subconjunctival GC injection. Artificial tears (Splash Tears, Sophia, Colombia) were administered daily to reduce possible complications of topical anesthesia. A daily ocular surface evaluation with a slit lamp was performed to detect possible corneal alterations associated with topical anesthesia.

Induction of OH and IOP Measurements

OH was induced in 10 eyes: G1 (5 eyes) and G3 (5 eyes). We used topical drops of prednisolone acetate (Prednefrin Forte® eye drops 10 mg/mL – Allergan, Brazil) applied twice a day, associated with subconjunctival injections of 0.5 mL of betamethasone acetate (Celestone Cronodose®, disodium phosphate, 3 + 3 mg/mL, Schering-Plough, Mexico) weekly in the right eye for 5 weeks to raise and maintain constantly elevated IOP [21, 22].

IOP was measured in both eyes using an applanation tonometer. Measurements were taken twice daily (at 7:00 a.m. and 4:00 p.m.) for all experimental animals across nine consecutive weeks. To obtain measurements, minimal head and neck restraint was applied to the animals to avoid excessive pressure on the eyelids and neck. Five readings were recorded for each eye and were averaged. The eye for starting the measurements was randomly selected and the same examiner conducted tonometry in all cases. Considering the normal IOP in rabbits, OH was considered when the readings were above 15 mm Hg [23].

Isolation and Culture of hWJ-MSCs

Human umbilical cord samples were collected from full-term births (caesarean and vaginal deliveries). Informed consent was approved by the Ethical Committee of Secretaria Distrital de Salud de Bogotá, and healthy donors signed it before collection under approval reference 2019EE44993. hWJ-MSCs were isolated as previously described [24, 25]. The umbilical cord fragments were washed with 0.9% saline solution containing 1% penicillin/streptomycin (10,000 U/mL), and the umbilical veins, arteries, and outer membranes were removed. Wharton's jelly was minced and cultured in flasks containing Dulbecco's Modified Eagle's medium low glucose (Gibco, Life Technologies, Carlsbad, CA, USA), 1% penicillin/streptomycin (10,000 U/mL) and supplemented with 10% hPL plus 8 IU/mL of heparin. The hWJ-MSC cultures were maintained at 37°C in a humidified atmosphere with 5% CO2. Confluent cultures (80%) were harvested, and hWJ-MSCs were expanded to the third passage and used in subsequent experiments.

Quality Control of hWJ-MSCs

Quality control of hWJ-MSCs was immunophenotypically characterized by labeling the cells with antibodies against CD90-APC, CD73-PE/Cy7, CD105-PE, CD274-PE, CD45-APC/Cy7, CD34-PerCP-Cy5.5, and CD31-PE (BioLegend, San Diego, USA). Inhibition of $TCD3^+$ was evaluated. In addition, the cell suspension was analyzed for sterility, endotoxins, and mycoplasmas. Flow cytometry analyses were performed using a FACSCanto II™ instrument (*BD, Franklin Lakes, NJ, USA*), and data were analyzed using the FlowJo vX.7.0 software package (*TreeStar, USA*).

Intravitreal Injection of hWJ-MSCs

Animals from G2 and G3 received a single intravitreal injection of the hWJ-MSC suspension at week seven of the study. hWJ-MSCs were supplied in single-dose cryotube vials and thawed previously. After general anesthesia and under sterile conditions, the hWJ-MSC suspension (1×10^5cells/100 µL) [16] was gently injected into the vitreous cavity with a 30-gauge needle through the pars plana of the globe in the direction of the optic disc. An intravitreal injection of sterile balanced salt solution was then applied to the untreated eye.

Aqueous and Vitreous Humor Collection

After anesthesia, povidone-iodine was instilled for 5 min and then flushed with saline. Aqueous humor samples (0.1 mL) were aspirated through limbal paracentesis using a 1 mL syringe with a 30-gauge needle at weeks 1, 3, and 9. For collection of the vitreous humor, a 27 G needle was used, and it was introduced 4 mm from the temporal corneal limbus, crossing the sclera in the direction of the ON head. As a result, 0.7–0.9 mL of vitreous humor was aspirated.

Analysis of Cell Characteristics in the Aqueous and Vitreous Humor

The samples were immediately stored at 4°C and sent to the laboratory. Aqueous humor samples were centrifuged for 5 min at 700 rpm and analyzed by flow cytometry (*FACSCanto II™ flow cytometry, BD Biosciences*™) to discriminate cell populations by size and granularity at 1 week. At 1, 3 and 9 weeks, supernatant samples were collected for analysis. After identifying the cell population, leukocytes were stained with fluorochrome-conjugated monoclonal antibodies specific for cell surface antigens: anti-CD3+ FITC (*anti-CD3 zeta antibody [H146-968] [FITC] [ab91493], Abcam, Cambridge, MA*) at 3 and 9 weeks. Analyses were performed using the software program FC EXPRESS 7 (*Cytosens*).

To measure the concentration of cytokines in the aqueous and vitreous humor, the supernatants of the previously centrifuged samples were thawed and analyzed by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions. Cytokines measured in aqueous humor were as follows: IL-6 (*Low Sample Volume Rabbit Interleukin 6 [IL6], ELISA Kit,* abx052084, *Abbexa, Cambridge, UK*); IL-8 (*Rabbit Interleukin 8/IL8 [CXCL8], ELISA Kit, abx155079, Abbexa, Cambridge, UK*); TNF-α (*Rabbit TNF-α [Tumor Necrosis Factor Alpha], ELISA Kit, E-EL-RB0011, Elabscience, USA*) and in vitreous humor: IFN-γ (*Mouse IFN-γ, ELISA set, BD OptEIA, 555138, BD Biosciences, CA*); IL-10 (*Rabbit IL-10 [Interleukin 10], ELISA Kit, E-EL-RB0487*);

and TGF-β (*Enzyme-Linked Immunosorbent Assay kit for Rabbit Transforming Growth Factor Beta 1 [TGF-β1], Uscn Life Science Inc., China*).

Immunohistochemistry

Animals were euthanized with an overdose of sodium pentobarbital (*Sigma-Aldrich®, 100 mg/kg, EUA*). The eyeballs were enucleated and marked with sutures to facilitate orientation in the dorsal and ventral areas. The eyecup was fixed in neutral-buffered 10% formalin for 24 h at room temperature. Tissues were embedded in paraffin, and 4-μm sections were cut through the ON. Immunohistochemistry was performed by deparaffinization of the retinal sections, followed by dehydration and antigen unmasking with TRS (pH 9, 1X) (*Dako^{MR} Target Retrieval Solution, High pH, 10x concentrate, Dako, EUA*). Endogenous peroxidase blocks were used, followed by incubation with the primary antibody. Washing was performed with TBS (*EnVision^{MR} FLEX Wash Buffer 20x, Dako, EUA*) and subsequent application of the secondary antibody. A second wash was performed, and the peroxidase-chromogen substrate was added (*EnVision^{MR} FLEX DAB+-Chromogen, Dako, EUA*). Counterstaining was performed using Mayer's hematoxylin. Subsequently, retinas were mounted flat on coverslips and examined under a light microscope. The markers used were acid fibrillar glial protein (GFAP) (*Rabbit Polyclonal GFAP Antibody, Novus Biologicals, NB300-141*), TCD3$^+$ (*ab11089, rat monoclonal to CD3 [CD3–12], 1:250; Abcam, Cambridge, MA*), TCD4$^+$ (*CD4 [GK1.5]: sc-13573; Santa Cruz Biotechnology, Inc.*), and TCD8$^+$ (*CD8 [UCH-T4]: sc-1181; Santa Cruz Biotechnology, Inc.*). Spleen was used as a positive control for T-lymphocyte labeling. Images of each section were analyzed. For the interpretation of immunostaining of retinal and ON sections for TCD3$^+$ cells and subpopulations, it was established as NIC: absence of T-cell infiltration and IC: presence of T-cell infiltration. Double-blind assessment techniques were used to reduce bias in histopathological studies.

Statistical Analyses

Data analysis was conducted using Jamovi version 2 (Jamovi, 2021). The results are presented as means, standard deviations, and interquartile ranges. The Shapiro-Wilk normality test was used to determine the distribution of continuous variables. Repeated-measures ANOVA was applied in cases of normal distribution, while Friedman ANOVA was used for non-normal distributions. If significant differences were detected using repeated-measures ANOVA and paired sample *t* test, post hoc analyses were carried out using the Tukey and Bonferroni tests. A check for the assumption of homoscedasticity was not necessary as this condition was consistently satisfied in our analysis.

Additionally, when statistically significant differences were identified in the Friedman ANOVA, the Durbin-Conover test was used to compare the groups. We employed one-way ANOVA or Kruskal-Wallis ANOVA to analyze the differences between the groups over the weeks based on the distribution. If one-way ANOVA revealed statistically significant differences, Levene's test for homoscedasticity was conducted. The post hoc analysis test type was determined based on the results (Tukey's test for equal variances and Games-Howell test for different variances). For the Kruskal-Wallis ANOVA, differences between groups were explored using the Dwass-Steel-Critchlow-Fligner test.

We utilized R 4.21 and GraphPad Prism (version 8) to analyze the data derived from immunohistochemistry. We conducted a categorical variable analysis to determine the frequency of TCD3$^+$, TCD4$^+$, TCD8$^+$, and GFAP infiltration in various retinal layers. Additionally, we employed the χ^2 test or Fisher's exact test to assess differences between groups, depending on the data distribution.

Results

Quality Control of hWJ-MSCs

hWJ-MSCs showed expression (≤80%) of antibodies such as CD90, CD73, CD105, and CD274, and absence of expression (≥2%) of CD45, CD34, CD31, and HLA-DR. The inhibitory capacity of TCD3$^+$ cells was ≤87%. The cells showed 99% viability and <0.45 EU/mL of endotoxin. The cells were free of mycoplasmas and other microorganisms (sterile cellular suspensions).

IOP and Ophthalmology Changes in the Experimental Model

The proposed experimental model produced early, consistent, and long-lasting hypertension even after drug discontinuation. hWJ-MSCs transplantation presented good safety profiles and few adverse events in the proposed experimental model.

The IOP significantly increased ($p < 0.001$, Kruskal-Wallis ANOVA) in G1 and G3 from week 3 of the study compared to that in the untreated eyes (online suppl. Table 1). The untreated eyes did not show significant differences between weeks ($p > 0.05$, Kruskal-Wallis ANOVA). The IOP in OD in the groups was G1: 25.22 ± 12.31 mm Hg [13.0–64.0], G2: 13.33 ± 3.32 mm Hg [7.0–25.0], and G3: 19.16 ± 4.47 mm Hg [11.0–42.0] at week 7. We observed a sudden increase in IOP in G1 compared with G3 after GC suspension at week 5 (online suppl. Table 1). In addition, all groups presented slightly higher IOP in the afternoon than in the morning. However, this was not significant, demonstrating that the animals were adequately acclimatized ($p > 0.05$, Kruskal-Wallis ANOVA).

A comprehensive ophthalmological examination was performed daily before and after the induction of OH to identify potential ophthalmological changes that could occur during the development of the proposed experimental model. No alterations were found in corneal curvature or irregularities in the corneal surface related to topical anesthesia.

During the 5 weeks of induction of OH, we did not observe damage at the level of the annexed organs of the eyeball or the presence of blepharospasm, epiphora, or

Table 1. Analysis of the lymphocyte's percentage in aqueous humor, before and after the intravitreal injection of hWJ-MSCs, and induction of OH

	Lymphocytes, % cells/μL				*p* value*
	control, mean (SD) [IQR]	G1, mean (SD) [IQR]	G2, mean (SD) [IQR]	G3, mean (SD) [IQR]	
Week 1	4.8 (0.8) [3.8–5.7]	5.1 (0.5) [4.4–5.6]	4.0 (2.4) [0.0–5.9]	3.5 (2.1) [0.0–5.2]	0.434[1]
Week 3	4.9 (0.2) [4.7–5.1]	20.7 (2.1) [17.6–22.2]	5.4 (1.1) [3.8–7.0]	19.0 (3.5) [13.3–22.7]	<**0.001**[1*]
Week 9	5.0 (0.2) [4.8–5.2]	48.4 (22.1) [22.0–68.7]	32.0 (5.2) [27.3–38.5]	34.0 (14.1) [16.8–54.1]	<**0.001**[1*]
p value*	0.8352	**0.01**[2*]	<0.001[2*]	<0.001[2*]	

G1, OH; G2, hWJ-MSCs; G3, OH + hWJ-MSCs; SD, standard deviation; IQR, interquartile range. *$p < 0.05$. [1]ANOVA one way. [2]ANOVA repeated measures.

photophobia that could indicate pain or discomfort in groups G1 and G3. However, from week 7 of the study, after the application of hWJ-MSCs, one animal from G3 presented with intense conjunctival hyperemia, mild corneal edema, rubeosis iridis, and iritis, changing its clinical characteristics at week 9 (less conjunctival hyperemia, gray-green iris, and corneal edema). In G2, one animal showed mydriasis without a response to light stimulation at week 9. There were no signs of uveitis, bacterial infections, or any abnormalities after intravitreal application in the other animals studied.

T-Cell Infiltration in the Anterior Chamber

We observed a significant increase in the number of infiltrating cells in the aqueous humor in G1, G2, and G3 groups over time (Table 1). In the post hoc analysis, we observed an increase in lymphocytes in G1 compared to that in weeks 1 and 3 ($p = 0.001$, Tukey's test, and $p = 0.002$, Bonferroni). In G2, greater cellular infiltration was observed when comparing weeks 1 and 9 ($p = 0.007$, Tukey's test, and $p = 0.01$, Bonferroni) and weeks 3 and 9 ($p = 0.005$, Tukey's test, and $p = 0.008$, Bonferroni). The same was observed in G3 when comparing weeks 1 and 3 ($p = 0.006$, Tukey's test, and $p = 0.008$, Bonferroni) and weeks 1 and 9 ($p = 0.021$, Tukey's test, and $p = 0.03$, Bonferroni).

In addition, the percentage of lymphocytes at week 3 was significantly lower than that at week 9 in all groups (G1: $p = 0.01$; G2: $p = 0.001$; G3: $p = 0.02$) (Fig. 1a). In the post hoc analyses, when evaluating week 3, a significant increase in lymphocytes was observed in G1 and G3 (both $p < 0.001$) when compared with the untreated eyes. The same increase in cell infiltration was observed in G1 and G3 (both $p < 0.001$) compared to that in G2. At week 9, greater lymphocyte infiltration was also demonstrated in G2 and G3 ($p = 0.001$ and $p = 0.03$, respectively) than in the untreated eyes.

Upon identifying the cell population, a general marker of $TCD3^+$ cells was used to detect these cells in aqueous humor samples. We observed significant cell infiltration in all groups at weeks 3 and 9 compared with the untreated eyes, which was further analyzed using the *post hoc* analysis proposed by Games-Howell (Table 2). At week 3, we observed a lower infiltration of $TCD3^+$ in the untreated eyes than in G1 and G3 ($p = 0.031$ and $p = 0.009$, respectively). Lower $TCD3^+$ infiltration was also observed in G2 than in G1 and G3 ($p = 0.0313$ and $p = 0.009$, respectively). At week 9, a lower infiltration of $TCD3^+$ was observed in the untreated eyes than in G1 ($p < 0.001$) and G3 ($p = 0.041$). However, when evaluating cellular infiltration over time, we observed a significant increase only in G1 ($p < 0.001$) at week 9 compared to that at week 1. When comparing the percentage of $TCD3^+$ cells between weeks 3 and 9, we observed a lower infiltration of $TCD3^+$ lymphocytes in week 3 compared to week 9 in all the studies, with statistical significance (G1: $p = 0.04$; G2: $p = 0.002$; and G3: $p = 0.05$) (Fig. 1b).

Intravitreal hWJ-MSCs Influence the Levels of Cytokines in the Eyes with OH

In this analysis, a decrease in the secretion of specific cytokines TNF-α, IL-6, IFN-γ, IL-10, and IL-8 was evident in G3 compared to G1. Moreover, we observed an increase in TGF-β in vitreous humor after applying hWJ-MSCs. We also measured TNF-α, IL-8, and IL-6 levels in the aqueous humor and IFN-γ, TGF-β, and IL-10 in the vitreous humor. We observed an increase in TNF-α, IL-6, and TGF-β levels in the OH group between weeks 3 and 9 in G1 (Table 3). However, only TNF-α levels were significantly different ($p < 0.001$). At week 3, we did not observe significant differences in TNF-α, IL-6, and TGF-β

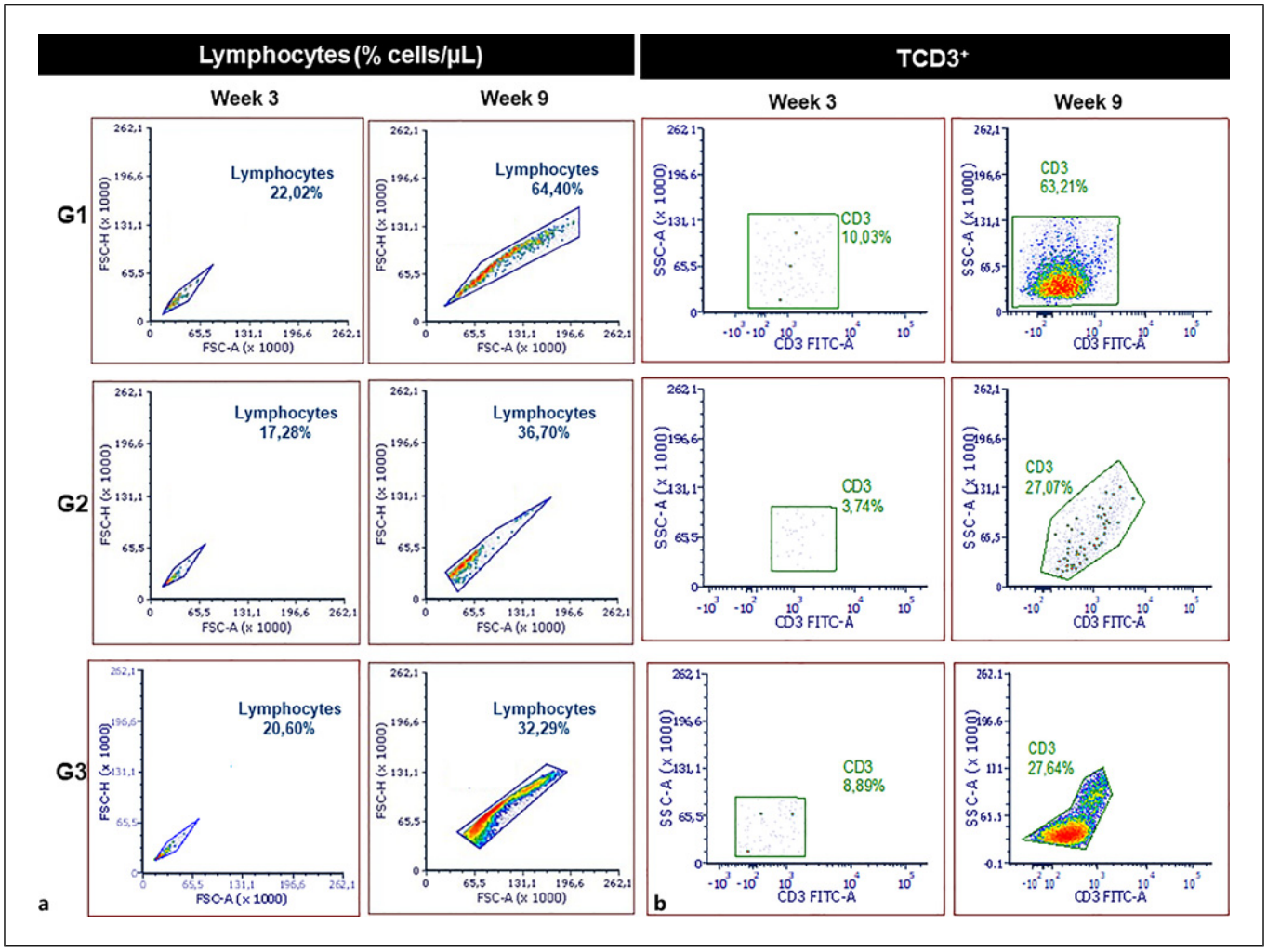

Fig. 1. Distribution of subsets of T cells *post*-OH, before and after the application of hWJ-MSCs at weeks 3 and 9. **a** Mononuclear cells isolated from aqueous humor samples were analyzed by multicolor flow cytometry for a general T-cell marker. The cytometer displays the following dot blot based on intrinsic cell characteristics: cell size and complexity, with a selected window for lymphocytes. **b** The percentages of TCD3$^+$ were significantly lower in the groups treated with hWJ-MSCs than in the untreated group. G1, OH; G2, hWJ-MSCs; G3, OH + hWJ-MSCs.

Table 2. Analysis of TCD3$^+$ lymphocyte´s percentage during the follow-up time between groups

	TCD3$^+$ lymphocyte's, % cells/μL				*p* value*
	control, mean (SD) [IQR]	G1, mean (SD) [IQR]	G2, mean (SD) [IQR]	G3, mean (SD) [IQR]	
Week 3	4.3 (0.3) [3.9–4.6]	14.8 (3.7) [10.0–18.1]	5.0 (1.0) [3.7–6.1]	15.2 (3.7) [10.6–18.9]	**0.002**[1*]
Week 9	4.1 (0.6) [3.2–4.7]	44.0 (21.7) [18.2–63.2]	23.9 (3.4) [18.9–27.1]	29.6 (13.2) [15.7–48.5]	<**0.001**[1*]
p value*	0.103[2]	<**0.001**[2*]	0.078[2]	0.634[2]	

G1, OH; G2, hWJ-MSCs; G3, OH + hWJ-MSCs; SD, standard deviation; IQR, interquartile range. *$p < 0.05$. [1]ANOVA one-way. [2]Student *t* test for repeated measures.

Table 3. Analysis between changes in cytokines at weeks 3 and 9 in G1

	Week 3, mean (SD) [IQR]	Week 9, mean (SD) [IQR]	*p* value*
TGF-β, pg/mL	30.5 (2.0) [28.2–32.3]	38.5 (20.4) [8.9–54.1]	0.518
TNF-α, pg/mL	21.6 (3.1) [18.4–25.8]	48.5 (0.3) [48.0–48.7]	<**0.001***
IL-6, pg/mL	18.3 (1.1) [17.0–19.6]	20.8 (3.6) [16.8–25.4]	0.333

ANOVA one way; ELISA analysis. G1, OH; SD, standard deviation; IQR, interquartile range; TGF-β, transforming factor beta; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6. *$p < 0.05$.

levels between G1, G2, and G3 and the untreated eyes (Table 4). However, an incremental non-significant trend for these cytokines was observed in G1 and G3 compared to untreated eyes but not in G2.

On evaluation of aqueous humor samples at week 9 (Table 5) (Fig. 2a), we observed that G1 and G2 significantly increased the levels of TNF-α ($p = 0.029$), whereas G3 reduced it by 22.54% compared to the untreated eyes. In addition, we found that G1 had significantly higher levels of this cytokine than G2 and G3 ($p < 0.001$). In the post hoc analysis, significant differences in TNF-α levels were found between the untreated eyes and all other groups ($p < 0.001$).

All groups showed a non-significant increase in IL-8 levels compared to the untreated eyes ($p = 0.06$), without significant differences between the groups. IL-6 levels increased in G2 ($p = 0.06$) and decreased in G3 ($p = 0.06$) compared to those in the untreated eyes; however, both trends were not significant. In the post hoc analysis, we observed an increase in IL-6 in G1 compared to that in the untreated eyes ($p = 0.04$) and G3 ($p = 0.005$).

At week 9, we found a non-significant increase in IFN-γ levels in the vitreous humor of G1 ($p > 0.05$) compared to the untreated eyes and a reduction in G2 and G3 (both $p = 0.03$) (Table 5) (Fig. 2b). No significant changes in TGF-β levels were observed in the groups compared to the untreated eyes. The concentration of TGF-β increased in the G3 group with respect to G1 and G2 groups, but the difference was not statistically significant. All groups showed significantly lower levels of IL-10 than the untreated eyes ($p < 0.05$). However, when comparing the groups, a significant reduction in the levels of this cytokine was observed in G2 compared to G1 ($p = 0.02$).

IOP Elevation Induces T-Cell Infiltration into the Retina and ON

When evaluating the infiltration of $TCD3^+/TCD4^+$ in the ganglion cell layer (GCL), it was observed that G3 presented infiltration, unlike G1, which did not present cellular infiltration. The retinal infiltration of $TCD3^+$ in all groups tended to belong more to the subpopulation of $TCD8^+$ cells than to $TCD4^+$ cells in various layers of the retina in the proposed experimental model. Likewise, all study groups showed strong GFAP expression in the retina and ON.

When evaluating the infiltration of $TCD3^+$ cells (Fig. 3a, 4a, b, c) in GCL, we observed that the only group that did not show cellular infiltration was G3, which was significant when compared to G1 ($p = 0.047$). Regarding the inner nuclear layer (INL) analysis, all groups exhibited infiltration, with no significant differences between the groups. When analyzing the ON, the G2 and G3 groups presented less $TCD3^+$ infiltration than the untreated eyes, which was statistically significant ($p = 0.037$). Additionally, G1 showed greater cellular infiltration than G2 and G3 ($p = 0.007$).

When evaluating $TCD3^+/TCD4^+$ cells (Fig. 3b, 4d, e, f), no evidence of infiltration was observed in the GCL, except for 1 case in G2. In the INL analysis, infiltration was mainly observed in G2 and G3, but no statistically significant differences were found. In the ON analysis, statistically significant differences were observed when comparing $TCD4^+$ infiltration, which was lower in the G2 and G3 groups than in the untreated eyes ($p = 0.037$ in both cases).

Regarding the $TCD3^+/TCD8^+$ analysis (Fig. 3c, 4g, h, i), the GCL in G1 exhibited higher infiltration than that in the untreated eyes ($p = 0.002$) and G2 and G3 ($p = 0.047$, for both). In the INL analysis, all groups showed a high infiltration of $TCD8^+$ without significant differences between the groups. In the ON analysis, low infiltration was observed, which was higher in the untreated eyes and G1 but without significant differences compared to the other groups.

Significant expression of GFAP (Fig. 3d, 4j, k, l) was observed in the GCL in G1 ($p = 0.002$), G2 ($p = 0.002$), and G3 ($p < 0.0001$) compared to that in the untreated eyes. When analyzing the INL, there was a significant

Table 4. Intergroup analysis of cytokines at week 3

	Control, mean (SD) [IQR]	G1, mean (SD) [IQR]	G2, mean (SD) [IQR]	G3, mean (SD) [IQR]	*p* value*
TGF-β, pg/mL	27.4 (0.7) [26.9–27.9]	30.5 (2.0) [28.2–32.3]	24.8 (4.1) [21.8–27.7]	30.7 (2.5) [28.9–32.5]	0.275
TNF-α, pg/mL	15.6 (0.6) [15.1–16.0]	21.6 (3.1) [18.4–25.8]	14.7 (3.8) [12.0–17.4]	21.4 (2.1) [19.9–22.9]	0.128
IL-6, pg/mL	15.4 (0.7) [14.9–15.9]	18.3 (1.1) [17.0–19.6]	16.1 (2.2) [14.6–17.7]	19.5 (0.6) [19.0–19.9]	0.069

ANOVA one way; ELISA analysis. G1, OH; G2, WJ-MSC; G3, OH + hWJ-MSCs; SD, standard deviation; IQR, interquartile range; TGF-β, transforming factor beta; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6. *$p < 0.05$.

Table 5. Intergroup analysis of cytokines at week 9

Cytokines	Control, mean (SD) [IQR]	G1, mean (SD) [IQR]	G2, mean (SD) [IQR]	G3, mean (SD) [IQR]	*p* value*
IFN-γ, pg/mL	97.7(37.6) [53.0–134.7]	971.9 (657.3) [249.5–1,688.1]	67.4 (42.7) [19.4–123.2]	164.9 (77.5) [120.2–280.7]	0.096
TGF-β, pg/mL	22.9 (11.3) [6.7–32.0]	38.5 (20.4) [8.9–54.1]	29.0 (20.6) [2.0–49.8]	50.3 (23.2) [33.3–83.7]	0.309
IL-6, pg/mL	14.5 (3.3) [10.4–18.4]	20.8 (3.6) [16.8–25.4]	17.9 (1.9) [15.4–20.0]	12.0 (2.7) [9.2–15.2]	**0.031***
IL-8, pg/mL	188.4 (228.0) [66.2–530.0]	368.7 (369.5) [58.8–888.8]	541.2 (764.7) [98.8–1,683.8]	324.1 (258.2) [82.5–690.0]	0.756
TNF-α, pg/mL	17.1 (0.4) [16.5–17.3]	48.5 (0.3) [48.0–48.7]	44.0 (1.6) [41.9–45.5]	13.2 (0.7) [12.6–14.2]	<**0.001***
IL-10, pg/mL	9.6 (0.7) [8.7–10.2]	11.4 (0.1) [11.3–11.6]	8.6 (0.8) [7.6–9.6]	9.6 (2.1) [6.9–11.9]	**0.003***

ANOVA one way; ELISA analysis. G1, OH; G2, hWJ-MSCs; G3, OH + hWJ-MSCs; SD, standard deviation; IQR, interquartile range; INF-γ, interferon-gamma; TGF-β, transforming factor beta; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; IL-8, interleukin 8; IL-10, interleukin 10. *$p < 0.05$.

GFAP expression in G3 ($p < 0.0001$) compared to that in the untreated eyes. G1 and G2 also showed expression but did not reach statistical significance when compared. Finally, the ON analysis showed evidence of GFAP expression in all groups in 100% of cases.

Discussion

In this study, we observed that therapy with hWJ-MSCs fostered an immunosuppressive environment in an experimental OH model produced by GCs by inhibiting proinflammatory cytokines and producing regulatory cytokines, such as TGF-β. Moreover, our experimental model showed predominant lymphocyte infiltration of $TCD3^+/TCD8^+$ cells in GCL, suggesting that cellular immunity, specifically by cytotoxic lymphocytes, could play a role in glaucomatous optic neuropathy in this animal model. However, this specific $TCD3^+$-/$TCD8^+$-cell infiltration was not observed in the groups treated with the cell therapy. Moreover, the evaluation of the INL showed not only infiltration of both $TCD3^+/TCD4^+$ and $TCD3^+/TCD8^+$ cells, but also strong GFAP expression, suggesting activation of the immune system in the studied groups. This is the first study to describe that intravitreal application of hWJ-MSCs can exert immunomodulatory effects on the ocular microenvironment in vivo in an animal OH model produced with GCs.

To carry out the OH experimental model, we use cell transplantation between different species. In this regard, we did not observe signs of clinical rejection after intravitreal transplantation of hWJ-MSCs, which leads us to think that in addition to the eye being considered an immunoprivileged site [26], the cells applied to the vitreous managed to remain alive *post*-transplant, promoting an additional immunosuppressive effect, avoiding rejection after transplantation in the experimental model of glaucoma within a few weeks of the study. We cannot rule out that an additional immunosuppressive effect

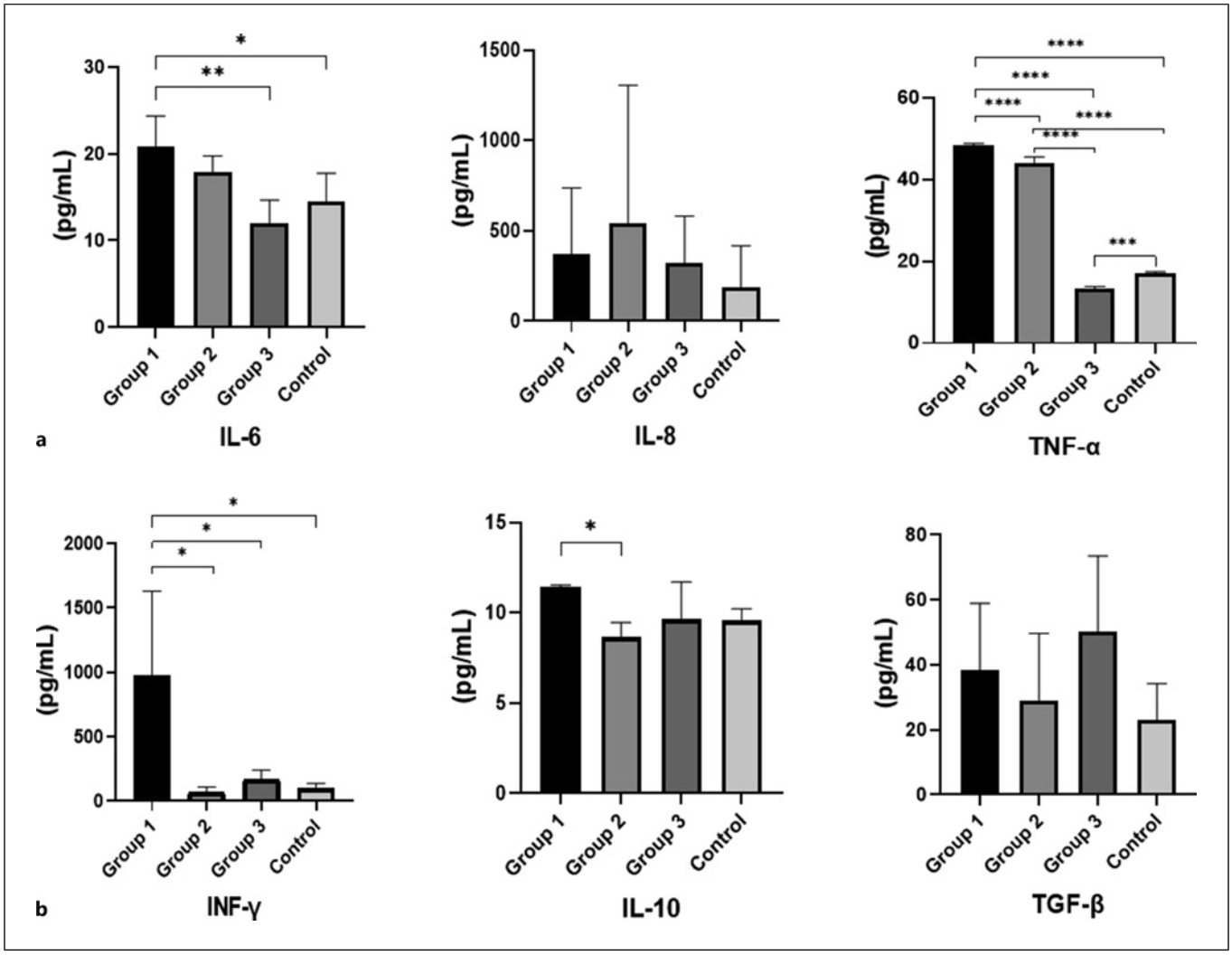

Fig. 2. Comparison of cytokines between the groups studied at week 9. **a** Cytokines in aqueous humor. **b** Cytokines in vitreous humor. ELISA analysis. G1, OH; G2, hWJ-MSCs; G3, OH + hWJ-MSCs. Control, untreated eye. Statistical test, ANOVA one way. INF-γ, interferon-gamma; TGF-β, transforming factor beta; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; IL-8, interleukin 8; IL-10, interleukin 10. *$p < 0.05$.

occurred since this was a model produced by GCs, even though the medications were discontinued.

At this point, one might wonder if we could observe any post-intravitreal transplant effects in just 2 weeks of study. In this sense, we corroborate with the study published in 2011, which observed changes in the retina of rabbits after 4 days of the application of human MSCs in the vitreous [16] and Bull et al. [27] who describe the ability of intraocular cells to survive in vivo and respond to their environment over a period of 2–3 weeks. The therapeutic effect time has not been established. However, prior studies have noted that these cells sustain improvements in macular hole thickness for up to 32 days [16] and retinal degeneration for approximately 70 days [28]. The current study observed immunomodulatory effects up to week 9 (56 days). However, the long-term impacts beyond this period remain unexplored due to the absence of further follow-up. Extended observation could yield additional insights into the behavior of these cells within the intraocular environment. The mechanism by which GCs increase IOP is not completely understood. However, studies have shown that the continuous use of topical GCs for 4 weeks induces the accumulation of glycosaminoglycan material in the trabecular meshwork, altering the extracellular matrix [29]. Moreover, GCs induce the activation and overexpression of TGF-β, with the

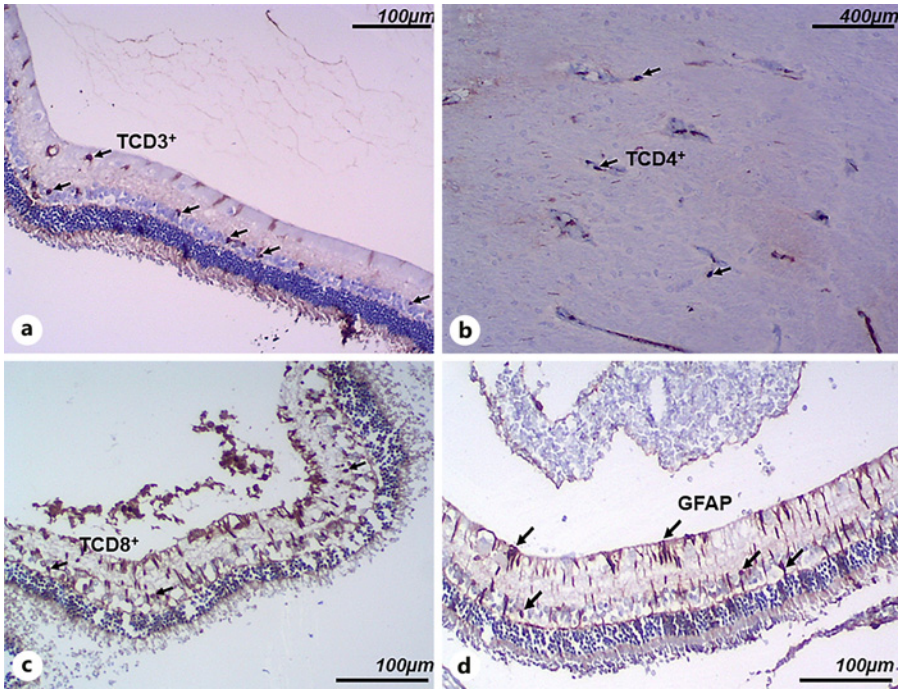

Fig. 3. Immunohistochemistry: New Zealand rabbit retinal immunostaining at week 9. **a** TCD3$^+$ cells positive immunostaining in the GCL and INL. **b** TCD4$^+$ cells positive immunostaining in ON. **c** TCD8$^+$ cells positive immunostaining in the INL. **d** GFAP expression in GCL and INL.

consequent inhibition of phagocytic activity in the anterior chamber [30], an increase in MMP-9, the formation of fibrosis, and consequently an increase in IOP [31–33].

The elevation of IOP in the posterior segment of the eye can lead to compression of the blood vessels and ON fibers. This in turn triggers axonal degeneration and apoptosis [34]. Beyond axonal changes, OH results in a diminished supply of neurotrophins [35]. This deficiency can lead to inadequate removal of glutamate from the extracellular space, which could represent an interaction whereby glial cell activation may play a dual role: on the one hand, they can provide support and have cell-protective capabilities [36], and can induce retinal ganglion cell death by secreting proinflammatory cytokines such as TNF-α and IL-1β [37].

As a result of glial cell activation and an elevation in vascular endothelial growth factor and intercellular adhesion molecule 1 (ICAM 1) in the retina, T-lymphocyte infiltration occurs through the retinal pigment epithelium. Although activated microglia control the transition from innate immunity to an adaptive immune response in the nervous system, it is not clear what is the cause and what is classified as an epiphenomenon for the manifestation of an autoimmune disease in glaucoma [38, 39]. In our study, the infiltration of intraocular T cells could be altered by the proposed model of OH, due to the dual effects of corticosteroids: on the one hand inducing IOP increase and on the other suppressing immune responses [40]. However, we observed not only an infiltration of T cells in the anterior segment in the OH groups, but also an increase in the levels of certain proinflammatory cytokines in both the aqueous and vitreous humors, suggesting that this experimental model can present paradoxical inflammation as a response to the alteration of intraocular homeostasis despite the immunosuppressive action of GCs.

Although T-cell-mediated immune responses may be beneficial and even necessary to optimally limit neurodegeneration within the central nervous system [41], the presence of lymphocytes may induce the recruitment of more circulating lymphocytes [29], as evidenced in our investigation. This observation may be due to the activation of residual macrophages and induction of the expression of IL-6 (dual action, proinflammatory, and anti-inflammatory), IL-1β, and TNF-α (proinflammatory) [33, 42, 43]. Even so, the overexpression of T lymphocytes could also be explained by the intravitreal application of hWJ-MSCs, which produce an active intraocular immune response in an effort to maintain homeostasis of the ocular microenvironment [44].

A significant increase in TCD3$^+$ cells was also observed compared to the eyes of the untreated eyes in the group treated with hWJ-MSCs without OH, suggesting that these cells not only produce inhibitory effects on T cells, but also have a stimulatory effect on some lymphocyte strains. An interesting finding in our experimental model was the presence of TCD3$^+$-cell

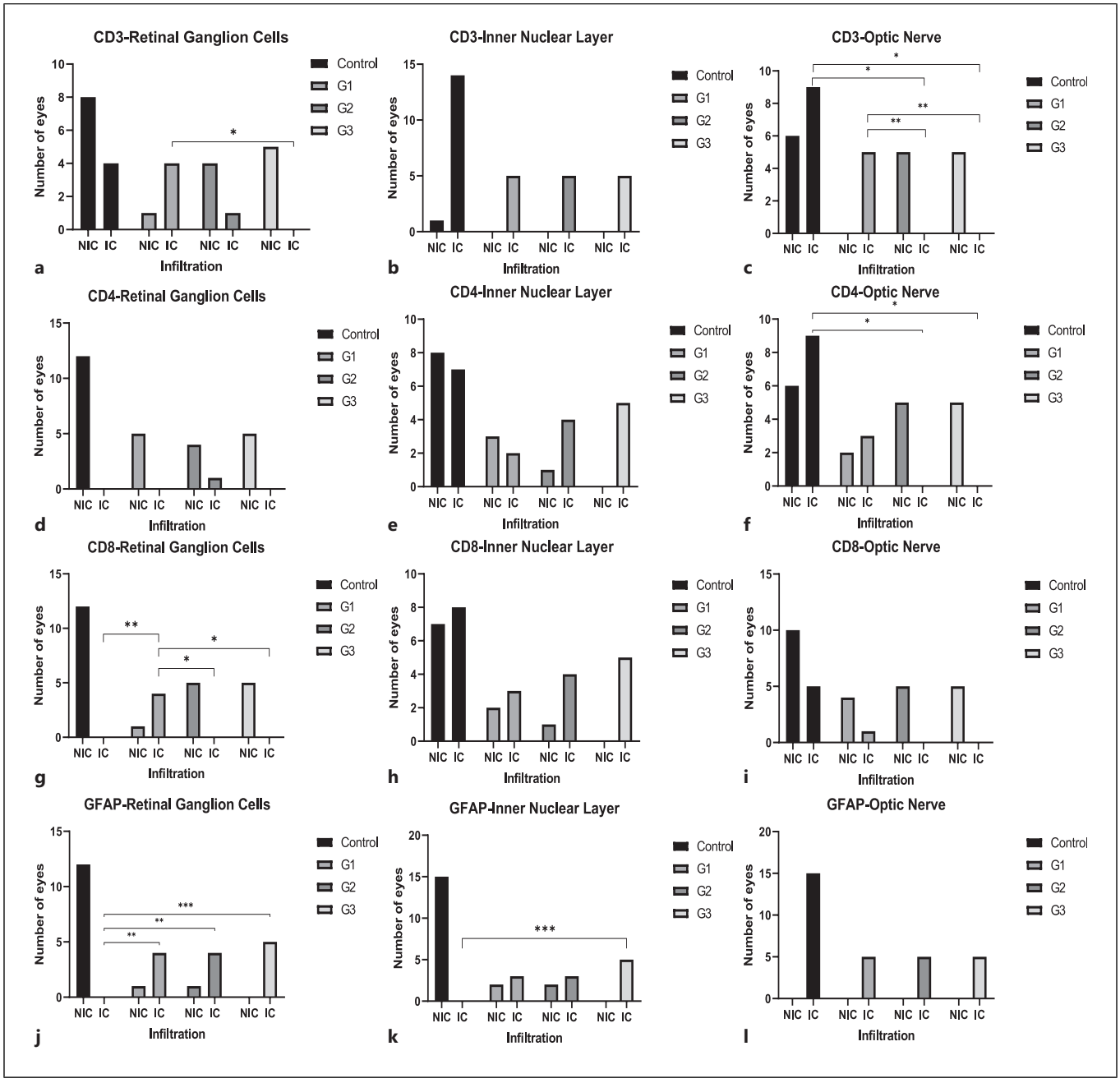

Fig. 4. Infiltration of lymphocytes in the retina and ON. **a–c** $TCD3^+$ (cluster of differentiation 3). **d–f** $TCD4^+$ (cluster of differentiation 4). **g–i** $TCD8^+$ (cluster of differentiation 8). **j–l** GFAP (glial fibrillary acidic protein). NIC, absence of T-cell infiltration; IC, presence of T-cell infiltration. All the analyses were done with the Chi or Fisher tests, depending on the data distribution. $*p < 0.05$.

infiltration in some retinal layers in the untreated eyes. Cellular infiltration has also been described in glaucoma models [45, 46].

We observed that GC-induced OH could also increase the concentrations of IL-6, IL-8, TNF-α, IL-10, and IFN-γ in both the aqueous and vitreous humors, as described in other forms of glaucoma [47–49]. We corroborated the findings of Yang et al. [50] by observing an increase in TGF-β levels in the OH group, suggesting that the increase in this cytokine may be the triggering factor

for the imbalance of certain cytokines in the ocular microenvironment.

The elevated concentrations of certain cytokines in glaucoma might be attributable to the activation of microglia, as indicated by the observed gliosis and GFAP overexpression in the OH groups. The activation of microglia is known to trigger the release of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6 [51]. Among these cytokines, the levels of IL-6, TNF-α, and IFN-γ decreased following the application of hWJ-MSCs in the OH group when compared to the group without cell therapy. This suggests that soluble factors secreted by MSCs may exert an immunomodulatory effect. Many of the factors produced by MSCs can inhibit Th1 lymphocytes and reduce IFN-γ and TNF-α levels [52]. Furthermore, these factors can promote the expansion of Tregs (highlighted by the markers TGF-β and IL-10) and Th2 [53], thus modulating the ocular microenvironment.

The literature suggests that hWJ-MSCs may express a higher level of immunomodulatory factors, such as TGF-β, than adult bone marrow-derived MSCs [53]. Other authors postulate that the suppressive effect of MSCs is particularly due to factors such as TGF-β1, IL-10, and IL-6 [11, 54–56]. In our study, we observed a lower concentration of TGF-β in the OH group than in the group that received cell transplantation. This indicates that hWJ-MSCs are capable of secreting factors that provide regenerative and restorative effects that inhibit the inflammatory reaction of cells in the ocular microenvironment [57, 58].

Contrary to what was observed by Mrahleh et al. [59] in an in vitro study of hWJ-MSCs, no increase in IL-10 concentration was observed in the cell therapy groups. However, this cytokine increased slightly in the OH group than in the untreated eyes. This increase in IL-10 associated with the increase in IL-6 has been described in POAG and may serve as an early biomarker of disease progression [56, 60]. The same tendency to increase IFN-γ levels was observed in the OH group compared with those treated with hWJ-MSCs. This finding can be attributed to the secretion of soluble factors involved in the immunosuppressive mechanisms of MSCs, such as IDO, which exerts an inhibitory effect on signal transduction in Th1 lymphocytes and is involved in the production of proteins necessary for the proliferation of lymphocytes [61].

One of the main cytokines altered in glaucomatous processes is TNF-α [62]. TNF-α is usually found in healthy eyes [63], explaining its presence in our study's untreated eyes. In addition, we observed that the OH group without hWJ-MSCs and the cell therapy group without OH significantly increased the concentration of this cytokine. This increase can be observed in stressful situations such as immune reactions, inflammation, ischemia, hypoxia, and oxidative stress [64]. In contrast, the OH group that received hWJ-MSCs showed a decrease in the level of this cytokine, which is consistent with other reports [59]. These findings demonstrated the potential immunomodulatory effects of these cells.

In our study, we observed an increase in IL-8 levels in all groups compared with that in the untreated eyes. This observation may be due to the characteristics of IL-8, a proinflammatory cytokine with proangiogenic, proliferative, and promotility activities, indicating neuroinflammation in the presence of elevated levels [69]. IL-6 is a cytokine with proinflammatory and anti-inflammatory characteristics, which could explain the increase in IL-6 after intravitreal application of hWJ-MSCs in the group without OH. A significant decrease in this cytokine was observed in the OH group that received cell therapy but not in the OH group. This alteration in the ocular microenvironment may be related to the anti-inflammatory property of hWJ-MSCs because of the suppression of the production of IL-6 and a reduction in the activation of circulating monocytes, causing the inhibition of some cytokines [70].

Currently, it has been hypothesized that glaucoma is an autoimmune disorder since the presence of antibodies against endogenous antigens [65] and T cells has been detected in glaucoma patients. The presence of T cells in the retina is unusual because the eye has barriers that prevent their entry [20]. However, as some authors have described in other OH models [20], we also observed infiltration of $TCD3^+$ cells in the retina, even in an immunosuppressed environment, owing to applying GCs.

When evaluating $TCD3^+$-/$TCD4^+$-cell infiltration, we observed infiltration of the INL in all groups, including the untreated eyes. The hWJ-MSC group without OH was the only group with $TCD4^+$-cell infiltration in the GCL. This may be due to the immunomodulatory effect of MSCs through the activation of immunoregulatory cells such as tolerogenic dendritic cells and regulatory T cells, which are capable of inhibiting antigen-specific T-cell responses [59].

In contrast to the results described by Yan et al. [56] in peripheral blood samples from glaucoma patients, in our study, we observed a significantly higher percentage of $TCD8^+$-cell infiltration in various layers of the retina

compared to untreated eyes. A promising finding was that when evaluating the GCL, we observed that the only group that presented an intense infiltration of $TCD8^+$ cells in this layer of the retina was the OH group. This finding may indicate that cellular immunity also plays an essential role in the initiation and/or progression of glaucomatous optic neuropathy [47]. These observations justify further studies to determine the importance of T-cell responses in neuroinflammation and neurodegeneration in glaucoma.

In the retina, both Müller cells and astrocytes and glial cells play an important role in retinal tissue homeostasis, regulating the immune response in the central nervous system [66]. In the early stages of glaucoma, glial cells undergo gliosis in response to an increased IOP. However, in chronic diseases, proliferative gliosis can accelerate neurodegeneration through the secretion of proinflammatory cytokines, increasing the inflammatory reaction and activating the apoptotic pathway [67, 68]. In our study, we assumed that the intravitreal application of hWJ-MSCs was capable of triggering a transient but massive infiltration of cells with Iba1+ markings (a protein highly expressed in microglia and macrophages) from the choroid to the retina, capable of altering the retinal structure [3]. We observed intense activation of glial cells, marked by the expression of GFAP, in the group of hWJ-MSCs without OH. Furthermore, intense GFAP expression was observed not only in the GCL, but also in the INL, and the ON was observed in all animals. This indicates that even without presenting toxic properties to the retina and ON, the transplant is not innocuous because it triggers the expression of GFAP in the group without OH with cell therapy.

One of the limitations of our study was the small sample size, which can reduce the statistical power. Another limitation was that we did not evaluate other cell populations, such as granulocytes and monocytes, in the aqueous and vitreous humor samples, which could be related to disease development. Some of the aqueous humor and vitreous humor samples from the first week of the study were lost due to misreading of the ELISA kits, reducing the number of cytokines evaluated at that time and consequently the sample size, which could influence the statistical analysis results of the study. Obtaining cytokine kits for rabbits was a challenge in addition to the limited collection of intraocular fluids. Although many antibodies originate in rabbits, there are not many antibodies with reactivity for this species. The initial idea was to use microarray kits to measure various cytokines, but the market lacks the production of these kits for the species studied, only for other species. For this reason, ELISA kits were randomly divided to be used between aqueous humor and vitreous humor.

Conclusion

Our study demonstrated that the intravitreal xenotransplantation of hWJ-MSCs promotes increased anti-inflammatory cytokine production while suppressing proinflammatory cytokine production in the ocular microenvironment of an experimental OH model induced by GCs. Moreover, even within this OH-immunosuppressed context, we noted predominant infiltration of $TCD3^+/TCD8^+$ lymphocytes in the retina. However, this infiltration decreased in animals subjected to cell therapy. These observations support that glaucomatous disease is not merely characterized by OH but also presents as an immune-mediated disorder resulting in T-cell infiltration into the retina. Therefore, the transplantation of hWJ-MSCs has emerged as a promising therapeutic option. These cells appear to modulate intraocular homeostasis, which may help delay disease progression.

Statement of Ethics

All procedures performed in this study were approved by the Animal Ethics Committee Escuela Superior de Oftalmología Instituto Barraquer de América (CEIA-ESO-IBA), letter C-20190226-1, dated 2019-02-26, and were in accordance with the Helsinki Declaration (1964) and its later amendments. This study protocol was reviewed and approved by the Ethical Committee of Secretaria Distrital de Salud de Bogotá, under approval reference 2019EE44993. Written informed consent in this study was obtained from all participants and from parents/legal guardians for all participants aged under 18. This study was conducted ethically in accordance with the World Medical Association Declaration of Helsinki.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Karine dos Santos Evangelho: investigation, methodology, visualization, and writing – original draft. Carlos Cifuentes-González: data analyses and writing – review and editing; William Rojas-Carabali: data interpretation and writing – review and editing; Clemencia De Vivero-Arciniegas: investigation and methodology; Mariana Cañas-Arboleda and Gustavo Salguero: methodology; Carolina Ramírez-Santana: writing – review and editing; and Alejandra de-la-Torre: project administration, investigation, and writing – review and editing. All authors have read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material files. Further inquiries can be directed to the corresponding author.

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